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Special Issue Reprint

Grassland Ecosystem Services

Research Advances and Future Directions for Sustainability II

Edited by
Michael Vrahnakis and Yannis (Ioannis) Kazoglou

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Grassland Ecosystem Services: Research Advances and Future Directions for Sustainability II

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Guest Editors

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This is a reprint of the Special Issue, published open access by the journal *Land* (ISSN 2073-445X), freely accessible at: https://www.mdpi.com/journal/land/special_issues/3LG5H086X9.

For citation purposes, cite each article independently as indicated on the article page online and as indicated below:

Lastname, A.A.; Lastname, B.B. Article Title. *Journal Name* **Year**, *Volume Number*, Page Range.

ISBN 978-3-7258-3719-9 (Hbk)
ISBN 978-3-7258-3720-5 (PDF)
https://doi.org/10.3390/books978-3-7258-3720-5

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About the Editors

Michael Vrahnakis

Dr. Michael Vrahnakis is a Professor of "Rangeland Science" in the Department of Forestry, Wood Sciences and Design at the University of Thessaly, Greece. He has 30 years of experience in higher educational institutes, specializing in rangeland management plans, the organization of agroforestry systems, and the management plans of protected areas. His current research interests are the management, utilization, and ecology of rangelands; the quantification of floristic diversity, agroforestry, rangeland conditions, and rangeland health; and the management and monitoring of protected areas. He has published over 200 peer-reviewed papers and articles in various international and national conferences, scientific and sectoral journals, articles, undergraduate books, and collective volumes. He has participated in several international and national scientific committees, and he is also a reviewer for international conferences and journals, as well as a member of academic and scientific committees of journals. He has successfully coordinated more than 15 research projects, in Europe and nationally, both competitive and self-funded, and he has participated in more than 20 other respective projects as a member of a research team. He has been an instructor at many national and international seminars on grassland management and has participated in scientific trips to several places of the globe. He has participated as an expert in thematic groups of Hellenic Ministries, and he has participated in corresponding thematic groups abroad. He is a member of many scientific organizations and associations and has participated in the administration of the Eurasian Dry Grassland Group, an international scientific organization (2009–2019).

Yannis (Ioannis) Kazoglou

Dr. Yannis (Ioannis) Kazoglou is an Associate Professor at the Department of Forestry, Wood Sciences and Design at the University of Thessaly, Greece, since April 2019. His research and teaching interests focus on biodiversity conservation and the positive effects that specific primary sector activities, such as extensive grazing, organic agriculture, breeding of autochthonous livestock, and wet meadow management, may have on the biodiversity of the EU Natura 2000 network of protected areas. An important part of his work aims at sustaining local communities and economies in remote areas and halting the loss of biodiversity due to land abandonment. Prior to his academic position, he actively participated in more than 25 research projects and applied restoration and monitoring projects in Greece (one of which was awarded among the five "Best of the Best" LIFE–Nature projects in the EU, out of a total of 398 projects ending between 2007 and 2008), in the transboundary Prespa Park (Albania, Greece, and North Macedonia) and in Azerbaijan. Between 2015 and 2018, he contributed to important lawmaking acts for the protected areas of Greece, for grazing management as the chairman of the Hellenic Range and Pasture Society and, later, as a special advisor of the Deputy Minister of the Environment. Over recent years, he has participated to the European CAP Network Focus Group on "Competitive and resilient mountain areas", as well as in national research projects aiming at identifying how grazing management can be beneficial for the conservation of specific habitat types and threatened butterfly and locust species within these protected areas. He has published more than 50 papers in peer-reviewed scientific journals, collective volumes, and national and international conference proceedings, and has been an active member of five scientific and non-governmental organizations.

Preface

This volume contains reprints of research and review articles published in the Special Issue "Grassland Ecosystem Services: Research Advances and Future Directions for Sustainability II" (Land journal, MDPI Publishing). The reprints discuss recent advantages and future directions on grassland ecology and management that are supported by research carried out in several grassland types around the globe. The volume accommodates fourteen (14) studies and two (2) literature review papers, covering a wide range of global rangelands from North, Central and South Europe, Central Asia, China, Australia, to Brazil, with the contribution of 79 authors.

In particular, the establishment of vegetation elements, such as *Calotropis procera* shrub in dry soils or Iran for restoration purposes or to exploit its therapeutic properties, seems to be favored by vermicompost coating of species' seeds that contain superabsorbent polymer (Dolatkordestani et al. 2023).

Also, in the drylands of Central Asia, animal husbandry depends on mixtures of crops and pastures around settlements and higher-elevation summer pastures. The typology developed by Azarov et al. (2024) that considers elevation, grazing period, cultivated area, and off-farm income, better captures the diversity of farming activities and household income in Kyrgyzstan compared to current classification models, and further helps to tailor policies and interventions suitable for enhancing sustainable livelihoods in mountain farming systems.

The study of insect sex-specific responses to grassland management may be a valuable tool to understand grassland dynamics. By modeling the responses of the abundance of male and female syrphids and their habitat preferences, we may draw conclusions related to the impact of grassland management intensity on their sustainability (Hussain et al. 2024, experiment carried out in Lower Austria).

The land transformation of tropical Cerrado, Brazil to croplands and pastures results in the degradation of the provision of ecosystem services due to reduced water cycling. The study of Glatzle et al. (2024) showed that the ecosystem services of Cerrado may be restored after the introduction of tree planting in integrated management schemes.

Valuable habitat types, like the Pannonian dry and sandy grasslands in Hungary, may suffer from several radical human interventions that may alter the structural components and their unique ecosystem services. However, military activity that continued until the late 1980's seems to have favored the conservation of Pannonian grasslands mostly due to their isolation and exclusion from potentially damaging land uses that often cause their disappearance (Bajnok et al. 2024).

The investigation of the effects of grazing intensity on compositional and functional diversity in grasslands is an intriguing research topic. This was the aim of the study conducted by Avramidou et al. (2024), at semi-humid grasslands of Northern Greece. The results support the "moderate grazing intensity" hypothesis, under which grassland floristic diversity is enhanced and shrubs are not allowed to invade; at the same time, high intensity grazing should be avoided to protect soils from erosion. The authors also suggest that the effects of grazing intensity on plant functional traits should further be studied in grasslands.

The diversity, ecological features, and conservation status of grassland habitats of the EU's interest in Calabria (Italy) were the focus points of the study of Morabito et al. (2024). The authors evaluated how species composition and Ellenberg's ecological indicators differentiated between the selected grassland habitat types. They found that temperature and moisture are the most significant ecological factors in differentiating habitat types, and diversity is strongly correlated with habitat

diversity as soil moisture increases.

Water-conserving turfgrasses are of high interest in arid urban environments, and their successful establishment is a challenging issue. Kazemi et al. (2024) conducted their research in Mashhad, Iran and suggested that to achieve turfgrass of high quality and to safeguard social and ecosystem services, warm-season turfgrasses adaptable to these environments must be used.

The hemiparasites of the genus *Rhinanthus* have a positive impact on grassland biodiversity. Levinsh (2024) reviewed the taxonomy, evolution, and geographic distribution of *Rhinanthus* taxa and gave insights on their reproductive biology, host range and selectivity, parasitic relationships, nutrition, ecological resilience, complexity of interrelationships with host plants, and their effects on plant diversity in grasslands. The author indicated the unremitting lack of knowledge about the signal exchange between the *Rhinanthus* parasites and the host plant.

The distribution of multi-component perennial grass roots into the Arenosols and their impact on SOC sequestration in temperate climate zones is of high research interest, especially under the question of fertilization. Tripolskaja et al. (2024) found that fertilized sites in Vilnius, Lithuania provided more favorable conditions for carbon accumulation in the soil and for the development of above-ground and below-ground grass biomass. Also, the biodiversity advantage of unfertilized grassland and the higher above-ground biomass under fertilization need to be considered in the phase of selecting the proper land management practices for sandy soils of low productivity.

Grasslands provide a wide range of ecosystem services, whose valuation methods can be grouped into three categories, i.e., ecological, sociocultural, and economic. Pergola et al. (2024) provided an overview of academic studies on grassland ecosystem services and on the most used economic evaluation methods. The difficulty in estimating individual services provided by grasslands from an economic point of view and the greater propensity to use physical, chemical, and biological indicators was highlighted.

The effect of grazing and its absence on plant and soil parameters in five study sites of steppe mount pastures of Armenia was studied by Navasardyan et al. (2024). The results indicated that grazing decreased the plant parameters and nitrogen and litter content across all sites, while it had no effect on the phosphorus and potassium content or the soil pH values. The authors suggested to introduce livestock rotation practices to achieve sustainable management of steppe pastures.

The study of Song et al. (2024) focused on grassland ecosystem services in the karst desertification control area in South China, and compared artificial restoration measures involving *Dactylis glomerata*, *Lolium perenne*, *Lolium perenne*, and *Trifolium repens* in a mixture, with natural restoration measures. The results showed that different vegetation restoration patterns significantly influenced grassland ecosystem services. Also, the mixture treatment was considered the best vegetation restoration practice for these environments.

Land use/cover changes significantly affect the function and distribution of grasslands, showing diverse patterns across space and time. Karatasiou et al. (2024) studied such changes by applying spatial and socioeconomic modeling in grasslands of Southern Greece, covering a period of 50 years. They found that grassland distribution was significantly impacted by climate change, with impacts varying by the period, scenario, and climate model used.

The study of the effects of local animal breeds on restored grassland plant communities is of high scientific interest. Szentes et al. (2024) studied such effects on Pannonian grasslands of northwestern Hungary restored by five different methods and grazed by Hungarian Grey cattle. The results indicated that returning to traditional land use improved the ecological status of all sites. Natural regeneration and the hay–mulch addition method were the most effective for conservation and grassland management.

Búzás and Bódis (2024) studied the effects of land use within a conservation context of a species-rich Hungarian fen meadow over 250 years. They found that the major global problems affecting the European fen meadow are drainage, agricultural intensification, the expansion of infrastructure networks, and the abandonment of farming. Mosaic, adaptive, small-scale landscape use is necessary for the long-term sustainability of European wet grasslands.

The overall message provided by these papers is that grasslands constitute a globally valuable land resource that supports a vast array of ecosystem services necessary for human and nature wellbeing. In addition, grassland managers must be equipped with adequate knowledge of the ecology of grasslands in order to succeed in their long-term sustainability to the benefit of nature and humanity.

We are thankful to all authors who kindly contributed to this reprint by presenting their findings, the reviewers who thoroughly reviewed the manuscripts and significantly improved their content, and the Section Managing Director Ms. Zita Zhang who supported us during the whole process of editing. Finally, we thank MDPI for offering us the opportunity to serve as Guest Editors in this Special Issue.

Michael Vrahnakis and Yannis (Ioannis) Kazoglou
Guest Editors

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Article

Effective Treatments for the Successful Establishment of Milkweed (*Calotropis procera* L.) under Water Deficit

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Abstract: The application of superabsorbents to soils and seed coatings is a pre-sowing seed treatment method that is commonly used to improve early vigor and establish stability and uniformity under water deficit conditions. To evaluate the interaction of seed coating and superabsorbent on *Calotropis-procera* L. (milkweed) under water deficit conditions, a greenhouse experiment was conducted. The experiment was conducted with four coating material levels (non-coated seeds and seeds coated with peat moss, vermicompost, and canola residue), four growth medium levels (soil, sand + soil, soil + 2 g superabsorbent, and soil + 4 g superabsorbent), and three field capacity regimes (25, 50%, and 100%) in a completely randomized design factorial arrangement with four replications. Reducing the field capacity from 100 to 25% led to decreased growth (shoot and root dry weights and leaf area) and chlorophyll content. The activities of SOD, CAT, APX antioxidant enzymes, and proline increased under drought stress. The use of superabsorbent polymers in growth media enhanced growth indices and chlorophyll content and decreased the activity of antioxidant enzymes and proline under water deficit conditions. The highest chlorophyll and growth indices were observed when 4 g of superabsorbent was added to the growth medium under drought stress. The application of 4 g of superabsorbent to the growth medium reduced the activity of antioxidant enzymes and proline. The use of seed coatings improved the growth indices, antioxidant enzyme activity, and chlorophyll content under drought stress. The most adaptive morphological and physiological responses to water stress were observed in the vermicompost-coated seeds. The vermicompost coating containing a superabsorbent polymer (4 g/kg soil) proved to be the best for establishing milkweed under mild (50% FC) and severe water deficits (25% FC).

Keywords: drought stress; antioxidant enzymes; drought; rangeland; superabsorbent

Citation: Dolatkordestani, M.; Taghvaei, M.; Mastinu, A. Effective Treatments for the Successful Establishment of Milkweed (*Calotropis procera* L.) under Water Deficit. *Land* **2023**, *12*, 1987. https://doi.org/10.3390/land12111987

Academic Editors: Michael Vrahnakis and Yannis (Ioannis) Kazoglou

Received: 13 September 2023
Revised: 26 October 2023
Accepted: 27 October 2023
Published: 29 October 2023

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1. Introduction

The significance of the rehabilitation and development of rangelands has recently been highlighted because of the increasing trend of their degradation under the effects of human factors and climate change, such as reduced rainfall [1–4]. In this regard, seeding projects and the application of superabsorbent polymers are of paramount importance for regenerating rangelands. Therefore, a great deal of time and money are spent on rangeland seeding projects [5,6]. Nevertheless, various factors, such as soil erosion and climate change, including increased average temperature and environmental stresses, particularly drought, can decrease seed emergence percentage in rangelands [7,8]. Iran is located in the Northern Hemisphere, between 46° and 64° E [2]. The geographical location, rainfall regime, and temperature conditions, as well as the annual rainfall of approximately 250 to 300 mm [1], make our country (Iran) one of the arid and semi-arid regions in the world.

Drought and water deficit are among the most significant factors limiting crop production worldwide. The most sensitive stages of plant development to drought stress are the germination and seedling stages [9–14]. In this context, decreased seed emergence percentage and seedling establishment in lentils (*Lens culinaris* Medik.), walnut (*Juglansregia* L.), and maize (*Zeamays* L.) under drought stress conditions have been reported [15–17]. Dryland soils are characterized by low organic matter content, low water-holding capacity, low infiltration, and low fertility, which lowers the percentage of seedling establishment due to low water maintenance. One way to improve seedling emergence and early seedling development is to use seed coating fillers [18] to increase the water stored in the soil for a longer period [19]. Organic materials, such as peat moss and vermicompost, can be used as soil water stores to increase the moisture around planted seeds.

Hence, it is of paramount importance to apply methods such as seed coating with organic material to reduce the effects of water stress and enhance the seed emergence percentage [20]. Some studies have reported increased seed germination uniformity and speed, as well as seedling establishment, under salinity and drought stress conditions using the seed coating method in rice, barley, rye, and wheat [21,22].

In addition, the use of superabsorbent polymers for soil amendment may improve moisture availability for germinating seeds and during later growth stages under water deficit conditions. Superabsorbent polymers are hydrophilic polymer gels that can absorb an amount of water many times their size [23]. After absorption, owing to the drying environment, water inside the polymer is gradually released, and the rhizosphere soil is kept wet for a long time without re-watering [24,25]. In this context, the use of superabsorbent polymers in soil significantly improved the germination of corn (*Zea mays* L.), pearl millet (*Pennisetum glaucum*), and oat *(Avena sativa* L.) seeds under drought stress [26–28].

Calotropisprocera L. (milkweed), which belongs to the family Apocynaceae, is an evergreen and perennial plant. This species is mostly distributed from the hot and desert areas of Southwest Asia and the Mediterranean region to the shores of Africa and southern Iran [29]. Milkweed is a unique species that plays an important role in the restoration of degraded land in arid and desert regions. Milkweed also has industrial and medicinal properties and is used as an anti-rheumatic, anti-diarrheal, and anti-fungal anticancer agent [30]. Milkweed plants are very important in revitalizing pastures and the pharmaceutical industry. This plant is propagated only by seeds. Milkweed produces many seeds, but its density is very low in arid regions. Its seeds are exposed to drought stress during the germination stage in spring, leading to a very low percentage of germination and seedling establishment because its seeds are sensitive to water stress during germination [31]. Little information is available regarding the use of a growing medium with superabsorbents or the use of organic fillers to reduce water stress and increase the percentage of plant establishment in arid lands. Our hypothesis is that the decrease in green percentage in the pasture is due to the type of growing medium or soil; that is, the percentage of silt is high, and the percentage of organic matter is very low, which cannot absorb a significant amount of water from accidental spring rain. Furthermore, the use of superabsorbent and organic matter can increase the absorption of spring rain; that is, the application of superabsorbent material can help the soil absorb a significant amount of rainwater, thus allowing it to provide water to the plant for a longer period of time, thereby reducing water stress during the emergence stage.

On the other hand, the use of organic material as a filler or seed cover can improve the amount of water absorption and retention time around the seed and increase the percentage of this plant's establishment in early spring. Hence, the aim of this study was to investigate the effects of the addition of superabsorbent polymers to growth media and the use of different types of organic matter seed coatings to help improve the early growth and establishment of milkweed under water deficit conditions.

2. Materials and Methods

2.1. Collection and Preparation of Seeds Coat

Five hundred fresh capsules of *Calotropis procera* L. were collected in August 2022 from one of its natural habitats in the rangelands of Sarbaz (61°25′ E and 26°63′ N, 892 m asl), Sistan, Iran. The environment consists of a degraded pasture with scattered cover of *Calotropis procera* L. trees and silty clay loam soils. One hundred milkweed fruits were dried, and the seeds were removed from the capsules and cleaned. Dried, uniform seeds were selected and disinfected in a carboxin–thiram solution (2 g L^{-1}) for 2 min.

After preparing the seeds, three organic compounds of peat moss, vermicompost, and canola residue were dried and sterilized at 100 °C for 30 min in an oven and then ground by a mill passed through a sieve with a 1 mm mesh (in this experiment, vermicompost and peat moss were prepared by Kian Pars Company, Teheran, Iran, and Miracle-Gro Company, Marysville, Ohio, USA, respectively, and canola residue was prepared by Shiraz University, Iran).

Subsequently, 2 g of each seed coating material was injected into a plastic half-circle (with a diameter of 5 cm), and the molds were dried at room temperature. They were maintained in a sterile environment until the beginning of the experiment (Figure 1). It should be noted that in the middle of one of the molds, there was a 4 mm deep hole for placing seeds. At the time of planting, two seeds were placed between the two halves of the molds and were planted in 5 L volume pots filled with growth medium "based on experimental treatments" (with one hole drilled in the bottom of each pot for drainage) (Figure 1), and one of them was removed. To apply the water stress, the field capacity of the soil was determined.

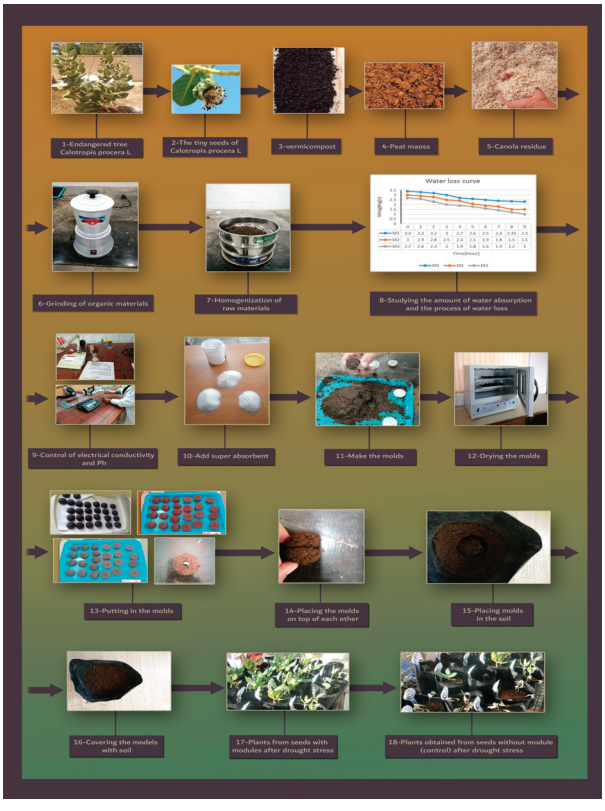


Figure 1. The steps of preparing seed coating.

The physicochemical features of the soils used in this study are listed in Table 1.

Table 1. Some of the physical and chemical properties of soil used in experiment.

Permanent Wilting Point (%)	Field Capacity (%)	Silt (%)	Clay (%)	Sand (%)	EC (d/Sm)	pH	Organic Matter (%)
11	28	53	33	14	2.1	7.1	0.268

After planting the seeds, the pots were placed in a greenhouse with 16/8 h day/night length at 25/15 °C day/night temperatures for a period of 20 days before the beginning of treatments.

2.2. Experimental Detail

This experiment was performed at the Research Greenhouse of the Department of Crop Production and Plant Breeding, School of Agriculture, Shiraz University, Shiraz, Iran (52°32′ E and 29°36′ N, 1810 m asl) in a completely randomized factorial design including four coating material levels (control, peat moss, vermicompost, and canola residue), four growth media levels (soil (S1), sand + soil 1:1 ratio (S2), 2 g superabsorbent per kg soil (S3), 4 g superabsorbent per kg soil (S4)), and irrigation at three levels (25, 50, and 100% field capacity) with four replicates. F1 superabsorbent polymer (a cationic amphoteric surfactant, as white crystalline fine powder (*potassium polyacrylate*), produced by the German Stockosorb® company, Essen, Germany) ISO 14001:2005 [32] was used in this experiment. The chemical characteristics of vermicompost, peat moss, and canola residues are listed in Table 2. Seeds coated with organic matter (peat moss, vermicompost, and canola residue) were sown in culture media. After 15 days of sowing, water deficit treatments (25, 50, and 100% of the field capacity) were carried out for three months and then the plants were harvested to determine indices. Every alternate day, to apply the field capacity, the pots were weighed, water treatments were applied to the weight base, and water was added to achieve the target soil moisture. The plants were harvested three months after the imposition of water deficit to record morphological and physiological parameters.

Table 2. Some chemical characteristics of vermicompost, peat moss, and canola residue used.

	Vermicompost Properties	Peat Moss Properties	Canola Residue Properties (Above-Ground Plant at Flowering)
pH	7.75	4.4	6.14
Electrical conductivity(EC)	3.8 (ds/m)	0.76 (ds/m)	3.27 (ds/m)
Organic matter	44.2 (%)	45.68 (%)	43.80 (%)
Organic carbon	113.48 (mg/kg)	192.19 (mg/kg)	46.46 (mg/kg)
Total nitrogen	21.7 (mg/kg)	12.21 (mg/kg)	3.7 (mg/kg)
Phosphorus	14,194 (mg/kg)	0.09 (mg/kg)	0.25 (%)
Potassium	10,000 (mg/kg)	0.03 (mg/kg)	1.4 (%)
Iron	3274 (mg/kg)	563 (mg/kg)	19 (mg/kg)
Zinc	112.3 (mg/kg)	104 (mg/kg)	15 (mg/kg)
Manganese	248.8 (mg/kg)	25 (mg/kg)	14 (mg/kg)
Copper	28.7 (mg/kg)	340 (mg/kg)	2.7 (mg/kg)

2.3. Measurements

2.3.1. Plant Morphology

At the end of the experiment, shoots and roots of the harvested plants were separated and placed in an oven (model no. ODWF24-SD) at 70 °C until a constant weight was recorded for shoot and dry weights. Leaves were separated from the harvested plants, and leaf area was measured using a leaf area meter (LI-3000C, LI-COR, Lincoln, Nebraska).

2.3.2. Leaf Free Proline Content

The leaf free proline content was determined as described by Bates et al. (1973) [33]. Fresh leaf samples (0.5 g) were mixed with 10 mL of a 3% sulfosalicylic acid solution. Two milliliters of the extract was mixed with 2 mL ninhydrin acid reagent (2, 2- dihydroxyindane-1,3-dione) and 2 mL acetic acid, and the mixture was incubated at 100 °C for 60 min. After incubation, the mixture was placed in a water bath, 4 mL of toluene was added, and the absorbance was read at 520 nm using a spectrophotometer (Biowave II model, UK). The leaf free proline content was determined from the standard curve.

2.3.3. Leaf Chlorophyll Content

Fresh leaf samples (1 g) were crushed with 15 mL 80% acetone, and the supernatant was collected after centrifugation at 3000 rpm for 15 min and diluted to 25 mL using 80% acetone. The absorption was measured using a spectrophotometer (20–120-UV model, Japan) at 645 and 663 nm. The chlorophyll content was calculated using the following equation:

$$\textit{Chlorophyll content}\ (\text{mg g}^{-1}\ \text{FW}) = [20.2\ (A) + 8.02\ (B) \times V/(\text{W} \times 1000)]$$

where A = absorption at 645 nm, B = absorption at 663 nm, V = final volume of the extract and acetone, and W = fresh leaf weight.

2.3.4. Activity of Enzymatic Antioxidant

Briefly, fresh leaf (0.5 g) samples were ground with liquid nitrogen in a Chinese mortar; 1 mL of 50 mM phosphate buffer containing 0.5 M EDTA polyvinyl and 2% polypyrrolidone was added to the extracted tissue and centrifuged at 14,000× *g* for 20 min at 4 °C [34].

The supernatant was used to measure ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD). APX activity was estimated as described by Nakano and Asada (1981) [35], CAT activity was determined following Beauchamp and Fridovich (1971) [36], and SOD activity was estimated following McCord and Fridovitch (1969) [37].

2.4. Statistical Analysis

Prior to analysis, data population normality was verified using the Kolmogorov–Smirnov and Shapiro–Wilk tests in SPSS (10.0) software (Table 3). For the kind of organic mater and superabsorbent amount experiment, the collected data were analyzed by analysis of variance using 'SAS' v. 9.4. The least significant difference (LSD) test was used for mean separation at $p \leq 0.05$.

Table 3. Data normality is determined by Kolmogorov–Smirnov and Shapiro–Wilk tests.

Variables	Kolmogorov–Smirnov			Shapiro–Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
EP	0.155	192	0.142 *	0.935	192	0.095 *
LA	0.197	192	0.151 *	0.912	192	0.123 *
LDW	0.135	192	0.091 *	0.937	192	0.071 *
SLA	0.090	192	0.161 *	0.957	192	0.008 *
SDW	0.067	192	0.200 *	0.987	192	0.568 *
RDW	0.095	192	0.066 *	0.965	192	0.066 *
R/S	0.078	192	0.200 *	0.969	192	0.044 *

* The lower limit of true significance; a Lilliefors Significance Correction; df = degree of freedom; Sig. = significance; EP = emergence percentage; LA = leaf area; SDW = shoot dry weight; RDW = root dry weight; R/S = root dry weight/shoot dry weight.

3. Results and Discussion

The ANOVA results (see table below) showed that the main effects of seed coating, growth media, and irrigation significantly influenced the measured traits. In addition, the interactions of seed coating × irrigation and growth media × irrigation had a significant effect on the measured traits at the 1% probability level. The interactions of seed coating ×

growth medium and seed coating × growth medium × irrigation had no significant effect on the measured traits (Table 4).

Table 4. Analysis of variance of the effect of seed coating, growth media, and irrigation and their interaction on the measured traits in milkweed.

Source of Variability	df	EP	LA	SLA	SDW	RDW	R/S	CHO	PRO	SOD	CAT	APX
C	3	124.82 **	13.92 **	0.73 **	17.91 **	12.39 **	0.005 ^{ns}	0.007 **	1.89 **	1230.18 **	987.05 **	2613.01 **
M	3	1470 **	242.42 **	0.017 ^{ns}	256.71 **	127.30 **	0.003 ^{ns}	0.06 **	73.07 **	35,186.41 **	4277.51 **	12,697.13 **
I	2	7376 **	8360.86 **	0.19 **	22,391.5 **	5204.56 **	0.26 **	18.25 **	2994.02 **	1,386,722.81 **	38,578.42 **	4,327,279.04 **
C × M	9	4.6 **	0.047 ^{ns}	0.010 ^{ns}	0.17 ^{ns}	0.07 ^{ns}	0.009 ^{ns}	0.0 ^{ns}	0.02 ^{ns}	8.27 ^{ns}	3.33 ^{ns}	3.10 ^{ns}
C × I	6	236.12 **	1.307 **	0.021 *	3.05 **	2.56 **	0.006 ^{ns}	0.002 **	0.63 **	318.65 **	247.45 **	42.22 **
M× I	6	2.98 *	53.76 **	0.054 **	74.09 **	30.25 **	0.043 ^{ns}	0.015 **	21.02 **	11,088.60 **	1066.64 **	246.09 **
C × M × I	18	4.50 **	0.063 ^{ns}	0.21 ^{ns}	0.14 ^{ns}	0.13 ^{ns}	0.023 ^{ns}	0.0 ^{ns}	0.04 ^{ns}	8.58 ^{ns}	1.42 ^{ns}	16.49 ^{ns}
Error	144	1.29	0.23	0.16	0.58	0.41	0.12	0	0.03	6.67	2.06	14.06
CV (%)	-	1.68S	1.1	12.2	3.02	4.21	20.12	2.12	1.65	4.36	3.02	6.12

**, *, and ns: significant at the 1%, significant at the 5% probability level, and not significant, EP = emergence percantage, LA = leaf area, SDW = shoot dry weight, RDW = root dry weight, R/S = root dry weight/shoot dry weight, CHO = chlorophyll, PRO = proline, SOD = superoxide dismutase, CAT = catalase, APX = ascorbate peroxidase, C = coat, M = media, I = irrigation.

3.1. Emergence Percentage, Leaf Area, Specific Leaf Weight, Shoot and Root Dry Weights, and Root/Shoot Dry Weights

Drought is one of the most important growth-limiting factors that reduces plant growth. Plant injury occurs under water shortages during any vegetative growth stage [38]. Impaired mitotic division due to reduced turgor pressure has been reported to reduce growth indices under drought stress. When there is a shortage of water in the soil, the water flow from the roots to the cells declines, leading to reduced cell division and elongation, resulting in decreased growth traits in the plant [39]. Another reason for the decreased growth indices in plants is the closed stomata, which reduce water loss under drought stress. Under these conditions, CO_2 uptake by stomata and dry matter production are reduced [40–42].

Our findings showed that reducing the field capacity from 100 to 25% led to decreased shoot and root dry weight, leaf area, specific leaf weight, and root/shoot dry weight in milkweed compared to 100% FC, leaf area, root dry weight, and shoot dry weight, which, respectively, decreased by 2.82-, 1.99-, and 2.27-fold at 25% of field capacity (Table 5). Similarly, the effects of drought on morphological traits have been reported in many experiments on crops such as apples and sour cherries [43,44].

Table 5. Effect of interaction of coat and media, coat and irrigation, media and irrigation on leaf area and shoot and root dry weights of milkweed under drought stress. S1 = soil, S2 = sand + soil 1:1 ratio, S3 = 2 g superabsorbent per kg soil, S4 = 4 g superabsorbent per kg soil (S3), and FC = field capacity.

	100% FC	**50% FC**	**25% FC**	
		Emergence Percentage		
Control	87.11 ± 1.5 ^{a}	68 ± 0.71 ^{b}	62.5 ± 0.81 ^{b}	**77.54 ± 10.55 ^{A}**
Canola residue	90.48 ± 1.12 ^{a}	69.5 ± 1.12 ^{b}	66.75 ± 1.08 ^{c}	**75.57 ± 10.60 ^{B}**
Peat moss	78.64 ± 1.45 ^{a}	72.75 ± 1.92 ^{b}	67 ± 0.71 ^{c}	**72.791 ± 4.75 ^{AB}**
Vermicompost	97.45 ± 1.545 ^{a}	75 ± 1.41 ^{b}	70.75 ± 0.82 ^{c}	**81.06 ± 11.71 ^{A}**
Mean	**88.42 ± 6.76 ^{A}**	**71.31 ± 2.74 ^{B}**	**66.75 ± 2.92 ^{C}**	
S1	86.94 ± 7.03 ^{a}	69.5 ± 2.29 ^{b}	65.75 ± 2.86 ^{b}	**67.66 ± 2.05 ^{B}**
S2	87.25 ± 6.45 ^{a}	71 ± 2.73 ^{b}	66.5 ± 3.57 ^{b}	**61 ± 0.82 ^{C}**
S3	89.5 ± 6.8 ^{a}	71.75 ± 2.86 ^{b}	66.75 ± 2.48 ^{c}	**69.66 ± 1.24 ^{B}**
S4	90 ± 6.81	73 ± 3.16	68 ± 2.82	**77.66 ± 2.05 ^{A}**
Mean	**88.42 ± 1.35 ^{A}**	**71.31 ± 1.26 ^{B}**	**66.75 ± 0.82 ^{C}**	

Table 5. *Cont.*

	100% FC	50% FC	25% FC	
		Leaf area (cm^2)		
Control	35.05 ± 0.11 ^{a}	21.77 ± 2.62 ^{bc}	11.86 ± 2.94 ^{cd}	**22.89 ± 9.49 ^{C}**
Canola residue	35.17 ± 0.11 ^{a}	22.22 ± 2.79 ^{b}	12.17 ± 3.02 ^{dc}	**23.19 ± 9.41 ^{B}**
Peat moss	35.44 ± 0.11 ^{a}	22.75 ± 2.76 ^{b}	12.63 ± 2.93 ^{c}	**23.61 ± 9.32 ^{B}**
Vermicompost	35.54 ± 0.13 ^{a}	23.55 ± 2.85 ^{b}	13.31 ± 2.96 ^{c}	**24.13 ± 9.08 ^{A}**
Mean	**35.3 ± 0.197 ^{A}**	**22.58 ± 0.65 ^{B}**	**12.49 ± 0.54 ^{C}**	
S1	35.29 ± 0.19 ^{a}	22.99 ± 0.78 ^{b}	12.82 ± 0.44 ^{c}	**23.70 ± 9.17 ^{B}**
S2	35.14 ± 0.22 ^{a}	18.03 ± 0.52 ^{bc}	7.61 ± 0.57 ^{d}	**20.26 ± 11.3 ^{C}**
S3	35.34 ± 0.19 ^{a}	23.88 ± 0.62 ^{b}	13.13 ± 0.65 ^{c}	**24.10 ± 9.08 ^{A}**
S4	35.45 ± 0.21 ^{a}	25.37 ± 0.72 ^{b}	15.41 ± 0.51 ^{c}	**25.41 ± 8.18 ^{A}**
Mean	**35.30 ± 0.11 ^{A}**	**22.56 ± 2.7 ^{B}**	**12.24 ± 2.8 ^{C}**	
		Specific leaf area		
Control	0.54 ± 0.001 ^{a}	0.488 ± 0.020 ^{b}	0.34 ± 0.154 ^{c}	**0.45 ± 0.081 ^{AB}**
Canola residue	0.53 ± 0.001 ^{a}	0.49 ± 0.017 ^{b}	0.43 ± 0.082 ^{b}	**0.48 ± 0.035 ^{AB}**
Peat moss	0.50 ± 0.001 ^{a}	0.49 ± 0.024 ^{b}	0.43 ± 0.072 ^{b}	**0.49 ± 0.041 ^{A}**
Vermicompost	0.53 ± 0.002 ^{a}	0.503 ± 0.012 ^{a}	0.451 ± 0.092 ^{b}	**0.49 ± 0.035 ^{A}**
Mean	**0.53 ± 0.018 ^{a}**	**0.49 ± 0.004 ^{a}**	**0.45 ± 0.017 ^{b}**	
S1	0.53 ± 0.002 ^{a}	0.45 ± 0.13 ^{b}	0.48 ± 0.15 ^{ab}	**0.48 ± 0.036 ^{B}**
S2	0.5 ± 0.002 ^{a}	0.49 ± 0.005 ^{b}	0.32 ± 0.011 ^{c}	**0.44 ± 0.092 ^{AB}**
S3	0.45 ± 0.002 ^{b}	0.50 ± 0.003 ^{a}	0.46 ± 0.009 ^{b}	**0.47 ± 0.022 ^{B}**
S4	0.55 ± 0.002 ^{a}	0.53 ± 0.008 ^{a}	0.50 ± 0.025 ^{ab}	**0.52 ± 0.021 ^{A}**
Mean	**0.51 ± 0.039 ^{A}**	**0.49 ± 0.028 ^{A}**	**0.44 ± 0.070 ^{B}**	
		Shoot dry weight (g)		
Control	66.55 ± 0.04 ^{a}	45.43 ± 3.7 ^{b}	28.45 ± 2.01 ^{c}	**46.79 ± 15.5 ^{AB}**
Canola residue	66.67 ± 0.04 ^{a}	45.84 ± 3.8 ^{b}	28.48 ± 3.00 ^{c}	**46.99 ± 15.6 ^{AB}**
Peat moss	66.75 ± 0.04 ^{a}	46.39 ± 3.5 ^{b}	29.41 ± 2.32 ^{c}	**47.51 ± 15.2 ^{A}**
Vermicompost	66.8 ± 0.51 ^{a}	47.30 ± 3.8 ^{b}	30.59 ± 2.40 ^{c}	**48.24 ± 14.7 ^{A}**
Mean	**66.68 ± 0.12 ^{A}**	**47.25 ± 0.72 ^{B}**	**29.23 ± 0.87 ^{C}**	
S1	66.69 ± 0.09 ^{a}	47.30 ± 0.55 ^{b}	27.22 ± 4.07 ^{cd}	**47.07 ± 16.1 ^{B}**
S2	66.63 ± 0.10 ^{a}	39.90 ± 0.71 ^{bc}	25.66 ± 0.56 ^{cd}	**44.06 ± 16.9 ^{C}**
S3	66.72 ± 0.10 ^{a}	48.56 ± 0.72 ^{b}	30.78 ± 0.82 ^{c}	**48.68 ± 14.6 ^{A}**
S4	66.75 ± 0.10 ^{a}	49.23 ± 0.83 ^{b}	31.47 ± 0.92 ^{c}	**49.15 ± 14.4 ^{A}**
Mean	**66.69 ± 0.044 ^{A}**	**46.27 ± 3.72 ^{B}**	**28.78 ± 2.41 ^{C}**	
		Root dry weight (g)		
Control	35.90 ± 0.04 ^{a}	27.75 ± 2.1 ^{b}	17.26 ± 1.96 ^{c}	**26.97 ± 7.62 ^{C}**
Canola residue	35.92 ± 0.04 ^{a}	28.17 ± 2.1 ^{b}	17.67 ± 1.97 ^{c}	**27.25 ± 7.47 ^{AB}**
Peat moss	35.95 ± 0.04 ^{a}	28.72 ± 2.1 ^{b}	18.25 ± 1.97 ^{c}	**27.64 ± 7.26 ^{AB}**
Vermicompost	36.03 ± 0.14 ^{a}	29.56 ± 2.2 ^{b}	18.84 ± 2.12 ^{c}	**28.14 ± 7.08 ^{A}**
Mean	**35.95 ± 0.05 ^{A}**	**28.55 ± 0.67 ^{A}**	**18 ± 0.59 ^{A}**	
S1	35.93 ± 0.02 ^{a}	29.022 ± 0.80 ^{b}	17.73 ± 0.31 ^{cd}	**27.56 ± 7.50 ^{ABb}**
S2	35.86 ± 0.02 ^{a}	24.86 ± 0.58 ^{bc}	14.84 ± 0.62 ^{c}	**25.18 ± 8.58 ^{C}**
S3	35.96 ± 0.02 ^{a}	29.9 ± 0.74 ^{b}	14.07 ± 6.85 ^{c}	**26.64 ± 9.22 ^{AB}**
S4	36.05 ± 0.12 ^{a}	30.42 ± 0.61 ^{b}	19.87 ± 0.70 ^{c}	**28.78 ± 6.70 ^{A}**
Mean	**35.95 ± 0.068 ^{A}**	**28.55 ± 2.18 ^{B}**	**16.62 ± 2.32 ^{C}**	
		Root/shoot dry weight		
Control	0.70 ± 0.0394 ^{b}	0.83 ± 0.006 ^{a}	0.835 ± 0.027 ^{a}	**0.7.88 ± 0.062 ^{B}**
Canola residue	0.72 ± 0.003 ^{ab}	0.82 ± 0.056 ^{a}	0.841 ± 0.02 ^{b}	**0.793 ± 0.052 ^{A}**
Peat moss	0.729 ± 0.001 ^{b}	0.82 ± 0.003 ^{a}	0.854 ± 0.02 ^{a}	**0.801 ± 0.055 ^{A}**
Vermicompost	0.729 ± 0.002 ^{b}	0.94 ± 0.01 ^{a}	0.854 ± 0.06 ^{a}	**0.841 ± 0.052 ^{A}**
Mean	**0.722 ± 0.018 ^{B}**	**0.872 ± 0.01 ^{A}**	**0.846 ± 0.02 ^{A}**	

Table 5. *Cont.*

	100% FC	50% FC	25% FC	
	Root/shoot dry weight			
S1	0.546 ± 0.0001 [b]	0.667 ± 0.06 [a]	0.623 ± 0.009 [a]	**0.584 ± 0.046 [B]**
S2	0.523 ± 0.03 [b]	0.637 ± 0.004 [a]	0.590 ± 0.017 [ab]	**0.621 ± 0.052 [A]**
S3	0.547 ± 0.001 [b]	0.659 ± 0.05 [a]	0.657 ± 0.008 [a]	**0.622 ± 0.053 [A]**
S4	0.548 ± 0.001 [b]	0.650 ± 0.04 [a]	0.669 ± 0.032 [a]	**0.623 ± 0.053 [A]**
Mean	**0.542 ± 0.01 [A]**	**0.653 ± 0.011 [A]**	**0.634 ± 0.031 [A]**	

For each variable, the mean (± standard deviation) values followed by the same letters (small letters for interactions and capital letters for means) are not significantly different using LSD at the 5% level.

According to our results, the highest emergence percentage, leaf area, specific leaf weight, shoot and root dry weights, and root/shoot dry weights were observed in S4 media, indicating that, in this study, the use of superabsorbent in growth medium enhanced growth indices under drought stress (Table 5). In the S4 growth medium, leaf area and shoot and root dry weights increased by 1.09-, 1.02-, and 1.04-fold compared to the control (Table 5).

It has been reported that superabsorbents may absorb water several times their own weight, providing water to plants under drought stress [45]. The consequences of soil moisture variability during plant establishment might be reduced by increasing the soil water-holding capacity (Patra et al., 2019). The use of hydrogel "superabsorbent polymers" (SAPs) to increase topsoil water-holding capacity (WHC) is a suitable management tool that absorbs a significant amount of water and improves the ability of the topsoil to store water. Therefore, plants exhibit a better growth performance under drought stress conditions. Patra et al. (2022) stated that superabsorbents reduced drought stress and enhanced dry matter production in bean [46].

Our findings showed that using a seed coat (peat moss, vermicompost, and canola residue) improved leaf area and shoot and root dry weights under drought stress, and the highest leaf area and shoot and root dry weights were achieved in the seeds coated with vermicompost, peat moss, and canola residue (Table 5). Compared to uncoated seeds, leaf area and shoot and root dry weights increased by 1.05-, 1.03-, and 1.04-fold in the vermicompost seed coating (Table 5).

Organic materials such as vermicompost have a strong water-holding capacity and ensure long-term plant water availability, in addition to increasing nutrient absorption by plants. Therefore, the use of organic matter as a seed coat helps plants to access water and nutrients under drought stress [47]. In agreement with our results, using seeds coated with organic matter enhanced the germination percentage and growth indices of wheat seedlings under drought stress [48].

3.2. Free Leaf Proline Content

One of the common responses of plants to mitigating drought stress is the production and accumulation of organic solutes, known as compatible solutes, such as proline [49]. Proline is believed to be one of the most important compatible solutes that plays a vital role in osmotic adjustment in plants subjected to drought stress [50]. Proline protects plants from drought by contributing to cellular osmotic adjustment, protection of membrane integrity, enzyme or protein stabilization, and reactive oxygen species [51].

Our results showed that reduced field capacity led to the accumulation of proline content in plants, with the highest proline content observed in 25% FC, i.e., 4.36-fold higher than that in the control plants (Table 6). Proline accumulation under drought stress has been reported in rose and coffee plants, consistent with our results. Previous studies have shown that proline accumulation is necessary to reduce the destructive effects of drought stress [50,51].

Table 6. Effect of different seed coating materials and growth media and their interaction on chlorophyll and proline contents of milkweed under drought stress.

	100% FC	**50% FC**	**25% FC**	
Leaf Chlorophyll Content (mg Chl g^{-1} FW)				
Control	1.62 ± 0.002^{a}	0.93 ± 0.039^{bc}	0.607 ± 0.055^{cd}	$\mathbf{1.071 \pm 0.44^{AB}}$
Canola residue	1.67 ± 0.002^{a}	0.94 ± 0.037^{b}	0.6205 ± 0.05^{c}	$\mathbf{1.078 \pm 0.43^{AB}}$
Peat moss	1.672 ± 0.002^{a}	0.95 ± 0.037^{b}	0.64075 ± 0.049^{c}	$\mathbf{1.088 \pm 0.43^{A}}$
Vermicompost	1.673 ± 0.003^{a}	0.96 ± 0.037^{b}	0.65425 ± 0.048^{c}	$\mathbf{1.098 \pm 0.42^{A}}$
Mean	$\mathbf{1.67 \pm 0.00^{A}}$	$\mathbf{0.95 \pm 0.012\ B}$	$\mathbf{0.63 \pm 0.018^{C}}$	
S1	1.66 ± 0.0005^{a}	0.95 ± 0.01^{b}	0.63 ± 0.012^{c}	$\mathbf{1.086 \pm 0.43^{AB}}$
S2	1.67 ± 0.0004^{a}	0.87 ± 0.05^{bc}	0.56 ± 0.025^{cd}	$\mathbf{1.024 \pm 0.47^{AB}}$
S3	1.67 ± 0.0004^{a}	0.96 ± 0.01^{b}	0.68 ± 0.021^{c}	$\mathbf{1.104 \pm 0.42^{A}}$
S4	1.67 ± 0.0012^{a}	0.97 ± 0.01^{b}	0.68 ± 0.013^{c}	$\mathbf{1.112 \pm 0.41^{A}}$
Mean	$\mathbf{1.67 \pm 0.00^{A}}$	$\mathbf{0.97 \pm 0.04^{B}}$	$\mathbf{0.63 \pm 0.05^{C}}$	
Free leaf proline content (μmol g^{-1} FW)				
Control	4.04 ± 0.012^{c}	9.54 ± 1.34^{b}	18.04 ± 1.81^{a}	$\mathbf{10.55 \pm 5.7^{A}}$
Canola residue	4.03 ± 0.007^{c}	9.4175 ± 1.34^{b}	17.7825 ± 1.89^{a}	$\mathbf{10.44 \pm 5.6^{A}}$
Peat moss	4.02 ± 0.011^{c}	9.2625 ± 1.32^{b}	17.4925 ± 1.94^{a}	$\mathbf{10.22 \pm 5.5^{A}}$
Vermicompost	3.92 ± 0.15^{c}	9.145 ± 1.31^{b}	17.0875 ± 1.97^{a}	$\mathbf{10.01 \pm 5.4^{A}}$
Mean	$\mathbf{4 \pm 0.04^{C}}$	$\mathbf{9.34 \pm 0.15^{B}}$	$\mathbf{17.08 \pm 0.35^{A}}$	
S1	4.03 ± 0.007^{d}	8.91 ± 0.19^{d}	17.95 ± 0.16^{b}	$\mathbf{10.29 \pm 5.76^{B}}$
S2	4.04 ± 0.010^{d}	11.6 ± 0.16^{b}	20.45 ± 0.34^{a}	$\mathbf{12.04 \pm 6.72^{A}}$
S3	4.02 ± 0.007^{d}	8.62 ± 0.12^{b}	16.65 ± 0.42^{b}	$\mathbf{9.755 \pm 5.20^{C}}$
S4	4.01 ± 0.021^{d}	8.22 ± 0.12^{b}	15.35 ± 0.47^{bc}	$\mathbf{9.186 \pm 4.66^{C}}$
Mean	$\mathbf{4.02 \pm 0.011^{C}}$	$\mathbf{9.34 \pm 1.33^{B}}$	$\mathbf{17.59 \pm 1.91^{A}}$	

S1 = soil, S2 = sand + soil 1:1 ratio, S3 = 2 g superabsorbent per kg soil, S4 = 4 g superabsorbent per kg soil (S3), chl = chlorophyll, FC = field capacity. For each variable, the mean (± standard deviation) values followed by the same letters (small letters for interactions and capital letters for means) are not significantly different using LSD at the 5% level.

The results showed that the highest proline accumulation was observed in the S2, S1, S3, and S4 growth media (Table 6). Proline accumulation in S2 media increased by 1.16-fold compared to that in the control (Table 6). Compared to other media, the S2 growth medium had a lighter texture owing to its sand. Thus, this condition had a lower ability to maintain water, and its plants experienced drought stress earlier than those in other growth media [52].

Accordingly, in this media (S2), proline accumulation in plants was higher. Proline accumulation in *Hammada salicornica* in sandy soils has been reported [53,54]. Our results showed the highest proline content in the control and in seeds coated with canola residue, peat moss, and vermicompost (Table 6). Hence, in the present study, seeds coated with three types of organic matter showed the lowest proline accumulation. It seems that, because of their ability to maintain water, coating seeds with organic matter causes them to experience less drought stress; hence, they have less proline accumulation [55].

3.3. Leaf Chlorophyll Content

Chlorophyll is one of the major chloroplast components for photosynthesis, and the relative chlorophyll content has a positive relationship with the photosynthetic rate. The decline in chlorophyll content under drought stress is considered a typical symptom of oxidative stress and may result from pigment photooxidation and chlorophyll degradation [39].

Our results showed that the reduction in field capacity from 100% to 25% resulted in decreased chlorophyll content in the milkweed, such that the lowest chlorophyll content was observed at 25% FC, i.e., a 2.65-fold decrease compared to the control (Table 6). In this regard, the chlorophyll content decreased significantly at higher water deficits in *Catharanthusroseus* L. [56], *Helianthusannuus* L. [57], and *Vacciniummyrtillus* L. [58].

According to the results, the highest chlorophyll content was obtained in S4, S3, S1, and S2 growth media, indicating an increase of 1.02-fold in chlorophyll content in S4 growth media compared to the control (Table 6). Furthermore, the S2 growth medium showed the lowest chlorophyll content, a 1.07-fold decline compared to the control (Table 6).

Our results showed that the application of a superabsorbent could help preserve chlorophyll under drought stress. It has been reported that because of its water retention, adding a superabsorbent to soil may prevent chlorophyll degradation under drought stress [23]. In media containing superabsorbents, increased chlorophyll accumulation has been reported in *Acaciavictoriae* L. and *Cynodondactylon* L. plants under drought stress [23,59,60]. In addition, the lowest chlorophyll content was observed in S2 growth media (Table 6). The amount of sand in this medium is the reason for this phenomenon, as the plant is exposed to less stress than in other growth media [52].

Our results showed the highest chlorophyll content in seeds coated with vermicompost, peat moss, and canola residue and the control (Table 6). The chlorophyll content in seeds coated with vermicompost, peat moss, and canola residue increased by 1.02-, 1.01-, and 1.006-fold, respectively, compared with the control plants (Table 6). In the present study, coating seeds with the three types of organic matter appeared to prevent chlorophyll degradation under drought stress. Enhanced chlorophyll content under drought stress has been reported in indica rice and bread wheat plants [21,61].

3.4. Activity of Enzymatic Antioxidant

Exposure of plants to water deficit conditions increases the production of reactive oxygen species (ROS), such as hydroxyl radicals and superoxide [62]. These ROS may initiate destructive oxidative processes such as lipid peroxidation, chlorophyll bleaching, protein oxidation, and damage to nucleic acids. Therefore, according to [61], ROS scavenging is the most important defense mechanism for plants to cope with stressful conditions. However, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) play key roles in scavenging activated species [63]. Modulation of the activity of these enzymes may be an important factor in the tolerance of various plants to drought stress [64].

The results indicated that reducing the field capacity from 100% to 25% enhanced the activity of SOD, CAT, and APX enzymes in milkweed; in 25% FC, the activity of SOD, CAT, and APX enzymes increased 4.55-, 2.99-, and 1.57-fold compared to the control (Table 7). Increased activity of antioxidant enzymes in different plants under drought stress has been reported in many studies. Sales et al. (2013) reported increased activity of APX and SOD enzymes in sugarcane under water-shortage conditions [65]. Increased activities of SOD, CAT, and APX enzymes under drought stress have been shown in *Amaranthustricolor* L. and *Pyruscommunis* L. plants [66,67].

Table 7. Effect of different seed coating materials and growth media and their interaction on activity of superoxide dismutase, catalase, and ascorbate peroxidase enzymes of milkweed under drought stress.

	Superoxide Dismutase Activity (Ug^{-1} FW)			
	100% FC	**50% FC**	**25% FC**	**Mean**
Control	80.44 ± 0.040 ^{c}	156.32 ± 25.36 ^{bc}	369.125 ± 42.45 ^{a}	**201.98 ± 122.19 ^{B}**
Canola residue	80.45 ± 0.041 ^{c}	160.42 ± 25.21 ^{b}	361.9375 ± 43.88 ^{a}	**200.98 ± 118.43 ^{B}**
Peat moss	80.45 ± 0.047 ^{c}	166.32 ± 26.04 ^{b}	367.125 ± 43.28 ^{a}	**204.68 ± 120.12 ^{A}**
Vermicompost	80.46 ± 0.053 ^{c}	171.82 ± 25.53 ^{b}	375.88 ± 41.31 ^{a}	**209.48 ± 123.48 ^{A}**
Mean	**80.45 ± 0.00 ^{C}**	**163.73 ± 5.89 ^{B}**	**368.51 ± 4.99 ^{A}**	
S1	80.46 ± 0.014 ^{e}	179.19 ± 5.29 ^{c}	377.44 ± 5.72 ^{ab}	**212.36 ± 123.48 ^{AB}**
S2	80.43 ± 0.11 ^{e}	199.03 ± 5.70 ^{bc}	429.14 ± 5.37 ^{a}	**236.23 ± 144.78 ^{A}**
S3	80.42 ± 0.008 ^{e}	149.125 ± 5.61 ^{d}	341.64 ± 9.75 ^{b}	**190.39 ± 110.55 ^{B}**
S4	80.41 ± 0.007 ^{e}	130.63 ± 6.01 ^{d}	313.34 ± 7.63 ^{b}	**174.78 ± 100.07 ^{C}**
Mean	**80.43 ± 0.021 ^{C}**	**164.50 ± 26.43 ^{B}**	**365.39 ± 43.27 ^{A}**	

Table 7. *Cont.*

Catalase activity (U g^{-1} FW)				
Control	24.20 ± 0.014 ^{d}	48.44 ± 12.59 ^{c}	65.12 ± 12.26 ^{ab}	**45.92 ± 16.79 ^{B}**
Canola residue	24.20 ± 0.014 ^{d}	52.25 ± 12.06 ^{bc}	69.75 ± 12.05 ^{ab}	**49.11 ± 18.83 ^{AB}**
Peat moss	24.21 ± 0.015 ^{d}	58.44 ± 12.62 ^{bc}	74.68 ± 12.41 ^{a}	**52.50 ± 21.05 ^{A}**
Vermicompost	24.22 ± 0.012 ^{d}	65.25 ± 12.10 ^{bc}	80.31 ± 12.04 ^{a}	**56.40 ± 23.63 ^{A}**
Mean	**24.21 ± 0.00 ^{C}**	**56.28 ± 60.04 ^{B}**	**72.49 ± 5.65 ^{A}**	
S1	23.45 ± 1.293 ^{d}	54.95 ± 5.86 ^{bc}	71.93 ± 5.37 ^{ab}	**50.11 ± 20.08 ^{AB}**
S2	24.235 ± 0.005 ^{d}	76.31 ± 5.63 ^{ab}	92.31 ± 5.57 ^{a}	**64.28 ± 29.06 ^{A}**
S3	24.205 ± 0.005 ^{d}	49.12 ± 6.67 ^{c}	65.37 ± 6.57 ^{b}	**46.23 ± 16.93 ^{B}**
S4	24.21 ± 0.007 ^{d}	43.81 ± 5.45 ^{c}	60.25 ± 5.17 ^{b}	**42.75 ± 14.73 ^{BC}**
Mean	**24.025 ± 0.33 ^{C}**	**56.048 ± 12.34 ^{B}**	**72.46 ± 72.49 ^{A}**	
Ascorbate peroxidase activity (U g^{-1} FW)				
Control	897.37 ± 0.96 ^{d}	1184.93 ± 15.29 ^{c}	1403.44 ± 27.34 ^{ab}	**1161.917 ± 207.23 ^{A}**
Canola residue	897 ± 0.82 ^{d}	1190.43 ± 16.09 ^{c}	1411.44 ± 26.34 ^{a}	**1166.207 ± 210.61 ^{A}**
Peat moss	898.18 ± 0.73 ^{d}	1198.5 ± 17.1 ^{c}	1420.44 ± 24.34 ^{a}	**1172.227 ± 213.83 ^{A}**
Vermicompost	898 ± 0.83 ^{d}	1209.5 ± 17.93 ^{c}	1435.44 ± 18.34 ^{a}	**1181 ± 220.35 ^{A}**
Mean	**897.64 ± 0.47 ^{C}**	**1195.84 ± 9.24 ^{B}**	**1417.53 ± 11.91 ^{A}**	
S1	898.93 ± 0.48 ^{c}	1198.12 ± 8.7 ^{b}	1413.06 ± 9.8 ^{a}	**1170.04 ± 210.82 ^{A}**
S2	872.93 ± 43.4 ^{c}	1221.56 ± 11.13 ^{b}	1456.62 ± 6.8 ^{a}	**1183.70 ± 239.78 ^{A}**
S3	896.93 ± 0.48 ^{c}	1188.75 ± 6.7 ^{b}	1405.93 ± 10 ^{a}	**1163.87 ± 208.54 ^{A}**
S4	896.81 ± 0.56 ^{c}	1177.43 ± 6.2 ^{b}	1387.31 ± 11 ^{a}	**1153.85 ± 200.93 ^{A}**
Mean	**891.41 ± 10.69 ^{B}**	**1196.49 ± 16.23 ^{AB}**	**1415.73 ± 25.41 ^{A}**	

S1 = soil, S2 = sand + soil 1:1 ratio, S3 = 2 g superabsorbent per kg soil, S4 = 4 g superabsorbent per kg soil (S3), FC = field capacity. For each variable, the mean (± standard deviation) values followed by the same letters (small letters for interactions and capital letters for means) are not significantly different using LSD at the 5% level.

In our study, the accumulation of antioxidant enzymes appeared to reduce damage caused by ROS production under drought stress [65]. The highest activities of SOD, CAT, and APX enzymes were observed in S2, S1, S3, and S4 growth media (Table 7). Compared with the control, the highest activities of SOD, CAT, and APX enzymes in the S2 growth media were 1.11-, 1.27-, and 1.01-fold greater, respectively (Table 7). Based on these results, the higher ratio of sand in S2 growth media was the reason for the increased activity of enzymes in this media; thus, in this media, the plants experienced drought stress earlier than in other media employed in the experiment [68].

The results showed that, compared to the control, the application of superabsorbent in soil reduced the activity of SOD, CAT, and APX enzymes (Table 7). This phenomenon is due to the ability of a superabsorbent to maintain water in the environment, providing more water to plants and reducing the severity of drought stress [69]. In line with our results, reduced activity of POD and CAT enzymes in *Acaciavictoriae* L. and SOD, CAT, POD, and APX enzymes in *Avenasativa* L. under the application of superabsorbents in soil has been reported under drought stress [28,59,60].

Our findings indicate that, compared to the control, the highest activities of SOD, CAT, and APX enzymes were observed in seeds coated with vermicompost, peat moss, and canola residue, respectively (Table 7). In addition, compared with the control, the activities of SOD, CAT, and APX enzymes in seeds coated with vermicompost increased 1.05-, 1.23-, and 1.01-fold, respectively (Table 7).

In this study, seed coating enhanced enzyme activity. Seed coating increases plant access to nutrients and water, contributing to increased enzyme activity to eliminate free radicals under drought stress. Enhanced antioxidant enzyme activity in rice and wheat plants has been reported under stress conditions [61,70].

4. Conclusions

Our findings showed that drought led to reduced growth indices in milkweed. During the emergence stage, the plant faces a lack of moisture because the soil in which it grows

cannot absorb a significant amount of spring rain and store it for a long time. The use of superabsorbents increases soil moisture and improves seedling growth in the emergence stage. In contrast, proline and antioxidant enzyme activities increase as defence mechanisms to reduce the destructive effects of drought stress. The application of organic seed carriers improved the growth indices under drought stress, especially leaf surface, leaf specific weight, and root-to-shoot ratio. The application of superabsorbents with coatings made with organic seed carriers decreased proline accumulation and enzyme activity under drought stress owing to improved plant access to water. Among the growth media treatments, the application of 4 g of superabsorbent to coatings made with vermicompost exhibited the best physiological and morphological performance under drought conditions. Seed coating with organic carriers improved growth indices and enhanced enzyme activity and chlorophyll content under drought stress. Among the seed coatings employed in the experiment, the vermicompost coating was superior to other coatings under drought conditions. Generally, seed coating with vermicompost, together with the use of a superabsorbent polymer (4 g per kg soil), was the best to establish milkweed under mild (50% FC) and severe water deficits (25% FC). Therefore, to increase the success rate in the establishment of this plant, instead of directly planting the seeds, the farmer can put the seeds of this plant inside seed balls made of vermicompost and superabsorbent and then plant them in the soil.

Author Contributions: Conceptualization, M.T. and M.D.; methodology, M.T. and M.D.; formal analysis, M.T. and M.D.; data curation, M.T., M.D., and A.M.; writing—original draft preparation, M.T.; writing—review and editing, M.T. and A.M.; supervision, M.T.; project administration, M.T.; funding acquisition, A.M. All authors read and agreed to the published version of the manuscript.

Funding: This study did not receive any external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available from the corresponding author upon request.

Acknowledgments: We would like to express our special thanks to the Department of Plant Production and Genetics, School of Agriculture, Shiraz University, and the University of Brescia for the initial financial support provided to conduct this study.

Conflicts of Interest: The authors declare no conflict of interest.

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A Proposed Typology of Farming Systems for Assessing Sustainable Livelihood Development Pathways in the Tien Shan Mountains of Kyrgyzstan

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Abstract: In Kyrgyzstan, most farming systems focus on animal husbandry, which depends on mixtures of crops and pastures around settlements and higher-elevation summer pastures. These farms face the problems of insufficient fodder production and pasture degradation due to overgrazing, resulting in low productivity of livestock and reduced household incomes. The spatial diversity of farms often hampers the development of interventions aimed at improving crop and animal productivity, as well as sustainable grassland management, while the absence of a comprehensive and systematic classification system that effectively encompasses the diverse range of livelihood strategies within farming systems presents a significant obstacle to the advancement of initiatives promoting sustainable livelihoods. This study aimed to develop a consistent typology of smallholder farms in the Tien Shan using multivariate analysis. By analyzing data from 235 farm-households and evaluating key classification variables, we identified two distinct farming systems, upper mountain farms and lower mountain farms, based on socioeconomic and agro-ecological characteristics. Our typology considers elevation, grazing period, cultivated area, and off-farm income and better captures the diversity of farming activities and household income compared to current classification models. These findings will inform and tailor policies and interventions suitable for enhancing sustainable livelihoods in Kyrgyzstan's mountain farming systems.

Keywords: pasture degradation; grasslands; agropastoralism; transhumance; subsistence; cluster analysis; principal component analysis; Central Asia

Citation: Azarov, A.; Sidle, R.C.; Darr, D.; Verner, V.; Polesny, Z. A Proposed Typology of Farming Systems for Assessing Sustainable Livelihood Development Pathways in the Tien Shan Mountains of Kyrgyzstan. *Land* **2024**, *13*, 126. https://doi.org/10.3390/land13020126

Academic Editors: Michael Vrahnakis and Yannis (Ioannis) Kazoglou

Received: 19 December 2023
Revised: 10 January 2024
Accepted: 12 January 2024
Published: 23 January 2024

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1. Introduction

In mountainous Kyrgyzstan, agricultural production takes place primarily on small family farms, which have been shaped by the political, social, and economic reforms that followed the collapse of the Soviet Union [1,2]. The breakup fragmented large state-owned agricultural enterprises that produced meat, wool products, and large-scale crops into more than 460,000 smallholder farms with an average size of two hectares [3–5]. While mountain agriculture in Kyrgyzstan has diverse characteristics due to different elevations, climatic regimes, and landscapes, extensive pastoral systems dominate the majority of highland areas [6,7]. These mountain pastoralists face various challenges to environmental and socioeconomic sustainability, putting both agricultural production and livelihoods at risk [8,9]. The rugged terrain increases vulnerability to climatic hazards, mudslides, landslides, and marginalization due to limited access to infrastructure, markets, and technology [10,11].

As more than 80% of the country's agricultural land consists of mountain pastures, smallholder farmers face additional sustainability challenges such as rangeland degradation and biodiversity loss, mainly due to overgrazing [12–15]. Various sources indicate that degraded pastures cover at least 30% of the country's mountain lands [16–21]. Satellite imagery further attributes this land degradation primarily to an increase in livestock numbers and unsustainable pasture use [22–25]. Current crop production in the midst of the challenging mountain climate results in insufficient quantities and diversity of crop species, which are mainly intended for animal fodder [6,15]. Insufficient fodder production due to increasing livestock numbers on smallholder farms, combined with poor pasture management, leads to further pressure on grazing lands. Inadequate nutrition further contributes to declining animal health, which affects the profitability of livestock production [26]. Limited household resources and management capacity have prevented farmers from expanding or diversifying both their farming practices and livelihoods throughout the three decades of the agricultural transition process [27].

Although the main contours of agricultural transformation have been captured in the literature, in-depth knowledge of the current functioning of mountainous farming systems and the socioeconomic context of smallholder livelihoods remains limited. Previous research has extensively documented the transformation process, focusing on the impact of land reform and resource management on the performance of emerging small farms [28–31]. Such studies relied on available statistical data from official sources to characterize farm types and production systems. As a result, the farm typologies were primarily based on farm size and land ownership and distinguished three main categories of farms: (a) household plots, (b) smallholder ('peasants') farms, and (c) large agricultural enterprises, cooperatives, agricultural stock companies, and state farms [32]. Household plots have no extensive arable land, with an average-size home garden being 0.12 ha and a herd of one livestock unit (livestock unit corresponds to one cattle, 0.80 horses or five sheep/goats), and are solely for subsistence. Despite their limited resources, they make up 34% of national agricultural production, being the most common type (≈800,000). In contrast, there are only a few large market-oriented agricultural enterprises with over 1000 ha; these are highly productive but contribute less than 1% of agricultural production. As a result, the majority of agricultural production (65%) comes from smallholder farms, with the farmers using most of the available arable land and pastures to support their livelihoods. While household plots and market-oriented enterprises are mostly homogeneous, grouping smallholder farms in one category is questionable. These smallholder farms focus primarily on livestock, with a particular emphasis on household subsistence, guided by a family-centered rationale. The strategy includes marketing surpluses to generate income to purchase goods not produced on the farm. Following the collapse of the Soviet regime, only a handful of farmers have expanded their operations, while the majority continue to operate with limited resources. Despite these challenges, smallholder farmers contribute significantly to the country's agricultural production and serve as critical links connecting rural areas to markets, fostering economic exchange, trade, and integration into broader economic systems [3].

A major shortcoming of the current system of farm typology in Kyrgyzstan is that it lacks socioeconomic and agro-ecological variables, which have been demonstrated to be essential for farm classification and exhibit specific farm characteristics in other countries [33,34]. Differences in the wide range of physical environments and agro-ecological zones are crucial because they directly influence the farming practices, resource availability, and ecological conditions unique to each region [35,36]. For instance, gently sloping lowlands and valleys with fertile soils benefit from well-developed irrigation systems. On the other hand, rainfed agriculture predominates in smaller areas of mountainous regions, with pastures located at higher elevations [37,38].

Other case studies on Kyrgyzstan's agricultural transition processes have established farm typologies based on resource capacities, including livestock numbers, non-farm activities, and farmers' livelihood strategies [27,39–41]. The latter include diverse activities

such as agricultural practices, off-farm employment, and off-farm diversification aimed at generating income. While these studies emphasize the importance of including additional factors in farm typology, they tend to simplify classifications using discriminant analysis and focus on distinguishing between resource-rich and resource-poor farming systems.

Given the existing limitations of farm typologies in the country, our study addresses a critical gap in existing farm typologies by introducing robust numerical clustering for smallholder farm classification that includes a broader range of socioeconomic and agro-ecological variables. The inclusion of diverse variables improves the classification methodology and provides a more nuanced understanding of farm dynamics [42–46]. The study conducts an in-depth analysis of production systems, socioeconomic performance, and specific constraints and opportunities for each identified farm type [47,48]. This approach not only contributes to the literature, but also enhances the effectiveness of agricultural interventions and policies for sustainable pasture management and livelihood development among smallholder farmers in mountainous regions of Kyrgyzstan.

2. Methodology

2.1. Study Site Characteristics

Data were collected from four rural districts in the central Kyrgyz provinces of Chuy and Naryn. The altitude in these areas ranges from 500 to 6000 m above sea level. In order to specifically study the typology of mountain agropastoral systems in Chuy province, villages below 1500 m above sea level in the plains were excluded. Attention was focused on the mountainous areas of Kemin and Jaiyl districts, located between 1500 and 2400 m, which are characterized by predominant agropastoral systems. In Naryn province, all villages fall within the elevation range of 1500–2400 m, which led to the selection of Kochkor in the north and At-Bashy in the south. The study included fifty villages of varied sizes located in high mountain valleys between 1500 and 2400 m above sea level. In Kochkor and At-Bashy districts, 36 out of 39 villages were selected, and, in Kemin and Jaiyl districts, nine out of eleven villages were included. Highland valleys in the overall study areas are characterized by a semiarid steppe climate with warm summers (from 10 to 12 °C) and long, cold winters (from −22 to −8 °C) with a lower average annual precipitation of 200 to 300 mm [35]. In total, 24,000 families live in the study area (Figure 1), reflecting the most densely populated and cultivated areas in the mountainous regions of Kyrgyzstan.

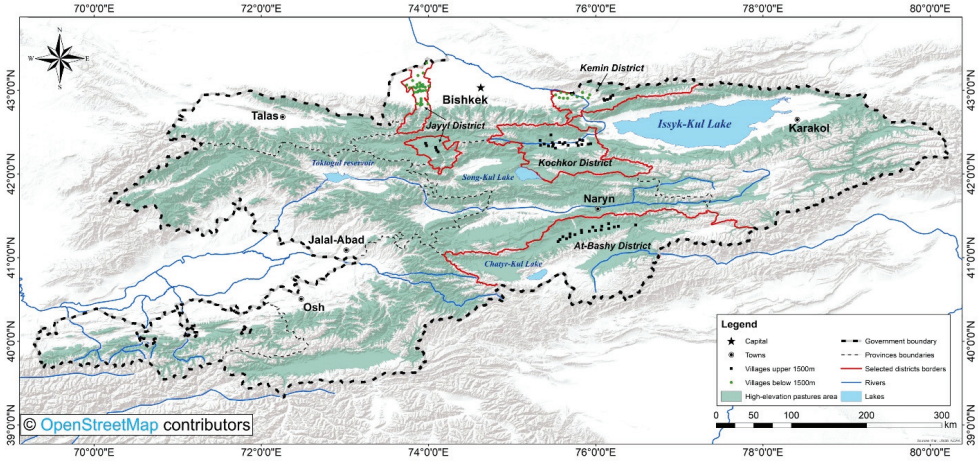


Figure 1. Study sites and selected villages located in four districts of Chuy and Naryn provinces of Kyrgyzstan.

2.2. Sampling and Data Collection

The sampling process excluded administrative centers and towns with populations of 8000–10,000 residents [49]. We used data collected in 2016, which represents a very appropriate sample to propose a new typology of smallholder mountain farming systems. Little has changed in recent years, and no new typology has been developed either. The early years of Kyrgyzstan's independence witnessed a significant transformation in the agricultural sector, with significant changes occurring in the first decade and a half. According to FAO and NSC reports [4,5], there has been a steady increase in the number of small farms. However, this numerical growth contrasts with the stagnation of the production systems within these farms, indicating a remarkable trend. The agricultural sector is moving towards greater fragmentation, mainly concentrated in rural households with limited production capacity. This pattern is particularly influenced by the absence of substantial government support, and a lack of investment in technology. This limited government involvement reflects Kyrgyzstan's classification as a low-income developing country [4]. As a result, the agricultural landscape reveals a complex interplay of economic factors and political choices, highlighting the need for a nuanced examination within the broader socioeconomic context.

In collaboration with local administrations, 235 randomly selected smallholder farmers were interviewed by the authors using a structured questionnaire on production systems, resource capacity, household income, off-farm activities, and remittances. To comply with statistical parameters, a narrower confidence interval of 90%, coupled with a margin of error of 6%, was established to ensure complete coverage of the population. Thus, farms in each district were selected based on an approximate proportion (2.0–2.2%) of the total population in each village. The decision to use a narrower confidence level was primarily motivated by the limited and resource-intensive data collection, especially considering the large size of the study area.

2.3. Data Analysis and Construction of a Farm Typology

Quantitative data collected from household surveys were processed and analyzed using the Statistical Package for Social Sciences (SPSS) version 21 (Armonk, NY, USA). Variables related to farm typology were classified to explore the diversity of smallholder farms in the study sites through multivariate analysis: (1) agro-ecological and socioeconomic, (2) land use, (3) labor, (4) livestock capacity, (5) production inputs, and (6) production methods. Data examination using box plots indicated positive skewness due to outliers at the 90th percentile of land holdings greater than 30 ha and livestock holdings greater than 40 livestock units. These outliers were discarded to improve the multivariate analysis and its generalization to the entire population. Out of the 235 households surveyed, two households had extremely large herds and cultivated areas and were therefore excluded from further data analysis.

Two multivariate techniques (principal component analysis (PCA) and cluster analysis) were used sequentially to generate a typology of the farms [50–54]. PCA was performed on standardized variables to condense all the information from the original interrelated variables into a smaller set of factors called principal components (PCs). The Kaiser–Meyer–Olkin (KMO) measure of sampling adequacy and the Bartlett test of sphericity were performed out to check the suitability of the data for PCA evaluation (KMO value 0.6 absolute minimum). Factors were rotated using the orthogonal Varimax method to subsume the correlated variables into a respective PC, making the loadings more pronounced and revealing a simple structuring of the variables into theoretically meaningful subdimensions. PCs were selected based on the eigenvalue ($\geq$1) criterion and explained by the collective variables [55]. Furthermore, correlated variables within a PC were represented by the variable with the highest loading coefficient [45].

Finally, correlation analysis was performed using Pearson's correlation coefficient to evaluate the relationship between the selected variables and to eliminate one of the two strongly related variables to avoid double weighting of factors [56]. The variable with the

largest standard deviation was selected for further analysis [42]. In the next step, the farms were grouped using agglomerative hierarchical cluster analysis based on four variables identified by PCA and Pearson's correlation matrix (see Supplementary Materials). Ward's method and Euclidean squared distance were used as metrics to establish clusters [57]. An independent *t*-test was used to compare the means of two independent groups to determine whether there were statistical differences. We then conducted a comparative analysis of household income, which consists of farm income and off-farm activities, to evaluate the economic performance of identified farm types. Farm income includes a variety of sources, including crop and livestock production systems, beekeeping, processing and sale of dairy products, and surplus produce from home gardens; in addition, some families earn income through service provision, using farm machinery (e.g., tractors) and providing herding services.

3. Results

3.1. Farming Systems Overview

Discussions with local government officials and farmers revealed that approximately 90% of the agricultural land in the study area relies on highland and nearby settlements for fodder. These sources are intensively used in the spring and fall, resulting in significant degradation from trampling and soil compaction due to insufficient winter forage. As livestock numbers increase, pressure on pastures escalates.

Livestock is the main economic activity and the main source of income for smallholder farmers. The average herd size is 16.3 livestock units and consists of almost equal numbers of fat-tailed sheep, local cattle, and horse breeds suitable for meat production. Most farmers use the services of professional herders whose grazing practices are based on the transhumance system. Each village has several dozen family herders who, after collecting all the livestock from the villagers, move to remote highland summer pastures ("jailoo" in the Kyrgyz language) and remain there throughout the summer. During the rest of the year, livestock graze on pastures, meadows, and arable land near settlements and are additionally fed with produced or purchased winter fodder. Animals often lose considerable weight during the winter due to lack of feed.

On average, farmers own 5.3 ha, half of which is non-irrigated (meadows). Most of the cultivated land is used to grow grass, fodder legumes for hay, and fodder cereals, mostly barley and, very rarely, oats. Potatoes, wheat, and vegetables are grown on smaller plots, mostly in kitchen gardens for home consumption. Farmers often use contractors for plowing and harvesting as few farmers own adequate machinery. Little fertilizer is used.

Income from off-farm activities contributes significantly to the total family income. Opportunities for off-farm employment and business are generally low; some farmers derive a large part of their income from off-farm sources. Pensions and salaries from public institutions account, on average, for more than half of total off-farm income. There is also seasonal internal migration (mostly in summer) to nearby larger towns. It is noteworthy that remittances from Russia, Kazakhstan, and other countries contribute a relatively small amount (12%) to total off-farm income, but their importance has increased in recent years. Markets are available in the district capitals, including the two largest livestock markets in Central Asia, where farmers sell mainly livestock and crop products. The average distance to markets ranges from 40 to 115 km, which creates some difficulties for more remote villages and increases the cost of transporting agricultural produce to market.

Table 1 presents eighteen classification variables selected to represent agro-ecological conditions, the resource base and use of farm households, agricultural production systems, and off-farm activities. These variables show significant variation, which is essential for a satisfactory classification of farm populations.

The PCA analysis (KMO = 0.668 and BTS $\leq$ 0.01) generated 18 PCs, out of which five PCs with eigenvalues >1, accounting for 64.4% of the variability, were selected. The first PC explains 20.4% of the data variability, while the second and third PCs explain 14.9% and 10.2%, respectively. The fourth and fifth components explain 7.6% and 6.3% of

the variance, respectively. The PCs were characterized according to the loading factors within each PC. PC 1 includes variables related to livestock production, i.e., herd size, number of horses, cattle, and sheep. The second PC includes variables related to crop production (cultivated area, fodder, and fallow land). The third PC includes a combination of variables such as elevation, which influences crop yield, the size of irrigated area, and the use of fertilizers. The fourth PC covers livestock production, but refers to local livestock management practices, including the grazing period. The fifth PC includes non-agricultural sources of income, such as total off-farm income and remittances (Table 2).

Table 1. Descriptive statistics for focused farms (n = 233).

Variable	Mean	SD	Min	Max	Median
Agro-ecology and socioeconomic					
Elevation of village (m a.s.l.)	1910	211	1600	2300.0	2000
Remittances (USD)	261	811	0.00	6276	250.0
Off-farm income (USD)	2249	2060	0.00	14,811	1882
Farm income (USD)	3797	4154	0.00	26,008	2510
Land use	4.90	2.93	0.00	16.60	4.50
Fallow (ha)	1.41	4.15	0.00	27.00	0.00
Cultivated area (ha)	5.32	5.08	0.00	30.00	4.08
Irrigated area (ha)	2.45	2.66	0.00	16.50	2.00
Labor					
Length of grazing (hired herder) (months)	5.87	0.88	3.50	7.70	5.60
Grazing period (months)	7.83	1.38	4.00	11.00	8.00
Livestock capacity and production methods					
Herd size (livestock units)	16.30	8.94	1.10	42.20	14.20
Horses (heads)	5.30	4.83	0.00	31.00	4.40
Sheep (heads)	3.80	4.04	0.00	36.00	4.80
Cattle (heads)	4.90	2.93	0.00	16.60	4.50
Fattened-up livestock (heads)	1.05	1.89	0.00	14.45	1.00
Production inputs and methods (crops)					
Fodder (grain) (t)	2.34	4.22	0.00	30.00	1.20
Fodder (hay) (kg)	690	751	0.00	8695	751
Usage of fertilizer (kg/ha)	20.40	73.25	0.00	500.0	0.00
Barley yield (t/ha)	1.26	1.35	0.00	6.00	1.05

Correlation was tested for five classification variables obtained from the PCA. For "cultivated area" and "herd size", a positive relationship (r = 0.70) was obtained. Therefore, the variable "cultivated area" was selected (see non-collinear variables in Supplementary Materials). Hierarchical clustering based on Ward's method was used to group similar farms. Figure 2 displays the dendrogram showing the range of cluster solutions resulting from Ward's method. The dotted line shows the cut-off point, which indicates a two-cluster solution. The 'Height' shows the agglomeration coefficient or distance between the clusters merged at each step.

Table 2. Rotated component matrix of classification variables with factor loadings grouped into five principal components (n = 233).

	Principal Component				
	1	2	3	4	5
Herd size	0.932				
Horses	0.795				
Farm income	0.699				
Sheep	0.672				
Cattle	0.659				
Fattened-up livestock	0.545				

Table 2. *Cont.*

	Principal Component				
	1	**2**	**3**	**4**	**5**
Cultivated area		0.883			
Fodder (grain)		0.824			
Fodder (hay)		0.623			
Fallow		0.548			
Usage of fertilizer			0.693		
Elevation of village			–0.647		
Irrigated area			0.624		
Barley yield		0.521	0.584		
Grazing period				0.904	
Length of grazing (hired herder)				0.901	
Remittances					0.843
Off-farm income					0.806

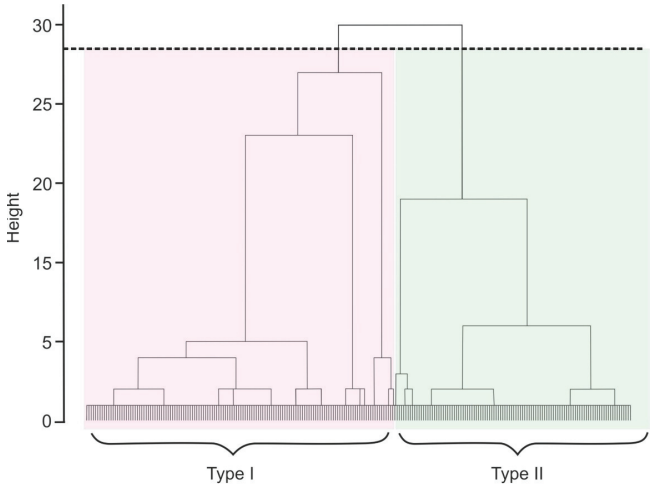


Figure 2. Dendrogram showing the range of cluster solutions resulting from Ward's method. The dashed line represents the cutoff point, which denotes a two-cluster solution: pink—Cluster I and green—Cluster II. The term 'Height' shows the distance between the merged clusters at each step.

Two main clusters of farming systems can be distinguished based on a distinct set of variables derived from PCA and correlation analysis:

- Cluster I: Upper mountain farms (UMF) located in high-elevation mountains between 2000 and 2400 m above sea level, mainly based on livestock and fodder production and characterized by a reduced grazing period and a low off-farm income;
- Cluster II: Lower mountain farms (LMF) located in medium-elevation mountain ranges between 1500 and 2000 m above sea level, based on livestock production fodder and other crops and characterized by a longer grazing period and a low off-farm income;
- Figure 3 shows the locations and distribution of the upper mountain farms located between 1500 and 2000 m a.s.l. in Kochkor and Kemin districts (red dots) and the lower mountain farms located above 2000 m a.s.l. in Suusmayr, At-Bashy, and Kochkor districts (green dots).

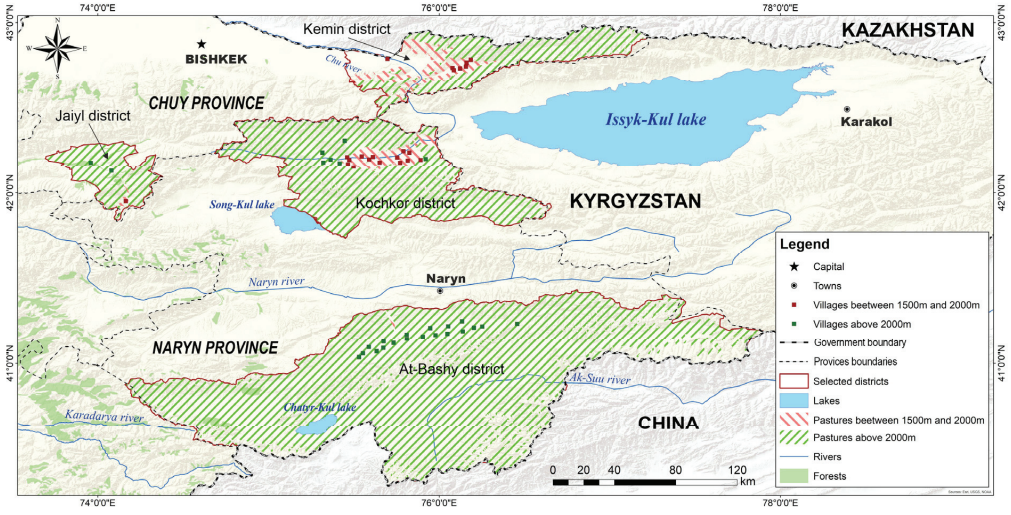


Figure 3. Distribution of the classified farming systems across central Tien Shan mountains.

3.2. Type I: Upper Mountain Farms (UMF)

UMF are the largest cluster, representing 54% of the farms surveyed. Livestock production is the most common economic activity and the main source of income (Table 3). The total herd size averages 16.4 livestock units, including roughly equal numbers of sheep/goats (35%), horses (30%), and cattle (31%). Farmers keep a small number of other animals such as yak (3%) and poultry (0.05%).

The average grazing period is 7.2 months due to environmental constraints during the cold season. Most farmers use the services of seasonal professional herders who stay on the high pastures one month less than the average total grazing period. Farmers generally try to keep their animals on pasture or cropland, when possible, to minimize the amount of feed needed for on-farm animals during the winter. Most farmers purchase supplemental feed during the winter or early spring due to a shortage of home-prepared winter feed and a lack of available pasture, highlighting the difficulties during this period.

On average, farmers have 8.4 ha of arable land. The proportion of non-irrigated land is 75%, contributing to the low productivity of agricultural land at higher elevations. About 2.5 hectares of fallow land was recorded per farm-household, mostly due to low fertility or remote locations and limited access to agricultural machinery. Farmers in some villages complained about the need to rehabilitate irrigation canals and the unreliability of water supplies from the mountains. Fodder crops account for the largest share of the cropland. Nearly 97% of the actual cultivated land (both irrigated and rainfed) is used to grow grass (47%), and sainfoin for hay making (27%), and fodder cereals, mostly represented by barley (22.5%). Potatoes, wheat, and vegetables are grown on the remaining land. Vegetables are mainly grown in home gardens for household consumption. Most of the non-irrigated land consists of rainfed lands or meadows with natural grasses used for hay production. On the irrigated land, legumes are typically grown, along with sainfoin and insignificant amounts of alfalfa. Sainfoin is a major crop on almost half of the farms, and its area has increased due to a proportional reduction of wheat, barley, and other crops in recent years. This increase has been driven by the high profitability of sainfoin cultivation due to low labor requirements (including minimal soil tillage) and the lack of harvesting equipment for cereals such as wheat or barley. Low yields and profitability of cereals continue to limit their cultivation. Wheat is mainly used for household consumption, while straw is fed to animals in winter. Barley is the most important crop, especially for concentrated animal feed and for its role in crop rotation.

Table 3. Farm and household characteristics of both farming systems (n = 233).

Variable	Unit of Measurement	Farming System Type	
		Upper Mountain (n = 125)	Lower Mountain (n = 108)
Elevation of village, location *	m a.s.l.	2200	1700
Grazing period *	month	7.2	8.5
Length of grazing (hired herder)	month	6.3	5.5
Herd size	livestock unit	16.4	16.2
Herd composition:			
Cattle	%	31	30
Sheep/Goat	%	35	34
Horses	%	30	35
Yak	%	3	<1
Poultry	%	1	<1
Farm size:	ha	8.4	4.9
Cultivated land *	ha	5.9	4.7
out of which irrigated land	ha	1.3	3.8
Uncultivated land	ha	2.5	0.2
Land use system:			
Meadows	%	47	17
Legumes for fodder	%	27	38
Barley	%	23	23
Wheat	%	<1	10
Potatoes	%	2	8
Other crops	%	<1	4
Farm income	USD	4430	6111
Farm income sources:			
Livestock	%	49	38
Crops	%	40	52
Other	%	11	10
Proportion of market sales in farm:			
Share of total farm product sales	%	26.5	35.2
Share of sales in livestock production	%	35.7	33.2
Share of market sales in crop production	%	17.3	37.2
Off-farm income *	USD	1933	2616
Off-farm income sources			
Public sector	%	18	25
Pensions	%	55	42
Business/employment	%	16	17
Remittances	%	11	16
Household income		6363	8727

* Variables of the final classification.

Livestock account for the largest portion of farm income, contributing about 49% compared to 40% from crops. Livestock sales are twice as large as crop sales, although the latter tends to fluctuate more. Notably, crops are grown for farmers' own animals, but farmers reported selling hay and other crops, including grain, to contractors immediately after harvesting due to cash shortages. This highlights the dependence of farmers on livestock sales, while the number of agricultural products sold to the market varies considerably among farm households. Additional farm income is generated from services provided by farm machinery (e.g., tractors) or herding services, beekeeping, processing, and sale of dairy products and crops, which account for 11% of farm income. Together, all sources of farm income account for 70% of household income.

A substantial share of the household income is derived from off-farm activities, averaging 30% of total household income. Pensions and salaries from public institutions account for more than 73% of the total off-farm income, followed by employment/private business (16%) and remittances (11%), reflecting poor access to off-farm employment and

business opportunities. It is noteworthy that remittances have not contributed significantly to off-farm income, but their role has been growing in recent years. Remittances are mainly from Russia and less frequently from other far-abroad countries.

3.3. Type II: Lower Mountain Farms

The average total herd size and structure in this cluster are similar to those of the UMF, except that LMF farmers keep more horses (35% of total herd size) because they require less labor and feed in winter. Horses graze for up to twelve months and do not require the presence of a herder. Farmers achieve slightly higher milk (mostly dairy cows) and meat yields because animals are in better condition at these lower elevations due to more abundant forage. LMF animals graze significantly longer, averaging 8.5 months, and animals suffer from feed shortages during the winter months, but to a lesser extent than UMF animals.

LMF farmers have an average of 4.9 hectares of arable land and 0.2 hectares of fallow land, significantly less than UMF farmers. On average, 80% of the cultivated land is irrigated, while the rainfed land is mostly meadow. Farmers produce a wider variety of crops, but most of the cropland is used for hay (50%) and feed grains, especially barley (23%). Wheat (10%), potatoes (8%), and cash crops, such as sugar beets and haricot beans, are also important. Crop yields are higher than in UMF due to favorable climatic conditions and better irrigation infrastructure (allowing two, very rarely three, harvests per year), accompanied by slightly increased use of chemical fertilizers. In contrast to UMF, vegetables and fruits grown in home gardens are mostly sold in local markets.

LMF farmers generate more income from livestock sales due to better market access and more intensive feeding. Despite a longer grazing period, average feed costs per livestock unit are higher because of the use of high-quality feeds (hay and concentrates). Similar to those on UMF, some farmers within the groups do not rely on additional sources of farm income (10%), while others are highly dependent and derive a significant portion of their income from beekeeping, herding and contracting services, and milk processing.

Pensions and salaries from public institutions are also an important source of household income for LMF farmers. Although LMF show a higher degree of market orientation (35.2%) compared to UMF, subsistence production continues to dominate. Crop production contributes significantly more to total farm income, despite the higher productivity of animal production.

Household income is one-third higher in LMF than in UMF (USD 8726 and USD 6363, respectively), reflecting better farming conditions, access to quality inputs, and off-farm opportunities.

4. Discussion

The official delineation of smallholder farms on the basis of size and legal status alone, without considering additional socioeconomic and agro-ecological parameters, neglects the diversity of smallholder farms [27,41]. While our theoretical classification approach using multivariate statistical methods to identify farm types is not new, it is a fundamental step in describing the socioeconomic conditions of farmers [48].

Our descriptive statistics reveal significant discrepancies with the official data on smallholder farms, especially in key variables such as the size of cultivated land. According to the NSC [5], the average size of smallholder farms is 2–3 ha, while our system shows averages of 5.3 ha in agropastoral areas and 0–0.12 ha in silvopastoral areas. Similarly, the official figures indicate an average of three livestock units per smallholder farm [5], while, in our system, the values range from 4.5 to 16.3 livestock units. Discrepancies are also observed in the distribution of cultivated crops; in agropastoral farms, fodder crops predominate, in contrast to the dominance of cereals (wheat) in the official statistics [4,5]. In addition, our analysis includes detailed insights into cropping and livestock systems, labor inputs, and economic performance, which are essential to classify farms with different means of production [58]. The inclusion of agro-ecological parameters influenced by

climate, topography, and land cover [59] further contributes to a nuanced division of farming systems, as evidenced by the "village elevation". Importantly, our results highlight the substantial contribution of off-farm activities to family income, an aspect overlooked in the official delineations of smallholder farms. By integrating off-farm and non-farm activities, we aim to comprehensively understand their roles and interactions, in line with similar farm typology studies [54,60,61].

For a better understanding of the diverse social, technological, and economic characteristics of mountain farms and their agro-ecological environment, we have classified farming systems using internal criteria such as the size of the farm and livestock systems and external factors such as elevation and off-farm income. Similar to other studies that have focused on developing typologies for livestock and mixed farming systems, our research considers the production and resource base of farms, including factors such as farm size, livestock numbers, grazing systems, and crop production. This consideration is crucial, as farm system management and production decisions play an important role in influencing both farm economics and environmental impacts [62–66]. Furthermore, the specific characteristics of livestock and mixed farming systems are determined by specific opportunities and constraints, which, in turn, are shaped by various factors beyond the farm-household scale, such as agro-ecological, elevation, climate, and soil characteristics [43,61,67].

While typologies are usually relevant within their development context, comparative categorization methods offer opportunities for their further development [48]. In mountainous regions of developing countries, smallholder farmers dominate livestock-based farming systems and have diverse livelihood strategies. This highlights the need to consider local context and diversity when developing interventions and policies to improve agricultural productivity and livelihoods in mountain areas [68]. Furthermore, there are similarities in the challenges faced by mountainous regions, particularly in Central Asia, including difficult terrain, long distances to markets, seasonal feed shortages, severe winters, and degradation of natural resources (pastures, forests) due to unsustainable management.

These challenges are also present in other mountainous regions of Central Asia [6,69–71], northwestern China [72], the northern Pakistani Hindu Kush Himalayas and Nepal [73–75], African highlands [76–78], and South American highlands [79,80]. In these studies, typical criteria for classifying small agropastoral systems in mountainous areas, in addition to farm size (including pasture and herd size), include elevation and agro-ecological conditions. For example, in arid and semiarid regions such as Pakistan, India, China, Nepal, and African countries, farms at higher elevations prioritize livestock production on extensive rangelands, emphasizing it as the primary livelihood strategy in highland conditions. In the mid-highlands, a mixed farming system prevails, with livestock and crop production as the main sources of income. At lower elevations, irrigated crop production becomes more important and animal husbandry becomes less important [81]. In the agropastoral systems we studied, this trend is clear to see; LMF operate more intensively and productively on arable land with improved irrigation practices, allowing for increased fodder production. They also grow a more diverse range of crops than UMF, highlighting the influence of agro-ecological conditions on farming practices.

Farm categorization in developed European mountain livestock systems often aims to refine existing typologies and provide a detailed description of farming activities and changes at farm level [82,83]. These studies include quantitative variables such as farm size, herd size, and animal specialization, including sheep, goats, and dairy cow specialization. Variables indicating the availability of different animal species are also commonly used [84–87]. In the proposed typology, a similar approach is possible, with clusters specializing in individual animals or intensive crop production. The typology of farming systems and subsequent analysis identify distinct categories of smallholder farms with different means of production and varying degrees of involvement in achieving livelihood goals. This insight into the social, technical, and economic context enables tailored recommendations to address specific issues and guide development trajectories [58].

Given the importance of livestock in UMF for both subsistence and financial security [88], ensuring an adequate supply of fodder is crucial. To increase fodder yields, support is needed to improve irrigation facilities, develop road infrastructure in remote areas (fallow), and engage agricultural contractors. At the production level, farmers should adapt farm management by increasing the use of productive inputs such as fertilizer, expanding the cultivation of legume fodder, using better quality seeds, adopting more efficient cultivation techniques, and improving irrigation systems. For instance, WOCAT (World Overview of Conservation Approaches and Technologies) has introduced and disseminated sainfoin cultivation practices in the highlands through demonstration studies. Improved and low-input cultivation practices have resulted in higher yields. Sustainable approaches are crucial as livestock numbers increase across the country [89], leading to pressure on pastures through issues such as overgrazing [90]. Small investments are particularly important as they are manageable for smallholder farmers who can only afford such adaptations. In addition, increased fodder stock allows farmers to keep animals indoors longer and prevents pasture degradation, especially during the wet early spring [26,91].

LMF, which are smaller but more mechanized (i.e., have easy access to contractors' services) than UMF, integrate livestock and crop production, focusing on income-generating fodder and diversified crops [92,93]. LMF also achieve significantly higher land productivity. Leveraging advantages such as irrigation, a warmer climate, fertile soils, and improved infrastructure, farms can expand cash crops including horticulture areas to increase production and support food security. Analysis shows that crop production is more profitable than livestock, highlighting the need for extension services to help farmers balance the benefits and costs of both. Substantial livestock production among LMF, together with insufficient fodder supply, also forces farmers to use pastures intensively in spring, which highlights the need for sustainable fodder strategies. The expansion of legume crops such as lucerne alone is not sufficient to provide feed for LMF. Silage production is currently neglected, but silage crops could offer a solution, with community silage production as a potential option [94]. Meeting the demand for fodder and cash crops can provide incentives for diversification or specialization, reducing reliance on subsistence production.

Our classification approach is not without limitations. Although early attempts at cluster analysis suggested a three-category division using a slightly different set of classification variables, resulting in a division of farms into three categories of resource rich, medium resource, and poor, we considered such a categorization simplistic and independent of multivariate analysis. Ultimately, our study characterized two types, supported by significant differences revealed by *t*-tests (see Supplementary Materials). This nuanced classification, which emphasizes differences in resource endowments and farm production systems, provides tailored recommendations for agricultural improvement. We found that resource-rich, medium-resource, and poor farms differ significantly. In contrast to official categorizations, our approach provides a more detailed understanding of smallholder farms. While the survey data were collected in 2016, Kyrgyzstan's status as a developing country and the continued socio-environmental stability in rural mountain areas support the relevance of these data. Recent official statistics, including FAO reports [4], indicate minimal changes in production in recent years, confirming the relevance of the study. Despite such limitations, the uniqueness of our study lies in the unprecedented collection of data before and after 2016. It provides a comprehensive picture of production systems and household economic outcomes, includes interviews with livestock and crop farmers, and includes off-farm income and resource bases. Thus, we affirm that our study provides valuable insights into the evolution of smallholder agropastoral households following the Soviet collapse.

5. Conclusions

This study aimed to fill the current gap in the typological delineation of farming systems in Kyrgyzstan, recognizing that similar gaps also exist in many mountain farming systems worldwide. The multivariate classification methodology developed in this study

offers clear advantages over typologies based on farm size and legal status, which do not consider the diversity among size classes and ignore the agro-ecological conditions as well as the socioeconomic situation of the farms. The methodology we used can be modified for different farm typology conditions, for example, by using different variables depending on the classification objectives. Our results suggest that two primary farm types have emerged in Kyrgyzstan's mountain areas, providing a basis for differentiating planning and extension intervention strategies to sustainably improve farm livelihoods. These are upper mountain farms (UMF), located at altitudes between 2000 and 2400 m above sea level, which focus primarily on livestock and fodder production. UMF are characterized by shorter grazing seasons and lower off-farm incomes. Lower mountain farms (LMF), located at mid-elevations between 1500 and 2000 m above sea level, are based on livestock, forage, and other crops. LMF are characterized by a longer grazing season and lower off-farm income. Although the agricultural production system of both identified farming systems can be characterized as low input low output, there is a clear difference between them. The altitude of the villages has a significant impact on the farmers, creating a clear divide between those in lower and higher zones. In the lower zones, with better access to off-farm activities and longer growing seasons, farmers have higher productivity, increased crop income, and improved food security. Their easy access to markets and technology, supported by strong infrastructure, boosts their agricultural efforts. Livestock also benefit, with increased productivity and minimal winter problems.

Challenges arise in higher regions. The shorter growing season and harsh climate limit farmers to a few crops and a short grazing season. Livestock face problems due to insufficient winter fodder, resulting in health problems and low income from livestock production. Located in remote and inaccessible places, farmers in these areas face geographic isolation and inadequate infrastructure, which affects their access to knowledge and technology.

To improve livelihoods in each cluster, we recommend tailored interventions that address specific challenges and leverage opportunities in each altitude zone. This focused approach ensures that the solutions provided are tailored to the unique realities of farmers in different topographical areas.

Based on our results, policy recommendations for improving smallholder farming systems in mountain areas include:

- Implement policies to facilitate smallholder and family farmer access to quality seeds and fertilizers. In addition, introduce new crop varieties to improve agricultural productivity;
- Provide extension services to farmers to encourage the adoption of sustainable grazing practices. Include education on the benefits of adopting newly improved livestock breeds to increase productivity;
- Provide resources to improve rural infrastructure, including irrigation systems, roads, sanitation, and access to clean water. This initiative aims to address rural underdevelopment and promote overall agricultural growth;
- Introduce a new methodology and redefine the criteria for smallholders and family farms in public policy. Work with government statistical agencies to develop an updated approach that ensures accurate representation and targeted support for these farming entities.

In addition, the following recommendations for future research are derived from the findings of our study:

- Conduct research focusing on cost-effective methods suitable for use by smallholder farmers to improve their fodder base. This may include advances in agricultural production technology, storage, and harvesting, with an emphasis on approaches that provide environmental benefits without requiring substantial investment;
- Undertake studies to explore conditions conducive to the adoption of income diversification strategies in farming systems. In particular, focus on off-farm activities such

as sustainable tourism or other value-added enterprises to provide additional sources of income for farmers;
- Examine grazing alternatives that will reduce ongoing land degradation and yet provide sufficient pasture use for cattle production.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/land13020126/s1; Table S1: Non-collinear variables used in hierarchical agglomerative cluster analysis; Table S2: Independent samples test.

Author Contributions: Conceptualization, A.A., V.V., D.D. and R.C.S.; methodology, R.C.S. and D.D.; software, A.A.; validation, D.D., V.V. and Z.P.; formal analysis, A.A.; investigation, A.A.; resources, Z.P. and V.V.; data curation, A.A. and D.D.; writing—original draft preparation, A.A. and R.C.S.; writing—review and editing, R.C.S., D.D. and V.V.; visualization, A.A.; supervision, Z.P., R.C.S. and V.V.; project administration, R.C.S.; funding acquisition, R.C.S. All authors have read and agreed to the published version of the manuscript.

Funding: The study was financed by the Internal Grant Agency of the Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague (IGA FTZ, project nos. 20233102 and 20233114). The Article Processing Charges were funded by the University of Central Asia, Graduate School of Development, Mountain Societies Research Institute.

Institutional Review Board Statement: Ethical review and approval were waived for this research due to the fact that data were collected anonymously and no personal identifiable information was gathered.

Data Availability Statement: Data are available upon reasonable request from the corresponding author.

Acknowledgments: We thank Samat Kalmuratov (Research Assistant at the Mountain Societies Research Institute) for his great help and assistance in organizing and conducting the survey.

Conflicts of Interest: The authors declare no conflicts of interest.

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Understanding the Dynamics of Sex-Specific Responses Driven by Grassland Management: Using Syrphids as a Model Insect Group

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Abstract: Grassland ecosystems, managed by various grassland managements strategies, are the world's most important land use. However, insect's sex-specific responses within the context of grassland management have never been considered before. Therefore, our aim was to expand the understanding to the dynamics of grassland managements that drive sex-specific responses by using syrphids as a model insect group. We hypothesize that (1) male and female syrphids exhibit differential habitat preferences in grassland managements, (2) abundance and activity of male and female syrphid levels are influenced by vegetation structure in grassland habitats. Extensive and intensive grassland exhibited significantly different male and female syrphid abundance compared to abandoned grassland. Surprisingly, grassland management had a significant impact on male syrphids richness only, not on female. Flower cover significantly increased male and female syrphid abundance and richness. However, plant height significantly increased female syrphid abundance and richness only. Interestingly, abandoned grassland supports a higher amount of unique female syrphids than male syrphids. The dynamics of grassland management are not unidirectional, but they are multifaceted and multidirectional. Considering the importance of sex-specific responses by insects can provide a more comprehensive understanding of dynamics of grassland managements.

Keywords: grassland management; insect; pollinator; biodiversity; abandoned grassland

Citation: Hussain, R.I.; Ablinger, D.; Starz, W.; Friedel, J.K.; Frank, T. Understanding the Dynamics of Sex-Specific Responses Driven by Grassland Management: Using Syrphids as a Model Insect Group. *Land* **2024**, *13*, 201. https://doi.org/10.3390/land13020201

Academic Editors: Michael Vrahnakis and Yannis (Ioannis) Kazoglou

Received: 25 December 2023
Revised: 4 February 2024
Accepted: 5 February 2024
Published: 7 February 2024

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1. Introduction

Grassland ecosystems constitute the most significant land use in the world, accounting for one-third of total land area [1,2]. Permanent grasslands account for 34% of Europe's total agricultural land area [3], and are defined as land that has been used to cultivate grass or herbaceous fodder, forage, or energy crops for five years or more [4]. Anthropogenic change is reshaping Europe's diverse grasslands [5,6], including land abandonment [7]. As a result, practically all European countries report a decrease in grassland area and a related loss of biodiversity [8,9]. This has raised concerns that European grasslands may be unable to provide key ecological functions such as pollination [10,11].

The conservation and enhancement of European grassland's biodiversity and ecological services has been prioritized in national and international policy and research programs [12,13]. In most European countries, biodiversity in grasslands is threatened by two opposing trends: intensification and abandonment [14,15]. Both have led to a reduction in the number of plant species [16]. Alternatively, low-intensity mowing has been utilized to preserve biodiversity, mainly insects, in semi-natural grasslands [17].

Insects constitute a significant proportion of terrestrial biodiversity [18], and their presence or absence can provide insights into the overall health and diversity of grassland habitats [19]. Changes in insect populations can reflect alterations in habitat conditions, including changes in vegetation structure, availability of food resources, and the presence of other insects [20]. Monitoring insect populations can therefore help to assess the overall biodiversity and ecological integrity of grasslands [21].

There are many studies on the abundance, richness, and composition of insects in grassland [22–25]. By studying these metrics, researchers gained insights into the ecological dynamics [26], community interactions [27], and responses of insects to various factors such as land management practices [28], habitat quality [29], and environmental changes [30]. These studies contribute to our understanding of grassland ecology and help for developing effective conservation and management strategies for maintaining insect biodiversity and ecosystem health in grassland habitats. However, it is important to note that insect's sex-specific responses within the context of grassland management have never been considered before.

Sex-specific responses in insects refer to the different behaviors, physiological characteristics, and ecological roles exhibited by males and females of the same insect species [31]. Grassland management, which involves modifying and maintaining the environment to support specific species or communities, can have significant impacts on insect populations, including their sex-specific responses [32]. In many insect species, males and females display different behaviors related to mating, foraging, and territoriality [33]. For example, male insects may exhibit courtship behaviors to attract females, while females might have specific behaviors related to egg-laying and parental care. Grassland management practices can influence the availability of resources for mating and reproductive success, impacting the overall population dynamics of the species [34].

We used syrphids as a model insect group to study sex-specific responses to different grassland management. Due to adaptability, mimicry and varied feeding habits, syrphids are a diverse family of flies within the order Diptera, and they are often abundant and easily recognizable in grassland habitats due to sexual dimorphism [35]. Grassland management practices significantly influence the life cycle of syrphids in various ways. Mowing, grazing, or burning can impact the availability of suitable habitats for syrphids at different life stages, affecting overwintering sites for adults, larvae, and pupae [36]. Studying syrphid sex-specific responses to grassland management is crucial for understanding how different management practices impact the behavior, ecological roles and abundance of males and females within the syrphid population. Referring to the last aspect, our aim was to expand the understanding of the dynamics of grassland management that drives sex-specific responses using syrphids. We hypothesize that (1) male and female syrphids exhibit differential habitat preferences in grasslands, and that (2) abundance and richness of male and female syrphids are influenced by vegetation flower cover and plant height in grassland habitats.

2. Materials and Methods

2.1. Study Sites

The field sampling was carried out in the alpine mountains, in the region of Amstetten, Scheibbs and Melk in the province of Lower Austria, spanning a total area of 19,186 km^2. Approximately 60% of the land is dedicated to cattle farming, which dominates the agricultural sector. To ensure comprehensive data collection, we carefully considered several factors when choosing our study sites. Primarily, we focused on capturing a uniform range of elevations within the alpine grassland to capture the full spectrum of syrphid diversity patterns in study sites [37]. This approach allowed us to account for potential variations in syrphid communities driven by different grassland types at the same elevation (~989 m). We chose two types of certified organic grassland (intensive and extensive), and abandoned grasslands that were located near to certified organic grassland (Figure 1). On south-facing slopes, we surveyed 10 replicates of each abandoned, extensive and intensive grassland

(n = 30). The selected extensive grasslands were not fertilized and only mown once or twice a year. Intensive grasses were mown four times per year at most. Management of the abandoned grasslands has been ceased for at least 10–20 years. All study sites were imbedded in a comparable landscape matrix and located in similar mesic and altitudinal zones. Vegetation in intensive grasslands was dominated by legumes and grasses indicating nutrient-rich conditions (e.g., *Trifolium repens Lolium perenne*), whereas that of extensive grassland was dominated by grasses and legumes indicating nutrient-poorer conditions (e.g., *Bromus erectus*, *Lotus corniculatus*), and the herbs *Ajuga reptans* and *Cruciata laevipes* were dominant in abandoned grassland.

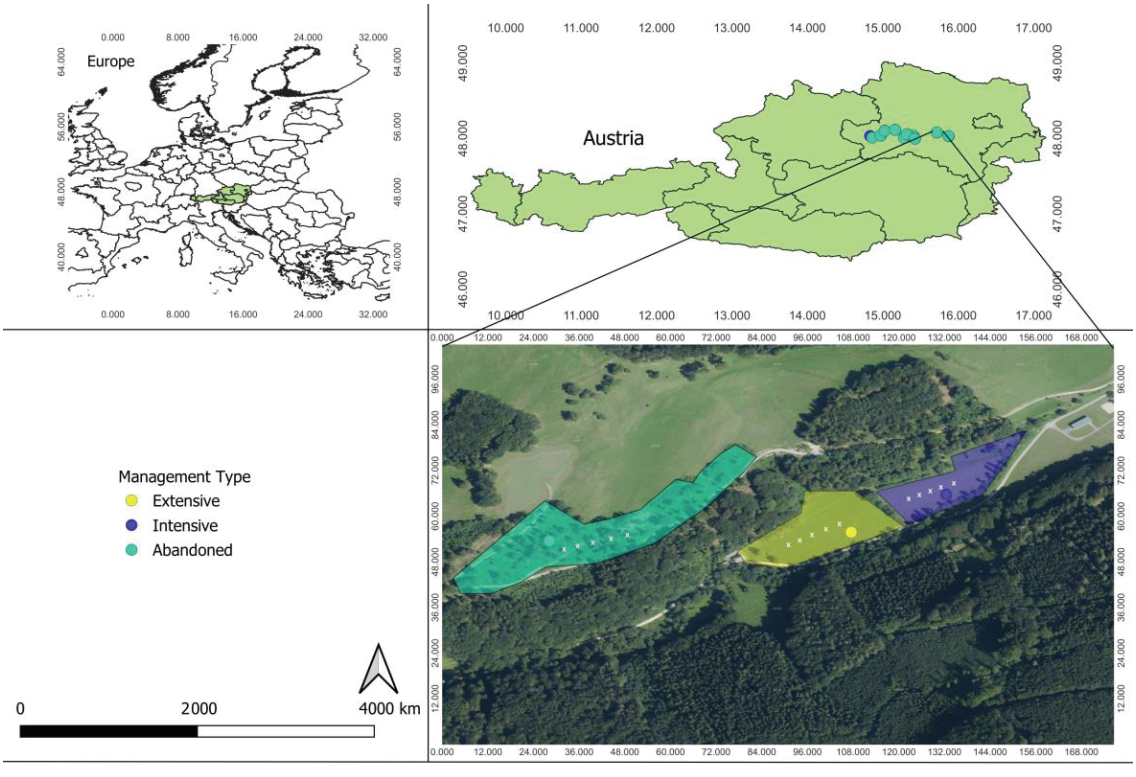


Figure 1. Map showing the locations of ten replicates of study sites, with an emphasis on the three types of grasslands that have been studied: extensive, intensive, and abandoned. The location of each sampling plot is marked with x. The three circles stand for each studied grassland type.

2.2. Syrphid Sampling

Syrphids were sampled eight times during the flowering season between May to August 2021 and 2022. We chose this time because it represents the period when most plants are in bloom, which indicates the maximum development of the vegetation [38]. Syrphids were sampled using two different methods: line transect and observation plot method [39]. For each study site (abandoned, extensive, intensive), we established three line transects (a total of 90 sweeps each), measuring 30 × 2 m each. Transects were placed with a minimum distance of 20 m between them, and towards the center of a study site. We used the observation plot method to create five 4 m^2 plots, each 10 m from a fixed starting point per study site. Each plot was examined for 10 min, and rapidly moving syrphids were captured using an entomological hand net. To prevent sampling bias, all study sites were randomly sampled between 9 a.m. and 6 p.m. Syrphid sampling was limited to sunny

days, as it is well known that the weather has a significant impact on syrphid activity [40]. Syrphids collected during observation plot and line transect method were sorted, based on sexes, and stored in 70% ethanol. Later, syrphid identification was carried out with the help of identification keys [41,42].

2.3. Vegetation Survey

Flower cover and plant height (from base until top) were measured as a predictor for syrphid abundance and richness between May and August 2021 and 2022. On each study site, we laid a wooden grid of 1 × 1 m size that was divided into 25 squares [43]. The proportion of total number of squares with at least one nectar-producing flower were counted. A measuring tape was inserted five times vertically up to the soil surface to measure plant height per study site.

2.4. Statistical Analysis

To test whether male and female syrphid abundance and species richness showed sex-specific responses to the grassland managements, the function glmer from package lme4 was used to create generalized linear mixed models (*GLMMs*) [44]. Poisson family *GLMMs* were applied because the response variables (abundance and species richness) were count data. We aggregated all plots (five observation plots) and transects (three line transects) for each study site, and replications were added as a group-level variable. The variable replication was used to fit the *GLMM* models with a varying-intercept group effect. The *dispersion_glmer* function of the *blmeco* package and the *dispersiontest* function from the *AER* package [45,46] were used to check for overdispersion (the residual deviation was more than the degree of freedom) showed by Poisson *GLMMs*. An additional observation-level random effect was added to a model in cases of overdispersion [47]. If there were significant differences across study sites, Tukey's post hoc pairwise comparisons were performed using *glht* function from package *multcomp*. Later, Nakagawa and Schielzeth's [48] method was used to determine marginal R^2 (R^2m) and conditional R^2 (R^2c) for mixed models using the *MuMIn* package.

To examine how syrphid species, based on sexes, overlapped among study grassland managements, we created Venn diagrams from *plotVenn* package that used *nVenn* algorithm. Furthermore, we used *GLMMs* to examine how studied predictor variables (flower cover and plant height) influenced sex-specific abundance and species richness of syrphids. The response variables were male and female syrphid abundance and species richness, and fixed factors were flower cover and plant height for each study site. Replications were added as a group-level variable. Additionally, using the R package *car*, we computed the variance inflation factors (VIFs) to check for multicollinearity between predictor variables [49]. In the models, any variables with a VIF over 5 were eliminated. We calculated our GLMs using these data as a basis.

Species assemblages in three grassland managements were calculated using Non-metric Multidimensional Scaling (NMDS) [50]. We pooled all sampling data (five observation plots and three line transects) for each study site. The species abundance was Hellinger-transformed to avoid the common zero problem. The function adonis in the R package vegan was used to generate a PERMANOVA (Bray–Curtis dissimilarities, 999 permutations) (as performed by Boetzl [51]). Using the function *betadisper*, data were examined for equal multivariate dispersion. Additionally, to check for variations in species assemblages amongst study sites (i.e., abandoned, extensive, intensive), multilevel pairwise comparison using *adonis* was performed (*pairwise.adonis*). The R program version 3.5.1 was used to carry out all statistical analyses [52], and model results were stated using the standards established by Zuur and Ieno [53].

3. Results

In the studied grasslands, we collected 2315 syrphid individuals from 93 species over the sample period (2021 and 2022). For the analyses, we only used males (1069 individuals

and 63 species) and females (1246 individuals and 70 species) that had clearly distinguished sex-specific characteristics (Appendix A Table A1). Out of the total male syrphids, 250 individuals and 39 species were in abandoned, 446 individuals and 38 species were in extensive, and 373 individuals and 37 species were in intensive grassland. Considering females, 309 individuals and 49 species were in abandoned, 466 individuals and 48 species were in extensive, and 471 individuals and 37 species were in intensive grassland. The most abundant male species were *Myathropa florea* in abandoned (28.40%), extensive (17.94%), and intensive grassland (12.87%). However, female *Melanostoma mellinum* was most abundant in abandoned (14.24%), extensive (22.96%), and in intensive grassland (32%).

Extensive and intensive grassland exhibited significantly different male and female syrphid abundance compared to abandoned grassland (Figure 2; Table 1). There was no significant difference between extensive and intensive grassland managements for male and female syrphid abundance and richness (Figure 2; Table 1). Flower cover within grassland management significantly increased male and female syrphid abundance and richness. Additionally, plant height significantly increased female syrphid abundance and richness (Table 2; Appendix A Figure A1).

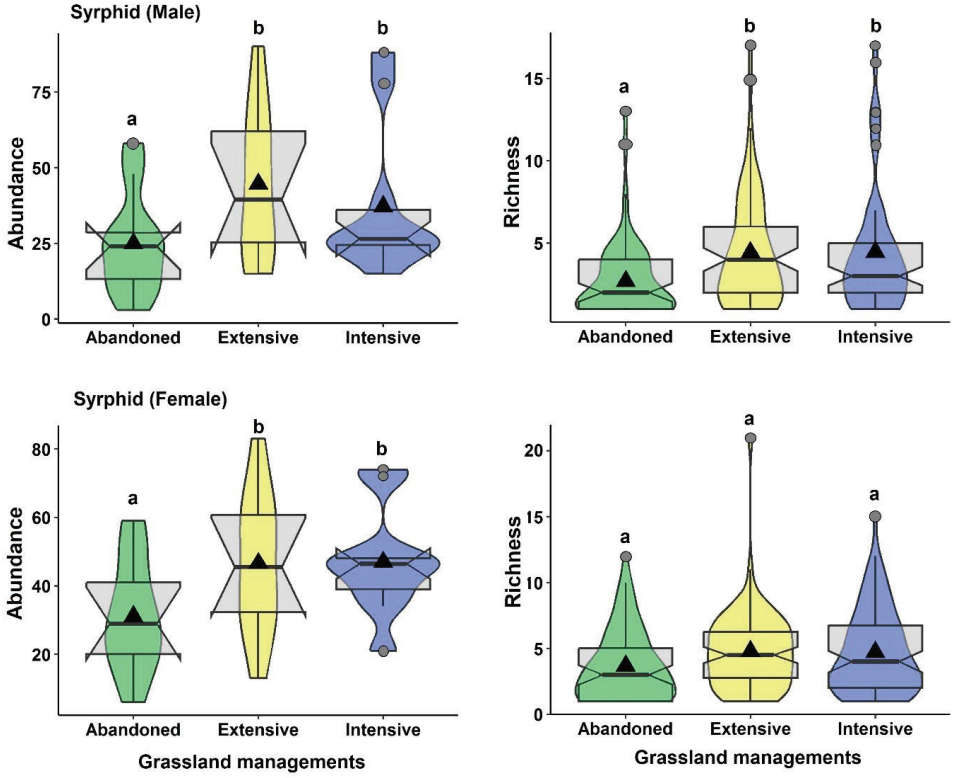


Figure 2. Effects of studied grassland managements on male and female syrphids abundance and species richness. Box-Whisker plots display the medians (—), mean (▲), notches, 25% and 75% percentiles, and outlier values (●). Box-Whisker plots with distinct letters differ significantly from one another ($p < 0.05$) using Tukey's post hoc pairwise tests.

Overall, about an equal amount of male and female syrphids were shared within grassland managements (32.3% syrphid male; 37.1% syrphid female; Figure 3). Interestingly, abandoned grassland supports a higher amount of female (22.9%) compared to male syrphids (14.5%). On the contrary, intensive grassland had a higher proportion of males

(14.5%) than females (5.7%) (Figure 3). Grassland management had similar male syrphid assemblages (Appendix A Figure A2 and Table A2). For female syrphid assemblages, abandoned grassland differed significantly from extensive grassland (Appendix A Figure A2 and Table A2).

Table 1. Generalized linear mixed effects models (GLMM) and Tukey's post hoc pair-wise testing for variations in male and female syrphids among studied grassland managements (abandoned—as intercept, extensive, and intensive). Significant *p* values are shown in bold.

Syrphid (Male)			**Estimate**	**Std. Error**	***z***	***p***	**95% CI**
abundance	GLMM	abandoned (Intercept)	1.009	0.114	8.796	<0.001	2.19–3.44
		extensive	0.462	0.148	3.109	0.002	1.19–2.13
		intensive	0.422	0.154	2.743	0.006	1.13–2.06
		Dispersion	0.96	R^2m	0.057	R^2c	0.726
	Tukey's post	extensive × abandoned	0.462	0.148	3.109	**0.005**	
	hoc pairwise	intensive × abandoned	0.422	0.154	2.743	**0.016**	
	comparisons	intensive × extensive	−0.039	0.144	−0.273	0.959	
richness							
	GLMM	abandoned (Intercept)	0.886	0.103	8.592	<0.001	1.98–2.97
		extensive	0.489	0.131	3.s718	<0.001	1.26–2.11
		intensive	0.493	0.135	3.652	<0.001	1.26–2.13
		Dispersion	1.277	R^2m	0.116	R^2c	0.406
	Tukey's post	extensive × abandoned	0.489	0.131	3.718	**<0.001**	
	hoc pairwise	intensive × abandoned	0.493	0.135	3.652	**<0.001**	
	comparisons	intensive × extensive	0.003	0.123	0.03	0.999	
Syrphid (Female)							
abundance	GLMM	abandoned (Intercept)	1.259	0.098	12.822	<0.001	2.91–4.27
		extensive	0.341	0.13	2.626	0.009	1.09–1.82
		intensive	0.305	0.129	2.357	0.018	1.05–1.75
		Dispersion	0.99	R^2m	0.044	R^2c	0.619
	Tukey's post	extensive × abandoned	0.341	0.13	2.626	**0.023**	
	hoc pairwise	intensive × abandoned	0.305	0.129	2.357	**0.048**	
	comparisons	intensive × extensive	−0.036	0.123	−0.296	0.953	
richness							
	GLMM	abandoned (Intercept)	1.212	0.085	14.239	<0.001	2.85–3.97
		extensive	0.255	0.112	2.266	0.023	1.04–1.61
		intensive	0.241	0.112	2.151	0.032	1.02–1.59
		Dispersion	1.18	R^2m	0.038	R^2c	0.311
	Tukey's post	extensive × abandoned	0.255	0.112	2.266	0.061	
	hoc pairwise	intensive × abandoned	0.241	0.112	2.151	0.079	
	comparisons	intensive × extensive	−0.014	0.107	−0.134	0.99	

Dispersion: 0.75 to 1.4; CI = 95% confidence intervals; R^2m = marginal R^2; R^2c = conditional R^2.

Table 2. Generalized linear mixed models (GLMMs) showing the effects of flower cover and plant height on male and female syrphids abundance and species richness. Significant *p* values are shown in bold.

Syrphid (Male)		**Estimate**	**Std. Error**	***z***	***p***	**95% CI**		
abundance	(Intercept)	0.977513	0.194999	5.013	<0.001	1.81–3.90		
	Flower cover	0.046236	0.009983	4.631	**<0.001**	1.03–1.07		
	Plant height	0.003303	0.002554	1.293	0.196	1.00–1.01		
	R^2m	0.121	R^2c	0.193	Dispersion	0.9784	VIF	1.003
richness	(Intercept)	0.776352	0.141685	5.479	<0.001	1.65–2.87		
	Flower cover	0.030096	0.007167	4.199	**<0.001**	1.02–1.05		
	Plant height	0.001823	0.001883	0.968	0.333	1.00–1.01		
	R^2m	0.099	R^2c	0.162	Dispersion	0.9572	VIF	1.005

Table 2. *Cont.*

Syrphid (Male)		Estimate	Std. Error	*z*	*p*	95% CI		
Syrphid (Female)								
abundance	(Intercept)	1.170764	0.139302	8.405	<0.001	2.45–4.24		
	Flower cover	0.039004	0.006846	5.697	**<0.001**	1.03–1.05		
	Plant height	0.005217	0.001911	2.73	**0.006**	1.00–1.01		
	R^2m	0.172	R^2c	0.232	Dispersion	1.006	VIF	1.001
richness	(Intercept)	0.834297	0.127584	6.539	<0.001	1.79–2.96		
	Flower cover	0.024706	0.005777	4.277	**<0.001**	1.01–1.04		
	Plant height	0.00496	0.00155	3.201	**0.001**	1.00–1.01		
	R^2m	0.139	R^2c	0.1975	Dispersion	0.906	VIF	1.003

Dispersion: 0.75 to 1.4; CI = 95% confidence intervals; R^2m = marginal R^2; R^2c = conditional R^2.; VIF= variance inflation factor.

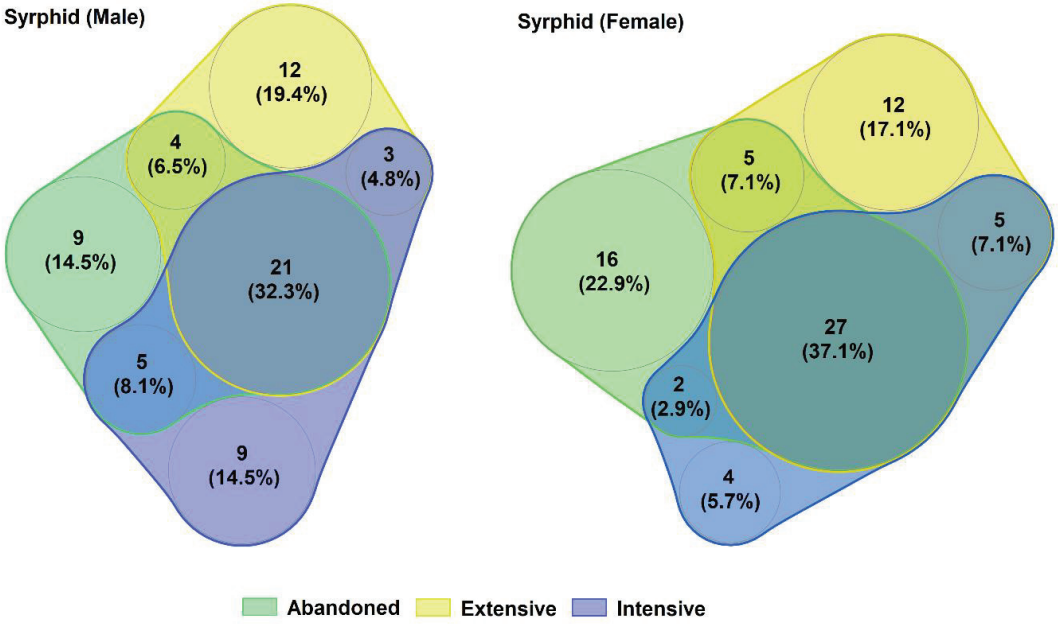


Figure 3. Venn diagrams showing the main overlaps and differences in male and female syrphids species among studied grassland managements. Numbers and percentages are calculated using the total dataset.

4. Discussion

It is known that extensive grasslands support high biodiversity, including an abundance of pollinators like syrphids [54]. This increase in biodiversity is more noticeable when pollinator diversity in extensive grassland was studied compared to abandoned grassland [55]. Furthermore, the high intensity of grassland management is expected to result in a decrease in pollinator diversity [56]. There could be several reasons why extensive and intensive grassland management had a similar effect on male and female syrphid abundance:

a. Scale and timing of management: The scale and timing of the grassland management practices may have aligned with the life cycle and movement patterns of syrphids. Syrphids have different activity periods [57], migration patterns [58], and breeding requirements [59]. If the grassland management practices consider these factors, it could have limited their extent in influencing syrphid male and female abundance.

b. Habitat connectivity: Syrphids often require suitable habitats in close proximity to each other to support their abundance [60,61]. If the grassland management practices create or maintain sufficient linear connectivity between different patches of suitable habitat, then it could have limited the movement and dispersal of syrphid males and females, ultimately equalizing their abundance.
c. Resource availability: Grassland management practices can directly influence the availability of resources that syrphids rely on, such as nectar, pollen, and suitable breeding sites [62]. For example, the maintenance of diverse plant communities in extensive grassland could enhance the availability of nectar and pollen resources, benefiting both male and female syrphids and potentially increasing their abundance in nearby intensive grassland.

Surprisingly, female syrphid richness did not differ among the grassland management practices. The richness of female syrphids could be influenced by a variety of factors, including flower cover, habitat structure, microclimate, and disturbance levels [60,63–65]. However, we do not know which factor led to a consistent pattern of female syrphid richness in the studied grassland management practices.

The increase in flower cover within grassland management practices can significantly enhance the abundance and richness of male and female syrphids. Increasing flower cover may lead to favorable foraging opportunities [66], and improves the availability of oviposition sites [67] for females. Females of some syrphid species exhibit preferences for specific plant heights based on their host plant choices. Certain syrphid species prefer taller plants as preferred oviposition sites [68]. Thus, grassland management practices, especially abandoned and extensive grassland, promoting taller plants may attract a greater number of species with specific host plant preferences, thereby potentially increasing the richness of female syrphids. Furthermore, a plant's height can support a greater number of prey insects, such as aphids (*Hemiptera*) and thrips (*Thysanoptera*) [69], which are an important food source for the larvae of female syrphids. More abundant prey availability can support larger populations of female syrphids as they actively search for suitable host plants to deposit their eggs [70]. Consequently, an increase in plant height can indirectly contribute to the abundance and richness of female syrphids through enhanced prey availability.

When grasslands are abandoned and left undisturbed, they can undergo changes in vegetation composition, structure, and ecological dynamics [71]. As abandoned grasslands undergo natural succession, with changes in plant communities over time, the syrphid assemblages can also change [72]. Early successional stages may attract certain syrphid species (i.e., *Baccha elongata*) that prefer open, grassy habitats, while later successional stages with increased shrub or tree cover may favor other syrphid species (i.e., *Chrysotoxum bicinctum*). Interestingly, all grassland managements exhibited similar amounts of unique male and female syrphid species. The presence and abundance of syrphid species in a particular area are influenced by the regional species pool as mentioned in [73,74]. It is possible that the grassland management practices were implemented within a region with a relatively consistent species pool, and the number of unique species may not vary significantly between the different management types. In such cases, the large number of species would remain similar regardless of the grassland management practices.

However, abandoned and extensive grasslands are often characterized by lower levels of human disturbance compared to intensive management practices [75]. Reduced disturbance can create more favorable conditions for certain female syrphids, as they may be more sensitive to disturbances during oviposition and larval development. The decreased disturbance levels in abandoned and extensive grasslands may attract a higher abundance or diversity of unique female syrphids, compared to intensive management types.

5. Conclusions

The dynamics of grassland managements are not unidirectional, but they are multifaceted and multidirectional. In many studies, insect species richness, abundance and assemblage patterns were taken into consideration. In our study, we found a sex-specific

response of syrphids to abandoned, extensive and intensive grasslands. Some limitations of understanding the dynamics of sex-specific responses lies in the potential lack of generalizability beyond the specific context of syrphid ecology and grassland ecosystems. While syrphids offer valuable insights into insect behavior and community dynamics, extrapolating findings to broader insect taxa or ecosystems may be challenging due to inherent differences in species composition, habitat characteristics, and management practices. Additionally, the complexity of grassland ecosystems, including spatial heterogeneity and temporal variability in environmental conditions, can introduce confounding factors that complicate the interpretation of sex-specific responses. Thus, considering and understanding these sex-specific responses is challenging for effective ecosystem management and conservation efforts. Studying the sex-specific responses can help in understanding the implications for population dynamics and colonization abilities of insects in fragmented landscapes. Considering the importance of sex-specific responses by insects can provide a more comprehensive understanding of dynamics of grassland managements. By doing so, we can gain deeper insights into population dynamics, species interactions, and conservation strategies for insect communities.

Author Contributions: Conceptualization, R.I.H. and T.F.; methodology, R.I.H.; study site selection, D.A. and W.S., formal analysis, R.I.H.; resources, J.K.F. and T.F.; writing—original draft preparation, R.I.H.; writing—review and editing, R.I.H., T.F., D.A., W.S. and J.K.F.; supervision, T.F.; project administration, T.F. and J.K.F.; funding acquisition, J.K.F. and T.F. All authors have read and agreed to the published version of the manuscript.

Funding: This study is part of the research project "Farmer Clusters for Realising Agrobiodiversity Man-agement across Europe (FRAMEwork)" which was funded by the European Union, grant number 862731.

Data Availability Statement: The original contributions presented in the study are included in the article/Appendix A, further inquiries can be directed to the corresponding author.

Acknowledgments: Special thanks to all the farmers and landowners for their permission to conduct investigations on their organic grassland farms. Thanks to Norbert Schuller for providing working materials and help in field sampling.

Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Table A1. List of studied male and female syrphid abundance and species richness in studied grassland managements. Abundance is given in numbers, while empty space indicates absence.

Syrphid (Male)	Species	Abandoned	Extensive	Intensive
1	*Baccha elongata*	1		
2	*Cheilosia bergenstammi*			1
3	*Cheilosia chrysocoma*	1		
4	*Cheilosia grossa*	1		
5	*Cheilosia impressa*	1	1	
6	*Cheilosia lasiopa*			1
7	*Cheilosia latifrons*		1	
8	*Cheilosia nasutula*			1
9	*Cheilosia soror*	1		
10	*Cheilosia sp.*	2	1	1
11	*Cheilosia variabilis*			1
12	*Cheilosia vernalis*	5		1
13	*Chrysogaster solstitialis*		1	
14	*Chrysotoxum bicinctum*	1		2

Table A1. *Cont.*

Syrphid (Male)	Species	Abandoned	Extensive	Intensive
15	*Chrysotoxum elegans*		1	
16	*Chrysotoxum festivum*		1	3
17	*Dasysyrphus albostriatus*		1	
18	*Episyrphus balteatus*	11	30	9
19	*Eristalinus aeneus*		2	
20	*Eristalis arbustorum*	1	8	
21	*Eristalis nemorum*		1	
22	*Eristalis pertinax*	1		
23	*Eristalis tenax*	19	28	31
24	*Eumerus ornatus*		1	
25	*Eumerus sp.*		1	
26	*Eumerus strigatus*			1
27	*Eupeodes bucculatus*			2
28	*Eupeodes corollae*	2	7	8
29	*Eupeodes lapponicus*	3	2	9
30	*Eupeodes latifasciatus*		1	1
31	*Eupeodes luniger*	1		3
32	*Eupeodes lapponicus*	2	3	2
33	*Leucozona laternaria*			1
34	*Melanostoma mellinum*	15	35	39
35	*Merodon aberrans*		1	
36	*Myathropa florea*	71	80	48
37	*Neoascia podagrica*	1		3
38	*Orthonevra nobilis*		1	
39	*Paragus haemorrhous*	1	1	
40	*Parasyrphus lineolus*		1	
41	*Pipizella sp.*	8		
42	*Pipizella viduata*	19	43	29
43	*Pipizella viduata*	1	2	3
44	*Pipizella virens*	15	42	23
45	*Platycheirus albimanus*	1	1	2
46	*Platycheirus podagratus*	1		
47	*Platycheirus sp.*			1
48	*Rhingia campestris*	3	1	
49	*Scaeva pyrastri*		2	2
50	*Scaeva selenitica*	5	1	6
51	*Sphaerophoria batava*	2		2
52	*Sphaerophoria interrupta*	6	6	16
53	*Sphaerophoria scripta*	13	60	34
54	*Sphaerophoria taeniata*	6	41	58
55	*Sphegina clunipes*	1		
56	*Syritta pipiens*	13	8	2
57	*Syritta pipiens*		1	
58	*Syrphus ribesii*	2	3	2
59	*Syrphus torvus*	7	21	17
60	*Syrphus vitripennis*	2	2	4
61	*Xanthandrus comtus*	1		
62	*Xanthogramma pedissequum*			1
63	*Xylota segnis*	3	2	3
Syrphid (Female)				
1	*Baccha elongata*	5		
2	*Brachyopa scutellaris*			1
3	*Cheilosia albitarsis*	3	4	1

Table A1. *Cont.*

Syrphid (Male)	Species	Abandoned	Extensive	Intensive
4	*Cheilosia antiqua*		1	1
5	*Cheilosia fraterna*	3		
6	*Cheilosia latifrons*		1	
7	*Cheilosia pagana*			2
8	*Cheilosia sp.*	1	2	2
9	*Chrysogaster solstitialis*		9	4
10	*Chrysogaster virescens*	1	2	
11	*Chrysotoxum bicinctum*	2	2	3
12	*Chrysotoxum cautum*	2		
13	*Chrysotoxum elegans*	1		
14	*Chrysotoxum festivum*	2	2	2
15	*Chrysotoxum octomaculatum*		1	
16	*Didea alneti*		1	
17	*Epistrophe eligans*	1		
18	*Episyrphus balteatus*	29	37	32
19	*Eristalinus aeneus*		3	
20	*Eristalinus sepulchralis*	1		
21	*Eristalis arbustorum*	5	6	1
22	*Eristalis nemorum*		1	
23	*Eristalis pertinax*	1		
24	*Eristalis tenax*	29	33	27
25	*Eumerus tuberculatus*		1	
26	*Eupeodes bucculatus*	1	1	1
27	*Eupeodes corollae*	2	2	3
28	*Eupeodes lapponicus*	4	3	4
29	*Eupeodes latifasciatus*		2	1
30	*Eupeodes luniger*	2	2	2
31	*Eupeodes nitens*			1
32	*Eupeodes lapponicus*	1		
33	*Helophilus hybridus*		1	
34	*Helophilus pendulus*		1	
35	*Helophilus trivittatus*		2	2
36	*Melanostoma mellinum*	44	107	151
37	*Melanostoma scalare*	4	4	8
38	*Meligramma cincta*		1	1
39	*Myathropa florea*	39	23	18
40	*Neoascia obliqua*	1		2
41	*Neoascia podagrica*	1	1	5
42	*Paragus sp.*	6	2	
43	*Parasyrphus annulatus*		1	
44	*Parasyrphus lineolus*	1		
45	*Philhelius pedissequus*	1		
46	*Pipiza noctiluca*	4	1	2
47	*Pipizella sp.*	1		
48	*Pipizella viduata*	16	34	22
49	*Pipizella viduata*	1	1	3
50	*Pipizella virens*	15	19	18
51	*Platycheirus albimanus*	9	9	16
52	*Platycheirus angustatus*		4	
53	*Platycheirus clypeatus*	1	1	
54	*Platycheirus scutatus*	1		
55	*Rhingia campestris*	9	1	2
56	*Scaeva pyrastri*	1	1	2

Table A1. *Cont.*

Syrphid (Male)				
	Species	**Abandoned**	**Extensive**	**Intensive**
57	*Scaeva selenitica*	3		1
58	*Sphaerophoria scripta*	29	99	94
59	*Sphaerophoria taeniata*		2	
60	*Syritta pipiens*	5	4	5
61	*Syrphus ribesii*	2	6	
62	*Syrphus torvus*	4	14	23
63	*Syrphus vitripennis*	4	6	6
64	*Volucella inanis*	1		
65	*Volucella pellucens*	2		
66	*Xanthogramma laetum*	1		
67	*Xanthogramma pedissequum*	2	1	
68	*Xylota segnis*	3	2	1
69	*Xylota tarda*	2		
70	*Xylota xanthocnema*		2	
71	*Xylota sylvarum*			1

Table A2. PERMANOVA (Pairwise-adonis) for male and female syrphids in studied grassland managements, i.e., abandoned, extensive and intensive grassland. Significant *p*-values (<0.05) are marked in bold.

Syrphid (Male)		**Df**	**Sum Sq**	**R^2**	**F**	***p***
Adonis	Management regimes	2	0.357	0.082	1.213	0.215
	Residual	27	3.976	0.917		
	Total	29	4.333	1		
Pairwise Adonis		F. Model	R^2	*p*	Adjusted *p*	
	abandoned × extensive	1.016	0.053	0.449	1	
	abandoned × intensive	1.431	0.073	0.127	0.381	
	extensive × intensive	1.162	0.06	0.318	0.954	
Syrphid (Female)						
Adonis	Management regimes	2	0.445	0.102	1.533	0.033
	Residual	27	3.919	0.897		
	Total	29	4.364	1		
Pairwise Adonis		F. Model	R^2	*p*	Adjusted *p*	
	abandoned × extensive	1.906	0.095	0.008	**0.024**	
	abandoned × intensive	1.681	0.085	0.048	0.144	
	extensive × intensive	0.841	0.044	0.621	1	

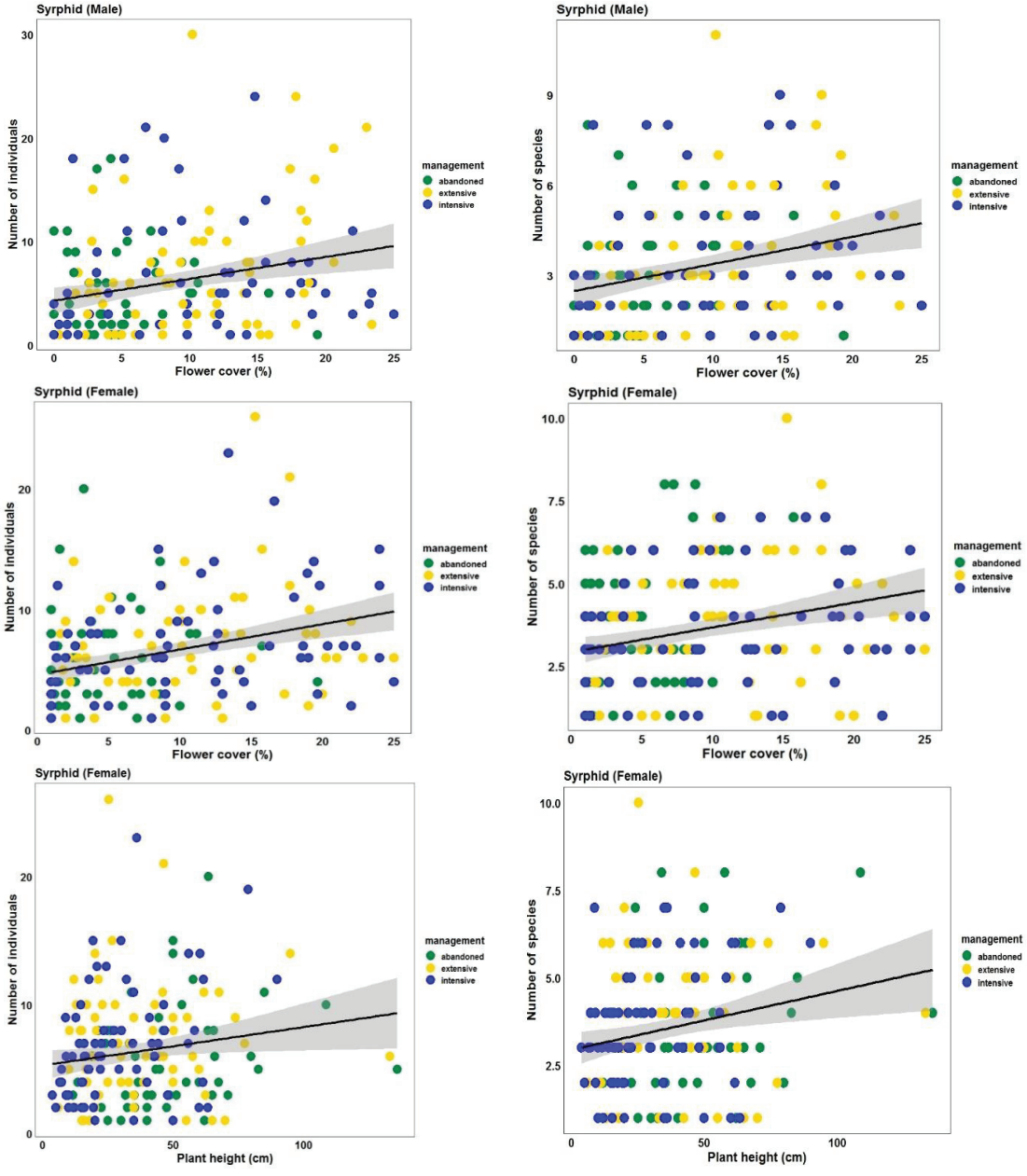


Figure A1. Linear regression showing positive significant relationships ($p < 0.05$) between number of male or female syrphid individuals and flower cover (%) or plant height (only female syrphids) for studied grassland managements.

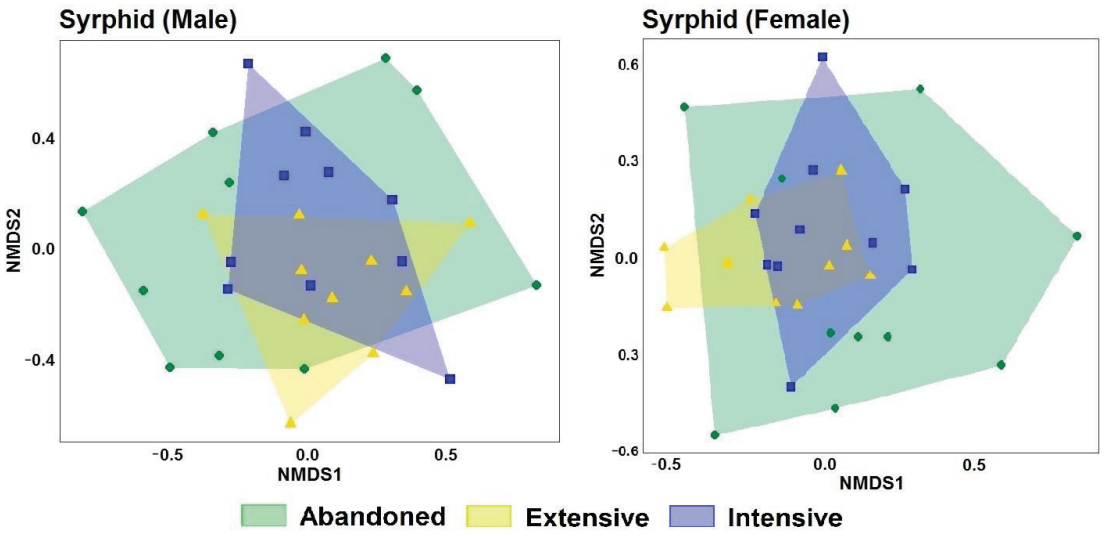


Figure A2. Non-metric multidimensional scaling (NMDS) for species assemblages of male and female syrphids in grassland managements.

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Integrated Land-Use Systems Contribute to Restoring Water Cycles in the Brazilian Cerrado Biome

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Abstract: Cerrado, constituting native Brazilian vegetation in the tropical and subtropical grasslands, savannas, and shrublands biome, has been extensively replaced by crop and pastureland, resulting in reduced water recycling to the atmosphere via evapotranspiration (ET). Re-introducing trees via integrated land-use systems potentially restores soil health and water-related processes; however, field data are scarce. During two years, we monitored soil moisture dynamics of natural Cerrado (CER), continuous pasture (COP), integrated crop-livestock (ICL), and integrated crop-livestock-forestry (ICLF) systems across 100 cm soil depth. Across years, mean soil moisture was highest for ICL, followed by COP and lowest in systems with trees (ICLF and CER). However, seasonal and spatial analyses revealed pronounced differences between soil layers and systems. COP and ICL mainly lost water from upper soil layers, whereas in ICLF, the strongest water depletion was observed at 40–100 cm depth, almost reaching a permanent wilting point during the dry season. CER was driest in the upper 40 cm, but water storage was highest below 60 cm depth. Our results suggest that compared to conventional land-use practices, integrated systems, including trees, increase water recycling to the atmosphere via ET and potentially compensate for the loss of key ecological functions of degraded or replaced Cerrado.

Keywords: soil moisture dynamics; soil profiles; seasonality; pasture; agroforestry

Citation: Glatzle, S.; de Almeida, R.G.; Pereira Barsotti, M.; Bungenstab, D.J.; Giese, M.; Macedo, M.C.M.; Stuerz, S.; Asch, F. Integrated Land-Use Systems Contribute to Restoring Water Cycles in the Brazilian Cerrado Biome. *Land* **2024**, *13*, 221. https://doi.org/10.3390/land13020221

Academic Editor: Michael Vrahnakis

Received: 10 January 2024
Revised: 7 February 2024
Accepted: 8 February 2024
Published: 10 February 2024

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1. Introduction

The Brazilian Cerrado biome, the second largest biome in South America, underwent and is still undergoing an expansion of agriculture for the last five decades [1,2]. About 50% of the natural Savannah vegetation has been replaced by monocultures of soybean, sugar cane, corn, coffee, or cotton, as well as by energy plantations and pastures [2–4]. Whereas rapid expansion of agricultural activities significantly increased crop yields and economic wealth in the region, it also contributed to soil degradation and altered water cycles [5].

Compared to undisturbed Cerrado vegetation, regions affected by deforestation and land use change were characterized by changed water pathways towards reduced evapotranspiration (ET) and relatively increased runoff and percolation [5,6]. Several studies reported potential consequences of land cover changes, including reduced annual precipitation and increased rainfall variability [6–8].

Strong seasonal variability of rain and, as a consequence, temperature greatly influence soil moisture dynamics in this ecosystem, resulting in reduced soil moisture availability during the dry season [9]. The natural savannah vegetation of the Cerrado is highly adapted to seasonal variability of soil moisture and has developed several coping strategies, such as

deep rooting systems, dormancy of the herbaceous layer, stomata control, leaf abscission, and the ability to extract water from soil at different depths [9]. Integrated crop-livestock systems (ICL) or integrated crop-livestock-forestry systems (ICLF) are management systems aiming at conserving soil resources while sustainably producing crops, livestock, and/or timber integrated into the same area employing intercropping or rotations [10]. Compared to conventional continuously cropped or grazed systems, integrated systems conserve soil quality by reducing erosion, maintaining or increasing soil organic matter, and improving soil structure and porosity, resulting in increased infiltration and water retention capacity [11,12]. Increased soil water infiltration and improved soil water storage of integrated systems potentially reduce drought impacts on agricultural production [13]. However, in comparison to deep-rooting natural Cerrado vegetation, pastures and integrated systems tend to extract water at shallower depths and can create a strong distortion in the vertical-spatial water distribution. On a transect within an ICLF system, large seasonal and vertical-spatial heterogeneity in soil moisture has been reported [14], and these effects of vegetation heterogeneity are expected to be more pronounced across and between systems. As the most important species of any pasture component, *Brachiaria brizantha*, mainly roots in the upper 30 cm of the soil [15]; the dynamic of moisture in the topsoil largely affects the productivity of the pasture and, thus, in addition to seasonal variability, dynamics of vertical-spatial variability of soil moisture within seasons needs to be taken into account when evaluating the effects of land-use change on water availability and -productivity. The soil moisture dynamics depend to a large extent on the concentration of soil organic matter in any soil layer, which, in turn, has been shown to be strongly affected by land-use change [16].

Nonetheless, information on seasonal and vertical-spatial variability of soil moisture as affected by land-use change from natural Cerrado to pasture or integrated systems remains scarce. Since land-use change also affects the quality and the physical characteristics of soils and, thus, soil moisture dynamics, it directly impacts long-term land productivity. On the other hand, actively growing vegetation also directly affects soil moisture via transpiration and evaporation, which renders comparing soil moisture dynamics across different land use types difficult as the transpiring biomass and the soil cover need to be factored in. Therefore, the objective of this study was to elucidate the seasonal and vertical-spatial variability of soil moisture of a Cerrado ecosystem as affected by different long-term land use management.

2. Materials and Methods

2.1. Experimental Area

The study was conducted on the long-term experimental sites of EMBRAPA Beef Cattle situated in Campo Grande in the state of Mato Grosso do Sul, Brazil (20°24′54.9″ S, 54°42′25.8″, altitude 530 m). The study area is characterized by a tropical savannah Aw climate (Köppen) with a mean annual temperature of 22.6 °C and mean annual rainfall of 1560 mm divided into a distinct rainy season and a distinct dry season (Figure 1). About 70% of the mean annual rainfall falls within the rainy season, and the remaining 30% within the dry season. Temperature, relative air humidity, solar radiation, wind speed, and precipitation were monitored with a nearby meteorological station operated by EMBRAPA Beef Cattle.

2.2. Treatments

All measurements were performed in four land use systems, defined here as treatments, namely: continuous pastures (COP), integrated crop-livestock system (ICL), integrated-crop-livestock-forestry system (ICLF), and natural Savannah vegetation, Cerrado (CER). Plots of the four treatments were arranged on three replications and located in close proximity (Figure 2). For the CER treatment, measurement plots were established in a large continuous Cerrado area.

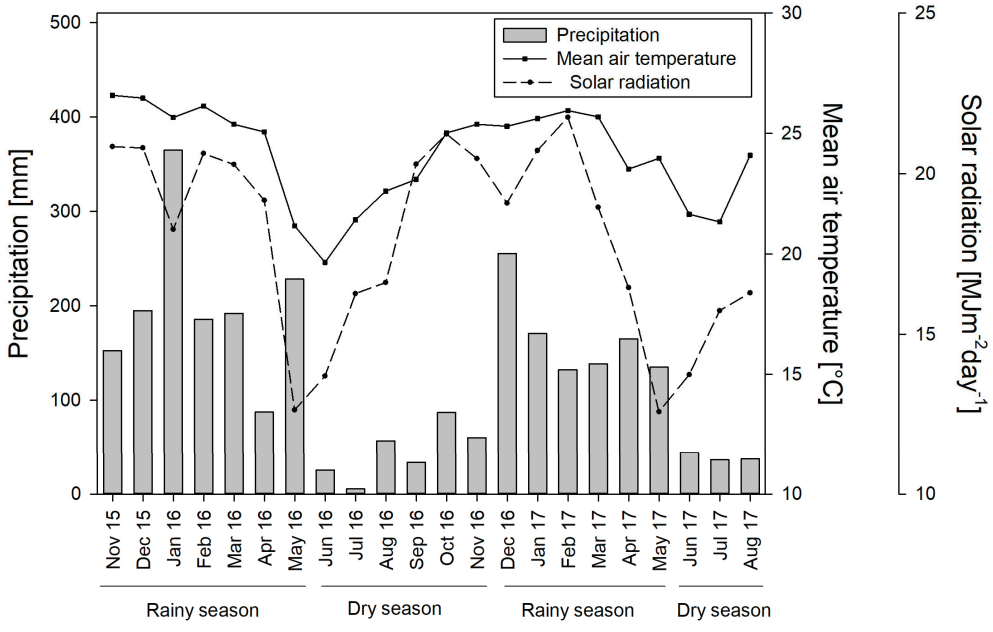


Figure 1. Monthly precipitation, mean air temperature, and mean daily solar radiation recorded over the experimental period at the EMBRAPA Beef Cattle station in Campo Grande, Mato Grosso do Sul, Brazil (20°24′54.9″ S, 54°42′25.8″, altitude 530 m) with a nearby meteorological station.

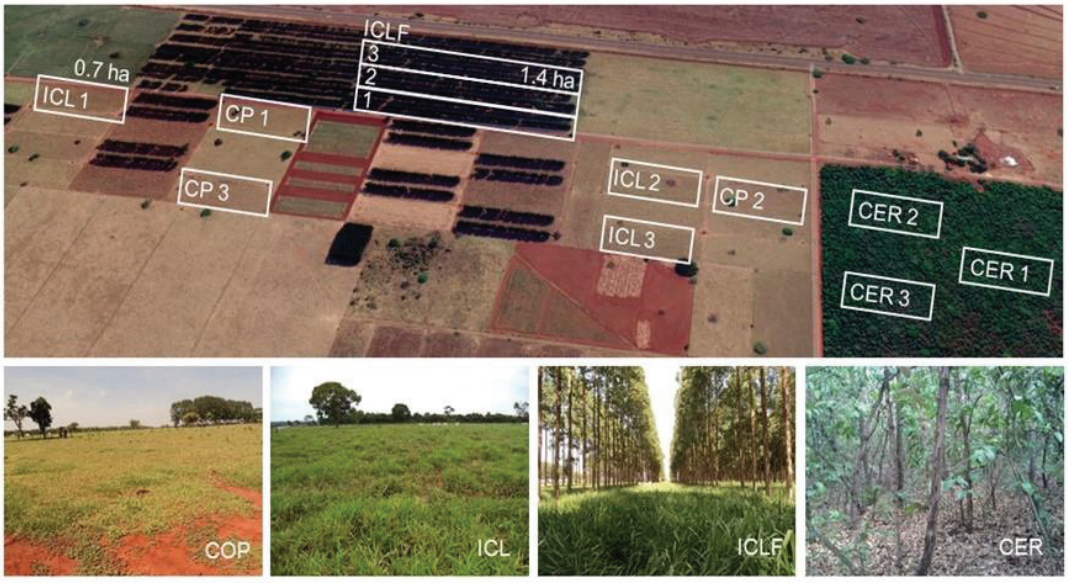


Figure 2. Top part: Map of the study area indicating the location of the four treatments and replications. CER: Cerrado (natural Savannah vegetation), ICLF: integrated-crop-livestock-forestry system, ICL: integrated crop-livestock system, COP: continuous pasture. Bottom part: Aspects of the respective treatments.

The plots of the treatments COP and ICL were part of a long-term management experiment that was established in 1995 and, thus, demonstrated management effects of more than 20 years. The COP treatment represented a typical degraded non-fertilized pasture often found in central west Brazil. The pasture consisted of *Brachiaria decumbens* cv. *Basilisk* under variable stocking rate with a minimum of two steers per plot and increasing stocking rate depending on forage availability. In ICL, rotation strategy was established with a cycle of 4 years, rotating Glycine max under no-till for one year, followed by *Brachiaria brizantha* cv. *BRS Piatã* pasture under variable stocking rate with annual fertilizer application of 50 kg N ha^{-1} (in the form of urea) and 300 kg NPK ha^{-1} (0:20:20) for three years. The ICLF was part of another long-term management experiment and was established in 2008. It consisted of a *Brachiaria brizantha* cv. *BRS Piatã* pasture lined with rows of 25 to 27 m high *Eucalyptus* (*Eucalyptus grandis* × *Eucalyptus urophylla*, H13 clone) trees with annual fertilizer application of 50 kg N ha^{-1} (in the form of urea) and 300 kg NPK ha^{-1} (0:20:20). The *Eucalyptus* seedlings were transplanted in east-west oriented rows. Each replication consisted of two single tree rows at a distance of 22 m and 2 m distance between the trees within each row, resulting in a tree density of 227 trees ha^{-1}. The ICLF followed a 4-year rotation strategy with *Glycine max* for five months, followed by 3.5 years of pasture. For this study, data were collected for the ICL and the ICLF treatment during the second and third years of the pasture cycle from November 2015 to August 2017. For the ICL and ICLF treatment, the cattle (Nellore heifers) grazed at varying stocking rates depending on biomass production to keep the height of the pasture at approximately 35 cm (put-and-take method).

2.3. Soil Characteristics

The soil of the study area has been classified as a Ferralsol [17] and a Typic Acrudox Oxisol [18]. From soil pits excavated in February 2017, undisturbed (coring rings 10 cm^3) and disturbed soil samples were taken for all four treatment plots in 10, 20, 30, 40, 60 and 100 cm depth, resulting in 3 replicates per depth. The undisturbed soil samples were used to determine soil bulk density (BD), and the disturbed soil samples to analyze pH measured in calcium chloride, soil organic carbon (SOC) using a C/N analyzer and soil texture using the pipette method (Table 1). Saturated hydraulic conductivity (Ksat), field capacity, and permanent wilting point were calculated using the computer program Rosetta Lite v. 1.1 within the software package HYDRUS 1D by PC Progress. Rosetta Lite v. 1.1 implements five hierarchical pedotransfer functions for the estimation of the van Genuchten water retention parameters (pF curve: field capacity and permeant wilting point) and the saturated hydraulic conductivity by using the measured soil texture and BD data [19].

Table 1. Soil characteristics for the treatments CER: Cerrado (natural Savannah vegetation), ICLF: integrated-crop-livestock-forestry system, ICL: integrated crop-livestock system, COP: continuous pasture. SCL: sandy clay loam, SC: sandy clay.

Depth [cm]	pH ($CaCl_2$)	BD [g cm^{-3}]	SOC [%]	Texture [%] Sand	Silt	Clay		Ksat [cm d^{-1}]
	Cerrado (CER)							
10	4.3	0.91	3.13	56	7	37	SC	107.1
20	4.3	0.94	2.08	53	8	39	SC	99.8
30	4.4	1.00	1.68	52	7	41	SC	83.4
40	4.6	1.05	1.59	51	7	42	SC	71.9
60	-	0.99	-	-	-	-	-	83.6
100	-	1.01	-	-	-	-	-	79.7
mean	4.4	0.98	2.12	53	7	40	SC	87.6

Table 1. *Cont.*

Depth [cm]	pH ($CaCl_2$)	BD [g cm^{-3}]	SOC [%]	Texture [%] Sand	Silt	Clay		Ksat [cm d^{-1}]
	Integrated-crop-livestock-forestry system (ICLF)							
10	5.4	1.24	1.83	56	6	38	SC	46.9
20	5.0	1.17	1.60	55	6	39	SC	55.5
30	4.8	1.11	1.35	54	7	39	SC	65.0
40	4.8	1.14	1.18	55	6	39	SC	60.3
60	-	1.14	-	-	-	-	-	60.3
100	-	1.10	-	-	-	-	-	67.2
mean	5.0	1.15	1.49	55	6	39	SC	59.2
	Integrated-crop-livestock system (ICL)							
10	5.2	1.25	2.39	53	7	40	SC	41.5
20	5.1	1.18	1.82	53	7	40	SC	51.8
30	5.0	1.21	1.50	53	8	39	SC	47.4
40	4.8	1.19	1.24	52	7	41	SC	49.1
60	-	1.18	-	-	-	-	-	50.6
100	-	1.10	-	-	-	-	-	64.1
mean	5.0	1.18	1.74	61	5	34	SC	50.7
	Continuous pasture (COP)							
10	4.9	1.34	1.75	64	5	31	SCL	49.1
20	4.8	1.24	1.51	60	6	34	SCL	57.5
30	4.7	1.28	1.29	61	4	35	SCL	52.1
40	4.6	1.24	1.05	59	4	37	SC	47.0
60	-	1.28	-	-	-	-	-	47.0
100	-	1.21	-	-	-	-	-	56.4
mean	4.8	1.27	1.40	61	5	34	SCL	52.4

2.4. Soil Moisture and Aboveground Biomass Measurements

For the treatments CER, ICL and COP in each replication, five fibreglass access tubes (DeltaT Type: ALT1) of 1 m length were vertically installed along a row with 2 m distance between the access tubes for volumetric soil moisture [vol%] measurements with a portable DeltaT FDR (frequency domain reflectometry) PR2/6 profile probe. In the replications of the ICLF treatment, the fibreglass access tubes were installed along a transect of five sampling points between the tree rows to account for the shading and soil moisture gradient caused by the trees. For the exact location of the access tubes between the tree rows, see Figure 2 in Glatzle et al. [14]. For the ICLF system, soil moisture values have been averaged from measurements taken along the transect between the tree rows to account for variability in soil moisture caused by the distance from the trees. FDR measurements were taken weekly in depths of 10, 20, 30, 40, 60 and 100 cm. The FDR soil moisture measurements were validated against gravimetric soil moisture measurements. Samples for gravimetric soil moisture measurements were taken with an auger right next to every FDR access tube at the same depths where the FDR probe measurements were taken.

Evapotranspiration data were calculated from changes in the volumetric soil moisture content in the respective layers and then added up and expressed in mm of evapotranspiration. Conversion from volumetric soil moisture content to mm was calculated as: Vol % in [m^3 m^{-3}] × depth [m] × 1000 = [l m^{-2}] = [mm]

In all four treatments, aboveground biomass (AGBM) [g DW m^{-2}] was quantified monthly at a total of 22 sampling dates (Dec 2015–Sept 2017). At each sampling date, biomass was collected at five sampling points in each replication (for ICLF along a transect between the tree rows). The grass layer was harvested on 1 m^2 and cut 5 cm above the ground. Furthermore, for COP and ICL, all dead material laying on the ground within the 1 m^2 was collected, and for ICLF, all dead material + litter from the *Eucalyptus* trees laying on the ground within the 1 m^2 was collected. Due to the non-existing grass layer, only litter was collected from 1 m^2 sampling points in CER. All harvested biomass samples were separated into green biomass, standing dead biomass, and litter-dried for 48 h at 70 °C and weighed to determine biomass as dry weight per area in g m^{-2}.

2.5. Data Analysis

Data were analyzed in a randomized block (3 replicates) design using the generalized least squares model of R [20]. For the analysis of soil moisture treatment, month and depth were considered as fixed effects. For the analysis of green AGBM and dead AGBM + litter, treatment and month were considered as fixed effects. As soil moisture was only measured at 10, 20, 30, 40, 60 and 100 cm depth, soil moisture values at 50, 70, 80 and 90 cm depth were obtained by linear interpolation of the measured data points.

3. Results

3.1. Seasonal Dynamics of Soil Moisture Content

Figure 3 shows the monthly dynamic of soil moisture content across the entire soil profile (0–100 cm) for all treatments over the experimental period. Monthly mean soil moisture content varied between 18 and 35 vol% and followed a clear seasonal pattern in all treatments. The dynamics of soil moisture content differed significantly ($p \leq 0.001$; Table 2) between treatments and seasons, with the integrated crop-livestock treatment being the wettest, varying between 35 vol% (wet season) and 25 vol% (dry season) (Figure 3).

The lowest monthly mean soil moisture content was observed in the natural Cerrado, with maximum values around 28–30 vol% during the rainy season and minimum values around 18–23 vol% during the dry season. The integrated crop-livestock treatment had, on average, about 5 vol% higher soil moisture content than the integrated crop-livestock-forestry treatment average during the rainy season. Averaged over the rainy season, CER had with 27.5 vol% the lowest soil moisture content, closely followed by ICLF with 27.7 vol%, followed by COP with 30.0 vol%, while ICL had with 32.3 vol% the highest soil moisture content. The different treatments follow the same sequence when averaged over the dry season but with a soil moisture level 10–15 vol% lower than in the rainy season.

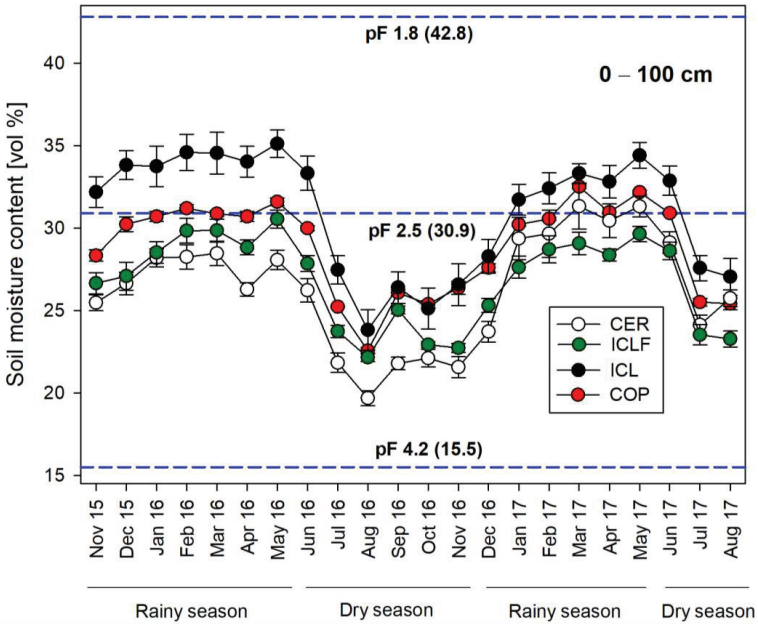


Figure 3. Monthly mean soil moisture content [vol%] for CER: Cerrado, COP: continuous pasture, ICL: integrated crop-livestock, and ICLF: integrated crop-livestock-forestry across 0–100 cm soil profile from Nov 2015 until Aug 2017. Bars represent standard errors with n = 3. Dashed horizontal lines indicate plant-usable field capacity between pF 1.8–2.5 (field capacity) and pF 4.2 (permanent wilting point).

Table 2. Analysis of variance for soil moisture content in the soil profile 0–100 cm. Df = degrees of freedom, SS = sum of squares, MS = mean square, n = 3.

Soil Moisture Content [vol%]	**SM = TxM + R**			
	Df	**SS**	**MS**	***p***
Treatment (T)	3	888	296	<0.001
Month (M)	21	2340	111	<0.001
Replication (R)	2	32	16	<0.001
Interaction (T × M)	63	199	3	<0.001
Residuals	174	226	1	

Figure 4 illustrates the vertical-spatial dynamics of soil moisture content for different soil profile depths (from 10 to 100 cm) for the entire experimental period for all four treatments. In general, soil moisture content increased with depth independent of season or treatment. As expected in all treatments and in all profile depths, soil moisture content was lower in the dry season and higher in the rainy season.

The vertical-spatial combined with the temporal resolution allows for a more detailed analysis of soil moisture dynamics in the various land-use systems. Where Figure 3 indicated that the natural Cerrado seems to be the least water-conserving land-use system, Figure 4—CER shows that particularly the topsoil down to 30 cm dries out severely, even during the rainy season (vol% values at or close to permanent wilting point), whereas the lower profile layers (>70 cm) maintain soil moisture values close to or at saturation. During the dry season, CER soils dried to a permanent wilting point down to about 70 cm below the surface. The removal of trees in the ICL and COP systems (Figure 4—ICL, COP) resulted in strongly different vertical-spatial soil moisture dynamic patterns. Except for the dry season, where the topsoil (10 to 30 cm) of the two systems dried to values close to the permanent wilting point, for the remaining time, soils stayed well saturated (vol% values between 25 and 40) from a depth of 10–15 cm below the surface for the entire remaining profile. Re-integrating trees into the system (ICLF—Figure 4—ICLF) changed the vertical-spatial dynamic of soil moisture towards the pattern observed in CER. The main difference is that the top soil stayed relatively well saturated during the rainy season and dried down to the permanent wilting point in the lower profile layers only during the driest months during the dry season.

Analysis of variance (Table 3) showed that the land-use system significantly affected soil moisture content, with this effect highly significant depending on the season (months). In addition, soil moisture content in combination with season was highly significantly affected by the position (depth) in the soil profile.

Table 3. Analysis of variance for soil moisture (SM). Df = degrees of freedom, SS = sum of squares, MS = mean square, n.s. = not significant at $p < 0.05$, n = 3.

SM	**SM = TxMxD + R**			
[vol%]	**Df**	**SS**	**MS**	***p***
Treatment (T)	3	6498	2166	<0.001
Month (M)	21	14657	698	<0.001
Depth (D)	5	23817	4763	<0.001
Replication (R)	2	117	58	<0.001
Interaction (T × M)	63	1261	20	<0.001
Interaction (T × D)	15	5623	375	<0.001
Interaction (M × D)	105	2701	26	<0.001
Interaction (T × M × D)	315	893	3	n.s. < 0.064
Residuals	1054	2738	3	

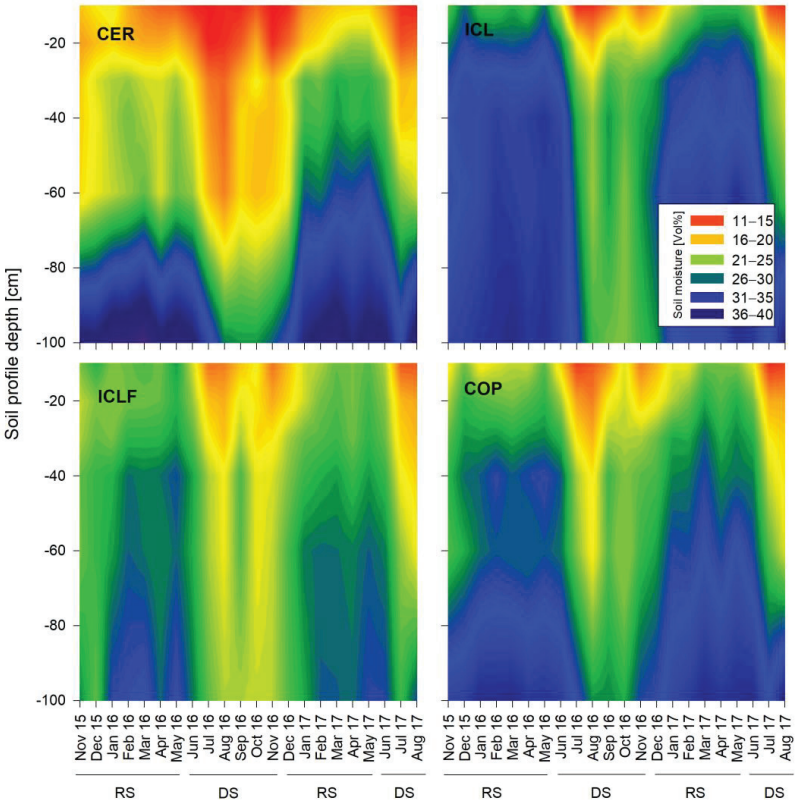


Figure 4. Soil moisture content [vol%] for 10 to 100 cm profile depth shown as monthly means for the four treatments: CER = Cerrado, ICLF = integrated crop-livestock-forestry, ICL = integrated crop-livestock, COP = continuous pasture. RS: rainy season, DS: dry season.

3.2. Soil Moisture Kinetics during Drying Events

In order to evaluate which of the treatments contributed to the local water cycle from which depth, we followed several dry spells during periods without rainfall in the different seasons with regular soil moisture measurements across the soil profile down to 100 cm of depth. We calculated the water lost from the different depths during the drying event and related it to the overall water loss from the respective treatments during the dry spell (Figure 5). During a period of 20 days without rainfall late in the rainy season (Figure 5a), the CER treatment lost 64 mm of soil moisture to evapotranspiration, with the largest share originating from the first 40 cm of the soil profile. All other treatments lost between 11% (COP), 15% (ICLF) and 18% (ICL) less water than the CER, and for the treatments including trees, the distribution of water evapotranspired from the different layers of the soil profile was more homogenous than in the CER. The differences between the treatments in the way water was lost through evapotranspiration became more manifest in periods without rain during the dry season. In the dry season, monthly rainfall ranges between 15–60 mm (Figure 1), but there are longer periods with no rain at all. In a 25-day period without rain early in the dry season (Figure 5b), all systems lost about the same amount of water. Meanwhile, in the CER, the topsoil did not hold much water (soil moisture values of less than 15%), soil moisture values below 30 cm depth were relatively high, and most water was lost from there. In all other systems, the top soil was covered with grass, and the top layer of the soil profile initially stored a high percentage of water with soil moisture values of 25 to 30 vol% in the top 10 cm.

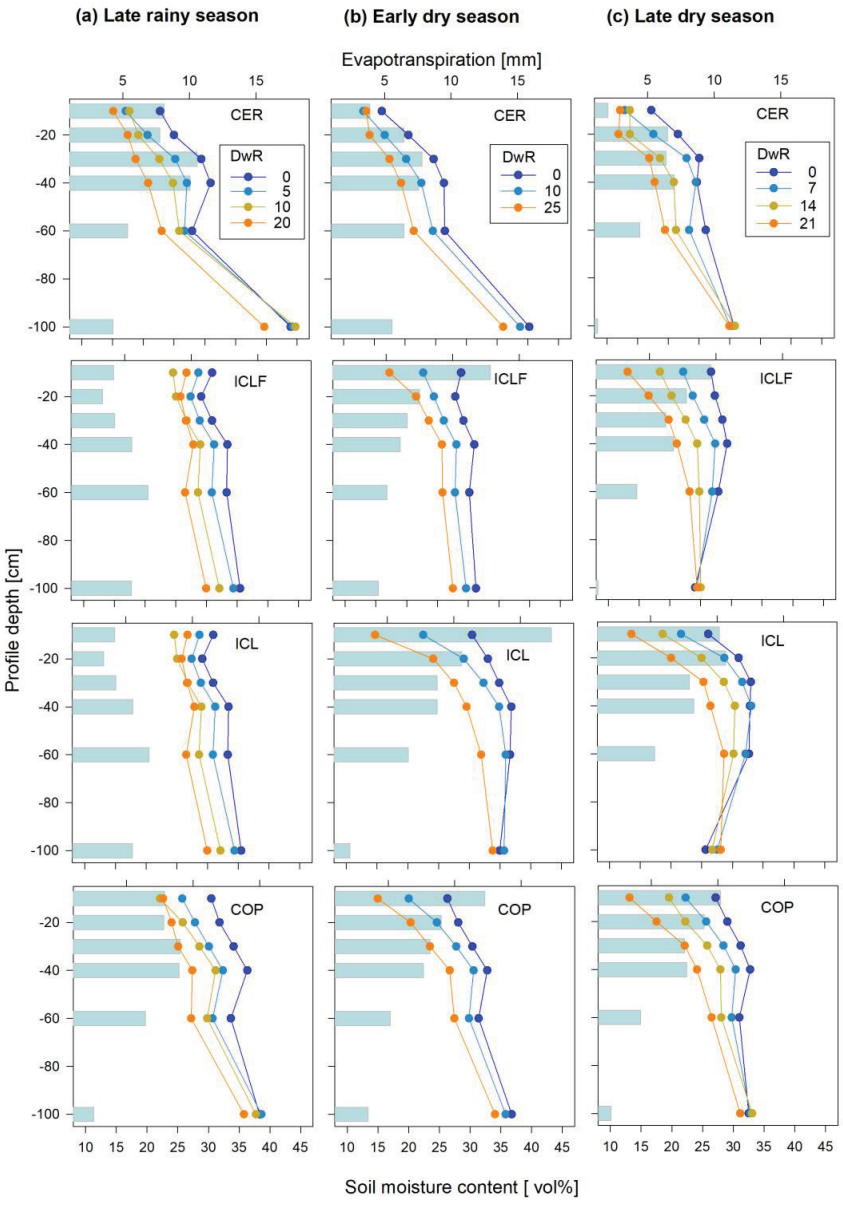


Figure 5. Soil moisture [vol%] kinetics shown for 10 to 100 cm profile depth during dry spells of at least three weeks without rain in three distinguished seasons for the four treatments CER = Cerrado, ICLF = integrated crop-livestock-forestry, ICL = integrated crop-livestock, COP = continuous pasture. Horizontal bars show the water amount of water lost to evaporation in mm from the different soil depths over the course of the respective dry spell. Data are shown as averages over three replications. Error bars have been omitted for clarity.

A period of about 3 weeks without rain late in the dry season (Figure 5c) resulted in reduced water losses in all treatments, mainly due to less water extraction for evapotranspiration from deeper soil layers, indicating a less active vegetation cover towards the end of the dry season. COP and ICL lost about the same amount of water (51 mm

and 49 mm, respectively), followed by ICLF (40 mm) and CER (34 mm). As in the drying period before, the strongest decrease in soil moisture content was observed in COP, ICL, and ICLF in the upper soil layers, whereas in CER, soil moisture decreased most strongly between 20 and 60 cm depth. During this period, hardly any water was lost at 100 cm depth in all treatments. On average, across the respective dry spells, the largest variation in soil moisture content was observed between 30 and 40 cm at CER, whereas in the other treatments, water was mainly lost from the topsoil.

3.3. Aboveground Biomass

Aboveground biomass (AGBM; from tree biomass, only litter was sampled) was monitored monthly for the entire experimental period and is shown for all treatments in Figure 6. In general, for all treatments, the total AGBM (green + dead + litter) was higher during the rainy season than during the dry season. Comparing the treatments on average over the experimental period, COP had 206 gDWm^{-2}, by far the lowest total AGBM, followed by ICLF with 607 gDWm^{-2} and ICL with 760 gDWm^{-2}. Comparing green AGBM on average over the measurement period, COP had 60 gDWm^{-2}, the lowest green AGBM, followed by ICLF with 168 g DW m^{-2} and ICL with 262 gDWm^{-2}. Comparing the dead + litter AGBM pool, COP (146 gDWm^{-2}) was lowest, followed by ICLF and ICL (439 and 497 gDWm^{-2}, respectively), while CER had 899 gDWm^{-2}, the largest amount of litter.

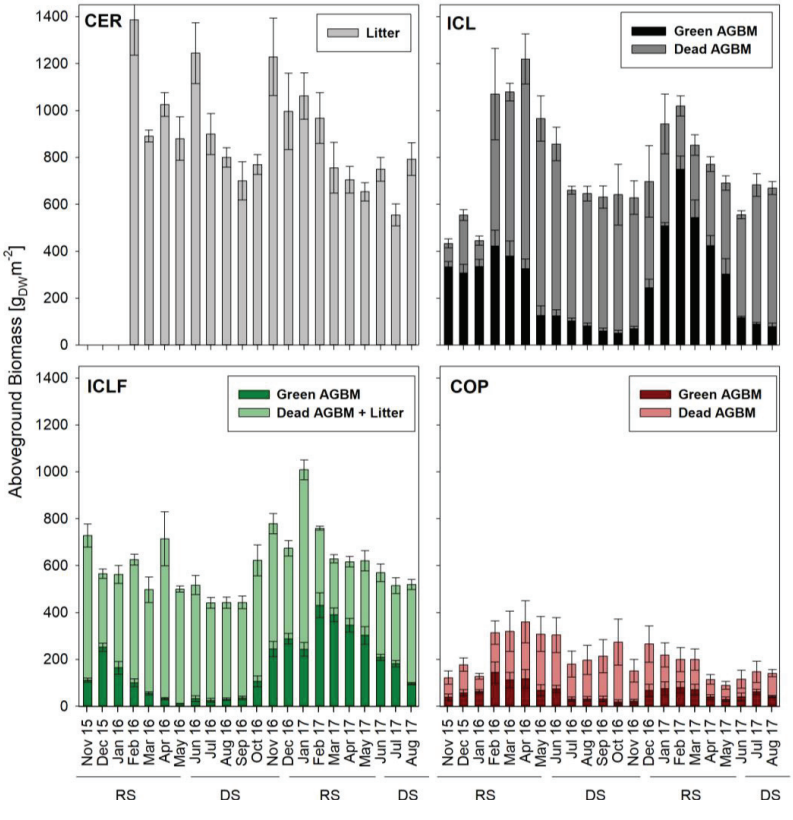


Figure 6. Time courses of aboveground biomass dry matter (AGBM) for the four treatments CER = Cerrado, ICLF = integrated crop-livestock-forestry, ICL = integrated crop-livestock, COP = continuous pasture. Stacked bars show the total AGBM, including standing green, standing dead and litter biomass (from tree biomass, only litter was sampled). Data are shown as averages over three replications. Error bars = Standard error of means.

4. Discussion

4.1. Soil Moisture Dynamics in the Different Land Use Systems

Monthly precipitation (Figure 1) and associated changes in soil moisture content to a depth of 100 cm for all 4 treatments were continuously measured for 22 months. Soil moisture content dynamics were strongly seasonal in all treatments, with high soil moisture content in the rainy season and significantly lower soil moisture content in the dry season relative to the rainfall regime. Intra-annual changes in soil moisture content were similar for the different treatments but on different levels. This indicates a higher turnover rate for precipitation in the dry season due to increased evapotranspiration. For both seasons, pooled over 1 m depth, soil moisture content was always lower in the treatments with trees, ICLF and CER, as compared to treatments without trees, COP and ICL. Due to rooting patterns, trees and shrubs often access deeper soil layers, resulting in increased water uptake and transpiration. It was shown for the ICLF system that soil moisture content, especially in deeper profile layers, decreased close to tree lines as compared to the central areas between the tree lines [14].

Comparing Cerrado (cerrado denso) and a natural grassland (cerrado sujo), Oliveira et al. [8] found higher evapotranspiration rates in the Cerrado due to differences in tree densities and leaf area, but also to differences in root distribution. Similar results of lower soil moisture content at a higher tree density were reported by Quesada et al. [9], who compared a densely wooded Cerrado with a burned Cerrado with a high grass density in central Brazil and by Herrera et al. [21] who compared a *Eucalyptus* plantation with a Savanah vegetation in Venezuela. In the current study, soil moisture content in CER was slightly lower than in ICLF on average over the experimental period, which was probably related to the higher density of woody vegetation and higher leaf area of the CER, resulting in higher transpiration rates.

In contrast, ICL showed similar amounts of aboveground biomass as CER, including a large share of standing dead biomass (Figure 6), but soil moisture content was always significantly higher in ICL than in CER. Since both systems had relatively high soil organic matter concentrations (Table 1), an indicator for increased available soil water capacity [22], differences in topsoil moisture content (Figure 3) could have been caused by the large quantity of dead AGBM in ICL serving as mulch in addition to the closed grass cover, which both reduce losses of soil moisture via bare soil evaporation [23,24]. Additionally, the CER canopy and thick litter layer intercept a large share of precipitation, especially of smaller rainfall events. As a result, some of the precipitation at CER directly evaporates from litter, leaf and stem surfaces and, consequently, does not contribute to soil moisture content.

In the COP, soil moisture content across the seasons (Figure 3) was always between CER (driest) and ICL (wettest), but biomass production was only about one-third of ICL. This indicates soil compaction and degradation resulting from deforestation, intensive cattle grazing, and machinery use [25–28], as also suggested by the relatively high bulk density and low hydraulic conductivity (BD and Ksat, Table 1).

Soil compaction may result in reduced growth of aboveground biomass as a consequence of limited root growth [29], which could be an explanation for the significantly reduced green AGBM in COP compared to ICL and ICLF. However, in contrast to COP, mineral fertilizer was applied in ICL and ICLF, comparisons of biomass growth between the systems are difficult and need to be considered with caution.

Soil moisture content and plant water availability strongly depend on soil properties and the retention capacity for water of the individual soil types. In order to compare soils for their level of water available to plants and their respective level of degradation, we calculated soil water retention curves for the different soils (Figure 7).

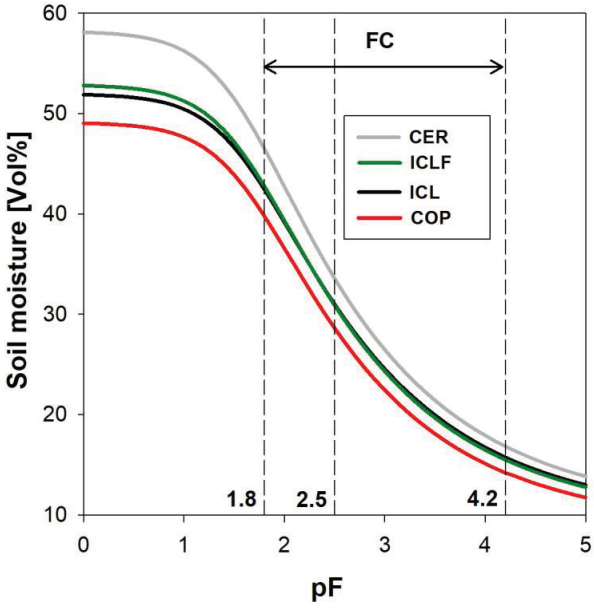


Figure 7. Water retention curves for the soils in the four treatments CER = Cerrado, ICL = integrated crop-livestock, ICLF = integrated crop-livestock-forestry, COP = continuous pasture. Curves were calculated using Rosetta Lite v. 1.1 within the software package HYDRUS 1D by PC Progress.

The water retention curves showed higher soil moisture content at the same pF values in the ICL and ICLF systems compared to COP, indicating the potential of integrated systems (ICL and ICLF) and other conservation systems (e.g., No-Till systems) to mitigate the effect of soil compaction in pasture systems and, thus, to restore degraded sites due to lower soil movement, the formation of biopores and input of soil organic carbon [30,31]. The potential of ICLF systems to improve the soil physical quality has also been described by Moreira et al. [32] and Assis et al. [33], who found improved soil physical quality in ICLF and ICL systems compared to degraded pastures in the Cerrado biome. In line with our findings, Nóbrega et al. [28] found significantly greater bulk densities and smaller Ksat and total porosity in the topsoil of a pasture that was converted from a Cerrado. In contrast to the results reported here, Feitosa et al. [34] found higher water retention at field capacity of soils under pasture in the Cerrado biome of northern Brazil, indicating potentially site-specific responses.

Due to the fact that our core experimental sites were established more than 25 years ago and were continuously maintained under the same management, we were able to capture a certain level of long-term processes reflected in parameters of soil texture, carbon content, and hydrological conductivity (Ksat) which could be clearly attributed to the respective land-use management. In general, our results confirm that tree components reduce average soil moisture content independent of seasons; however, regarding seasonal dynamics in different soil depths, differences in water extraction patterns were found between the systems.

4.2. Seasonal and Vertical-Spatial Soil Moisture Variations

In general, soil moisture content was lowest in the top layers of the profiles and increased with depth. These moisture profiles changed in the dry season, where relatively low soil moisture content was found in greater depths. However, soil moisture content in the observed profiles varied widely between treatments. While the topsoil was driest in CER in all seasons, soil moisture content in the topsoil layers showed only little variation

between the other treatments, with COP having the lowest soil moisture content during the peak of the dry season and ICL having higher soil moisture content during the rainy season (Figure 4). In contrast, in profile layers deeper than 30 cm, soil moisture content was, on average, the lowest in ICLF. However, the differences between ICLF and CER in soil moisture content in the deeper soil layers were strongly seasonal, with a large difference during the rainy season and similar values during the dry season. COP and ICL had, on average, soil moisture contents in the deeper soil layers similar to CER, but during the dry season, soil moisture content was higher in COP and ICL than in CER.

The upper layers of the soil in the Cerrado have been described as relatively dry, with values below the permanent wilting point during the dry season [35] and a study comparing the hydrology of catchments predominated by either Cerrado or pasture found significantly higher streamflow values in the pasture catchment indicating higher evapotranspiration of the forest [28], confirming results from Dias et al. [36]. Despite the extensive land conversion from Cerrado to pasture in recent years, studies directly comparing soil moisture content between the two land use types are scarce. With a lower bulk density and a higher share of organic matter, the topsoil of CER should have a higher water retention than the other land use systems. Further, evaporation from the topsoil has been found to be lower in the forest than in an open pasture [37], as the tree canopy strongly limits the radiation reaching the ground and the soil is covered with a layer of litter. A layer of litter, which was present in CER year-round, not only strongly limits evaporation [37] but also intercepts a substantial share of rain in the Cerrado [38]. Interception from Cerrado canopy was estimated to be around 20% of total precipitation [6], which in turn is directly contributing to higher evapotranspiration of Cerrado systems compared to other land-use types.

We are aware that litter can also lead to errors when measuring soil moisture with permittivity sensors [39], such as the one used in our study. The access tube needs to be in close contact with the surrounding soil for a precise FDR measurement (Delta-T Devices Ltd., Cambridge, UK, 2016), and in CER, the upper 2–3 cm of the access tube was in contact with a layer of litter in a stage of decomposition, which impeded the required close contact, the precision of the soil moisture measurements at 10 cm depth can be questioned. However, soil moisture content at 20 cm depth in CER was also lower than in the other land use types, and its measurement was not affected by the layer of litter. As evaporation and litter-related measurement errors can be, thus, excluded as causes for the lower values of soil moisture content in CER, a possible explanation could be the soil-specific characteristics found in Ferralsols under native Savannah vegetation. Natural ferralsols have a granular macrostructure due to the gibbsitic (aluminium hydroxide) mineralogy [40], resulting in a high amount of macropores and micropores [41]. The stable soil structure is based on the bonds between negatively charged clay minerals and positively charged Aluminum hydroxide. Juhász et al. [42] found in the topsoil under native Cerrado vegetation (cerradão) low soil, bulk density and low water retention caused by high organic matter content and high total macroporosity. According to this study, water infiltration, conductivity, and retention depend on the shape of the pores and, consequently, on soil structure. The pore shapes found in the topsoil of these natural Ferralsols favour water infiltration and conductivity, which is a disadvantage to retention. Furthermore, natural Ferralsols were found to have more macropores under native perennial vegetation due to the formation of biopores [43]. The high total macroporosity, low water retention, and higher saturated hydraulic conductivity in the topsoil of CER (Table 1) could explain the low soil moisture content values in this treatment. Moreover, the slope of the CER retention curve (Figure 7) is steeper compared to the other treatments, indicating that soil moisture is less retained. In the pasture systems without trees COP and ICL, water was mainly lost from the upper 40 cm of the soil, while in CER, the largest share of water lost, in the upper 1 m of the soil was depleted between 20 and 40 cm depth (Figure 5). In ICLF, most of the water was extracted below 40 cm at the end of the wet season, but with increasing water deficit, a larger share of water was lost from the upper part of the soil. In the pasture, it can be expected that the largest share of water is lost from the upper part of the soil, as more than

80% of the roots of *Brachiaria brizantha* are found in the upper 30 cm of the soil [15]. For the ICLF system, soil moisture content values have been averaged from measurements taken along a transect between the tree rows to account for variability in soil moisture caused by the distance from the trees [14]. Bosi et al. [13] found the largest fluctuation in soil moisture content in the upper 30 cm of the soil in an ICLF system. However, they also described a fast soil water withdrawal closer to the trees, indicating a higher water uptake by the trees than by the pasture. Even though *Eucalyptus* has a large share of its root system in the top layers of the soil, its roots grow far beyond the depth of 1 m [44]. The varying depth of the zone of the largest water extraction found in our study (Figure 5) indicates a shift in water uptake to greater depth during the dry season. As with decreasing soil moisture content from the end of the wet season to the end of the dry season, water extraction at 1 m depth in ICLF diminished, and a larger share of water was probably extracted from greater depth. In a study from south-eastern Brazil, deep water uptake by *Eucalyptus* roots accounted for a relatively small proportion of the total water uptake, but during the dry season, trees increased the share of the water taken up near the water table at 12 m depth [45]. Similar effects were reported for the Cerrado. Even with the highest root density being found in the topsoil in a Cerrado forest and more than 90% of the fine roots in the upper 1 m of the soil, deep water uptake accounted for 83% of the total water use during the dry season [8]. However, as soil moisture content was much lower in ICLF than in CER between 40 and 100 cm during the rainy season (Figure 4), *Eucalyptus* trees probably take up more water in the upper part of the rooted subsoil (40–100 cm) than the Cerrado vegetation during periods of high water availability. Further, as soil moisture content was much lower in ICLF than in CER at 1 m depth throughout the year (Figure 4), we hypothesize that the shift in water uptake to greater depth in response to low soil moisture content is taking place earlier in CER resulting in water conservation. This aspect of seasonal dynamics of the water balance between natural Cerrado and ICLF systems needs to be investigated in more detail if systems' sustainability should be assessed, especially since other essential ecosystem processes related to water availability may have changed. *Eucalyptus* trees are by far the most dominant tree species used in ICLF systems, and the potential of other (native) tree species to be used in ICLF systems appears not to have been fully evaluated yet. However, by contributing to increased evapotranspiration, *Eucalyptus* or other trees may directly compensate for reduced water recycling to the atmosphere that has been reported from other land use systems established in previous Cerrado areas [5,46]. From this perspective, integrated systems, including trees, are closer to the natural Cerrado than pasture and other integrated systems. In view of the serious consequences caused by altered water cycles because of land use change, ICLF systems should be considered as an additional option to mitigate previous land use change effects in the context of sustainable intensification.

5. Conclusions

Integrated systems may offer a good alternative to traditional pastures in terms of soil health and sustainable water management. Trees as components of integrated land use systems indicate the potential of restoring natural ecosystem functions with regard to water recycling to the atmosphere and consequentially suggest the possibility of partly compensating for Cerrado deforestation. However, the complexity of integrated systems and natural climate variability need further in-depth analysis of seasonal and spatial soil water dynamics in order to improve our understanding of ecological functions and processes towards sustainable land use management.

Author Contributions: Conceptualization, S.G., D.J.B., M.G. and M.C.M.M.; Formal analysis, S.G. and M.P.B.; Investigation, S.G. and M.P.B.; Methodology, S.G., M.G. and F.A.; Project administration, M.G.; Resources, R.G.d.A., D.J.B., M.G. and F.A.; Supervision, M.G. and F.A.; Writing—original draft, S.G. and S.S.; Writing—review and editing, S.G., M.G. and F.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Anton & Petra Ehrmann-Stiftung Research Training Group "Water—People—Agriculture (WPA)" at the University of Hohenheim, Germany.

Data Availability Statement: All data supporting the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments: Help and support of the field and laboratory staff, the students, and interns from EMBRAPA Beef Cattle is gratefully acknowledged.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Military Activity Impact on Vegetation in Pannonian Dry Sandy Grasslands

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Abstract: The conservation of dry sandy grasslands is a global issue because of the restoration and conservation of endangered ecosystems to provide a sufficient amount of forage under warming and drying climatic conditions. Our aim was to explore the impact of military activity on sandy grasslands in Hungary. The sample areas chosen were the Little Hungarian Plain (I. and II.) and the Great Hungarian Plain (III.), consisting abandoned, restored areas; still-active military exercise fields; and shooting ranges. In each sample area, six to ten coenological surveys were made. Based on our survey, the closed natural sandy grassland was documented only in the Little Hungarian Plain and were found rich in species. The open sandy grassland was described in all studied sites, *Festuca vaginata* appeared in all of them, while *Festuca pseudovaginata* was observed only in the Great Hungarian Plain. In the open sandy grassland, the natural vegetation had the highest cover value (78.8%), the sowed grassland area had the least cover value (53.3%), while the III. sample area was also poor in coverage (56.5%) but consisted of a natural species composition favorable for restoration. Our results confirmed the indirect role of military activity in the successful habitat conservation of Pannonian dry sandy grassland ecosystems.

Keywords: conservation restoration; coenology; diversity; *Festuca vaginata*; *Festuca pseudovaginata*; human disturbance

Citation: Bajnok, M.; Penksza, K.; Fűrész, A.; Penksza, P.; Csontos, P.; Szentes, S.; Stilling, F.; Saláta-Falusi, E.; Fuchs, M.; Melenya, C.; et al. Military Activity Impact on Vegetation in Pannonian Dry Sandy Grasslands. *Land* **2024**, *13*, 252. https://doi.org/10.3390/land13020252

Academic Editors: Michael Vrahnakis and Yannis (Ioannis) Kazoglou

Received: 25 January 2024
Revised: 15 February 2024
Accepted: 16 February 2024
Published: 18 February 2024

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1. Introduction

The conservation of dry sandy grasslands is a global issue, since their biodiversity is critically vulnerable to fragmentation. The effects of climate change have resulted in increasing significance of these drought-tolerant ecosystems. They represent an important genetic pool too, as biodiversity has been declining in grasslands of the Pannonian region [1–3]. Biodiversity loss is caused by anthropogenic influences, but also by the improper use or lack of land management [1]. Global warming necessitates the restoration and conservation of endangered ecosystems which are still able to provide a sufficient amount of forage under warming and drying climatic conditions too. The Pannonian endemic dry, sandy grasslands have a low nutrient content in general. Nevertheless, despite their

low nutrient content and biomass value, these areas are used for grazing in the long term. The ecosystem of these habitats can provide nutrients even in the context of global warming. However, the application of sustainable management practices is necessary for their maintenance, as complete abandonment can cause biodiversity reduction and a decreased number of species. According to several research studies, this decrease in biodiversity is often accompanied by a decrease in plant biomass [2,4–18]. Appropriate grazing or mowing are important management practices for grasslands, especially in low-quality Pannonian grasslands, as the amount of surface phytomass has a positive effect on the number of species even with a slight disturbance [14]. The enhancement in productivity can contribute to species richness, contingent upon various factors such as geographical location [13], habitat expansion [11], fragmentation [7], biocoenosis type [6], and habitat successional condition [11].

As in the study of sandy grassland restoration [16], the amount of target species in sandy grassland was found to be higher in older than in younger fallows. By definition, fallows are arable areas that have been abandoned for less than 10 years, whereas grasslands are areas that have been treated as grassland for more than 10 years. The species are considered "target species" that can be dominant characteristic species in natural grasslands and that are also important for grassland and nature conservation management purposes. Most of the target species were already sown in young fallows, but only a few new target species appeared in older fallows [9]. According to the study by Rehounková and Prach [16], the appearance of new target species in sandy areas is probably a propagule-limited process, which is influenced by the species composition of adjacent grasslands [5,6]. This statement is confirmed by the experiences of restoration of the Homoktövis Nature Conservation Area in Hungary [7] too. Therefore, activities of habitat restoration are important to reduce isolation and increase the expansion of patches of grassland habitats. The final aim of restoration is to reach the habitats' original condition to the closest extent possible [19]. The relevance of habitat restorations by active intervention has been increasing [20–22], especially in natural and semi-natural areas of highly transformed environments [21].

In the process of grassland restorations, sown grasslands has gained more importance [2,5], representing one of the most common interventions of habitat reconstruction today. However, the sowing of grasslands can be carried out in various ways, including the adding of hay or the application of seed mixtures of different diversities [23–25]. The selection of seed mixtures is an important issue for nature conservation, as in ideal cases, species compatible with the landscape and the most suitable for the environmental conditions of the area are collected [26,27]. When seed mixtures are sown, different seed mixtures with low diversities (2–8 species) [28,29] or high diversities (9–40 species) are used [25,29], if the determination of dominant species is conducted in controlled conditions. In addition, burning has also become common in regeneration maintenance works [30–32].

Countries worldwide dedicate vast areas to military exercises. Due to the nature of the activity, these military training grounds often exceed 100,000 hectares individually. The perception of these areas is Janus-faced, as on the one hand, the destructive nature of military training can cause significant environmental damage, while on the other hand, the restrictions applied to these areas makes it impossible to pursue other (non-military) human activities [33]. Based on the literature, evaluation of the harmful environmental effects of activities on training grounds can be grouped into the following main research areas: ground water pollution, soil degradation (compaction) and soil pollution (especially due to heavy metal contamination), the entry of pollutants into the food web, and ecotoxicology [34]. Soil compaction and structure degradation are central issues regarding vegetation, as decreased pore space and the related reduced hydraulic conductivity directly affect plant life [35]. Furthermore, the uptake of toxic metals by the roots and shoots of the grass, forb, and shrub species increases the risk of these substances entering the food chain [35,36]. The biomass of the vegetation can also be reduced significantly on military training grounds by the off-road maneuvers of tanks and other heavy military vehicles, and the bare surfaces may enhance soil erosion 30 times higher than reference plots [37].

However, the wide variety of damages caused by military training, coupled with their irregular distribution in space and time, creates a wide variety of microhabitats with diverse local ecological conditions [38,39]. A striking example of microhabitat diversification is related to explosion craters that increased species diversity in the Nowa Dęba (SE Poland) military training area [40]. This increased plant diversity on military training grounds was documented in other localities as well, and these vegetation units often harbored populations of endangered plant species [33,39]. This special phenomenon is explained by the "heterogeneous disturbance hypothesis", which is related to Connell's intermediate disturbance hypothesis [40]. Warren's hypothesis emphasizes the importance of multiple kinds and frequencies of disturbance, as well as its varying severity, periodicity, and size. These factors are also suggested to be considered during conservation activities on abandoned military training grounds.

In the present research, we investigated vegetation types that grow on sandy soils with poor nutrient content and with questionable economic potential. However, as they are well adapted to drought, their potential expansion in the future may increase their economic importance as pasture and forage provider habitats. Among these vegetation types, the most typical ones are the calcareous perennial open sandy grasslands (*Festucetum vaginatae* Rapaics ex Soó 1929 em. Borhidi 1996), which grow in very dry and nutrient-poor areas.

Based on the reviewed literature, we sought to answer the following question in our research: Can military activity serve as a special option of habitat conservation in natural and semi-natural sandy grasslands of Hungary? This option can be considered special, as vegetation conservation does not belong to the task of military activities, so nature conservation in military areas is conducted in an indirect way. Thus, our aim was to investigate the impact of military activities on highly variable endemic arid vegetation types in the context of climate change using coenological surveys.

2. Materials and Methods

2.1. Description of the Study Areas

The surveys were carried out in two locations in the Little Hungarian Plain region (I. Military Shooting Range of Gönyű, and II. Military Exercise Area of Győrszentiván) and in one location in the Great Hungarian Plain (III. Tatárszentgyörgy) (Figure 1).

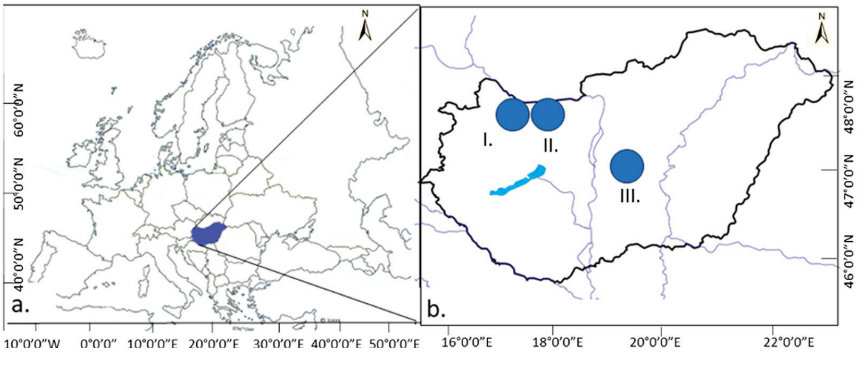


Figure 1. Sample areas (I.: Little Hungarian Plain Gönyű (above sea level: 103 m), II.: Little Hungarian Plain Győrszentiván (above sea level: 116 m), III.: Great Hungarian Plain Tatárszentgyörgy (above sea level: 95 m)). (**a**). Europe, (**b**). Hungary.

The selected study areas are particularly valuable because they belong to the Pannonian endemic grasslands [3].

In the two military areas of the Little Hungarian Plain (Gönyű, localization: 47.733300, 17.833300; Győrszentiván, localization: 47.697863, 17.736483), habitat reconstruction and grassland establishment were carried out using different methods, and military activity is

inactive at present. In the area of the Great Hungarian Plain (Tatárszentgyörgy, localization: 47.081500, 19.369669), military activities are still active.

In the Military Shooting Range of Gönyű, three vegetation types were surveyed: an undisturbed vegetation type, a burnt area (which was burnt for 3 years and abandoned 5 years before the time of the survey), and a manually sown grassland.

In the Military Exercise Area in Győrszentiván, a sown grassland patch was established with a dual purpose. One of the aims was to establish a grassland with a high nature conservation value, supplemented with increased grassland management utilization as well.

In the sample areas, the studied vegetation types (associations) were the following with the objectives of the grassland management:

I. Military Shooting Range of Gönyű. Its main purpose was the habitat restoration and establishment of *Festucetum vaginatae*.
   - I.1. FvGE: *Festucetum vaginatae*, an original, natural, calcareous, open sandy grassland, unmanaged.
   - I.2. FvGEÉ: *Festucetum vaginatae*, an original, natural, calcareous, open sandy grassland, but burnt for 3 years and abandoned 5 years before this study, unmanaged.
   - I.3. FvGS: *Festucetum vaginatae*, a manually sown semi-natural grassland, where an artificial sandy ridge, rampart, was established.

II. Military Exercise Area of Győrszentiván. Its main purpose was to establish a habitat through grassland establishment for economic and nature conservation values.
   - II.1. FvGYV: *Festucetum vaginatae*, a sown semi-natural grassland.
   - II.2. FrGYV: *Festucetum rupicolae*, a sown semi-natural grassland.
   - II.3. FrGYI: *Festucetum rupicolae*, a natural grassland.

III. Military Exercise Area of Tatárszentgyörgy. There is still active military activity (military exercises) here.
   - III.1. FpT: *Festucetum pseudovaginatae,* natural or semi-natural grassland.
   - III.2. FvT: *Festucetum vaginatae,* natural or semi-natural grassland.

In the areas of the Little Hungarian Plain (Gönyű, Győrszentiván), military activities were terminated in 1989. The restoration of the area was planned in 2006, and then the reclamation of the grasslands started in 2008 and was carried out in two phases, using two 5-year plans. Our survey was performed in 2018, 5 years after the termination of the restoration project. Based on this, the natural sandy grassland (FvGE) was already a natural grassland before the initial works in 2006. In the natural grassland that was burnt for 3 years (2010–2013), the activity had been abandoned for 5 years by 2018. On the other hand, in the two studied sown grassland areas (Gönyű—FvGS, Győrszentiván—FvGYV, FrGYV), the intervention was carried out in 2015, 3 years before the study period. Deforestation was applied in the *Festucetum rupicolae* natural grassland (FrGYI) and the invasive woody species were also removed in 2015. The only still-active military exercise area of our study is situated in the Great Hungarian Plain (Tatárszentgyörgy).

Among the natural vegetation types in the sample areas, the calcareous open sandy grassland is *Festucetum vaginatae* Rapaics ex Soó 1929 em. Borhidi 1996, which can be found in all sample areas of the three studied locations. Thus, a comparison of this vegetation type was also conducted, including the effects of the different management practices.

2.2. Field Methodology

In the field surveys, we used 2 × 2 m quadrats in each sample area and in each vegetation type in 2018. During the vegetation period, the modified method of Braun–Blanquet was followed [41], where vegetation cover is given as a percentage. Each sampling quadrat was chosen randomly in typical patches of the studied vegetation type. In each patch, 6–10 coenological surveys were carried out in each vegetation period and repeated at the same sample location. All occurring vascular plant species were noted down and their cover values were estimated. Finally, the cover values of the species in the three vegetation

periods (May, June, September) were analyzed together. Species names were provided based on the nomenclature of Király [42].

2.3. Statistical Analysis

To describe the mean and deviation of vegetation cover of the studied areas, we averaged the cover of species in each sample area and then we worked with these data in further steps. For each treatment, we ordered the plant species into different categories (characteristic species in open sandy grassland, characteristic species in closed natural sandy grassland, weeds, disturbance-tolerant species) in order to prepare clear diagrams. The differences in species number between territories were analyzed using one-way ANOVA. We used an agglomerative cluster analysis technique (Ward's method by PAST) with a fusion algorithm as a combinatorial method (minimizing increase in variance), and the correlation was used as the comparative function. In the analyses, the Kruskal–Wallis test was used for the variables of the one-way analysis of variance (ANOVA). Tukey's HSD test (post hoc test) was used to perform pair-wise multiple comparisons. The data structure of the coenological surveys was analyzed with detrended correspondence analysis (DCA) ordination using Bray–Curtis Distance Indices to visualize the species composition of the *F. vaginata*, *F. rupicola*, and *F. pseudovaginata* series in the three sites. All statistical analyses were performed using the PAST program [43,44].

3. Results

In order to analyze data easily, we classified the surveyed data into four coenosystematic groups (Table 1) [3]:

1. Characteristic species in open sandy grassland (Festucion vaginatae, Corynephoretalia, Festucetalia vaginatae & rupicolae, and Festucetalia vaginatae & valesiacae).
2. Characteristic species in closed natural sandy grassland (Brometea, Festuco-Brometea, Festucetalia valesiacae) refer to the natural condition of the grasslands.

The other two groups include the species that indicate disturbance:

3. Weeds.
4. Disturbance-tolerant species (Figure 2).

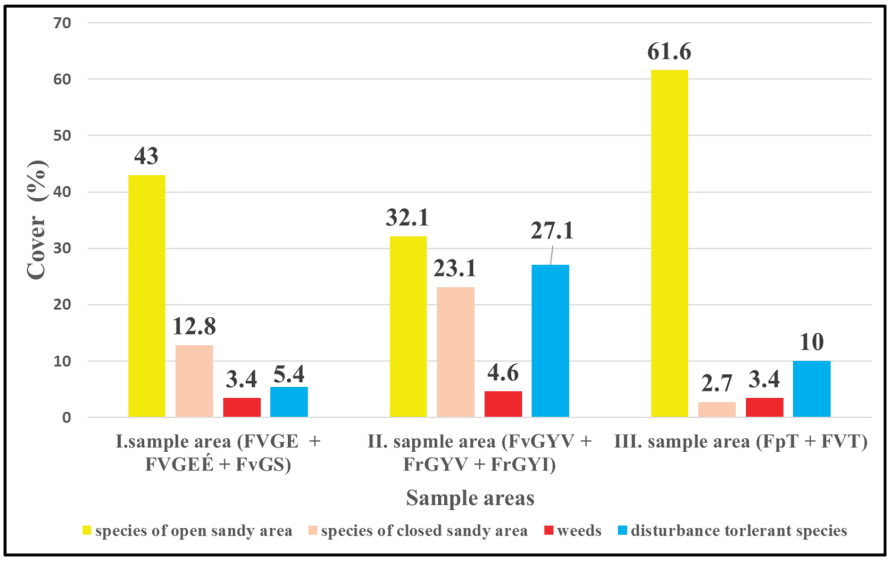


Figure 2. Comparison of the sample areas of the three locations based on the mean cover in % of species belonging to the four vegetation categories.

Table 1. The recorded species of the four coenosystematic groups.

Characteristic Species in Open Sandy Grassland		Characteristic Species in Closed Natural Sandy Grassland		Weed Species		Disturbance-Tolerant Species	
Acinos arvensis (Lam.) Dandy	*Adonis vernalis* L.	*Arabis hirsuta* (L.) Scop.	*Arabis turrita L.*	*Ambrosia artemisiifolia* L.	*Anthemis ruthenica* M. Bieb.	*Achillea pannonica* Scheele	*Anchusa officinalis* L.
Allium sphaerocephalon L.	*Alyssum alyssoides* (L.) L.	*Arrhenatherum elatius* (L.) P. Beauv. ex J. Presl & C. Presl	*Asparagus officinalis* L.	*Apera spica-venti* (L.) P. Beauv.	*Agropyron repens* (L.) P. Beauv.	*Arabidopsis thaliana* (L.) Heynh.	*Bromus tectorum* L.
Alyssum tortuosum Willd.	*Artemisia campestris* L.	*Astragalus onobrychis* L.	*Berberis vulgaris* L.	*Camelina microcarpa* Andrz. ex DC.	*Capsella bursa-pastoris* (L.) Medik.	*Calamagrostis epigejos* (L.) Roth	*Bothriochloa ischaemum* (L.) Keng
Arenaria serpyllifolia L. (* A–D: 1–20%)	*Bromus squarrosus* L.	*Bromus erectus* Huds. (A–D: 4–10%)	*Carex humilis* Leyss.	*Carduus acanthoides* L.	*Carex stenophylla* Wahlenb.	*Centaurea micranthos* (Griseb.) Hayek	*Dactylis glomerata* L. (A—D: 2–10%)
Carex liparocarpos Gaudin	*Centaurea arenaria* M.Bieb. ex Willd.	*Dianthus giganteiformis* subsp. *pontederae* (A.Kern.) Soó	*Dorycnium germanicum* (Gremli) Rikli	*Cenchrus incertus* M. A. Curtis	*Crepis rhoeadifolia* M. Bieb.	*Crataegus monogyna* Jacq.	*Cynodon dactylon* (L.) Pers.
Cerastium semidecandrum L.	*Dianthus serotinus* Waldst. & Kit.	*Euonymus europaeus* L.	*Festuca arundinacea* Schreb. (A—D: 2–8%)	*Crepis tectorum* L.	*Descurainia sophia* (L.) Webb ex Prantl	*Eryngium campestre* L.	*Erysimum diffusum* Ehrh.
Erophila verna (L.) CHEV.	*Euphorbia seguieriana* Neck.	*Festuca rupicola* Heuff. (A—D: 2–15%)	*Festuca rubra* L. (A—D: 2–10%)	*Echium vulgare* L.	*Erodium cicutarium* (L.) L'Hér.	*Euphorbia cyparissias* L.	*Festuca pseudovina* Hack. ex Wiesb. (A—D: 2–15%)
Festuca pseudovaginata Penksza (A—D: 10–25%)	*Festuca vaginata* Waldst. & Kit. ex Willd. (A—D: 8–65%)	*Filipendula vulgaris* Moench	*Helianthemum ovatum* (Viv.) Dunal	*Lamium amplexicaule* L.	*Lithospermum arvense* Thunb.	*Gagea pusilla* (F.W.Schmidt) Sweet	*Galium verum* L.
Fumana procumbens (Dunal) Gren. & Godr.	*Gypsophila fastigiata* L.	*Helictotrichon adsurgens* (Schur ex Simonk.) Conert	*Helictotrichon pubescens* (Huds.) Pilg.	*Melandrium album* (Mill.) Garcke	*Reseda lutea* L.	*Hypericum perforatum* L.	*Knautia arvensis* (L.) Coult.
Gypsophila paniculata L.	*Helichrysum arenarium* (L.) Moench	*Koeleria cristata* (L.) Pers. em. Borbás ex Domin	*Linaria genistifolia* (L.) Mill.	*Setaria viridis* (L.) P. Beauv.	*Taraxacum officinale* F. H. Wigg.	*Lotus corniculatus* L.	*Ononis spinosa* L.
Holosteum umbellatum L.	*Iris arenaria* Waldst. & Kit.	*Linum austriacum* L.	*Muscari racemosum* Mill.	*Tribulus terrestris* L.		*Ornithogalum umbellatum* L.	*Plantago lanceolata* L.
Iris pumila L.	*Koeleria glauca* (Spreng.) DC.	*Peucedanum oreoselinum* (L.) Moench (A—D: 2–15%)	*Phleum phleoides* (L.) H.Karst.			*Poa angustifolia* L. (A—D: 1–15%)	*Rumex acetosella* L.
Koeleria majoriflora (Borbás) Borbás ex Domin	*Medicago minima* (L.) Bartal.	*Polygonatum odoratum* (Mill.) Druce	*Potentilla arenaria* Borkh.			*Salsola kali* L.	*Scabiosa ochroleuca* L.
Minuartia glomerata (M.Bieb.) Degen	*Minuartia verna* (L.) Hiern	*Stachys recta* L.	*Teucrium chamaedrys* L.			*Stellaria media* (L.) Vill.	*Stipa capillata* L.
Myosotis stricta Link	*Oxytropis pilosa* (L.) DC.	*Thalictrum minus* L.	*Thesium arvense* Horv.			*Trifolium campestre* Schreb.	*Veronica arvensis* L.

Table 1. *Cont.*

Characteristic Species in Open Sandy Grassland		Characteristic Species in Closed Natural Sandy Grassland		Weed Species	Disturbance-Tolerant Species	
Plantago indica L.	*Poa bulbosa* L. (A–D: 1–10%)	*Verbascum lychnitis* L.	*Thymus glabrescens* Willd.		*Vicia angustifolia* L.	*Vicia hirsuta* (L.) Gray
Polygonum arenarium W et K.	*Sanguisorba minor* Scop.	*Veronica spicata* L.			*Vicia tetrasperma* (L.) Schreb.	
Saxifraga tridactylites L.	*Secale sylvestre* Host					
Silene conica L.	*Silene otites* (L.) Wibel					
Solidago virgaurea L.	*Stipa borysthenica* Klokov ex Prokudin (A–D: 2–25%)					
Veronica dillenii Crantz	*Vincetoxicum hirundinaria* Medik.					
Viola kitaibeliana Schult.						

* A–D: abundance–dominance values (extreme values in percentage) of the important species.

Among the study sites, the I. sample area had the lowest mean cover value. Examining all vegetation types of sample areas of the three locations together, the III. sample area, where military activity is still active, belongs mainly to the open grassland category (61.6%). At the same time, characteristic species of open sandy grassland were dominant in the I. and II. sample areas, too. The I. sample area had a quite high mean cover (43%), and it was followed by the II. sample areas with 32.1%. The closed grasslands of I. and II. sample areas were rich in species. The proportion of species of natural closed grassland was highest in the II. sample area (23.1%) but was quite high in the I. sample area (12.8%) as well.

Weeds were found with low cover (3.5–4.6%) in all three sample areas, reaching the highest proportion in the II. sample area (4.6%). The proportion of disturbance-tolerant species was low in the I. and III. sample areas. The highest mean cover value of the disturbance-tolerant species (27.1%) was found in the sown grassland areas of the II. sample area, followed by the III. sample area with around 10% and the recultivated area of the I. sample area (FvGS) with only 5.4%.

Based on the sample areas and the coenological records, it can be seen from the figure of DCA (Figure 3) that the records of the III. sample area are well distinguished. Among these, the most distinguishable are the records of the *Festucetum pseudovaginatae* vegetation type of area III (FpT). The records of the *Festucetum vaginatae* (FvT) of area III. are close to but also distinguishable from the sample areas (I. and II.) of the Little Hungarian Plain. The original sown *Festucetum vaginatae* (FvGE) and the original but burnt open *Festucetum vaginatae* (FvGEÉ) grassland records form a complete group, whereas the records of the closed grassland *Festucetum rupicolae* (FrGYV and FrGYI) are separated significantly in terms of number of species, vegetation cover, and statistical comparison.

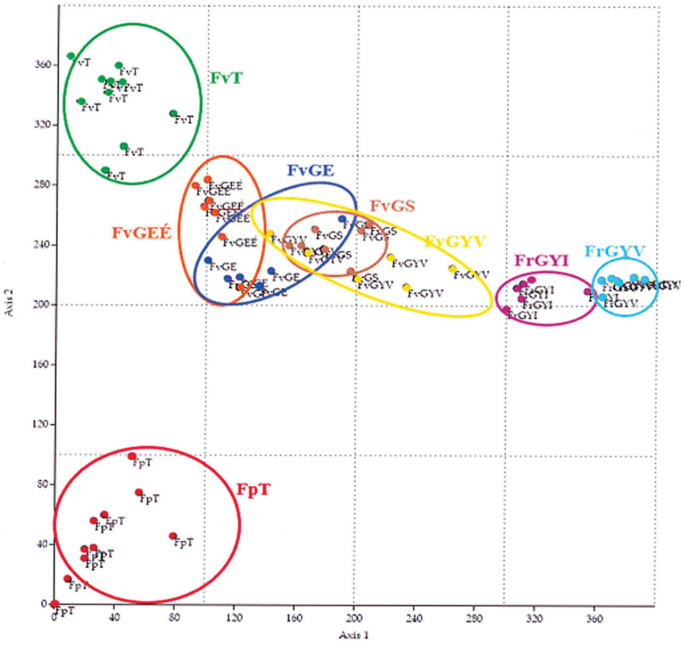


Figure 3. DCA analysis of the coenological records.

Focusing on the five areas with the vegetation type classification of *Festucetum vaginatae* association showed that the sown rampart of the I. sample area (FvGS) had the highest proportion of weed species with 10.5% (Figure 4). It was followed by the sown grassland of the II. sample area (FvGYV) with 4.2%. However, only a minimal mean cover value (0.3%) can be seen in the III. of weeds. The proportion of disturbance-tolerant species was high

in the sown rampart of the I. sample area (7.8%) (FvGS) and in the original vegetation of the I. sample area (7.3%) (FvGE), but the highest mean cover value (12%) was in the sown grassland of the II. sample area (FvGYV). The number of species that belonged to the open grassland category was generally high, around 50%, except for the vegetation type of the sown rampart of the I. sample area (29.2%) (FvGS), and it was highest in the III. sample area, where military activities are still active (54.4%). In terms of species of closed grassland, the richest were the original vegetation of the I. sample area (22.5%). It was followed by the burnt grassland of the I. sample area with 8.2% (FvGEÉ), and then the sown rampart of the I. sample area (FvGS) with 7.8%. The sown grassland of the II. sample area (FvGYV) had a lower cover value in terms of the characteristic species of closed grassland (7%), but the III. sample area (FvT) had almost no species that are characteristic of closed grasslands (0.3%).

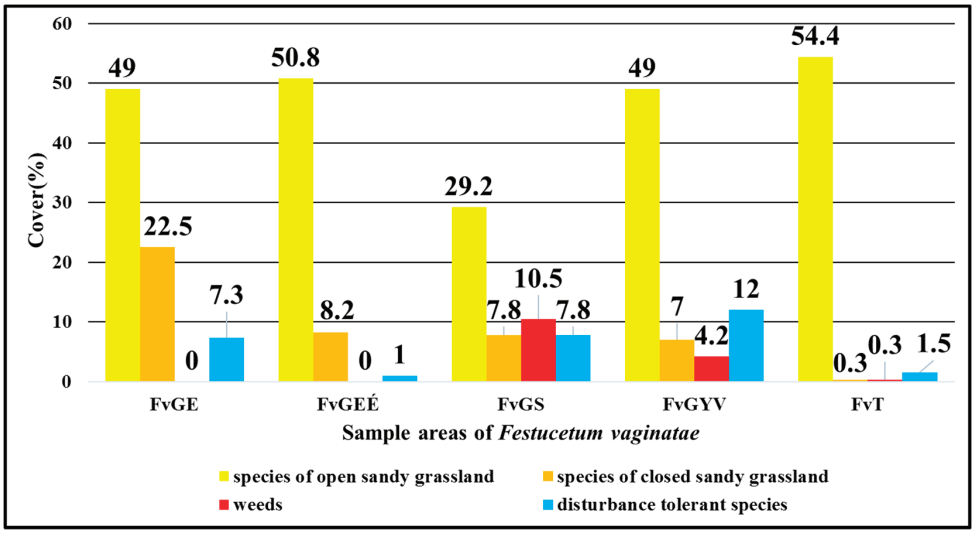


Figure 4. Comparison of *Festucetum vaginatae* vegetation types in the three sample areas based on the mean cover in % of the species belonging to the four vegetation categories.

Based on the classification analysis of the *Festucetum vaginatae* vegetation types (Figure 5), the groups of records that belonged to each sample area were organized into individual clades. The clade of the Great Hungarian Plain area in Tatárszentgyörgy (FvT) was distinctly separated from the other studied locations. Based on the clade, the two sown grasslands (FvGYS and FvGI) were close to each other, and the two areas of the Little Hungarian Plain (Gönyű, Györszentiván) were not classified by geographical separation, but by management. The lowest difference between the quadrats was shown by the sown rampart of area 1 in Gönyű (FvGS).

According to the recorded data of the *Festucetum vaginatae* vegetation type, the vegetation of Tatárszentgyörgy (III. sample area) was distinguishable from the other sample areas (Figures 2–4). The highest average number of species was found among the records of natural grassland at 23. The average number of species in the burnt area (FVGEÉ) was significantly lower at 12, and the average number of species of the III. sample area in Tatárszentgyörgy (FvT) was the lowest at 7, whereas the number of species in the manually sown rampart (FvGS) in Gönyű was 22. Similarly, the number of species in the sown grassland (FvGYV) of the II. sample area in Győrszentiván was lower at 15 (Figure 6).

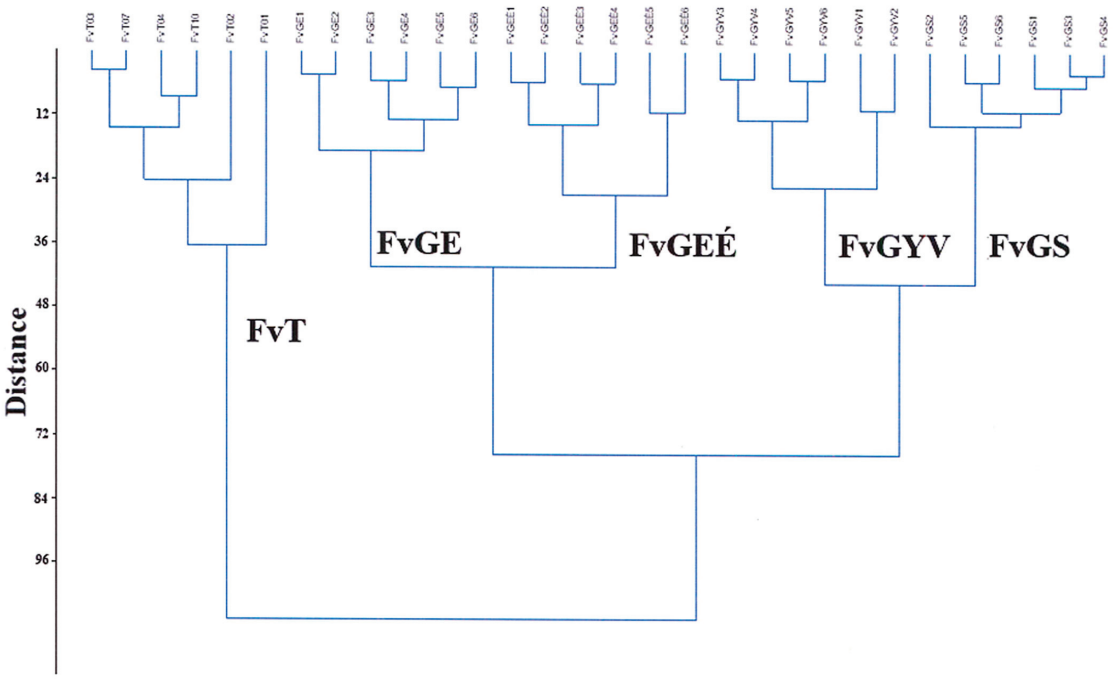


Figure 5. Hierarchical classification of the records of the vegetation type *Festucetum vaginatae* in the three sample areas.

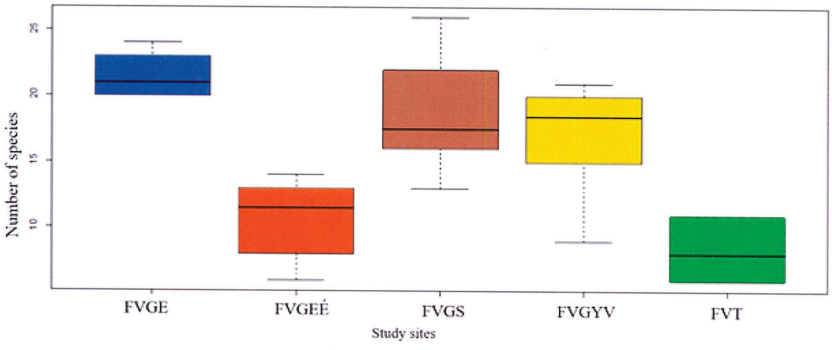


Figure 6. Number of species of the surveyed vegetation types.

4. Discussion

Among the three sample areas, the I. sample area had the lowest mean cover values in the vegetation, reflecting the fact that among the studied vegetation types, the manually sown areas with low species cover reduce the value of total cover of the area. In the Little Hungarian Plain, the abundance of species in closed grasslands is higher than the species richness in the sample area of the Great Hungarian Plain. This can be the result of the specific species composition of closed grasslands (*Poa angustifolia*, *Festuca rubra*, *Festuca arundinacea*) that were used for grassland sowing [3,45]. In addition, there is also a climatic context, because the grasslands of the Little Hungarian Plain have more mesophilic species and vegetation types, indicating Atlantic environmental conditions [1]. The reason is that the climate of the Little Hungarian Plain within the Pannonian Basin is cooler than in the Great Hungarian Plain [46].

Among the studied grasslands, the sown rampart of the I. sample area (FvGS) and the sown grassland of the II. sample area (FvGYV) are very similar to the study of sandy fallows, where the amount of target species (plant species of open sandy vegetation) is greater in older fallows than in younger fallows. Moreover, the target species that are typical in open sandy grasslands (*Festucion vaginatae*, *Corynephoretalia*, *Festucetalia vaginatae* & *rupicolae*, and *Festucetalia vaginatae* & *valesiacae*) include most of the species at the start of succession. On the contrary, in our study target species were already documented at the beginning of the restoration period [9]. The results of the study by Rehounková and Prach [16] are similar to the results of other studies on grasslands [9,22,47]. These studies suggest that the appearance of open sandy grassland species in the sandy grassland is probably a propagule-limited process, which can be influenced by the species composition of adjacent grasslands. Therefore, characteristic species could appear rapidly in our sample areas.

During the military activity, significant natural propagule sources and patches of native grassland survived from where species could grow again easily and rapidly. The fallows which do not have suitable propagule sources can provide target species that may grow very slowly. Furthermore, it can impede the growing of target species by microhabitat limitation due to the growth of perennial biomass [33,36]. Our present studies rejected the possibility of microhabitat limitation, because both fires and active military activity resulted in low numbers of species, but the occurring species were composed of characteristic plant species of open sandy grassland vegetation.

The dominant grass species (*Festuca*, *Stipa*) have made the territorial conquest. Their short generation time and faster adaptability are confirmed in the present study. Hence, they may become dominant at the start of succession, where they occupy mainly open, disturbed areas first [48]. This is due to species with a short life stage responding quickly to changes and disturbances, reacting much faster than perennial species. This result refers to natural annual species and pioneer species. The present study also confirms the results that during succession, all of these species with short life stages are replaced over time by perennials [49] due to their better competitive ability [45,47].

Looking at the species numbers, the effects of the current active military activity would indicate a decline in the vegetation. However, based on the data and the analysis of the vegetation composition, the conserved open sandy grassland in the Great Hungarian Plain (FvT) shows natural conditions. It provides the occurrence of pioneer species of the open surface. In addition, it supports the appearance and maintenance of an endemic association of *Festucetum pseudovaginatae* [50], which is unique to the Great Hungarian Plain. It contains high proportions of species of open sandy grasslands, so it is achieved to conserve the naturalness of the vegetation, although not as an intended result of the military activity.

The investigation of species that occur in critical habitats in extreme conditions is also important in the context of climate change as a major vulnerability to species extinction [51,52]. The rise in global temperature may lead to various risks [51,53], and the best prediction for the near-future is 1.5 °C (2021–2040) [54]. The climatic factors significantly influence the composition of vegetation, which reflects global climate change [55]. The vegetation of extreme arid habitats is important in terms of climate change because these habitat types will probably expand, and their conservation is an international priority [56]. In the investigated habitat type, mechanical factors also affect the vegetation [57,58], degrading the area, but nevertheless, based on the investigations, the vegetation of the III. sample area tolerated the active military activities well. The role of the dominant species in the open sandy grasslands (*Festuca vaginata* and the endemic *Festuca pseudovaginata*) might be enhanced by this condition, as endemic species can adapt better to climate change [59–63]. Based on our study, we were able to confirm that these endemic, extreme-drought-tolerant, perennial Festuca species and the studied vegetation types were well adapted to extreme drought and tolerated the extreme human disturbance, with changes in soil and climate in the micro-conditions.

In the year of our study, after the 5 years of restoration works were finished, the vegetation in the two sample areas (I. and II. sample areas) in the recultivated and sown

Little Hungarian Plain became more natural. This is due to the propagule sources near the study areas, from where the natural grassland could spread and grow. So, overall, to answer the question, the military activity indirectly supported the regeneration of the open sandy grassland vegetation of the studied area. In summary, active military activity resulted in the conservation of open grassland conditions, which might help the growth of the relatively low number of species of the potential, native, rare, and endemic open sandy grasslands of the Pannonian Basin.

5. Conclusions

Despite the previous hypothesis that military activity can cause only vegetation degradation, based on the results of our research, the activities in military areas can beneficially affect the dry, sandy grassland habitats. The military exercise areas in the open sandy grasslands of the Pannonian region played a significant role in the vegetation survival, as these areas were isolated from potentially damaging land uses that often cause their certain disappearance. Despite their relative undisturbedness, these areas may be threatened by numerous factors (invasive species, weeding, off-road motorcycling, etc.) that could destroy their remnant natural values over a few decades.

Long term surveys and well-established nature conservation management plans of the subjected areas can help to restore and maintain these valuable, endemic vegetation types. Cooperation between nature conservation and national defense agencies can define the requirements for military land use in the future and set objectives for nature conservation in the affected areas. Despite the different practices applied, all management methods were suitable for conserving and restoring the natural condition of the studied grasslands. The results of the coenological records will provide a useful part of long-term monitoring databases.

Moreover, the present work indicates that these types of grasslands in different regions around the world will tackle the challenges related to food supply requirements due to the effect of global warming that will significantly influence the production capacity of grasslands. Thus, the present results on grasslands' vegetation components and species ratios can be subject to caveats. Hence, based on the results and conclusions, the sample areas are affected by several dependent factors. With all of the aspects considered carefully, these data are suitable to support the development of nature conservation practices in the dry sandy grasslands of Hungary in the future.

Author Contributions: Conceptualization, K.P., M.B. and J.H.; methodology, K.P. and S.S.; software, P.P., C.M. and J.H.; formal analysis, Z.W. and F.S.; investigation, K.P., A.F., E.S.-F., S.S., M.F., D.B. and J.H.; writing—original draft preparation, M.B., A.F., E.S.-F., K.P., J.H. and P.C.; writing—review and editing, M.B., A.F., K.P., P.C. and J.H.; supervision, Z.W. and K.P.; funding acquisition, K.P. and Z.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by OTKA K-125423 and OTKA K-147342.

Data Availability Statement: Data are contained within the article..

Conflicts of Interest: The authors declare no conflicts of interest.

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Diversity Levels under Different Grazing Intensities in Semi-Wet Grasslands

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Abstract: The biodiversity of grasslands has been shaped by long-time interaction between natural processes and human activities, such as grazing. Traditional grazing management by animals contributes to the maintenance of high biodiversity in grasslands. However, changes in land use in recent years such as the abandonment of animal husbandry or intensification of grazing, pose a threat to grasslands biodiversity. The aim of the present study was to investigate the compositional and functional diversity in semi-humid grasslands of northern Greece under different grazing intensities. Three sites with similar vegetation and composition but different grazing intensities were selected in Taxiarchis University Forest of Mountain Holomontas. The three grazing intensities were (1) ungrazed, for more than 40 years, (2) moderate grazed, and (3) highly grazed. The ungrazed area showed an increase in vegetation cover and a decrease in bare ground. In comparison to grazed areas, the ungrazed one showed lower levels of grasses and legumes but higher abundances of woody and broad-leaved species. The highest values of all the studied diversity indices (Shannon–Wiener (H), Simpson Diversity Index, Jaccard, and species abundance) were recorded at moderate grazing intensity. Differences were recorded between the grazed and ungrazed areas in all of the quantitative traits (specific leaf area, leaf dry matter content, stem dry matter content, vegetative and reproductive plant height, leaf nitrogen, and phosphorus concentration). Protecting sensitive mountainous grasslands from both overgrazing and abandonment requires a balanced and sustainable management approach.

Keywords: grassland ecosystems; grazing intensity; diversity; cover; composition; functional traits

Citation: Avramidou, E.; Karamichali, I.; Tsiripidis, I.; Abraham, E.M. Diversity Levels under Different Grazing Intensities in Semi-Wet Grasslands. *Land* **2024**, *13*, 488. https://doi.org/10.3390/land13040488

Academic Editor: Shiliang Liu

Received: 29 January 2024
Revised: 28 March 2024
Accepted: 3 April 2024
Published: 9 April 2024



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1. Introduction

Grasslands occupy approximately 54% of terrestrial ecosystems [1,2] and are the largest natural ecosystem on earth. Grasslands in Greece represent around 40% of the total surface of the country, a number that defines them as the largest land-use resource [3]. Grasslands play a crucial role in supporting biodiversity. Specifically in Europe, 18.1% of endemic plant species are found in grasslands [4] as well as a large proportion of vertebrate and invertebrate species [5].

The biodiversity of grasslands has been shaped by the long-term impact of human activities, such as grazing [6,7]. Particularly, extensive traditional management has played an important role in the high biodiversity of Mediterranean grasslands [8]. In recent years, however, changes have been observed in the way they are managed, either due to the abandonment of animal husbandry, mainly in the mountainous areas, or due to intensification [9,10]. Those changes pose threats to their biodiversity [4].

Mediterranean grasslands, often developing in shallow soils, are highly vulnerable to both management changes and climate variations. Grazing in these ecosystems affects the structure and function of plant communities, leading to alterations in species composition.

As a result, grazing is a critical factor influencing their diversity, which serves as an indicator of their condition and stability.

The initial assumption regarding the impact of grazing intensity on floristic diversity was that species diversity increases under moderate grazing intensity. Later, Huston [11] and Kondoh [12] argued that moderate grazing intensity enhances floristic diversity in high-productivity grasslands, while reducing it in low-productivity ones. According to this hypothesis, Milchunas et al. [13] added the "grazing history" factor. Nevertheless, research on grasslands, regarding the impact of grazing intensity on floristic diversity has yielded conflicting results, such as an increased, decreased, or insufficient response [14,15]. According to the theoretical model proposed by Pulungan et al. [16], floristic diversity increases with moderate grazing intensity when (i) grazing is the primary disturbance factor for the plant community and (ii) species composing the plant community differ in their response to grazing.

Classical assessments of ecosystem floristic diversity were based on species composition and were expressed through various indices. However, plant species within a community differ in morphology, physiology, and reproduction, consequently influencing the ecosystem functions differently. This led to a shift in research towards functional diversity. According to Ma et al. [17], the floristic diversity of plants is defined as the range and dispersion of plant characteristics within a community, landscape, or ecosystem that are functionally related to growth, reproduction, and survival. It is considered a significant component of biodiversity. Recent studies have shown that the functional traits of species in a plant community are more effective tools for predicting the response of the community to disturbances such as grazing, compared to species composition. Plant functional types classify species into groups with similar combinations of functional traits, exhibiting similar reactions or sensitivities to environmental or anthropogenic disturbances [18,19]. For classifying species based on their response to grazing, a group of functional characteristics related to the distribution, establishment, and survival of species in the community has been proposed and used [20]. However, the value of these functional traits as predictive tools for the response of the plant community to grazing in different vegetation types and climatic conditions is still under investigation [21]. The results of this research indicate that, for the same functional traits, species show different responses under different environmental conditions [22]. Based on these data and for the improvement in the approach to functional diversity, Diaz et al. [22] have suggested the use of specialized functional traits for each ecosystem.

The use of functional traits to describe and classify plant species into groups based on their response to grazing, in order to predict the stability of plant communities and the functioning of ecosystems, is constantly increasing. However, research regarding the relationship between species diversity and the diversity exhibited by functional characteristics within species is limited [23].

The aim of the present study was to detect the effect of different grazing intensities on species composition and floristic and functional diversity in mountainous semi-humid grasslands of northern Greece. The research focused mainly on the effect that the abandonment of grazing has on the grassland ecosystem, as a rapid decline in livestock farming has been recorded in mountainous areas in recent decades.

2. Materials and Methods

2.1. Study Area

The research was conducted in Taxiarchis University Forest located on the southern and southwestern slopes of Mountain Cholomontas (40°23′–40°28′ N, 23°28′–23°34′ E), at an altitude of approximately 800 m. The total area of the University Forest is approximately 5.800 ha and it is estimated that approximately 4.620 ha are freely grazed mainly by goats and sheep (Management Plan of the University Forest of Taxiarchis-Vrastama 2012–2021) (Figure 1).

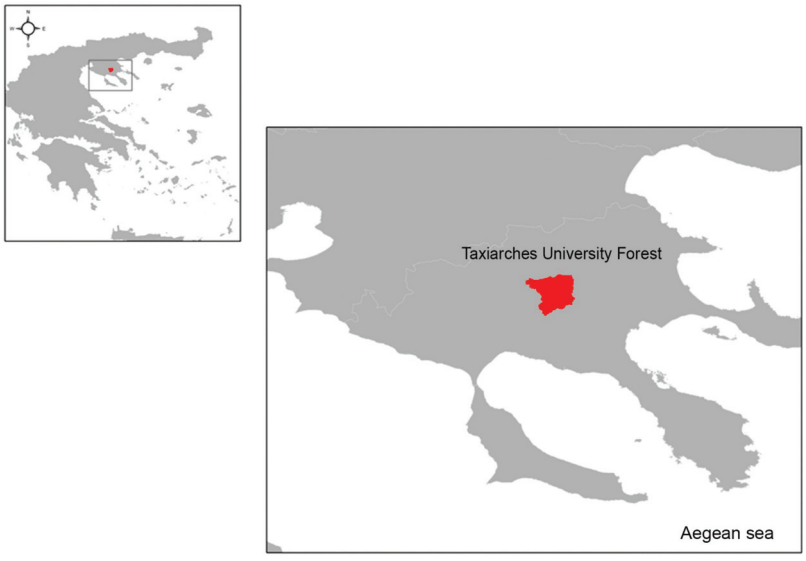


Figure 1. Map of the research area (1:10,000).

The climate of the study area is characterized as terrestrial Mediterranean with short, warm, and dry summers and mild winters. The main feature of the climate is the significant fluctuations in rainfall, with peaks in November–December and May–June. The average air temperature is 11.3 °C, the average maximum air temperature is 15.1 °C, and the average minimum air temperature is 7.1 °C. The coldest months are January and February, with an average temperature of 2.0–2.5 °C, while the warmest months are July–August, with an average temperature of 20.9–21.4 °C.

The average monthly temperatures for each year of measurements (2013, 2014, and 2015) were 12.61 °C, 12.45 °C, and 12.08 °C, respectively, and the precipitation amounts were 749.9 mm, 1320.7 mm, and 822.8 mm, respectively. However, during the months of measurements (May and June), the temperature range and precipitation varied. In 2013, the average monthly temperatures for May and June were 17.64 °C and 19.34 °C; in 2014, they were 14.7 °C and 19.01 °C and in 2015, they were 16.41 °C and 17.72 °C. Similarly, the average precipitation for the year 2013 was 88.6 mm and 65.4 mm for each month, for 2014 it was 52.3 mm and 41.3 mm for each month, and for 2015 it was 48.6 mm and 110 mm for each month. It is observed that while 2014 had the highest total precipitation, during the critical months of May–June (Supplementary Materials), it had the lowest rainfall compared to the other two years. Additionally, relatively low precipitation was recorded in the autumn and winter of 2013 (Supplementary Materials). In contrast, in 2015 (Supplementary Materials), the highest rainfall was recorded in May and June, with high rainfall also during the autumn and winter of 2014 (Supplementary Materials).

Three sites with similar vegetation and composition but different grazing intensities were identified: (1) ungrazed (forage utilization percentage = 0% for more than 40 years), (2) moderate grazed (FUP 30–50%), and (3) highly grazed (FUP > 60%) (Management Plan of the University Forest Taxiarhis–Vrastamon 2012–2021) (Figure 2). The ungrazed area belongs to a private owner and has not been grazed for more than 40 years. Each selected area for all three grazing treatments corresponds to approximately 2 hectares (Figure 2). The FUP was estimated by the Ocular estimate-by-plot method [24]. Forage utilization is defined as the amount or percentage of the current growth of forage that has been removed by grazing animals and can be applied to single plants, plant groups, or the grazing land as a whole [25].

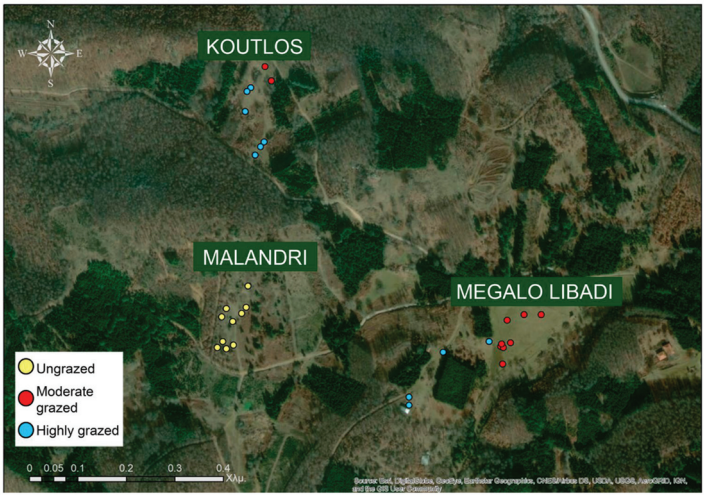


Figure 2. Map of the three grazing intensities. Each dot represents the permanent transect lines for each grazing intensity.

2.2. Vegetation Cover and Composition

In each site, 10 permanent transect lines of 25 m long were taken randomly and measurements of the cover of the vegetation were taken using the line-point method [26]. Species composition was derived from the measurements of cover, after deducting the dry matter, soil, and stones. Plant species that could not be identified in the field were collected and transported to the laboratory. Flora Europaea [27–31] and Flora Hellenica [32,33] were used to classify the collected plant samples into taxonomic units. For the nomenclature of all the taxa, papers of Dimopoulos et al. [34,35] were used as well as the database created from these papers and published on the website https://portal.cybertaxonomy.org/flora-greece/intro (accessed on 29 January 2024). For the determination of species composition, plant species were classified into grasses (Poaceae), legumes (Fabaceae), forbs, which include the herbaceous plants of all the other families, and woody species. Furthermore, the grazing effect on the cover of each dominant species in ungrazed plots (ΔGE dc) was expressed as

$$\Delta\text{GE dc} = \frac{(\text{G} - \text{UG})}{(\text{G} + \text{UG})}$$

where G and UG are the coverage percentages of the species in moderate or highly grazed areas and in the ungrazed area, respectively. As the index approaches 1, species are favored by grazing, while the smaller the index is, grazing reduces the presence of the species [36]

Also, the forage value index was calculated according to Klapp-Stahlin [37,38] after being equalized by the percentage of cover of each species. The forage value index assesses the favorability of a plant species while they grow in their natural state within the plant communities.

2.3. Floristic Diversity

The measurements of floristic diversity were made along the transects, which were used to measure cover and species composition based on the line and point method. In each plot, 10 transect lines of 20 m were placed at random locations and then 0.5×0.5 m sampling frames were used. Four frames were placed at 5 m, at 10 m, at 15 m, and at 20 m Then, on a special form, the species and the number of individuals of each species were recorded. In case that plant species could not be identified in the field, samples were collected for their identification in the Laboratory as above (Section 2.2).

Moreover, the species number, the diversity indices of Shannon–Wiener, Simpson, evenness, β-diversity, equitability, as well as a Jaccard index, were estimated for each grazing intensity.

The diversity indice of Shannon–Wiener was estimated by the following formula:

$$H = -\Sigma pi * lnpi$$

where H = diversity indice of Shannon–Wiener, Σpi = the sum of the number of species, and pi = the proportion of individuals of i species in the total sample.

The diversity indice of Simpson was estimated by the following formula:

$$D = \Sigma\left(\frac{n}{N}\right)$$

where D = diversity indice of Simpson, n = the total number of individuals of a particular species, and N = the total number of individuals of all species.

The evenness of plant species (J) was estimated by the following formula:

$$J = \frac{H}{Hmax} = \frac{H}{lnN}$$

where J = eveness, H = the value of diversity index, and Hmax = the maximum possible value of the diversity index (H), which is calculated as the logarithm of the number of species per frame in each transect.

β-diversity was estimated by the following formula:

$$\beta = \frac{ST}{Smean}$$

where ST = total number of species for each grazing intensity and Smean = the mean number of species which recorded in each transect for each grazing intensity [36,39].

The Jaccard index was estimated by the following formula:

$$Cj = j/(a + b - j)$$

where j = the number of species common to both sites, a = the number of species in site A, and b = the number of species in site B.

2.4. Functional Traits of Plant Species

The protocol described by Cornelissen et al. [40] was followed in order to measure the functional traits of species. During the field measurements, plant samples were collected in order to identify and determine species composition in the study area and at the three grazing intensities. The plant samples, after being dried, were identified based on Flora Europea [27–31]. Plant nomenclature was based on the site http://portal.cybertaxonomy.org/flora-greece/intro (accessed on 29 January 2024).

The following plant traits were investigated: specific leaf area (SLA), leaf dry matter content (LDMC), stem dry matter content (StDMC), leaf phosphorus concentration (LPC), leaf nitrogen concentration (LNC), vegetative plant height (VPH), reproductive plant height (RPH), plant defense mechanisms against grazing, life form, life cycle, and photosynthetic pathway.

Specific leaf area is the one-sided area of a fresh leaf divided by its oven-dry mass expressed in m^2kg^{-1} or correspondingly in mm^2mg^{-1} [40]. Leaf dry matter content is the dry-oven mass (mg) divided by its water-saturated fresh mass (g), expressed in mg g^{-1} [40]. Leaf nitrogen and phosphorus concentration are total amounts of N and P, respectively, per unit of dry leaf mass, expressed in mg g^{-1} [40]. Stem dry matter content is the dry-oven mass of a section of a plant's main stem divided by the mass of the same section when still fresh. It is expressed in mg mm^{-1} [40]. The vegetative and reproductive height is

the shortest distance by the upper boundary of the main photosynthetic and reproductive tissues on a plant and the ground level, expressed in meters [40].

Specific leaf area (SLA) (Section 3.3.1), leaf dry matter content (LDMC) (Section 3.3.1), stem dry matter content (StDMC) (Section 3.3.1), leaf phosphorus concentration (LPC) (Section 3.3.2), leaf nitrogen concentration (LNC) (Section 3.3.2), vegetative plant height (VPH) (Section 3.3.3), and reproductive plant height (RPH) (Section 3.3.3) are quantitative (continuous) characteristics and were measured on the experimental areas; all the other measurements were carried out in the Laboratory of Grassland Ecology of AUTH. Plant defense mechanisms against the grazing, life form, life cycle, and photosynthetic pathway (Section 3.3.4) are qualitative (categorical) characteristics and were gathered from bibliographic sources and field observations. Data collection was performed based on the species composition for each grazing intensity.

To identify the dominant herbaceous species, those that collectively accounted for 80% of the species composition on each grazing intensity were calculated. According to Garnier et al. [41], this assumption ensures a satisfactory description of ecosystem properties. Also, Grime [42] emphasizes that the functioning of an ecosystem is significantly influenced by the dominant species participating in its composition.

The leaf surface was measured using the software Image-Pro 6.3 for Windows (Figure 3). The specific program scans the area of the leaves and then it automatically calculates the perimeter, area, size, and other parameters. Before carrying out the measurements, the accuracy of the Hewlett–Packard Scanner was checked and verified by using pieces of paper of known area and similar shape and size according to the leaves that were measured.



Figure 3. Leaf surface of *A. canina* was measured using the software Image-Pro 6.3 for Windows.

2.5. Statistical Analysis

All data concerning plant cover, species composition, and all floristic diversity indices were analyzed statistically by using a two-way ANOVA in SPSS ver. 21 for Windows package [43] based on the general linear model (general linear models—GLM) with main plot factor grazing intensity and sub-factor the year of measurements. Vegetation transects for coverage and composition measurements were considered replications. If statistically significant differences were found, the averages were checked with the test of least significant difference—LSD (least significant difference) [44]. All comparisons were made at the 5% significance level ($\alpha = 0.05$).

A principal component analysis (PCA) and a dendrogram were conducted to explore the basic (dominant) components in species composition of all grazing intensities. PCA was based on the correlation matrix. For the analysis, all plant species participating in the composition of the grasslands with a percentage of 5% and above were used, covering the

three years of measurements and all grazing intensities. The statistical package vegan, part of R [45], was employed for the analysis.

3. Results

3.1. Plant Cover and Species Composition

Plant cover was lower and bare soil was significantly higher in the highly grazed area for the three years of measurements, compared to the areas with moderate grazing intensity and the ungrazed ones (*p*-values = 0.017 and 0.010). However, it must be emphasized that even in the highly grazed area, plant cover was relatively high (90.9%, 87.6%, and 94.9%) for 2013, 2014, and 2015, respectively. Grazing intensity overall did not significantly affect litter percentage (*p*-values = 0.161). However, the percentage of litter was significantly higher in the highly grazed area compared to the other two in year 2015 (Table 1).

Table 1. Means of vegetation cover (%) for three grazing intensities for the years 2013, 2014, and 2015.

	Grazing Intensity								
	2013			2014			2015		
Category	Highly grazed	Moderate grazed	Ungrazed	Highly grazed	Moderate grazed	Ungrazed	Highly grazed	Moderate grazed	Ungrazed
Plant cover (%)	90.9 b	95.9 a [1]	95.3 ab	87.6 a	91.1 a	91.3 a [1]	94.9 b	98.4 a	99 a [1]
Litter (%)	5.8 a	3.6 a	4,2 a	7.2 a	6.4 a	8.5 a	4.1 a	1.10 b	1.0 b
Soil (%)	3.3 a	0.5 b	0.5 b	5.2 a	2.50 ab	0.20 b	1.0 a	0.5 ab	0.0 b

[1] Means on the same line followed by the same letter are not statistically significantly different at the $\alpha = 0.05$ level.

The percentage of woody species was affected by grazing intensity and was significantly higher in the ungrazed area (*p*-values = 0.000), while woody species either did not participate at all or participated in a small amount to the grazed areas for the three years of measurements. The percentage of grasses and legumes was generally lower in the ungrazed area compared to grazed ones for the three years of measurements (*p*-values = 0.000 and 0.000). The percentage of forbs in the ungrazed area was statistically significantly higher (*p*-values = 0.001) in relation to the moderate and highly grazed area for the three years of the measurements. According to the results, the lack of grazing affects species composition, mainly by favoring the forbs and woody species (Table 2).

Table 2. Means of participation of functional groups (grasses, legumes, forbs, and woody) in species composition (%) for three grazing intensities for the years 2013, 2014, and 2015.

	Grazing Intensity								
	2013			2014			2015		
Functional groups	Highly grazed	Moderate grazed	Ungrazed	Highly grazed	Moderate grazed	Ungrazed	Highly grazed	Moderate grazed	Ungrazed
Grasses (%)	63.53 a [1]	64.00 a	28.90 b	59.15 a [1]	54.76 a	39.86 a	69.88 a	74.64 a [1]	50.44 b
Legumes (%)	16.05 a	11.23 a	7.50 a	12.24 a	8.44 b	0.65 c	17.65 a	4.16 b	1.72 b
Forbs (%)	20.41 b	23.17 b	40.77 a	24.61 a	34.58 a	34.19 a	12.36 b	19.36 ab	28.46 a [1]
Woody (%)	0.0 b	1.60 b	22.83 a	0.0 b	2.21 b	25.29 a	0.11 b	1.84 b	19.38 a [1]

[1] Means on the same line followed by the same letter are not statistically significantly different at the $\alpha = 0.05$ level.

PC1 and PC2 axes together accounted for 67.78% of the total variance (Figure 4). Plant species formed four distinct cycle groups. The species formed in the red circle represents the ungrazed area, the green circle is the highly grazed area, the blue circle is the moderately grazed area, and, finally, the creation of a new group in the purple circle. The presence of woody vegetation and bracken in species composition was pronounced in the ungrazed area (red color), with the dominant species being *Rubus sanctus* Schreb., *Rumex acetosella* L., and *Pteridium aquilinum* (L.) Kuhn. subsp. *aquilinum*. Bracken (*Pteridium aquilinum* (L.) Kuhn.

subsp. *aquilinum*) contributed to the composition of the ungrazed area (average) by 2.48%, 4.09%, and 5.21% for the years 2013, 2014, and 2015, respectively, while woody vegetation contributed to the composition of the ungrazed area by 22.83%, 25.29%, and 19.38% for the years 2013, 2014, and 2015, respectively. Additionally, other species with a strong presence in the ungrazed area included *Agrostis canina* L. subsp. *canina*, *Thymus sibthorpii* Benth., *Anthoxanthum odoratum* L., and *Bromus squarrosus* L. subsp. *squarrosus*. Dominant species in moderate grazing intensity were *Vulpia muyros* (L.) C.C. Gmel., *Chrysopogon gryllus* (L.) Trin., *Bromus hordeaceous* L., *Festuca ovina* aggr., and *Rumex acetosella* L., while those recorded in the high grazing intensity were *Cynodon dactylon* (L.) Pers., *Lotus corniculatus* L., *Plantago lanceolata* L., and *Lolium perenne* L.

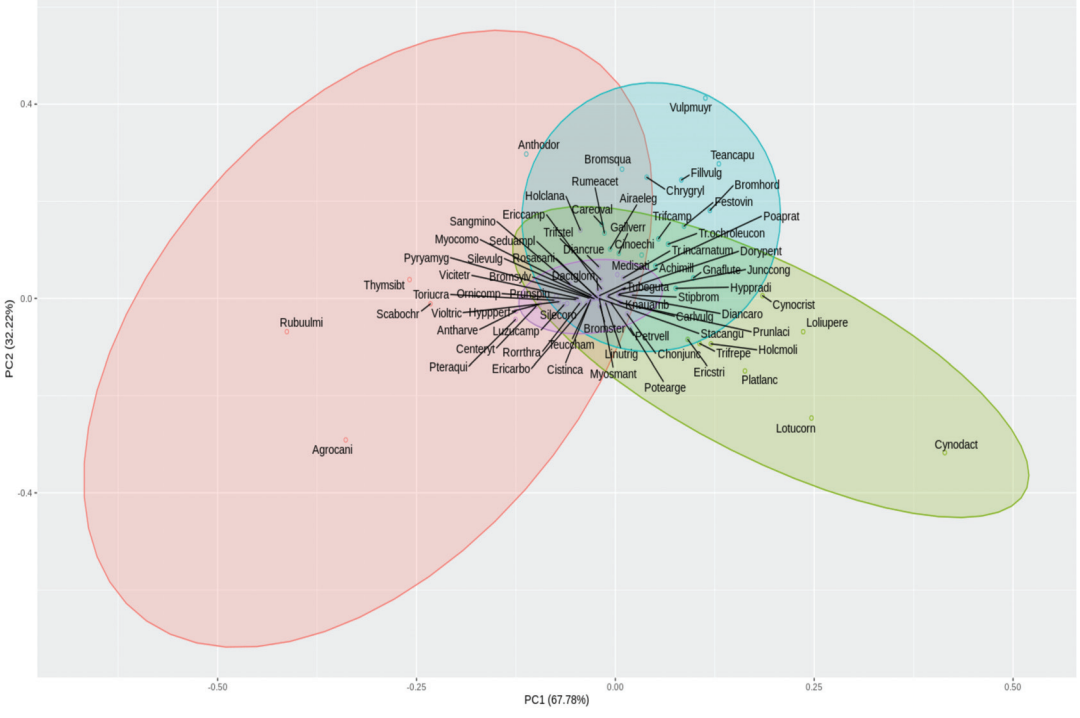


Figure 4. Principal component analysis (PCA) for the three grazing intensities based on species composition with a participation rate of at least 5%. PC1 and PC2 axes together accounted for 67.78% of the total variance. Plant species formed four distinct cycle groups. The species formed in the red circle represent the ungrazed area, the green circle is the highly grazed area, the blue circle is the moderately grazed area, and, finally, the creation of a new group in the purple circle. Antodor: *Antoxanthum odoratum* L., Vulpmuyr: *Vulpia muyros* (L.) C.C. Gmel., Bromsqua: *Bromus squarosus* L., Bromhord: *Bromus hordeaceous* L., Bromster: *Bromus sterilis* L., Rumeacet: *Rumex acetosella* (L.), Holclana: *Holcus lanatus* L. subsp. *lanatus*, Holcmoli: *Holcus mollis* subsp. *mollis*, Sangmino: *Sanguisorba minor* Scop, Ericamp: *Eringium campestre* L., Careoval: *Carex ovalis* Gooden, Airaeleg: *Aira elegans* Roem. and Schult, Chrygryl: *Chrysopogon gryllus* (L.) Trin., Fillvulg: *Fillipendula vulgaris* Moench., Teancapu: *Taeniatherum caput-medusae* (L.) Nevski, Trifcamp: *Trifolium campestre* Schreb., Trifochr: *Trifolium ochroleucon* Huds., Trifinca: *Trifolium incarnatum* L., Trifrepe: *Trifolium repens* L., Trifstel: *Trifolium stellatum* L., Festovin: *Festuca ovina* aggr., Poaprat: *Poa pratensis* L., Myossylv: *Myosotis sylvatica* Hoffm., Pyryamyg: *Pyrus spinosa* Forssk., Thymsibt: *Thymus sibthorpii* Benth., Rubuulmi: *Rubus sanctus* Schreb., Agrocani: *Agrostis canina* L. subsp. *canina*, Scabochr: *Scabiosa ochroleuca* L., Silevulg: *Lychnis vulgaris* (L.) Desr., Lotucorn: *Lotus corniculatus* L., Cynodact: *Cynodon dactylon* (L.) Pers., Lolipere: *Lolium perenne* L., Cynocris: *Cynosurus cristatus* L., Platlanc: *Plantago lanceolata* L.,

Potearge: *Potentilla argentea* L., Myosmant: *Myosotis sylvatica* Hoffm., Cistinca: *Cistus creticus* L. subsp. *creticus*, Ericarbo: *Erica arborea* L., Pteraqui: *Pteridium aquilinum* (L.) Kuhn. subsp. *aquilinum*, Vicitetr: *Vicia tetrasperma* (L.) Schreb., Ericstri: *Euphrasia stricta* J.F. Lehm, Seduampl: *Sedum amplexicaule* subsp. *tenuifolium* (Sm.) Greuter, Rosacani: *Rosa canina* L., Bromsylv: *Brachypodium sylvaticum* (Huds) P. Beauv. subsp. *sylvaticum*, Toriucra: *Torilis ucranica* Spreng., Violtric: *Viola tricolor* L. auct. Fl. graec, Antharve: *Anthemis arvensis* L., Centeryt: *Centaurium erythraea* Rafm., Rorrthra: *Rorripa thracica* (Friseh) Fritch., Luzucamp: *Luzula campestre* (L.) D.C. subsp. *campestris*, Hyppperf: *Hypericum perforatum* L., Ornicomp: *Ornithopus compressus* L., Prunspin: *Prunus spinosa* L., Silecoro: *Lychnis coronaria* (L.) Desr., Teuccham: *Teucrium chamaedrys* L. subsp. *chamaedrys*, Dactglom: *Dactylis glomerata* L., Diancrue: *Dianthus cruentus* Griseb., Gallverr: *Gallium verum* L. subsp. *verum*, Cinoechi: *Cynosurus echinatus* L., Medisati: *Medicago sativa* (L.), Achimill: *Achillea millefolium* L., Gnaflute: *Helichrysum luteoalbum* (L.) Rchb., Stipbrom: *Achnatherum bromoides* (L.) P. Beauv., Tubeguta: *Tuberaria guttata* (L.) Fourr, Knauambi: *Knautia ambigua* Boss and Orth., Linutrig: *Linum trigynum* L., Petrvelu: *Petrorhagia velutina* (Guss) P.W., Carlvulg: *Carlina vulgaris* L. subsp. *vulgaris*, Chonjunc: *Chondrilla juncea* L., Stacangu: *Stachys angustifolea* M. Bieb., Prunlaci: *Prunella laciniata* (L.) L., Diancaro: *Daucus carota* L., Hyppradi: *Hypochaeris radicata* L., Junccong: *Juncus conglomeratus* L., Dorypent: *Dorycnium germanicum* (Gremli) Rikli.

The species that are associated with the fourth group (purple circle) mainly comprised forbs (*Moenchia mantica* L. Bartl., *Linum trigynum* L., *Potentilla argentea* L., *Chondrilla juncea* L., *Stachys angustifolia* M. Bieb., *Carlina vulgaris* L. subsp. *vulgaris*, *Knautia ambigua* Boss and Orth., *Daucus carota* L., *Juncus conglomeratus* L., *Achillea millefolium* L., *Dianthus cruentus* Griseb., *Sedum amplexicaule* subsp. *tenuifolium* (Sm.) Greuter, *Myosotis sylvatica* Hoffm., *Lychnis coronaria* (L.) Desr, *Torilis ucranica* Spreng., *Viola tricolor* L. auct. Fl. graec, *Hypericum perforatum* L., *Centaurium erythraea* Rafm, *Rorripa thracica* (Friseh) Fritch., *Teucrium chamaedrys* L. subsp. *chamaedrys*, and *Cistus creticus* L. subsp. *criticus*) in ungrazed and moderately grazed area. The listed species included in the above grazing intensities and grasses, such as *Bromus sterilis* L., *Achnatherum bromoides* (L.) P. Beauv., *Dactylis glomerata* L., and *Luzula campestris* (L.) D.C.; legumes, such as *Dorycnium germanicum* (Gremli) Rikli, *Trifolium incarnatum* L., *Medicago sativa* (L.), *Trifolium stellatum* L., *Ornithopus compressus* L., and *Vicia tetrasperma* (L.) Schreb.; and some woody species, like *Rosa canina* L., *Pyrus spinosa* Forssk, *Vicia tetrasperma* (L.) Schreb., *Prunus spinosa* subsp. *dasyphylla* (Schur) Domin, and *Erica arborea* L.

Out of these species, 12 are common in all three years of measurements, namely *D. cruentus* Griseb., *T. stellatum* L., *D. glomerata* L., *L. coronaria* (L.) Desr, *B. hordeaceus* L., *P. spinosa* subsp. *dasyphylla* (Schur) Domin, *O. compressus* L., *V. tricolor* L. auct. Fl. graec, *H. perforatum* L., *R. thracica* (Friseh) Fritch., *E. arborea* L., and *T. chamaedrys* L. (Supplementary Materials).

The results of the hierarchical clustering analysis are shown in Figure 5. The first group represents the ungrazed area for the years 2013, 2014, and 2015, suggesting that species composition for those years was similar and formed a distinct cluster. The second group primarily includes data from the years 2013 and 2014 based on moderate grazing intensity. This also indicates that species composition in these two years and under moderate grazing was similar and formed another distinct cluster. The third group mainly comprises data from the years 2013 and 2014 according to high grazing intensity. Similar to the previous group, this suggests that species composition in these two years and under high grazing intensity was similar and formed a separate cluster. The last group represented data from the year 2015, including both high and moderate grazing intensities.

Table 3 shows the effect of grazing intensity on the plant cover of dominant species of the ungrazed area (ΔGE dc) for the three years of measurements (mean value). According to the results, *C. gryllus* (L.) Trin and *L. corniculatus* L. are affected differently in the two grazing intensities. *C. gryllus* (L.) Trin. increases its presence in moderate grazing intensity while it decreases its presence in high grazing. In contrast, *L. corniculatus* L. decreases under

moderate grazing and increases under high grazing intensity. *A. canina* L. subsp. *canina* and *A. odoratum* L. decrease as grazing intensity increases, while all other species increase.

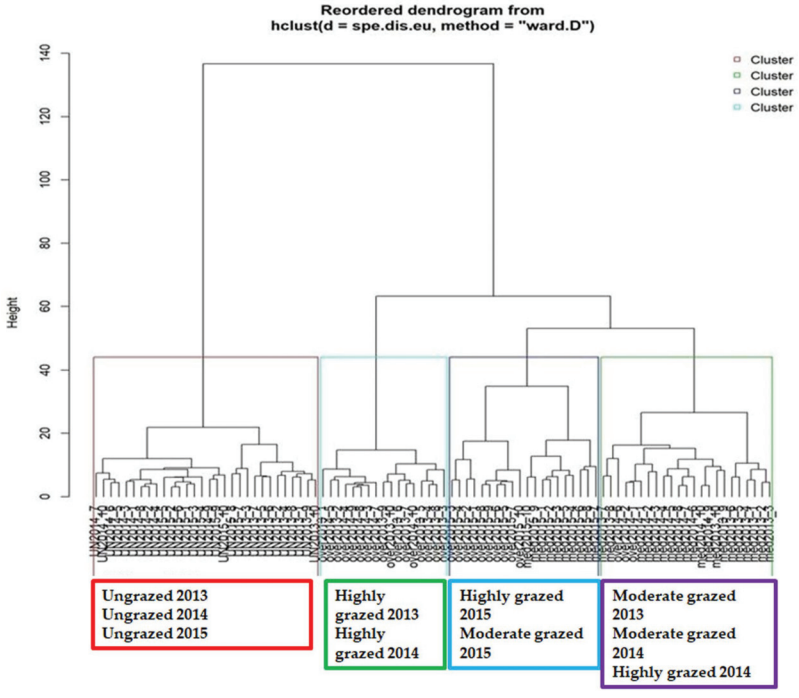


Figure 5. Dendrogram depicting the distance of plant species that participated in species composition at a rate of at least 5% for the three grazing intensities.

Table 3. Effect of grazing intensity on dominant species in the ungrazed area for the three years of measurements (2013, 2014, and 2015).

	ΔGEdc(M) [1]	ΔGEdc(G)
Chrysopogon gryllus (L.) Trin.	0.97	−0.54
Agrostis canina L. subsp. *canina*	−0.33	−0.82
Plantago lanceolate L.	0.72	0.82
Festuca ovina aggr.	0.85	0.74
Bromus hordeaceous L.	0.94	0.63
Trifolium repens L.	0.98	0.83
Cynodon dactylon (L.) Pers.	1.00	1.00
Lotus corniculatus L.	0.15	0.96
Lolium perenne L.	1.00	1.00
Anthoxanthum odoratum L.	−0.23	−0.61
Cynosurus cristatus L.	1.00	1.00

[1] G and UG are the coverage percentages of the species in the grazed and in the ungrazed area, respectively.

Table 4 presents the forage value index (FVI). The forage value index is used in order to assess the quality of forage for grazing animals. The highest FVI values are observed at high grazing intensity for the years 2013 and 2015. This suggests that, in these years, the vegetation provides the most favorable forage for animals. In contrast, in the year 2014, the highest FVI was associated with moderate grazing intensity, indicating that the vegetation in this area consisted of species with the highest quality for grazing animals. However, when you aggregate the data for the three years of measurements, FVI is the same (4.9) for both moderate and high grazing intensity implying that, when taking into account the

overall forage quality across these three years, both moderate and high grazing intensity areas offer similar forage quality.

Table 4. Forage value index (FVI) for the three grazing intensities and the three years of measurements.

	Year of Measurements			
Grazing intensity	2013	2014	2015	Means
Ungrazed	3.1	2.8	3.6	3.2
Moderate grazed	4.7	5.0	4.9	4.9
Highly grazed	5.0	4.2	5.4	4.9

3.2. Floristic Diversity

Floristic diversity according to the Shannon–Wienner index was statistically significantly higher in moderate grazing intensity for the years 2013 and 2015 compared to ungrazed and highly grazed intensities (p-values = 0.000 and 0.002). In 2014, there were no statistically significant differences (p-value = 0.900) between the three grazing intensities (Table 5), although there was a tendency for higher floristic diversity in the moderate grazing intensity. The differences that exist between years can be attributed to different weather conditions (temperature and rainfall), which affect species composition and floristic diversity.

Table 5. Means of floristic diversity (Shannon–Wiener index) for the three grazing intensities for the years 2013, 2014, and 2015.

	Year of Measurements			
Grazing intensity	2013	2014	2015	Means
Ungrazed	1.40 a	1.46 a [1]	1.62 a [1]	1.49 a [1]
Moderate grazed	1.78 b	1.51 a	1.87 b	1.72 b
Highly grazed	1.45 a	1.50 a	1.85 b	1.60 b
Means	1.56 b	1.49 b	1.78 a [1]	

[1] Means on the same column followed by the same letter are not statistically significantly different at the $\alpha = 0.05$ level.

As it concerns the Simpson index, floristic diversity was statistically significantly higher in the moderate grazing intensity (p-values = 0.000, 0.015, and 0.005) for all years of the measurements compared to the other two grazing intensities, in which the differences were also statistically significant for the year 2013, while in the years 2014 and 2015, the two other grazing intensities did not differ statistically significantly from each other (Table 6). Simpson's index, regardless of grazing intensity, had the lowest value in 2015 compared to the other two years. Indicatively, the closer the Simpson index is to zero, the greater the diversity tends to be.

Table 6. Means of plant diversity (Simpson index) for the three grazing intensities for the years 2013, 2014, and 2015.

	Year of Measurements			
Grazing intensity	2013	2014	2015	Average
Ungrazed	0.37 a	0.33 a [1]	0.27 a	0.32 a [1]
Moderate grazed	0.23 c	0.26 b	0.22 b	0.24 b
Highly grazed	0.28 b	0.28 a	0.21 b	0.26 b

[1] Means on the same column followed by the same letter are not statistically significantly different at the $\alpha = 0.05$ level.

Eveness (J) showed the highest value at high grazing intensity for all three years and differed statistically significantly (p-values = 0.002, 0.004, and 0.004) (Table 7). The closer to

one the value of evenness is, the more similar the ecosystem is considered to be, in terms of plant species.

Table 7. Means of evenness of plant species (J) in the three grazing intensities for the years 2013, 2014, and 2015.

	Year of Measurements			
Grazing intensity	2013	2014	2015	Average
Ungrazed	0.72 a	0.73 a [1]	0.79 a [1]	0.75 a [1]
Moderate grazed	0.75 a	0.72 a	0.79 a	0.75 a
Highly grazed	0.80 b	0.80 b	0.82 b	0.81 b
Average	0.76 ab	0.75 a [1]	0.80 b	

[1] Means on the same column followed by the same letter are not statistically significantly different at the $\alpha = 0.05$ level.

The number of species (S) and individuals per plot (N) were statically significantly higher in moderate grazed areas compared to highly grazed and ungrazed ones for the three years of measurements (Table 8) (p-values (S) = 0.000, 0.003 and 0.001, p-values (N) = 0.000, 0.000 and 0.022). Additionally, in 2015, the number of species and individuals of the species per plot showed higher values compared to years 2013 and 2014 for all grazing intensities.

Table 8. Means of the number of species (S) and number (N) of individuals per plot for the grazing intensities for the years 2013, 2014, and 2015.

	Year of Measurement			
Grazing intensity	2013	2014	2015	Mean
Ungrazed	7.48 (62) b 64.06 b	7.95 (46) ab 51.45 b	8.55 (44) b 80.75 b	7.99 b 65.42 b
Moderate grazed	11.40 (56) a 88.08 a	8.83 (74) a 71.83 a	11.28 (48) a 101.93 a	10.50 a 87.28 a
Highly grazed	6.45 (29) b 51.13 c	6.85 (41) b 59.70 b	9.70 (39) b 93.12 ab	9.84 a 91.93 a
Mean	8.44 b 67.76 b	7.87 b 60.99 b	9.84 a 91.93 a	

Means on the same column followed by the same letter are not statistically significantly different at the $\alpha = 0.05$ level. Number of species per plot. Number of individuals of species per plot.

The Jaccard similarity index for the years 2013, 2014, and 2015 is presented in Table 9. Moderate grazing intensity had a higher similarity with high grazing intensity for the three years of measurements, while the ungrazed and the highly grazed had the lowest similarity for the years of measurements. During the years 2014 and 2015, the moderate grazed area showed high uniformity in species composition with the highly grazed area.

Table 9. The Jaccard similarity index between different grazing intensities (ungrazed, moderately grazed, and highly grazed) for years 2013, 2014, and 2015.

2013	**Ungrazed**	**Moderate Grazed**	**Highly Grazed**
Ungrazed	-	0.396	0.22
Moderate grazed	0.396	-	0.392
Highly grazed	0.22	0.392	-
2014	Ungrazed	Moderate Grazed	Highly Grazed
Ungrazed	-	0.367	0.278
Moderate grazed	0.367	-	0.588
Highly grazed	0.278	0.588	-
2015	Ungrazed	Moderate Grazed	Highly Grazed
Ungrazed	-	0.397	0.338
Moderate grazed	0.397	-	0.631
Highly grazed	0.338	0.631	-

As it concerns β-diversity for the three years of measurements, the ungrazed and moderate grazed area had a statistically significantly higher value compared to the highly grazed area (*p*-values = 0.001, 0.001, and 0.001) (Table 10).

Table 10. Means of β-diversity at the three grazing intensities for the three years of measurements (2013, 2014, and 2015).

	Year of Measurements		
Grazing intensity	2013	2014	2015
Ungrazed	5.36 a	5.69 a	5.99 a
Moderate grazed	4.94 a	5.74 a	5.18 a
Highly grazed	3.53 b	4.71 b	4.09 b

Means on the same column followed by the same letter are not statistically significantly different at the $\alpha = 0.05$ level.

3.3. Functional Traits of Plant Species

3.3.1. Specific Leaf Area, Leaf Dry Matter Content, and Shoot Dry Matter Content

Specific leaf area (SLA) was statistically higher in the ungrazed area compared to grazed ones (Table 11). On the other hand, leaf dry matter content (LDMC) was statistically lower in the ungrazed area compared to the areas that were grazed. Stem dry matter content (StDMC) presented its higher value in the moderately grazed area compared to the ungrazed and highly grazed areas but these differences were not statistically significant (Table 11).

Table 11. Means of specific leaf area (mm^2/mg) (SLA), leaf dry matter content (mg/g) (LDMC), and shoot dry matter content (mg/g) (StDMC) of the dominant species for the three grazing intensities.

Grazing Intensity	**SLA**	**LDMC**	**StDMC**
Ungrazed	345.40 a	65.66 b	327.30 a
Moderate grazed	220.74 b	339.42 a	352.24 a
Highly grazed	225.55 b	322.70 a	344.67 a

Means on the same column followed by the same letter are not statistically significantly different at the $\alpha = 0.05$ level.

3.3.2. Vegetative and Reproductive Height

Vegetative height (VH) and reproductive height (RH) were higher in the ungrazed area compared to the grazed ones. The lowest value was recorded in the highly grazed area (Table 12).

Table 12. Means of vegetative (VH) and reproductive (RH) plant height (cm) of dominant species for the three grazing intensities.

Grazing Intensity	VH	RH
Ungrazed	26.66 a	41.70 a
Moderate grazed	14.90 b	38.84 a
Highly grazed	2.19 b	8.71 b

Means on the same column followed by the same letter are not statistically significantly different at the $\alpha = 0.05$ level.

3.3.3. Leaf Nitrogen and Phosphorus Concentration

In the ungrazed area, the leaf nitrogen concentration (LNC) was 1.24 mg/g, in the moderately grazed it was 1.29 mg/g, and in the highly grazed it was 1.7 mg/g but they did not differ statistically. Similarly, leaf phosphorus concentration (LPC) did not statistically differ among the grazing intensities (Table 13).

Table 13. Leaf nitrogen (LNC) and phosphorus (LPC) concentration (mg/g) at the three grazing intensities.

Grazing Intensity	N	LNC	LP
Ungrazed	10	1.24 a	0.58 a
Moderate grazed	10	1.30 a	0.51 a
Highly grazed	10	1.70 a	0.76 a
Mean	10	1.35	0.57

Means on the same column followed by the same letter are not statistically significantly different at the $\alpha = 0.05$ level.

3.3.4. Qualitative (Categorical) Traits

The biotic form, life cycle, and photosynthetic pathway were the qualitative traits of the dominant species that were measured. The biotic forms of the dominant species included therophytes, hemicryptophytes, phanerophytes, and hamaephytes. Figure 6 depicts the number of groups for each of the three different grazing intensities. In the ungrazed area, all groups appeared but hemicryptophytes were the most abundant consisting of the species *L. campestris*, *A. canina* L. subsp. *canina*, *P. lanceolate* L., *S. minor* Scop., and *H. perforatum* L. Phanerophytes had the lowest participation with the species *R. sanctus* Schreb. Increased grazing intensity had as a result, especially in the moderate grazed area, namely the disappearance of phanerophytes, while the hemicryptophytes increased in number (10), and also the therophytes (9). In highly grazed areas, hemicryptophytes were the biotic form that was only found and was compromised of the species *P. lanceolate* L., *H.* L. subsp. *lanatus*, *A. canina* L. subsp. *canina*, *L. perenne* L., *C. dactylon* (L.) Pers, *L. corniculatus* L., and *C. cristatus* L.

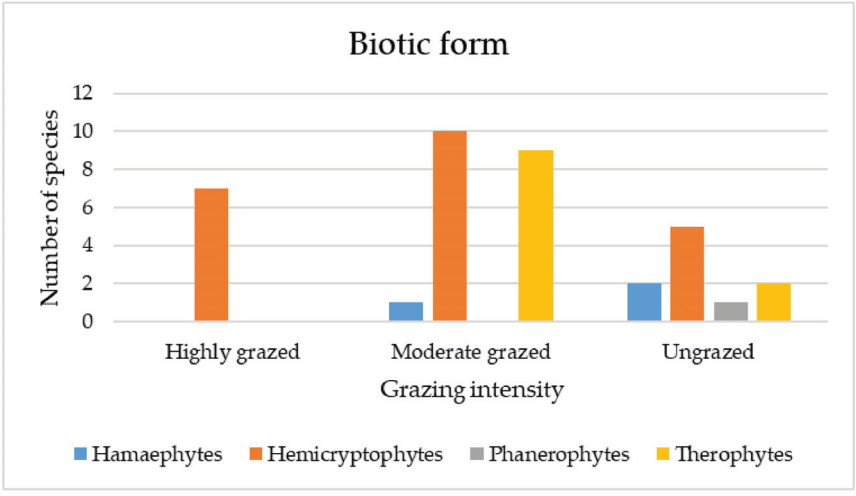


Figure 6. Distribution of species according to their biotic form.

In total, 1 out of the 10 species (*A. arvensis* L. and *T. gutata* (L.) Fourr) followed the annual life cycle in the ungrazed area, 9 out of 20 species in the moderate grazed area (*T. incarnatum* L., *T. ucranica* Spreng, *G. verum* L., *C. cristatus* L., *V. muyros* (L.), *B. hordeaceous* L., *L. trigynum* L., *C. echinatus* L., and *G. luteoalbum* (L.)), and 1 out of 7 species (*C. cristatus* L.) in the highly grazed area (Figure 7).

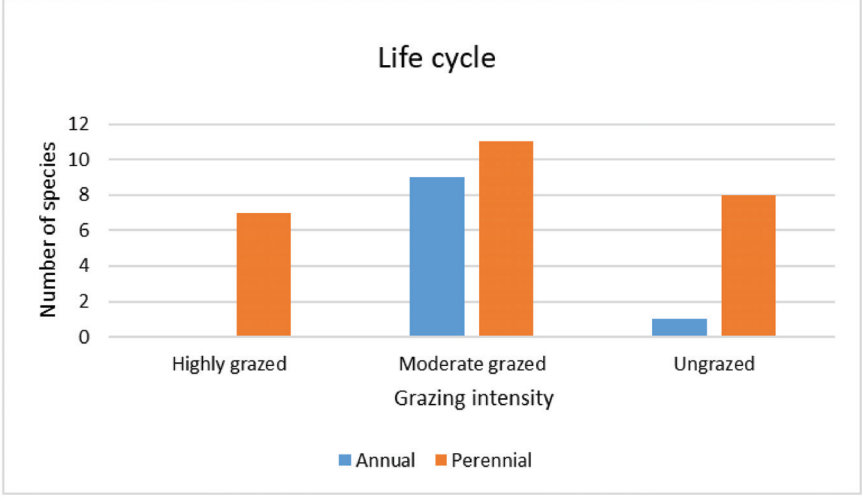


Figure 7. Distribution of species according to their life cycle.

The majority of species followed the C3 photosynthetic pathway, while only two grasses, *C. gryllus* (L.) Trin. And *C. dactylon* (L.) Pers., followed the C4 photosynthetic pathway (Figure 8).

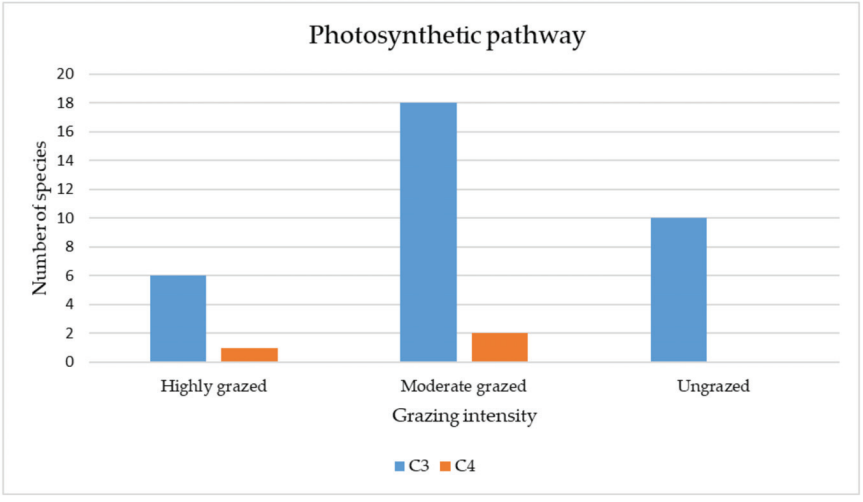


Figure 8. Distribution of species according to their photosynthetic pathway.

Also, the majority of species presented some defense mechanisms against grazing mainly by hairs, spines, and felt at all the grazing intensities (Figure 9).

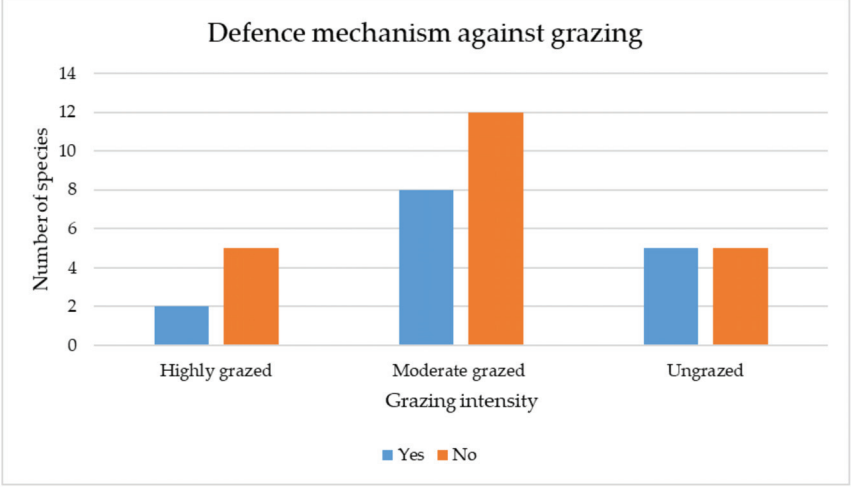


Figure 9. Distribution of species according to their defense mechanism against grazing.

4. Discussion

4.1. Plant Cover and Species Composition

The percentage of plant cover and bare soil was affected by grazing intensity and the year of measurements. The lowest percentage of plant cover and the highest of bare soil were recorded in the area that was highly grazed for all the years of measurements. Additionally, the lowest percentage of plant cover was recorded in 2014, the second year of measurements. The year effect is probably related to the variation in weather conditions from year to year, mainly in temperature and precipitation, during the growing season. From this point of view, 2014 was the year with the lowest precipitation in this particular period (May and June) (Supplementary Materials). Consequently, high intensity of grazing reduces plant cover and this negative effect is amplified in periods of drought.

Souther et al. [46], in a study that was conducted in semi-humid grasslands in Colorado considering how the long-term effect of grazing affects plant cover, concluded the same results as in the present study. In a meta-analysis by Eldridge et al. [47], conducted with data from grasslands of arid, semi-arid, humid, and semi-humid regions, concluded that the effect of grazing on plant cover was strong in arid and semi-arid areas, while it was relatively mild in humid and semi-humid areas. Determinant factors for the intensity of the grazing effect were the weather conditions and especially the variation in the percentage of precipitation from year to year. This is also confirmed in the present study. Even in highly grazed areas, plant cover was particularly high. Additionally, the smooth relief of the area may also have contributed to this.

The year of measurements and the intensity of grazing affected species composition in grasslands in the study area. Regarding the effect of years, the high amount of precipitation during the months of May and June 2015 combined with the high amount of precipitation during the autumn and winter of the previous year, seems to favor grasses, whose participation rate increased at all grazing intensities (Supplementary Materials). On the contrary, in the same year, the percentage of forbs in species composition decreased. It appears that there is a consistent pattern where species composition in the year 2015, regardless of grazing intensity (high or moderate), is similar. This might indicate that species composition or ecological factors in 2015 led to vegetation that was less affected by grazing intensity compared to the patterns observed in 2013 and 2014. Weather conditions are varying among the years and are considered a factor that strongly influences species abundance and composition [47,48]. Additionally, weather conditions have a more decisive role than grazing in the species composition of certain grassland ecosystems [48]. However, there are reports according to which weather conditions had no effect on species composition [49].

The decline in woody species in areas grazed by goats, such as in the study area, is expected. Goats generally prefer woody species [50–52], which participate in high percentages in their diet [53]. A similar decrease in woody species participation with the increase in grazing intensity is reported in both semi-arid [54–56] and semi-humid [57] grasslands. Grasses appear to be favored in the grazed areas, while forbs are favored in the long-term ungrazed areas. Legumes generally had low rates of participation in species composition, with a lower presence in the ungrazed area compared to the grazed one. Souther et al. [46] also reported similar results with the absence of grazing in semi-humid grasslands in Colorado about increasing forbs and also Scott-Shaw and Morris [58] in South Africa. On the contrary, in different geographical areas and climatic zones, it is reported that as grazing intensity increases, forbs increase and grasses decrease [59–62]. The dominance of grasses in the grazed areas in the present study (Table 2) could be attributed to a combination of factors related to the diet of the grazed animals (sheep and goats), the resistance of plant species to grazing, and their interactions with other species of the plant community and also with ecological factors such as soil properties. Additionally, in a study conducted by Abraham et al. [63] in the same area, it is reported that grass abundance is associated with high soil N content, which is also associated with high grazing intensity [64].

As they concern species response index to grazing (Table 3), the results showed that *A. canina* L. subsp. *canina* and *A. odoratum* L. decrease as grazing intensity increases, *C. dactylon* L. (Pers.), *L. perenne* L., *C. cristatus* L., *L. corniculatus* L., *P. lanceolata* L. and *T. repens* L. are favored by grazing, while *C. gryllus* (L.) Trin. is favored in moderate grazing intensity but decreased in high grazing intensity. *L. perenne* L. is concerned as a trampling and grazing tolerant species [65,66] as is *C. cristatus* L. [66]. Also, *C. dactylon* (L.) Pers. has creeping growth and is reproduced by stolons and rhizomes, characteristics that confer resistance to grazing [67]. Regarding *A. canina* L. subsp. *canina*, there is no literature related to its resistance to grazing. Among *Agrostis* species, *A. stolonifera* is reported as resistant to grazing [66] while *A. capillaris* is reported as susceptible [65] and/or resistant [68,69].

L. corniculatus L., *P. lanceolata* L., and *T. repens* L. have morphological and reproductive characteristics that make them resistant to grazing.

Changes in species composition occur through grazing selectivity, which is generally reported to lead to a reduction in desirable grazing species and their replacement by other less desirable or undesirable species [59,60].

4.2. Floristic Diversity

Moderate grazing intensity appears to enhance floristic diversity in semi-humid grassland ecosystems according to the results of the present study. Similar results were reported by Torok et al. [70], in a study carried out in different types of grassland ecosystems in Hungary. The Shannon index and the number of species had their highest value at moderate grazing intensity.

In a study carried out in a steppe in China by Ren et al. [71], it was reported that floristic diversity based on the Shannon–Wienner index was not affected by grazing intensity. Skornik et al. [72], in a study conducted in grasslands in the Northern Adriatic at four grazing intensities, recorded the highest number of species at light grazing intensity and the lowest at high grazing intensity. On the other hand, according to Zhang et al. [73], floristic diversity based on the Simpson index increased with increasing grazing intensity in alpine grassland ecosystems in Qinghai Province, China. Similarly, Haarmeyer et al. [74] documented an increase in species abundance under high grazing intensity in grasslands in South Africa.

According to previous studies, the highest floristic diversity is recorded under light or moderate grazing intensity and any increase in grazing intensity has a negative effect on species richness. A meta-analysis of 116 published papers [75] regarding the effect of grazing intensity on species abundance and the Shannon–Wienner diversity index in temperate grasslands reported the same results. The authors mentioned that the results of the meta-analysis are consistent with the intermediate disturbance hypothesis.

A long-term absence of grazing (more than 40 years) in the present study appears to have reduced floristic diversity. Similar results were reported by Song et al. [76] in alpine grasslands in China where the short-term (2–4 years) and long-term (9–11 years) abandonment of grazing was also studied. Plant diversity based on the Simpson index in short-term grazing exclusion remained stable, while in the long-term, grazing exclusion decreased. Also, a decrease in the number of species was recorded in the mountainous grasslands of Slovakia after 46 years [77] and 10 years of abandonment of grazing [72]. Also, in a study conducted by Wang et al. [78], the abundance of species declined after five years of grazing exclusion in grasslands on the Tibetan Plateau. In contrast, in a meta-analysis performed by Xiong et al. [79], short-term exclusion of grazing (<5 years) increased the number of species in alpine and temperate steppes but nothing similar was observed in alpine grasslands. Grazing in natural ecosystems causes changes in the composition and structure of vegetation [15,36,80,81] and finally in floristic diversity.

The intermediate disturbance hypothesis (IDH) [82–84] has been widely used to describe the relationship of disturbance, such as grazing, with the floristic diversity of natural ecosystems. According to the hypothesis, moderate grazing intensity results in higher floristic diversity compared to the absence of grazing or high grazing intensity [13,84]. However, the conflicting results regarding the effect of grazing on the floristic diversity of grasslands indicate that the IDH does not hold true in all cases and the impact depends on various factors such as weather conditions, grazing history, type of grazing animal, and ecosystem productivity [13,15]. According to a meta-analysis by Gao and Carmel [85], IDH applies only to wet and semi-wet areas- like those of the study area, while it does not apply to dry areas. Furthermore, their analysis suggests that the absence of grazing reduces floristic diversity in wet and semi-wet areas while increasing it in dry areas. This could be attributed to differences in vegetation structure and species composition. Thus, in wet and semi-wet grasslands of the Mediterranean region, such as those in the study area, vegetation is relatively dense, with high plant growth rates, and mainly dominated by tall

to medium-height grasses. Such species were also dominant in the study area's grasslands (see Supplementary Materials). Competition for light is intense in these plant communities and grazing may contribute to reducing it and creating openings where less competitive species can establish, such as low grasses and forbs [22].

This effect is more intense under moderate grazing, resulting in an increase in floristic diversity [36]. On the contrary, the abandonment of grazing leads to the dominance of more competitive species results in the decrease in floristic diversity. Also, floristic diversity was affected by the year of measurements. The effect of the year refers to the differentiation in weather conditions from year to year, mainly temperature and precipitation. Ren et al. [71] in Mongolian steppes, Moradi et al. [86] in Iran, Xiong et al. [79], and Wu et al. (2012) in alpine meadows in China reported that floristic diversity was more affected by weather conditions than by grazing intensity. Conversely, Yan et al. [87] reported that the floristic diversity of grasslands in Mongolia was not affected by weather conditions (rainfall and temperature) but by grazing intensity. According to recent reports, the floristic diversity of grasslands is mainly affected by the amount of rainfall [88] and lesser by temperature. A study that took place in Tibet in 116 grassland sites indicates that species richness and Shannon diversity index are positively related to precipitation [88]. Furthermore, precipitation during the growing season appears to have a greater impact compared to the total annual precipitation [71,86]. Accordingly, the results of the present study indicate that higher diversity based on Shannon–Wiener, Simpson indices, and species richness (regardless of grazing intensity) was recorded in 2015, which was the year with the highest precipitation during the growing season (Supplementary Materials).

The Jaccard similarity index showed the highest value in highly grazed grassland compared to the ungrazed area and the one with moderate grazing intensity. A meta-analysis of Liu et al. [89] and Li et al. [90] for different grassland ecosystems, including semi-humid ones, concluded that the Jaccard similarity index is higher in grazed sites compared to the ungrazed ones. This is mainly attributed to the dominance of the most competitive species in the ungrazed grasslands. On the other hand, Ren et al. [71] in grasslands in Mongolia and Yan and Lu [91] in alpine grasslands in the Tibetan Plateau did not observe any variation in the Jaccard similarity index between grazed and ungrazed areas. Similarly, Pueyo et al. [92] in grasslands in northern Spain and at six grazing intensities observed no change in the Jaccard similarity index but a decreasing tendency when increasing grazing intensity.

In the semi-humid grasslands such as in the study area, the lowest value for β-diversity was recorded in highly grazed areas, indicating a greater homogeneity of plant communities compared to the ungrazed ones. This tendency was not observed in moderate grazed areas. This may be attributed to different degrees of environmental differentiation (soil, micro-topography, etc.) of the areas with moderate and high grazing intensity. How grazing affects plant community composition and floristic diversity depends on many environmental and management factors. In addition to these factors, the small-scale differences of each area play a decisive role in which way grazing will affect the plant community, i.e., the homogeneity or heterogeneity in terms of soil, microtopography, etc. If those differences are small, the role of grazing is decisive and there is a tendency to homogenize composition [91,92]. On the contrary, when those differences are great, the role of grazing is then limited.

In conclusion, the results of the present study are consistent with the intermediate disturbance hypothesis [84], since all diversity indices were superior at moderate grazed areas. However, these changes in floristic diversity seem to be influenced and varied by the weather conditions of each year as well.

4.3. Functional Traits of Plant Species

Specific leaf area (SLA) in the plant community presented its highest values in the ungrazed area, compared to the grazed ones. This practically means that the plants in the ungrazed area tended to have larger leaves with lower weight. In contrast, leaf dry

matter content (LDMC) and stem dry matter content (StDMC) presented their lowest value in the ungrazed area compared to grazed areas. However, it must be emphasized that stem dry matter content (StDMC) is a functional trait that has not been particularly studied and the literature references were limited [93]. Similarly, a trend of increasing specific leaf area in wet ungrazed grasslands in Southwest Germany was also observed by Kahmen and Poschold [94] and Wang et al. [95], in semi-arid grassland ecosystems in China. As it concerns LDMC, the results of this research are in agreement with those of Louault et al. [96] and Cruz et al. [97] in wet grassland ecosystems in France and Brazil, respectively. Additionally, Jascke et al. [98], in a study carried out in semi-arid grassland ecosystems in the Tibetan Plateau and in two grazing intensities (high and light), did not observe any difference in SLA due to grazing intensity as Peco et al. [99] in semi-arid grassland ecosystems in central Spain.

The higher value of SLA in the ungrazed area can be attributed to plant species' strategy in order to absorb more solar radiation, under shade conditions, created due to the density of the plant community [99–101]. Lower values of SLA at highly grazed areas are related to greater leaf resistance [15] and reduced palatability [100], which is also a strategy in order to avoid grazing.

In contrast, Streit et al. [102], in dry grasslands in southern Brazil, reported a decrease in SLA in the ungrazed area, while LDMC did not show any clear tendency related to grazing intensity. Golodets et al. [103], in semi-arid grasslands in northern Israel, reported that SLA and LDMC had their lowest values in the ungrazed area (area protected from grazing). However, a decrease in SLA in the ungrazed area and an increase in LDMC are reported in a number of studies [15,99,104,105]. The changes occurring in the above traits after the abandonment of grazing indicate that the photosynthetic rate of plants decreased and that their growth rate retarded [105].

SLA and LDMC depict plant strategies for absorbing nutrients from the soil and then utilizing them appropriately. Contrasting results presented in the aforementioned studies could be explained by the different grassland habitats that were examined (arid and semi-arid), the various species within the plant communities, and the distinction in the survival strategies employed by the various species within the plant community [102]. After long-term exclusion of grazing, there is a spread of woody vegetation, increasing the density of plant cover and reducing available solar radiation to some small plant species, which attempt to develop their leaves [102]. Conversely, plant species under high grazing intensity produce a greater number of leaves in order to compensate for the loss of leaf mass. Thus, plant communities subject to long-term and high-intensity grazing are characterized by plant species with small surface areas and large leaf masses in semi-arid grassland ecosystems [95]. These grazing intensity responses facilitate the ability of each plant to acquire resources to recover its losses and ensure its growth [41] and thus directly reflect the ability of plants to adapt to the environment [105].

The vegetative (VH) and reproductive height (RH) of the species presented their highest value in the ungrazed area compared to grazed ones. Plant height is considered as the functional trait that can best predict the response of plants to grazing [22,106]. Generally, the studies show that plant height increases in the ungrazed area [15,21,94,95,98,99,104,105,107]. Grime [108] reported that the increase in plant height in the ungrazed area is related to the competition of plants for light in order to survive.

The leaf nitrogen (LNC) and leaf phosphorus concentration (LPC) of the plants in the present study had the tendency for higher values in the highly grazed area compared to the other two areas. Janscke et al. [98] and Wang et al. [105] reported the same results regarding leaf nitrogen concentration studying semi-arid grassland ecosystems in China. However, Golodets et al. [103], in semi-arid grassland ecosystems in northern Israel, observed that leaf phosphorus concentration increased in the ungrazed area while leaf nitrogen concentration was not affected by grazing intensity. The tendency for higher values in leaf nitrogen concentration at high grazing intensity may be due to the presence of animals that, with their urine and feces, increase the soil nitrogen content [89,109]. Also, grazing animals,

as they move into the grasslands, accelerate the rate of decomposing dry foliage causing an increase in soil nitrogen [78]. Therefore, the increase in soil nitrogen may be the main reason for the increase in leaf nitrogen concentration. This increase in soil fertility due to the presence of nitrogen also causes an increase in leaf phosphorus concentration [105,110]. Due to the increase in soil fertility, plant species develop their shoot at a faster rate and the concentration of phosphorus in the leaves also increases.

Hemicryptophytes predominated in the three grazing intensities. Castro et al. [104], in the grasslands of Portugal, reported that hemicryptophytes predominated in the ungrazed area. In fact, they emphasize that after a 15-year abandonment of grazing, the hamaephytes decreased, while after 30 years of abandonment of grazing, hemicryptophytes prevailed. Janscke et al. [98], in a study carried out in China, reported that no change in the biotic form of plants was observed in relation to grazing intensity. De Bello et al. [21], in another study, reported that phanerophytes predominated in ungrazed areas, mainly due to the increase in shrubs. However, this was not observed in the present study since phanerophytes appeared only in the ungrazed area. The presence of hemicryptophytes in the highly grazed areas is attributed mainly to special conditions of the mountainous grasslands since the combination of the high altitude with the climatic conditions (strong winds and cold winters) had, as a result, the seeds of the plant species found in dormancy, to be closer to or in the soil [111,112].

Perennial species dominated over annual species at all three grazing intensities. De Bello et al. [21], in grasslands in Spain and at three grazing intensities, reported that perennial species prevailed over annuals, after long-term exclusion of grazing. Bonet and Pasas [113] referred to the fact that moderate grazing intensity may favor annual species, which partly happened in the present research, although the percentage of participation of annual and perennial species was approximately the same. In contrast, Jascke et al. [98] reported that perennial species were not affected by grazing intensity, indicating that perennial species may not be so sensitive to grazing in dry grasslands such as those studied in their study (China), a finding that is also in agreement with the results of Díaz et al. [114]. In the present study, grasslands are considered as humid and according to Díaz et al. [114], in dry grasslands with a long history of grazing, the effect of grazing intensity does not significantly affect the proportion of annual and perennial species.

From the results of the present study, it appears that for all grazing intensities, the percentage of species that followed the C3 photosynthetic pathway was higher than those that followed the C4 photosynthetic pathway. Particularly, in the ungrazed area, all the species followed the C3 photosynthetic pathway. The results from the present study are in agreement with the results of Karakosta [115] in the same study area since all the species in the ungrazed area followed the C3 photosynthetic pathway. More generally, plants that follow the C3 photosynthetic pathway predominate in mountainous grasslands (higher altitudes) where temperatures are lower [3]. Cornelissen et al. [40] reported that compared to C3 plants, C4 plants tend to predominate in warm, sunny, and relatively dry environments.

The majority of species exhibited a defense mechanism against grazing across all three grazing intensities. However, in the ungrazed area, the presence or absence of a defense mechanism is almost the same. The density of the herbaceous layer, due to the intense presence of woody species, provides protection to herbaceous plants against grazing [116], allowing less adapted species to prevail. According to Papadimitriou [93], in the ungrazed area, taller species overshadow shorter ones, offering them protection from grazing. In areas with high grazing intensity, dominant species try to develop avoidance mechanisms, such as low height [22]. Additionally, the lower value of specific leaf area is likely associated with high leaf resistance and low palatability, serving as another defense mechanism against grazing in areas with high grazing intensity [93].

The study of plant functional traits facilitates the understanding of the survival and performance mechanisms of plants in different grassland ecosystems [117]. Additionally, useful conclusions about the response of grassland ecosystems to abiotic and biotic distur-

bances can be drawn through functional traits. Numerous studies indicate that plant biotic form and height are directly influenced by grazing intensity [21,98,106,118]. However, they emphasize that plant life form, height, and reproductive strategies can be considered examples of adaptation not only to grazing but also to climatic conditions. The combination of plant height, plant life form, and leaf mass is suitable for studying the response of species to grazing, while specific leaf area cannot be used as a trait response to grazing [119]. According to the present study, it appears that plant life form is not affected by grazing intensity, while plant height, specific leaf area, and leaf dry matter content can be used to study the response of plant communities to grazing in the studied grasslands.

5. Conclusions

The main disturbance in grasslands is grazing. For the mountainous grasslands such as those in the present study, the management goal must be the application of moderate grazing intensity. The absence of grazing for more than 40 years resulted in the invasion of shrubs, the increase in density of plant community, and decrease in floristic diversity. Tall competitive grasses dominated and legumes declined, which may have contributed to the reduction in nutritional value. Management measures must aim at enhancing species composition and then applying moderate grazing intensity. On the contrary, in highly grazed areas, sites with bare soil are observed, which also can lead to soil erosion. In this case, grazing animals should ideally be removed from those grasslands for a reasonable period of time in order to allow plant species to recover and then moderate grazing intensity must be applied.

In conclusion, grazing appears to be a crucial factor in shaping plant community and floristic diversity in grasslands. However, the way it influences their formation and the correlation between them is also influenced by many other factors such as climate, soil, and grazing history, which vary in each specific grassland. What should be further studied in these ecosystems is the role of climatic conditions, the specific topography, and the intensity of grazing in the study of plant functional traits. The combined study of the above levels of diversity can be a useful tool for exploring the intensity of grazing in grasslands and contributing to their sustainable management.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/land13040488/s1. Figure S1: Hyothermic diagram 1973–2015; Figure S2: Hyothermic diagram 2013; Figure S3: Hyothermic diagram 2014; Figure S4: Hyothermic diagram 2015; Table S1: Species composition list 2013-2014-2015; Table S2. Defense mechanism against grazing.

Author Contributions: Conceptualization, E.M.A. and E.A.; methodology, E.M.A. and I.T.; software, E.A. and I.K.; validation, E.M.A. and I.T.; formal analysis, E.A.; investigation, E.M.A. and E.A.; resources, E.A.; data curation, E.M.A.; writing—original draft preparation, E.A.; writing—review and editing, E.M.A.; visualization, E.A.; supervision, E.M.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Data are available from the corresponding author upon request.

Conflicts of Interest: The authors declare no conflicts of interest.

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Diversity and Ecological Assessment of Grasslands Habitat Types: A Case Study in the Calabria Region (Southern Italy)

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Abstract: Grasslands differ in many types depending on the diversity of climatic conditions and substrates. Due to the great wealth of species found in semi-natural grasslands, they conserve an essential part of the biodiversity of the European Union (EEC 43/92), defined as habitats of community interest. Using the region of Calabria (southern Italy) as a case study, this study aims to evaluate how species assemblages and a set of indicators applied to them can be used to analyse and characterize the diversity, ecological features and conservation status of grassland habitats. Vegetation analysis was carried out using the phytosociological method, and habitat ecological characteristics were defined by Ellenberg's ecological indicator adapted to the Italian flora. Multivariate analysis of the surveys by means of cluster analysis and Principal Components Analysis (PCA) made it possible to define eight habitat groups according to EEC Directive 43/92: 6110 Rocky or basophilous calcareous grasslands of the Alysso-Sedion albi, 6170 Alpine and subalpine calcareous grasslands, 6220*a Pseudo-steppe with grasses and annuals of the *Thero-Brachypodietea* dominated by a *Lygeum spartum*, 6220*b Pseudo-steppe with grasses and annuals of the *Thero-Brachypodietea* dominated by *Hyparrhenia hirta*, 6210* Semi-natural dry grasslands and scrub facies on calcareous substrates (*Festuco-Bromometalia*) (*important orchid sites), 6230* Species-rich Nardus grasslands, on siliceous substrates in mountain areas (and submountain areas, in Continental Europe), 6410 Molinia meadows on calcareous, peaty or clayey-siltladen soils (*Molinion caeruleae*), 6420 Mediterranean tall humid herb grasslands of the *Molinio-Holoschoenion*, 6430 Hydrophilous tall herb fringe communities of plains and of the montane to alpine levels. Temperature (T) and oisture (U) are the most statistically significant ecological factors in differentiating different habitat types. Analysis of diversity, assessed through Shannon and Evenness indices, showed that it is strongly correlated with habitat diversity as soil moisture increases. The study of ecological characteristics, correlated with biodiversity indices, helps to characterize grassland habitats, providing guidance for the implementation of active conservation policies for these habitats.

Keywords: biodiversity; naturalness; Calabria region; grassland habitat; alien species; disturbing species

Citation: Morabito, A.; Musarella, C.M.; Spampinato, G. Diversity and Ecological Assessment of Grasslands Habitat Types: A Case Study in the Calabria Region (Southern Italy). *Land* **2024**, *13*, 719. https://doi.org/10.3390/land13060719

Academic Editors: Michael Vrahnakis and Yannis (Ioannis) Kazoglou

Received: 25 March 2024
Revised: 27 April 2024
Accepted: 13 May 2024
Published: 21 May 2024

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1. Introduction

Grasslands contain a high percentage of the world's biodiversity, despite only a part of the continents being covered by natural or semi-natural grasslands [1–3]. These ecosystems account for 28% of natural and semi-natural habitats, ranking after forests in terms of importance [4]. In the Italian territory, grasslands cover about 6.2% of the surface, and about 3.6% is mapped as open grasslands by APAT [5].

Generally, semi-natural grassland habitats embedded in agricultural landscapes support floristic diversity and a variety of plant communities [6–8], being characterized by a high number of species, many of which are endemics, and are important ecosystems for maintaining biodiversity [9–11]. These habitats, therefore, have a high conservation value, particularly due to their high biodiversity and related ecosystem services [12,13]. The maintenance of grassland habitats and their associated species has been an important issue

in nature conservation for decades [14]. Today, numerous studies analyse the ecological and floristic features of natural and semi-natural Palearctic grasslands (accessed on 6 November 2023). Understanding and analysing the ecological factors that influence biodiversity is essential for designing biodiversity management and conservation plans that are consistent with the floristic and ecological characteristics of habitats [15–17].

The remarkable species richness of grasslands is threatened across Europe due to global changes, fire and biological invasions [18–24]. The decline in the diversity and connectivity of grasslands in Europe has increasingly become a major issue for their conservation. Changes in land use have the greatest impact on the degradation and loss of different habitat types [25–27]. Over the last century, a continuous loss of species-rich grassland habitats, influenced by humans with conversion to intensive arable or grassland, or abandonment to evolve into forests, has been recognized in Europe [28,29]. In this perspective, beta-diversity, defined as the amount of turnover in species composition [30], is important as a measure of species diversity on a regional scale, to be used as a basis for conservation [31]. The conservation and sustainable use of a given territory must take into account models that consider biodiversity as a factor in ecosystem stability and functioning [32–34].

Some studies show that the species composition of grasslands varies to a different extent depending on environmental characteristics [35–37]. Various ecological factors affect the species diversity of grasslands habitats [38]; among them, the most important are climate [39,40], altitude and, above all, soil moisture conditions [41,42]. In particular, the humidity is one of the most important ecological factors that influence plant species, the diversity and the floristic composition of grasslands habitats, as highlighted by Zelnik et al. [43]; there have also been numerous research studies carried out in the last decade in Europe on wetlands and the role of ecological factors [44–53]. Some studies state that nutrient availability within the soil influences species richness in various plant communities [54,55].

Grazing management is another factor that influences the structure and function of these ecosystems [56–58], changing their plant diversity. Lozano et al. [59] suggest that grazing pressure has a negative effect on the presence of endemic species, whereas in alpine environments, grasslands with higher floristic diversity are generally associated with environments with forms of management that involve the removal biomass, such as mowing and grazing [60,61].

The use of ecological indices can point out anthropogenic impact and thus allow the degree of naturalness or degradation of a habitat to be assessed based on species composition [62].

This study aims to assess the conservation status of grassland habitats by analysing the diversity, naturalness, and ecological characteristics of the plant communities. The grassland spread in the Calabria region was used as a case study. In particular, the study aims to (1) identify the different habitat types through the species assemblage; (2) define the main ecological factors responsible for variation in the floristic composition of grassland habitats vegetation; (3) assess the biodiversity and naturalness of the different habitat types; (4) evaluate the relationship between species diversity and ecological factors. The results of this study can be useful and applied in the management of grassland habitats for biodiversity conservation purposes.

2. Materials and Methods

The study area was the Calabria region, which, as indicated in the Carta Natura [63], occupies 24,375 ha, or 2.4% of the regional territory. For the photointerpretation of the habitats, arc map 10.7 was used, with reference system WGS84 UTM Zone 33, (False_Easting: 500000,00000 False_Northing: 0.00000; Central_Meridian: 15.00000; Scale_Factor: 0.99960; Latitude_Of_Origin: 0.00000; Linear Unit: Kilometres). The analysis has affected all grassland habitats depicted on the nature map.

These habitats are distributed throughout the Calabrian territory, from sea level to the mountain range, affecting different environmental contexts characterized by different ecological conditions [27].

The analysis of grassland habitats was based on the vegetation relevés carried out in the period 2018–2021, using the phytosociological method of the Zurich-Montpellier school, considered to date the most suitable method for analysing vegetation [15,64,65]. A total of 61 unpublished relevés with 490 species were carried out (Figure 1) (Supplementary Materials Table S1). The matrix of phytosociological relevés was subjected to multivariate analysis to define coenological groups on an actual statistical basis. To this end, the abundance-dominance data of the individual species reported with the Braun-Blanquet scale were converted with the scale of numerical values proposed by Van der Mareel. The software used for the organization of raw data and subsequent statistical analyses was Microsoft Excel 2010; PAST version 4.13; R 4.1.1 R Core Team 2021.



Figure 1. Study area: Calabria region. The red dots and numbers indicate the location of the vegetation relevés.

The cluster analysis of the relief matrix used the average bond criterion (UPGMA) and the Chord distance algorithm to identify homogeneous floristic compositions.

To highlight the ecological characteristics of plant species, the Pignatti–Ellenberg indicator value was used in the version modified by Pignatti [66–68] for the Italian territory. The

values of the ecological indicator of continentality (C), substrate reaction (R), temperature (T), humidity/water availability (U), light (L) and nutrients (N) were taken into account.

The PCA was performed taking into account the Ellenberg ecological indicator attributed to each species in the survey matrix to show the distribution of values for ecological characteristics.

The nomenclature of the species follows the "Portal to the flora of Italia" [69], while syntaxonomic references of plant communities take into account several studies [70]. Manuals for the interpretation and monitoring of habitats of community interest in Italy were used for the interpretation of community habitats according to EEC Directive 43/92 [71,72].

Grassland biodiversity was assessed using the Shannon–Wiener H^+ [73] and Evenness (J) indices.

$$H^+ = -\Sigma pi \ln pi \quad (1)$$

where pi is the relative presence value of the ith species.

$$J = H/Hmax, \quad (2)$$

where Hmax = ln S and S = species richness.

This index depends on the abundance and uniform distribution of species [74]. J' varies between 1 when the species of the community are equally distributed and 0 when only one species is present.

The Shannon index (H^+) has also made it possible to evaluate Naturality (Na) by taking into account not only the presence of alien species [74–77] but also the indicators of disturbance, which emphasise the anthropic impact on the community.

$$Na = H^+ \text{ (without alien and disturbing plant species)}/H^+). \quad (3)$$

The Na index varies from 0 to 1, where 0 indicates that plant diversity consists entirely of alien and disturbance species, while 1 indicates the absence of the latter in phytocoenosis [74–78]. The alien species were extrapolated from the "Portal of the flora of Italy" [69], while for the disturbance species the syntaxonomic references of the plant communities [70] belonging to the anthropic vegetation classes *Stellarietea mediae* Tüxen, Lohmeyer & Preising ex Von Rochow 1951 and *Galio aparines-Urticetea dioicae* Passarge ex Kopecký 1969 were taken. Most disturbance species characterize various alliances: *Securigero securidacae-Dasypyrion* villosi Cano-ortiz, Biondi & Canoex Cano-ortiz, Biondi & Canoin Biondi et al., 2015 [*Bromopsis erecta* (Huds.), *Dasypyrum villosum* (L.) P.Candargy), *Fedio graciliflorae-Convolvulion cupaniani* Brullo & Spampinato 1986 [*Avena barbata* Pott ex Link, *Galactites tomentosus* Moench, *Sonchus oleraceus* L., *Geranium dissectum* L.]; *Fumarion wirtgenii-agrariae* Brullo in Brullo & Marcenò 1985 [*Calendula arvensis* L. (Raf.) Nyman, *Rumex bucephalophorus* L., *Fumaria agraria* Lag]; *Trifolio medii-Geranietea sanguinei* Müller 1962 [*Senecio scopolii* Hoppe & Hornsch; *Asphodelus ramosus* L.]; *Scleranthion annui* (Kruseman & Vlieger 1939) Sissingh in Westhoff, Dijk, Passchier & Sissingh 1946 [*Anthemis arvensis* L., *Agrostis castellana* Boiss. & Reut., *Muscari comosum* (L.) Mill.]; *Convolvulo arvensis-Agropyrion repenti* Görs 1966 [*Picris hieracioides* L., *Dactylis glomerata* L., *Bunium bulbocastanum* L.]; *Inulo viscosae-Agropyrion repentis* Biondi & Allegrezza 1996 [*Reichardia picroides* (L.) Roth, *Pallenis spinosa* (L.) Cass., *Daucus carota* L., *Verbena officinalis* L.]; *Cerastio arvensis-Cynosurenion cristati* Blasi, Tilia, Rosati, Del Vico, Copiz, Ciaschetti, Burrascano 2012 [*Anthoxanthum odoratum* L., *Lotus corniculatus* L., *Plantago lanceolata* L.]; *Resedo albae–chrysanthemenion coronarii* Cano-Ortiz, Biondi & Cano In Biondi, Allegrezza, Casavecchia, Galdenzi, Gasparri, Pesaresi, Poldini, Sburlino, Vagge & Venanzoni 2015 [*Reseda alba* L.]; *Echio plantaginei-Galactition tomentosae* O. Bolòs & Molinier 1969 [*Sherardia arvensis* L., *Plantago lagopus* L., *Lotus ornithopodioides* L.]; *Digitario ischaemi-Setarion viridis* Sissingh in Westhoff, Dijk, Passchier & Sissingh 1946 (*Digitaria sanguinalis* (L.) Scop.; *Hordeion leporini* Br.-Bl. in Br.-Bl., Gajewski, Wraber & Walas 1936 corr. O. Bolòs 1962 [*Carduus pycnocephalus* L., *Anacyclus clavatus* (Desf.) Pers., *Rostraria cristata* (L.) Tzvelev, *Glebionis coronaria* (L.) Spach]; *Hypochoeridion achyrophori* Biondi & Guerra 2008 [*Linum strictum* L., *Euphorbia exigua* L.]; *Taeniathero-Aegilopion geniculatae* Rivas-

Martínez & Izco 1977 [*Trifolium stellatum* L., *Trifolium scabrum* L.]; *Holco mollis-Pteridion aquilini* Passarge (1994) 2002) [*Pteridium aquilinum* (L.); *Holcus lanatus* L.]; *Ridolfion segeti* Negre ex Rivas-Martinez, Fernandez-Gonzalez & Loidi 1999 [*Phalaris paradoxa* L., *Trigonella sulcata* (Desf.) Coulot & Rabaute]; *Caucalidion platycarpi* Tüxen ex Von Rochow 1950 nom. mut. Rivas-martinez, T.E. Diaz, Fernandez-Gonzalez, Izco, Loidi, Lousã & Penas 2002 [*Lysimachia arvensis* (L.) U. Manns & Anderb.]; *Veronico agrestis-Euphorbion peplus* Sissingh ex Passaggio 1964 [*Sonchus asper* (L.) Hill].

To verify the significance of the differences observed between the different habitat groups and the average values of the ecological indicator, the Mann–Whitney test was used. Finally, a correlation analysis (PAST version 4.13 [79,80]) was applied between the value of Naturalness (Na) and the values of the ecological indicator of temperature (T) and humidity (U) of the identified habitat types. Person statistics were used, and, finally, the linear regression model by Durbin–Watson and Breusch–Pagan tests were applied [81–83].

3. Results

3.1. Cluster Analysis to Define Habitat Types

Cluster analysis of the grassland vegetation relevés matrix produced the dendrogram in Figure 2, where the abscissa shows the distinctive number of each relevés, while the ordinate shows the scale of similarity. The first subdivision is at a similarity level of 1.4; the subsequent subdivisions (similarity coefficient 1.3-1.2-1.1) show us eight groups of different grassland habitats.

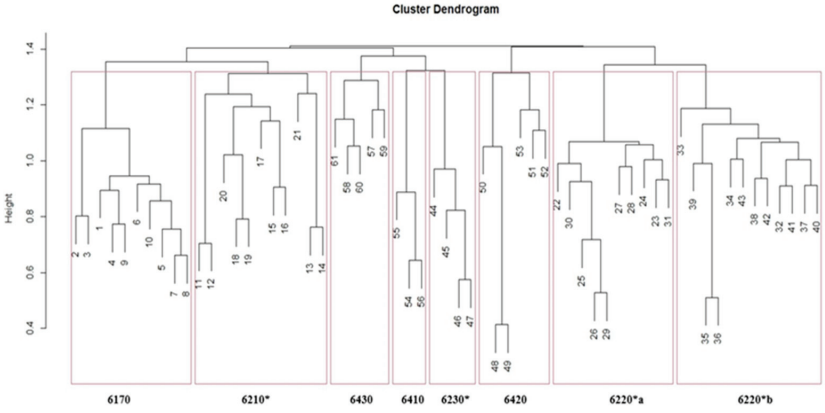


Figure 2. Dendrogram derived from cluster analysis on grassland relevés (Chord as distance coefficient and UPGMA as clustering algorithm) showing 8 habitat groups: 6220*a: Pseudo-steppe with grasses and annuals of the *Thero-Brachypodietea* dominated by *Lygeum spartum* L., 6220*b: Pseudo-steppe with grasses and annuals of the *Thero-Brachypodietea* dominated a *Hyparrhenia hirta* (L.) Stapf, 6230*: Nardus grasslands rich in species, on siliceous substrates in mountain areas; 6210*: Semi-natural dry grasslands and scrubland facies on calcareous substrates (*Festuco-Brometalia*) (*important orchid sites).

The first group (Rel. 1–10), according to "Italian interpretation manual of the 92/43/EEC Habitats Directive" [71], belongs to the habitat 6170: Alpine and subalpine calcareous grasslands, the second group (Ril 11–21) belongs to habitat 6210*: Semi-natural dry grasslands and scrubland facies on calcareous substrates (*Festuco-Brometalia*) (*important orchid sites), the third group (Rel. 22–31) to the habitat 6220*a: Pseudo-steppe with grasses and annuals of the *Thero-Brachypodietea* dominated by *Lygeum spartum* L., the fourth group (Rel. 32–43) to habitat 6220*b: Pseudo-steppe with grasses and annuals of the *Thero-Brachypodietea* dominated by *Hyparrhenia hirta,* the fifth group (Rel. 44–47) fits to the habitat 6230*: Nardus grasslands rich in species, on siliceous substrates in mountain areas (and submountain areas, in Continental Europe), the

sixth group (Rel. 48–53) belongs to habitat 6420: Mediterranean tall humid herb grasslands of the *Molinio-Holoschoenion*, the seventh group (Rel. 54–56) belongs to habitat 6410: Molinia meadows on calcareous, peaty or clayey-siltladen soils (*Molinion caeruleae*), and the eighth group (Rel. 57–61) belongs to habitat 6430: Hydrophilous tall herb fringe communities of plains and of the montane to alpine levels.

Habitat 6220* is characterized as grassland of small grasses rich in therophytes, predominantly open, thermo- and meso-Mediterranean bioclimate, xerophilous; oligotrophic soil communities are located on base-rich substrates, often calcareous. In loose soil, we find the habitat dominated by *Hyparrhenia hirta* [84], while on clayey soils, we find the habitat dominated by *Lygeum spartum* [85]; in both, it is possible to find some interesting species [86]. Habitat 6230* [87,88] includes grasslands dominated by *Nardus stricta* which develop on acid soils in rather flat areas from the hills to the mountain range; in acidic soils, they can derive from siliceous or carbonate rocks through leaching. The grasslands of habitat 6210* [71,89,90] are predominantly of secondary origin and characterized by a high density of species. Hemicryptophytes dominate these communities and can develop on different types of substrate. In Italy, these grasslands, excluding the North, show their greatest presence along the Apennine chain. Habitat 6170 is located on limestone substrates [91,92] and frames the grasslands present in the Alps and the Apennines above the forest limit. Habitat 6410 includes wet semi-natural grasslands characterized by *Molinia caerulea* [93,94] and is located from the plain to the upper limit of the forest. Habitat 6420 includes high size grasslands characterised by Mediterranean reeds and other hygrophilous species [95,96] and is present in retrodunal systems but also in indoor wetlands. Habitat 6430 is characterized by hygrophilous and nitrophilous species (megaforbes), which settle mainly along the edge of waterways [97,98].

3.2. Ecological Factors Analysis

The PCA was carried out taking into account the Ellenberg ecological indicator value, which showed the distribution of relevés on the basis of ecological characteristics and explained 81.3% of total dataset variation with the first two components, with PC1: 60.9% and PC2: 20.4% (Figure 3).

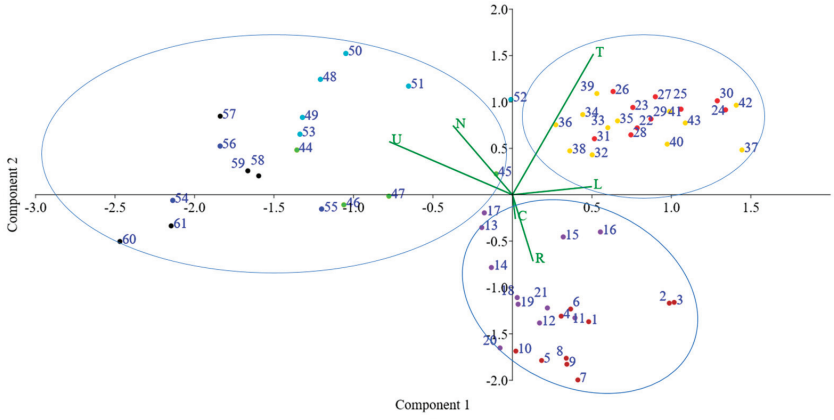


Figure 3. Biplot of relevés dispersion on PCA related to Ellenberg ecological indicator. The numbered points are the relevés of the various habitat types: in purple is the habitat 6210*, brown is the habitat 6170, light green is the habitat 6230*, red is the habitat 6220*a, yellow is the habitat 6220*b, sky blue is the habitat 6410, black is the habitat 6430, blue is the habitat 6420. The green lines indicate the direction of maximum correlation of the Ellenberg indicator values. (U = humidity; C = continentality; R = substrate reaction; T = temperature; L = light; N = nutrients).

The PCA highlights three habitat groups. The habitats 6230*, 6410, 6420, and 6430 are related to the ecological factors of moisture (U) and nutrients (N); these are in fact habitat

types located in the mountainous belt, where there is a climate with greater water availability. The habitats 6220a* and 6220*b are arranged along ecological gradients of temperature (T) and light (L), confirming that these habitats are characterised by herbaceous, perennial, thermophilous vegetation that settles in the driest areas of the Mediterranean [70]. Habitats 6170 and 6210* are distributed on the basis of the ecological indicator of continentality (C) and substrate reaction (R); they are in fact habitats located in the mountainous belt on highly permeable basic pH limestone substrates.

The overall table of this assessment is Supplementary Materials Table S2.

The ecological features of the eight identified habitats are shown in Figure 4 by boxplots with the values of the ecological indicator of humidity (U), temperature (T), light (L), nutrient (N), continentality (C) and substrate reaction (R). To quantify the separability of the various habitat groups considering the mean values of the ecological index, the Mann–Whitney test was applied.

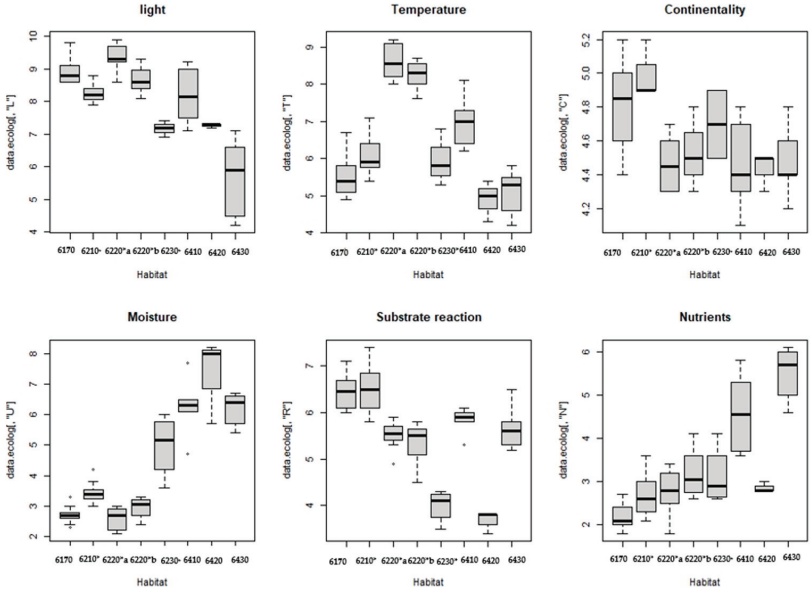


Figure 4. Box plot representing the Ellenberg indicator values of each habitat: 6170 Alpine and subalpine calcareous grasslands; 6210* Semi-natural dry grasslands and scrubland facies on calcareous substrates (*Festuco-Brometalia*) (*important orchid sites); 6220*a Pseudo-steppe with grasses and annuals of the *Thero-Brachypodietea* dominated a *Lygeum spartum* L., 6220*b Pseudo-steppe with grasses and annuals of the *Thero-Brachypodietea* dominated by *Hyparrhenia hirta*; 6230* Nardus grasslands rich in species, on siliceous substrates in mountain areas; 6420 Mediterranean tall wet grass grasslands of the *Molinio-Holoschoenion*; 6410 Molinia meadows on calcareous, peaty clayey-siltladen soils (*Molinion caeruleae*); 6430 Hydrophilous tall grass fringe communities of plains and of the mountain to alpine levels.

The ecological factor light (L) is greatest regarding 6220*a and 6220b*, while lower values are observed in the habitat 6420; significant differences occur for this ecological indicator between habitat 6220*a and habitats 6170 (p-value = 0.000) and with habitat 6210* (p-value = 0.000); other significant differences are observed between habitat 6220*b and habitat 6430 (p-value = 0.04).

The ecological indicator temperature (T) is higher in habitats 6220a*and 6220*b and lower in habitats 6170, 6420 and 6430, as shown by the dissimilarity between habitat 6170 and habitats 6220*a (p-value = 0.00) and 6220*b (p-value = 0.00); other statistically significant differences are shown between habitat 6220*a and habitats 6210* (p-value = 0.00); 6420

(*p*-value = 0.05); and 6430 (*p*-value = 0.05). Finally, habitat 6220*b also shows differences with habitat 6210* (*p*-value = 0.00) and 6430 (*p*-value = 0.04).

The Ellenberg indicator values of continentality (C) are highest in habitat 6210* and 6170, while they are lower in habitats 6220*a 6220*b, 6410 and 6430. These differences are significant because habitat 6210* differs from habitats 6220*a (*p* value = 0.00), 6220*b (*p* value = 0.00), 6410 (*p* value = 0.01) and 6430 (*p* value = 0.03).

The indicator values of moisture (U) are higher in habitats 6430, 6420 and 6410, while they are lower in habitats 6220*a, 6220*b, 6170 and 6210*. Statistically significant differences are observed between habitat 6170 and 6210* (*p*-value = 0.0097), 6410 (*p*-value = 0.0314;) and 6430 (*p*-value = 0.0490;). Other significant differences are found between habitat 6210* and habitats 6220*a (*p*-value = 0.00), 6220*b (*p*-value = 0.03), 6410 = (*p*-value = 0.02) and 6430 (*p*-value = 0.04). Finally, the humidity factor differentiates habitat 6220*a with 6410 (*p*-value = 0.03), 6220*a with 6430 (*p*-value = 0.04), habitat 6220*b with 6410 (*p*-value = 0.03), and 6220*b with 6430 (*p*-value = 0.03).

The indicator values of substrate reaction (R) show other significant differences between habitat 6170 and habitats 6220*a (*p*-value = 0.00); 6220*b (*p*-value = 0.00). Analysis shows that habitat 6210* differs from habitats 6220*a (*p*-value = 0.00) and 6220*b (*p*-value = 0.00), confirming that the grasslands of this habitat develop on calcareous and basic or sub-acid substrates [70].

The nutrient ecological indicator (N) in wet grasslands 6430 and 6410 differs significantly from other habitats. In particular, habitat 6170 differs from habitat 6220*b (*p*-value = 0.00) and habitats 6410 (*p*-value = 0.03) and 6430 (*p*-value = 0.05), and also habitat 6220*a has significant differences to habitats 6410 (*p*-value = 0.03) and 6430 (*p*-value = 0.05), and habitat 6220*b differs from habitat 6430 (*p*-value = 0.04).

Overall, this analysis showed that the ecological indicator humidity (U) and nutrients (N) are the most important that influence the composition of the species in the analysed dataset, while the factors of light (L) and substrate reaction (R) are less important. This confirms the clear difference between dry and wet grasslands. The overall table of this assessment is a Supplementary Materials Table S3.

3.3. Plant Diversity and Naturalness

The biodiversity values of the grassland habitats compared (Figure 5) show that the total Shannon index (H^+) is higher in grassland habitats characterized by species with higher humidity values (U), including 6230*, 6430, 6420 and 6210*, while the Shannon index (H^+) calculated on disturbance and alien species has higher values for the driest grassland habitats, such as 6220*a and 6220*b, characterized by species with higher Ellemberg indicator value of temperature (T) and light (L). Equipartition (J) is higher in habitats with higher humidity index (U) (see Supplementary Materials Table S4).

The naturalness values (Na) for the different habitat types (Figure 6), which take into account the ratio between the values of total H^+ and H^+ without disturbance and alien species, are found to be at a maximum level in habitats 6230*, 6410, 6430 and 6420, while habitats 6220*b, 6220* and 6170 show lower values of naturalness (Supplementary Materials Table S5).

Figure 7 shows the contribution to the biodiversity of alien and disturbance species in the different habitat types, taking into account the relationship between the total H^+ value and the H^+ value of the alien and disturbance species. The graph shows that maximum values are found in habitats 6220*b, 6220* and 6170, confirming that in these habitats disturbance and alien species contribute more to the value of biodiversity than habitats 6230*, 6410, 6430 and 6420, which show lower values and are therefore characterised by greater biodiversity (Supplementary Materials Table S6).

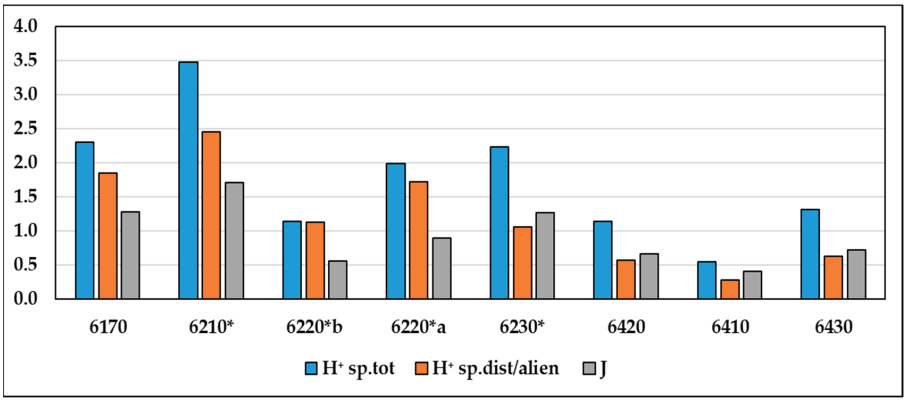


Figure 5. Values of biodiversity indices for each habitat type (H^+ = Shannon total species, J = Evenness, H^+ sp. dist/alien = Shannon alien and disturbance species).

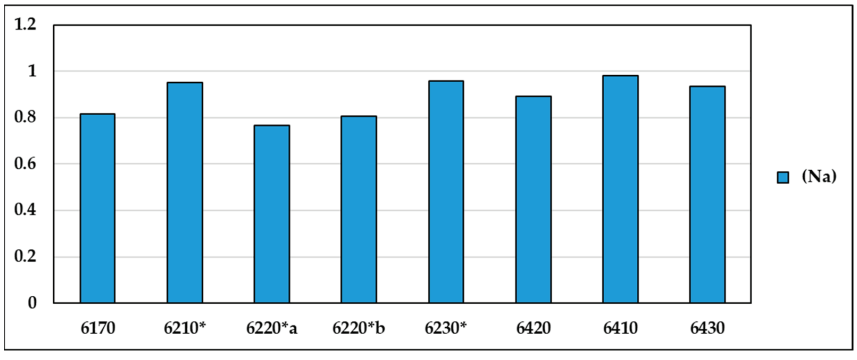


Figure 6. Values of the naturalness index (Na) for the various habitat types.

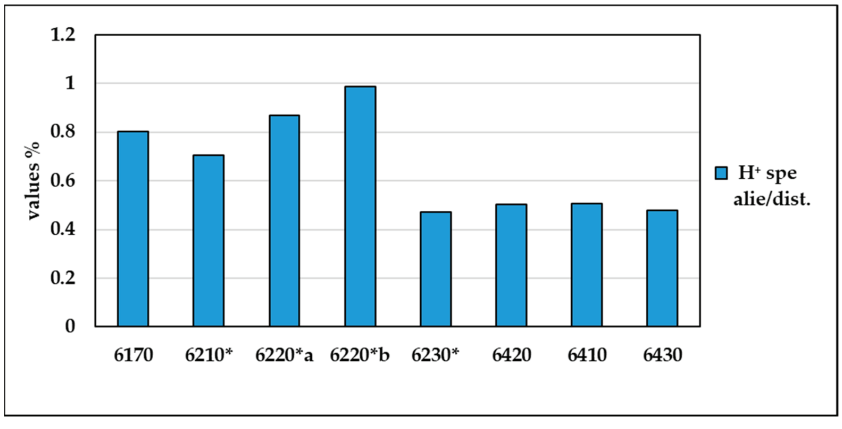


Figure 7. Percentage values of the H^+ of alien and disturbance species on total biodiversity in the various types of habitat.

3.4. Relationship between Diversity and Ellenberg Indicator Values

The linear regression model (Figure 8) between the naturalness index (Na) and the ecological temperature indicator (T) shows a significant slope value < 0.05 (0.000046817), correlation index r = −0.84 and regression index r^2 = 0.71 (Supplementary Materials Table S7).

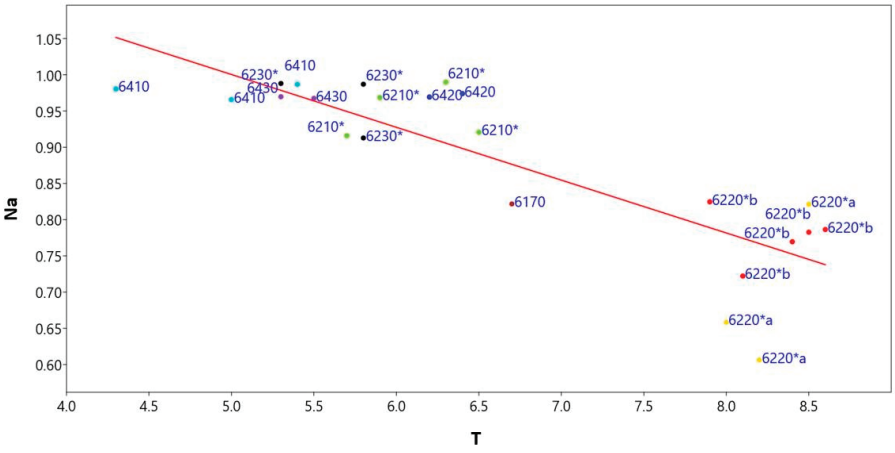


Figure 8. Linear regression model between the naturalness index (Na) and the Ellenberg indicator values of temperature (T) of the representative relevès for each habitat type: in yellow 6220*a, in red 6220*b, in black 6230*, in green 6210*, in brown 6170, in dark blue 6420, in blue 6410, in violet 6230*.

To check the autocorrelation analyses, the Durbin-Walson statistical test was applied, which assumes a value of 1.82 and a probability of autocorrelation of 32%, while the variance of the residuals and thus the homeschedasticity was calculated using the Breusch–Pagan test, with a value of 3.01 and a probability of homeschedasticity of 0.08%. This analysis shows that habitats' naturalness decreases with increasing temperature, as low-lying habitats are most impacted by anthropogenic activity and are often close to urbanized areas.

The linear regression model between the naturalness index (Na) and the Ellenberg indicator values of moisture (U) (Figure 9) shows a significant slope value < 0.05 (0.000000010994), correlation index r = −0.87 and regression index r^2 = 0.75 (Supplementary Materials Table S8). In order to verify the autocorrelation analyses, the Durbin–Watson statistical test was applied, which assumes a value of 1.51 and a probability of autocorrelation of 0.09%, while the variance of the residuals and thus the homeschedasticity was calculated using the Breusch–Pagan test, with a value of 1.40 and a probability of homeschedasticity of 23%. This analysis agrees with the previous one; in fact, it shows that the naturalness of habitats increases with increasing humidity, an ecological indicator that characterizes habitats at higher altitudes with low values of the temperature index, located in the mountain belt where most of the protected areas of the region are located and where the anthropic pressure is lower.

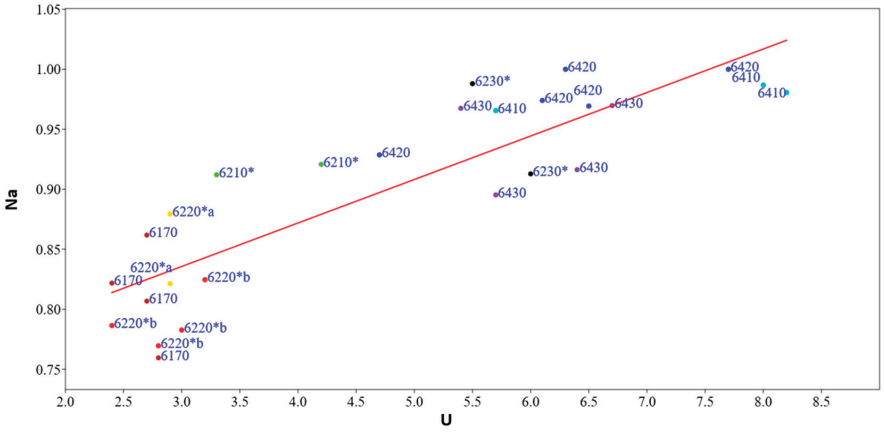


Figure 9. Linear regression model between the naturalness index (Na) and the Ellenberg indicator values of humidity (U) of the representative relevés for each habitat type: in yellow 6220*a, in red 6220*b, in black 6230*, in green 6210*, in brown 6170, in dark blue 6420, in blue 6410, in violet 6230*.

4. Discussion

Cluster analysis on grasslands vegetation allowed us to identify eight different groups attributed to as many habitats of EEC Directive 43/92 in accordance with the interpretation manual [71]. These groups are in agreement with the alliances recognized in the phytosociological literature [70,99].

Principal component analysis (PCA) allowed us to recognize three groups in the matrix of phytosociological relevés based on ecological factors: habitats 6210* and 6170 are characterized by species that prefer base-rich substrates, also showing high values of continentality; habitats 6410, 6430 and 6420 are those with the greatest availability of moisture and nutrients in the soil; and habitats 6220*a and 6220*b are the most thermophilous ones as they are located at low altitudes in purely Mediterranean bioclimatic conditions [86].

Box plots of Ellengerg indicator values confirm ecological differences between habitats. In fact, the box plots show a significant difference for the continentality indicator between habitat 6210* and the other habitats, which is related to the fact that the typical vegetation of this habitat, referring to the class *Festuco valesiacae-Brometea erecti* Br.-Bl. & Tüxen ex Br.-Bl. 1949, characterizes the continental grasslands of Central and Eastern Europe, which develop in areas with low annual rainfall, in agreement with Biondi et al. [70]. This habitat, in the analysis carried out, is similar to 6170, characterized by the vegetation of the *Ranunculo pollinensis-Nardion strictae* Bonin 1972 alliance distributed in the mountain range on calcareous or dolomitic substrates and compacted soils rich in organic substances, often in stations characterized by prolonged snowfalls which guarantee greater soil humidity [100].

The high biodiversity values of habitats 6230*, 6430, 6420 and 6210* are associated with the high value of the Ellember indicator moisture value (U) and this is consistent with [101], which emphasises high biodiversity values for semi-natural wet grassland habitats.

The ecological factors that contribute to the differentiation of habitat types are mainly temperature (T) and humidity (U), which play an important role in defining the floristic composition of the identified grassland habitats. Indeed, grassland habitats are widespread in the region from sea level to the mountain belt and occupy a wide altitudinal range, where temperature and humidity factors play a fundamental role in the ecological characterisation of the different habitat types.

The nutrient factor (N) showed significant differences between the findings belonging to habitat type 6430, characterized by nitrophilous communities, and those belonging to groups 6220a and 6220b; this could be due, in agreement with the evidence of other studies [102,103], to a higher soil moisture influencing nutrient availability. In fact, habitat

6430 presents higher humidity values, but, above all, high values of naturalness and diversity are characterized by a variable phytocoenosis consisting also of species from other communities [104], some of which are rare and threatened, which, in agreement with other studies, guarantees a greater conservation value for the habitat [105].

Overall, the analysis performed, in agreement with some studies [106], shows that the most important ecological gradients influencing the diversity of grassland vegetation are moisture and nutrients. Furthermore, the results show that grassland habitats characterised by species with higher moisture values are more natural.

This is evident in habitat 6410, of grasslands with high floristic diversity and a very broad ecology developing on mesotrophic, oligotrophic, moist and cool soils [70,107]. The vegetation of this habitat is linked to the water table, which determines the differentiation of species in these environments [101].

A Mediterranean, thermo-xerophilous vegetation characterizes the 6220*a and 6220*b habitat types; they grow at low altitudes in the driest stations where ecological factors such as temperature and brightness are greater and therefore reduce water availability within the soil [86]. These habitats are the least natural, as intensive land use at low altitudes, associated with fires, soil disturbance and anthropisation, plays a fundamental role in structuring grassland plant communities [86,108], affecting diversity and naturalness and causing changes in biodiversity, both in terms of a reduction in species richness and the replacement of typical species by disturbed ones [109,110].

Many of the species typical of grasslands are threatened with extinction [110], and the decrease in surface area and quality shows that grasslands are now among the most endangered European habitats [18,111].

5. Conclusions

Our study shows that the plant assemblage composition is the best tool to characterize grassland habitats from an ecological and typological point of view, indeed the analysis of grassland vegetation can provide information on ecological factors that influence the species composition and distribution of plant communities. In particular, the species diversity indices (Shannon, Evenness) applied in this study show that naturalness is greater in wet than dry grasslands. However, knowledge of land use and the environmental context of ecosystems varies due to the interaction between the effects of human activity and other environmental factors. Ecological indicators, such as those used here, can therefore be equally effective to define patterns and processes of change and are a useful tool for identifying areas of high natural value. To counter the continuing loss of grasslands and their typical species, it is important to understand the causes of vegetation change, which are likely to differ between different habitat groups. Therefore, the results of this study showed that the diversity and naturalness indices applied, together with the assessment of ecological factors, are useful tools for implementing both an appropriate interpretation and a management and conservation strategy under EEC Directive 43/92. Understanding the ecological features and assessing habitat biodiversity allows us to interpret evolutionary dynamics and predict future scenarios due to climate change [112,113].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/land13060719/s1, Table S1: matrix of phytosociological relevés; Table S2: Relevés' dispersion biplot on PCA in relation to ecological factor; Table S3: The mean values of the ecological index and the non-parametric Kruskal–Wallis test; Table S4: Values of biodiversity indices for each habitat type (H^+ = Shannon total species, J = Evenness, H^+ sp. dist/alien = Shannon index of alien and disturbance species); Table S5: Values of the naturalness index (Na) of habitat types; Table S6: Percentage values of the H^+ index of alien and disturbance species on total biodiversity in the various types of habitat; Table S7: Linear regression model between the naturalness index (Na) and the values of the ecological temperature factor (T); Table S8: Linear regression model between the naturalness index (Na) and the values of the ecological factors of humidity (U) of the representative surveys for each type of habitat.

Author Contributions: Conceptualization, A.M., C.M.M. and G.S.; methodology, A.M.; software, A.M.; analysis, A.M.; data curation, C.M.M.; writing—original draft preparation, A.M. and C.M.M.; writing—review and editing, A.M., C.M.M. and G.S.; visualization, G.S.; supervision, G.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partially supported by "Nature Map System" project signed between the AGRARIA Department—Mediterranean University of Reggio Calabria and the Territory and Environment Department of Calabria Region, Sector 5, Parks and Protected Natural Areas, under the Regional Operational Program (ROP) 2014/2020—Action 6.5.A.1—Actions provided for in the Prioritez Action Framework (PAF) and in the Natura 2000 Network Management Plans, scientific manager GiovanniSpampinato.

Data Availability Statement: The original contributions presented in the study are included in the article.

Conflicts of Interest: The authors declare no conflicts of interest.

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Quality and Establishment of Some Water-Conserving Turfgrass Species for Sustainable Development and Some Ecosystem Services in Arid Urban Environments

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Abstract: Turfgrasses are essential landscape plants with social, environmental, and aesthetic services for urban ecosystems. However, more is needed to know how to establish them so that they can benefit from their ecosystem services in urban environments. This research examined some quality and morphological and physiological factors for the establishment and social and environmental service assessment of three warm-season turfgrasses, including Kikuyu grass (*Pennisetum clandestinum*), bermuda grass (*Cynodon dactylon*), and buffalo grass (*Buchloe dactyloides*), compared to the cool-season grass of tall fescue (*Festuca arundinacea* Schreb.). The experiment was split-plot in time, based on a randomized complete block design with eight replications. The main plot was the season with four levels, and the subplot was the four turfgrass species types. The results indicated that seasons and turfgrass types and their interaction significantly impacted most measured variables ($p \leq 0.01$). Some quality measurements like turf density, color, texture, coverage, and quality after clipping and establishment confirmed the superiority of *Buchloe dactyloides* over the other species. Also, kikuyu grass showed higher turfgrass density, more potential for weed control, and higher coverage and growth rate but also showed invasiveness features. Tall fescue had the lowest visual aesthetic compared with the other turfgrass species. Warm-season turfgrasses adaptable to the ecology of the region should be used compared to tall fescue to achieve better turfgrass quality and social and ecosystem services for the sustainable development of arid urban environments.

Keywords: warm-season grass; cool-season grass; green space; landscape; ecosystem services

Citation: Kazemi, F.; Golzarian, M.R.; Rabbani Kheir Khah, S.M. Quality and Establishment of Some Water-Conserving Turfgrass Species for Sustainable Development and Some Ecosystem Services in Arid Urban Environments. *Land* **2024**, *13*, 721. https://doi.org/10.3390/land13060721

Academic Editors: Michael Vrahnakis and Yannis (Ioannis) Kazoglou

Received: 22 March 2024
Revised: 16 May 2024
Accepted: 16 May 2024
Published: 21 May 2024

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1. Introduction

Despite the water shortage in many cities worldwide, irrigation of urban landscapes, especially turfgrass species, accounts for a considerable percentage (between 40% and 70%) of urban water consumption [1]. Conversely, urban green spaces are a fundamental infrastructure that positively affects cities' sustainability through their ecological, social, and economic benefits [2,3]. The cities' main sustainability performances in ecology, biodiversity, urban heat island mitigation, and recreation depend on conserving and developing urban green spaces [4,5]. Turfgrasses are one of the essential components of urban green spaces [6,7], providing many ecosystem services that other vegetation types offer. Functional, aesthetic, recreational, social, and economic services, as well as physical and mental health effects, are among the ecosystem services of turfgrasses in urban environments previously receiving emphasis [7]. Turfgrasses have a special aesthetic significance and offer a unique surface for leisure sports and activities [7,8]. Based on the literature, if lawns are managed extensively, they have high capacities to prevent soil erosion due to their high soil coverage and density [9], reduction of surface runoff, contribution to carbon sequestration [10], and adding biodiversity values to urban environments [11,12]. Despite

such benefits, significant debates exist about expanding and maintaining them in arid urban landscapes due to their high water consumption [7]. To achieve sustainability in urban turfgrass management, researching, establishing, and adapting water-conserving turfgrasses is very important. One of the most effective strategies to reduce water consumption in turfgrass species is to select varieties and cultivars adapted to the region's climatic conditions [13]. Turfgrasses used in urban green spaces belong to the Poaceae family, which includes many different species and ecotypes [6]. Such variation can provide high adaptability for the species to different climatic conditions. Despite this, turf breeders still try to develop cultivars that can grow satisfactorily in various climates, soils, and environmental conditions [14].

The warm- and cool-season grasses comprise the two main turfgrass categories. While cool-season grasses grow most actively in temperatures between 16 and 24 °C, warm-season turfgrasses sprout and grow best in warm months at temperatures between 27 and 35 °C [15]. The most prevalent warm-season grasses, which are more drought-resistant than cool-season grasses, include bermuda grass, buffalo grass, bahia grass, zoysia grass, and St. Augustine grass [16]. Water-restricted countries are more likely to create these types of lawns because warm-season turfgrasses use less water than cool-season turfgrasses in desert climates. In temperate climatic regions, warm-season turfgrass species undergo browning and winter dormancy due to low temperatures, causing a clear seasonal pattern [17]. Law et al.'s study [18] examined the growth rate, clipping practices, and environmental impacts of warm- and cold-season grass species, and the greenhouse gas fluxes from turfgrass systems. In comparison to C_4 turfgrasses, which had mean CO_2 flux rates ranging from 0.273 to 0.361 g CO_2-C m^{-2} h^{-1}, C_3 turfgrasses had the greatest mean CO_2 flux rates, varying from 0.373 to 0.431 g CO_2-C m^{-2} h^{-1}. Turfgrasses classified as C_4 were more likely to be CH_4 sources, whereas C_3 grasses were frequently CH_4 sinks. The drought resistance, salt tolerance, and fertilizer response of warm-season turfgrasses have all been examined [19,20]. Local study is necessary to find warm-season turfgrasses that can remain green for a long time throughout each region's cold seasons.

The grass industry in many countries relies on the National Turfgrass Evaluation Program (NTEP) developed by Morris and Shearman [21] for the quality assessment of turfgrass in urban landscapes. Grass reformers, researchers, and experts in this field use the data obtained based on the NTEP to determine the degree of adaptation of the cultivars [22]. However, image-based systems are also emerging [23]. The evaluation process of the lawns is based on the visual estimation of a set of qualitative indicators such as color, density, softness, growth habit, and uniformity of the texture by scoring the grasses by a number between 1 and 9 by a group of experts, and the numbers above six are considered acceptable lawn qualities [21–23]. Studies have been carried out on the establishment and quality of turfgrasses worldwide. For example, Mortazavi and Rabbi [24] examined the ecological adaptation of some exotic turfgrass cultivars (*Poa pratensis*, *Lolium perenne*, and *Festuca rubra*) in Zanjan, Iran. The results showed that these varieties had different germination rates, cover, uniformity, texture, seasonal colors, and growth rates under drought conditions. Their ecological adaptation in arid regions should be examined under drought conditions. Saeedi Pooya [22] examined the compatibility of *Lolium perenne* var. Yarand, *Lolium perenne* var. Chadegan, *Festuca arundinacea* var. Chadegan, and *Lolium multiflorum* var. Chadegan in pure and mixed sowings in different seasons under the climatic conditions of Mashhad. Salehi and Khosh-Khui [25] also made qualitative evaluations of color, density, and uniformity in single-seed and mixed plantings of cool- and warm-season turfgrasses. In this research, the turfgrasses—perennial rye grass, kentucky blue grass, common bermuda grass, and creeping red fescue—in monoculture or in mixtures of 1:1 (by weight) and 1:1:1:1 (by weight) and two sport turfgrasses—BAR 11 (Barenbrug Co., Dandenong, Australia) and MM (Mommersteeg Co., Hilvarenbeek. The Netherlands)—were used. According to their results, a mixture of kentucky bluegrass (*Poa pratensis*) and bermuda grass (*Cynodon dactylon*) was the most suitable lawn mixture for Shiraz climate conditions.

Akbarzadeh [26] investigated the growth responses of buffalo grass and tall fescue under three regulated deficit irrigation levels (40, 70, and 100% of the water requirement). Compared to tall fescue, the results showed that buffalo grass had better performance and minor damage to its morphological and physiological traits under drought stress. Buffalo grass varieties were more resistant to water stress and weeds than tall fescue. Therefore, tall fescue is recommended in water-limited and cold winter areas, especially for over-seeding purposes. In Mediterranean settings, Martiniello and Andrea [27] evaluated the quality and adaptation rate of cool-season turfgrasses, including creeping red fescue, tall fescue, perennial rye grass, and kentucky blue grass. Kentucky blue grass and creeping red fescue, compared with tall fescue and perennial rye grass, were less successful in quality, color, and turfgrass cover.

In contrast, perennial rye grass and tall fescue's rate of climate adaptability was much more than that of kentucky blue grass in Mediterranean conditions. Johnson [28] reported the differences in the growth speed of fescue and buffalo grass. He suggested that fine fescue (*Festuca rubra* ssp. *rubra* L. 'Vista', *F. ovina* var. glauca Lam. 'Minotaur', *F. rubra* ssp. *commutate* Gaud. 'Jamestown II') covered 100% of the plots and gradually prevailed on the buffalo grass. Volterrani [29] stated that bermuda grass cultivars showed good adaptability to the climatic conditions of Italy. Although these cultivars are yellow in winter, this problem could be solved by planting them mixed with cool-season turfgrasses. Some management techniques can increase the period of greenness, color, density, and cold tolerance of these cultivars. Whitman et al. [30] showed that the cultivation of mixed warm-season grasses significantly reduced maintenance needs such as lawn mowing and the use of pesticides compared to single cultivations of these grasses.

This study will investigate the establishment and quality of three single-seed water-conserving warm-season turfgrass species and their seed mixes compared with the cool-season grass of tall fescue during four seasons of a year to suggest the most suitable species for future sustainable urban landscaping.

2. Materials and Methods

2.1. Experimental Design and Site Description

This field experiment was performed in Mashhad, Iran (elevation 989 m; mean annual rainfall 255.2 mm) in an arid to semi-arid climate region. Long-term maximum and minimum temperatures average 22 °C and 8.9 °C, respectively (Table 1).

Table 1. Monthly meteorological parameters of the experimental area.

	2015						**2016**					
	July	**August**	**September**	**October**	**November**	**December**	**January**	**February**	**March**	**April**	**May**	**June**
Total precipitation (mm)	0	0	0.1	0	15.6	16.4	16.5	18.1	16.3	48	87.8	44.6
Average temperature (°C)	28.8	29.6	26.8	21.4	17	8.8	5.4	6.8	7.8	11.7	15	23
Maximum temperature (°C)	40	40.6	39.8	33.4	35.9	22.4	23.5	22.2	27.4	29.1	32.6	36.8
Minimum temperature (°C)	14.6	18.6	13.7	8.6	3.6	0.6	−5.6	−4.5	−5	0.7	2.2	13
Relative humidity (%)	13.5	15.5	20.6	45.9	68.1	98.1	72.6	34	33.2	69.5	53.8	40.4

This study was conducted as a split-plot arrangement based on a randomized complete block design experiment with eight replications. Seasons of the year (summer, autumn, winter 2015, and spring 2016) were considered as the main plot, and four turfgrass species, including kikuyu grass (*Pennisetum clandestinum*), bermuda grass *(Cynodon dactylon*), buffalo grass (*Buchloe dactyloides*), and tall fescue (*Festuca arundinacea*) (Figure 1) were considered as subplots. Also, a mixture of these three types of grass with similar seed percentages were considered as subplots. However, as kikuyu grass was highly invasive, the plots with a mixture of the three warm-season grasses were shortly over-dominated by kikuyu grass,

and our turfgrass analysis only showed the characteristics of this grass species. Therefore, this study eliminated these mixed turfgrass plot data from further analyses to avoid biases. Over-domination of kikuyu grass was considered one of the measurements of the invasive nature of this turfgrass species.



Figure 1. A schematic view of the experimental design (G1: kikuyu grass, G2: bermuda grass, G3: tall fescue, G4: buffalo grass, G5: mixture of the three warm-season grass types and R1–R4: Blocks or Replications).

2.2. Description of the Studied Species

2.2.1. Kikuyu Grass (*Pennisetum clandestinum*)

This grass species was introduced to Western Australia in the early 1920s. It is native to the central regions of Africa. However, the plant species now exists in many parts of the world, and in countries like Australia, New Zealand, and South Africa, it is considered one of the most popular grasses. This species is cultivated as a pasture plant for livestock grazing due to its high nutritional value (very digestible, low fiber, high protein, and good taste). However, it was initially considered a weed due to its fast coverage and aggressiveness [31]. Kikuyu grass spreads by large creeping fleshy rhizomes and stolons that sometimes grow up to 2 m. Its stolons produce a wide aerial section in each node, which sometimes reaches 60 cm in length. Among these features are fast regeneration power after picking or wear, high competition power with weeds, high growth rate and fully developed growth system, good tolerance to drought, heat, and salinity, covering power with high density, and stability. It is excellent for soil erosion control and is relatively shade-resistant [32,33]. This grass species, like bermuda grass, has a particular photosynthetic pathway that enables it to absorb carbohydrates at a high rate and grow rapidly during high solar radiation intensity and warm temperature periods. However, unlike bermuda grass, it maintains its constant growth rate under lower temperatures [33]. The optimal temperature for its growth is 18–30 °C, but it can maintain its active growth and color at lower (10 °C) and higher (38 °C) temperatures [33]. It is dormant in winter, starts growing in spring, and grows rapidly in summer and early autumn [31].

2.2.2. Bermuda Grass (*Cynodon dactylon*)

This grass is resistant to salinity, drought, and flooding stress and can compete with weeds. It quickly produces thatch and needs to be defoliated continuously [26]. This grass has a strong creeping and transverse growth feature, and its establishment speed is good. Its expansion range is extensive, and it shows excellent tolerance for environmental stresses [29]. Also, this grass is adaptable to a wide range of weather conditions and cannot only adapt to hot season areas but can also be established in areas where cold climate grasses are limited due to drought and salinity stress [26]. Its optimal growth temperature range is 27 to 35 °C. The grass leaves turn brown in winter. This species originates from the Middle East (Southwest Asia) [34].

2.2.3. Buffalo Grass (*Buchloe dactyloides*)

This species, native to semi-arid regions of North America [8,35], is a low-growth grass species with a soft texture that is remarkably resistant to hot and dry conditions [26]. It is an effective soil erosion controller, requiring minimal maintenance and surviving under low fertilization and watering regimes. It is worth noting that it will turn light brown if not watered during the summer, but its quality will be significantly improved when watering is resumed.

2.2.4. Tall Fescue (*Festuca arundinacea*)

This species is drought-tolerant and has deep and strong roots [36]. It is one of the cold-season perennial herbaceous grasses with high resistance to foot rot in optimal growth conditions (spring and autumn) [36]. It is a coarse-textured grass that grows in masses and remains green throughout the year. This grass has excellent resistance to heat, shade-sun, and drought and is compatible with a wide range of soil conditions [37]. It also shows a terrific response to increased irrigation and fertilization. Long grass is incompatible with other grasses and acts as a weed, especially in combination with fine-textured grasses [37]. The tall fescue used in this experiment was the variety native to Fereydon Shahr in the Isfahan province of Iran. This area is a mountain region with cold winters, cool summers, and an altitude of 2490 above sea level.

2.3. Planting and Maintenance

The turfgrass species' seeds were planted in plots of 1×1 m^2 covered with a thin layer of leaf compost and manure. Irrigation was carried out based on reference evapotranspiration (ET0). Reference evapotranspiration (ET0) was measured using an evaporation pan class A, which was installed within the experimental site. The amount of evaporation was calculated using the following formula:

$$ET0 = ECA \times K_p \quad (1)$$

where ET0 was reference evapotranspiration (mm/day), ECA was the amount of evaporation from the Class A evaporation pan (mm/day), and K_p was the pan index, which was 0.77 for the Mashhad climate condition. The irrigation interval was once per day in the establishment stage, and it was then reduced to once every other day during the active growing period of the lawns.

The planting rate of the seeds was calculated considering their pure live seeds (PLS) by weight. Based on this calculation, the rate of the seedlings was 30 g/m^2 for buffalo grass, 10 g/m^2 for bermudagrass, 15 g/m^2 for Kikuyu grass, 50.5 g/m^2 for tall fescue, and 15.5 g/m^2 for the mixed turf according to the seed sizes, purity, and germination percentage. The mixture of the three warm-season grass types (kikuyu grass, bermuda grass, and buffalo grass) was sown with similar percentages of the species (based on 1000-seed weight and plot size, the quantity of seed used for the seed mixture was 7.8 g for buffalo grass, 3 g for bermuda grass, and 4 g for kikuyu grass).

The soil texture was loam, pH = 7.21, cation exchange capacity = 6.6 meq/100, and organic matter was 0.9%. The weed species, including narrow and broad leaves, were hand-pulled during the one year of this study. The soil was amended using 5% cow manure at the planting time. No chemical fertilizers were applied during the experiment as the aim was to identify the best established low-input turfgrass species for the study area.

2.4. Measured Factors

Visual quality was assessed using a visual scoring scale of 1–9, as introduced by the National Turfgrass Evaluation Program (NTEP) of the USA [21]. One was the lowest or poorest rating score, and nine was the highest or best rating score. A rating of six or greater was considered acceptable. Visual quality was evaluated 12 times during the experiment in monthly intervals. Chlorophyll and carotenoid content were measured in the laboratory in

the middle of each season, and growth parameters were also measured in the field at the end of each month.

The overall plot color is represented by the seasonal color. On a scale of 1 to 9, 1 being straw brown and 9 being dark green, the ratings were given. Depending on the damage caused by diseases, insect pests, nutritional deficiencies, environmental stresses, and the capacity to retain color when seasonal changes occur, seasonal color can be utilized to successfully distinguish color variations in lawns (Figure 2). Assessing color is essential for measuring how warm-season grasses respond to cold stressors and how cool-season grasses respond to heat stressors [21]. Turfgrass density (visual estimate of living plants or tillers per unit area) was used using a 1–9 scaling system, with nine equaling maximum density [21]. The texture's visual rating was also based on the one to nine rating scale, with 1 equaling the coarse texture and 9 equaling the fine texture. Quality after clipping and general quality were also evaluated using the 1 to 9 scaling system (1 = poorest, 9 = best quality). A rating of six or above is generally considered acceptable.



(**A**) (**B**) (**C**)

Figure 2. Example images of scored color of buffalo grass based on NTEP method: (**A**) score 8, (**B**) score 5, (**C**) score 1.

Other traits such as uniformity, quality after clipping, resistance to weeds, and cold resistance were also evaluated using a 1 to 9 rating scale (1 = poorest, 9 = best) [21]. Visual ratings require consistency to ensure their merit. Therefore, total traits according to NTEP were measured every month, almost in the middle of the month. In the present study results, data from the three months in each season were averaged and reported as the seasonal effects. Four evaluators, including two men and two women, evaluated each plot in the measurement times, and the scores were averaged to enhance the accuracy of the results. To enhance the accuracy and consistency of the visual quality assessments using the NTEP method, a workshop on how to apply the method was held for the evaluators. The evaluators also reviewed the NTEP guideline and had access to example images of the different scored turfgrass species from our previous research work to use them as comparison images and references in their turfgrass evaluations and scorings. Some scored images for the color of buffalo grasses based on the NTEP method have been provided in Figure 2.

The coverage percentage can be quantified by the spread of stolon, rhizomes, and the primary tillering of the plants that occur after germination. A turfgrass is considered established if it covers more than 90% of the ground surface [23].

Also, to measure the leaves' chlorophyll content, 0.25 g of the fresh leaves were crushed in a mortar with 5 mL distilled water, and then they were mixed in balloons with distilled water. Then 0.5 mL of the resulting mixture was taken and mixed with 4.5 mL methanol and then was centrifuged for 15 min with 3500 rotations per minute. The solution was taken and used in a spectrophotometer, and the light absorbance at wavelengths of 470, 653, and 666 nm was red. The chlorophyll concentrations were obtained from the following equation [38].

$$\text{Chl a } (\mu g/mL) = (15/65 \times \text{A } 666) - (7/34 \times \text{A } 653) \quad (2)$$

$$\text{Chl b } (\mu g/mL) = (27/05 \times \text{A } 653) - (11/21 \times \text{A } 666) \quad (3)$$

$$\text{Carotenoid } (\mu g/mL) = (1000 \times \text{A } 470) - (2/860 \times \text{Chl a}) - (129/2 \times \text{Chl b})/245 \quad (4)$$

$$\text{Chl c } (\mu g/mL) = \text{Chl a} + \text{Chl b} \quad (5)$$

Growth parameters were also measured, including plant height with a ruler and leaf width with a caliper. The fresh weight of the leaves was measured with a digital scale, and their dry weight was also measured and recorded with the same scale after placing the leaves in an oven at 55 °C until a constant weight was obtained [5].

2.5. Statistical Analysis

JMP8 software was used to undertake analysis of variance (ANOVA) in the data. When an ANOVA revealed statistically significant treatment effects ($p \leq 0.05$), Tukey's test was used to compare the means. Excel package from Microsoft Office 365 was the program used to create the graphs.

3. Results

The results of the analysis of variance (Tables 2 and 3) indicated that season and turfgrass types significantly affected all the measured variables reported in these tables ($p \leq 0.05$). Also, the interaction effect of the season and turfgrass types was significant for all the measured traits ($p \leq 0.05$) except for total chlorophyll and carotenoids.

Table 2. Analysis of variances (mean squares) related to visual quality assessment of the turfgrasses in different seasons.

Source	d_f	Color	Density	Turf Texture	Uniformity of Turf	Weed Score	Resistance to Cold	General Quality	Coverage
Block	3	1.28	2.92	0.67	4.27	3.16 *	2.95	2	6.66 **
Season	3	141.79 **	11.25 *	22.28 **	11.51 *	11.78 **	123.38 **	158.59 **	21.83 **
Error a	9	1.55	2.45	3.27	3.37	1.25	1.26	0.98	1.02
Grass	3	69.94 **	69.49 **	9.02 *	79.22 **	51.11 **	24.60 **	21.97 **	188.74 **
Grass × season	9	22.15 **	14.68 **	19.88 **	12.25 **	8.26 **	23.01 **	30.25 **	23.13 **
Error b	36	1.78	3.2	2.13	2.53	2.16	1.59	1.09	2.48

**, *, significant at 1 and 5% probability levels, respectively.

Table 3. Analysis of variances (mean squares) related to morphological and physiological traits of the turfgrasses in four different seasons.

Source	d_f	Quality after Clipping	Height	Leaf Width	Fresh Weight of Clipping	The Dry Weight of Clipping	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Carotenoids
Block	3	2.64	54.45 **	0.009	79.611 *	3.5	2.05	0.87	3.69	0.4
Season	3	5.57 *	568.24 **	0.1 **	12,520.8 **	1025.34 **	6.05 *	5.16 **	10.40 **	1.43 *
Error a	9	1.36	7.31	0.006	13.18	4.02	1.72	0.31	1.18	0.31
Grass	3	3.21 *	404.41 **	1.51 **	11,626 **	248.25 **	6.03 **	16.79 **	18.62 **	2.98 **
Grass × season	9	4.27 **	136.48 **	0.1 **	7165.38 **	236.42 **	1.83*	1.27 **	1.77	0.6
Error b	36	1.08	13.19	0.008	65.33	5.45	0.88	0.41	1.03	0.43

**, * show significant at 1 and 5% probability levels, respectively.

Tall fescue had a darker color than the other turfgrasses during the four seasons of this study (Figure 3a). Based on Figure 3b, the highest turfgrass density was related to kikuyu grass in the four seasons of the study except for spring, and the lowest density belonged to tall fescue in the summer season.

The uniformity of the turfgrasses was significantly affected by seasons (Table 2). The best and worst uniformity of the turfgrasses were observed in kikuyu grass, buffalo grass,

bermuda grass, and tall fescue. The most uniform lawn type was buffalo grass in spring, and the least uniform grass was tall fescue in the summer season (Figure 3d).

The coarsest (worst) and the finest (best) leaf textures were observed in the winter and spring, respectively. Buffalo grass had the most delicate leaf texture among the four turfgrass types. However, the other turfgrasses had no significant difference in texture, although bermuda grass had a more acceptable texture than the other turfgrass species.

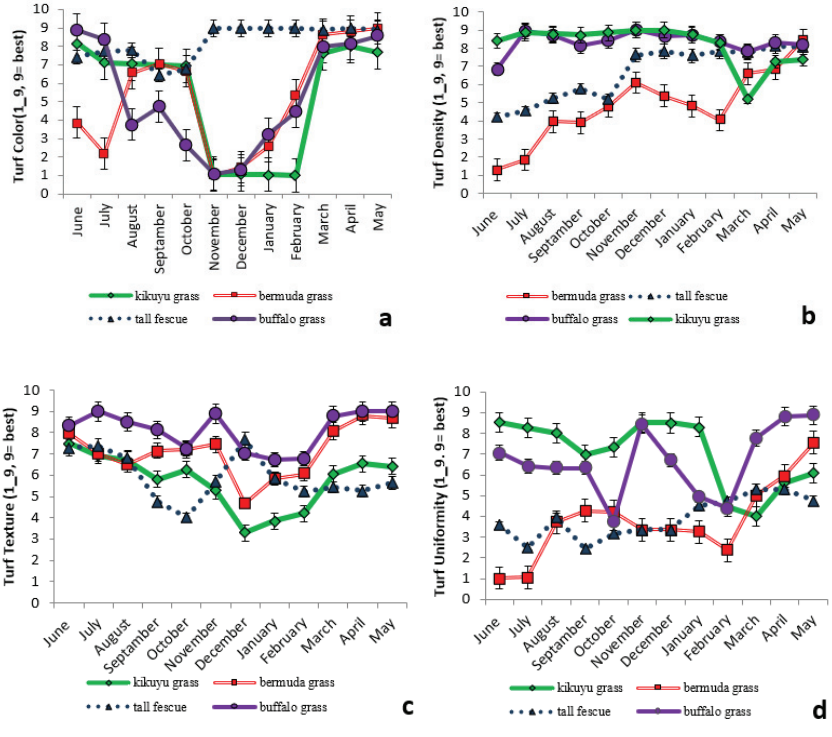


Figure 3. Monthly changes in color (**a**), density (**b**), texture (**c**), uniformity (**d**) in turfgrass species. Visual merit scores (1 = poorest, 9 = best) were measured according to NTEP, and during summer, autumn, winter 2015, and spring 2016. Error bars represent ± 1 standard error.

In this experiment, kikuyu grass and buffalo grass showed the fewest weeds compared to tall fescue and bermuda grass. The results showed that these four grass types were more robust against the weeds in the spring (Figure 4). The most common weeds included *Cyperus rotundus*, *Plantago major* L., *Datura stramonium* L., *Portulaca oleracea*, and *Chenopodium album*. In the winter, tall fescue was the most resistant to cold (maintaining plant color and active growth), and kikuyu grass was the most sensitive species. Buffalo grass was also a species minorly tolerant of autumn's cold weather (Figure 4).

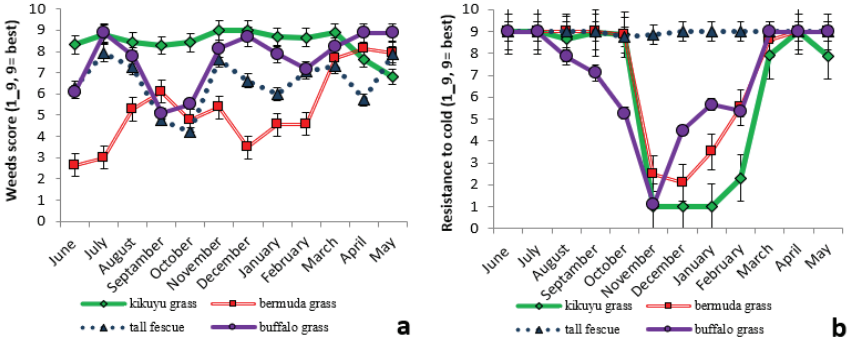


Figure 4. Monthly changes on weed attack score (**a**), cold resistance (**b**), in turfgrass species. Visual merit scores (1 = poorest, 9 = best) were measured according to NTEP and during summer, autumn, winter 2015, and spring 2016. Error bars represent ± 1 standard error.

The general quality of kikuyu grass was better than that of the other turfgrass species. Turf quality followed the sequence of kikuyu grass ≥ buffalo grass > tall fescue > bermuda grass. Generally, the quality of the turfgrasses was the best in the spring season. The seasonal effect on the turfgrasses' quality followed the order of spring > summer > autumn > winter. Results indicated that buffalo grass, kikuyu grass, and bermuda grass had higher visual attributes in the spring. All the turfgrass types had the lowest quality in winter except for tall fescue and buffalo grass, which had the most inferior quality in autumn. The turfgrass qualities in winter were tall fescue ≥ buffalo grass > bermuda grass > kikuyu grass (Figure 5a). The coverage percentage of the turfgrasses was generally higher in buffalo and kikuyu grasses. These two turfgrass types established and covered many plots during the summer, autumn, and winter. In the spring of the second year, buffalo grass had an excellent cover percentage, but kikuyu grass did not maintain an acceptable cover percentage. The other two turfgrass species, tall fescue and bermuda grass, had an increasing trend and reached an adequate coverage percentage level (in the spring of the second year) (Figure 5b).

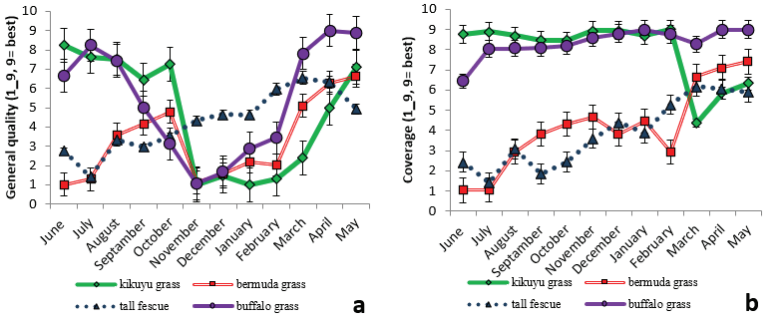


Figure 5. Monthly changes in general quality (**a**), coverage (**b**) in turfgrass species. Visual merit scores (1 = poorest, 9 = best) were measured according to NTEP, and during summer, autumn, winter 2015, and spring 2016. Error bars represent ± 1 standard error.

The mean quality after clipping was the lowest in tall fescue grass compared to the other turfgrasses, and buffalo grass had the best performance. According to Figure 6, among the seasons, autumn was the season in which the turfgrasses had the lowest quality after clipping.

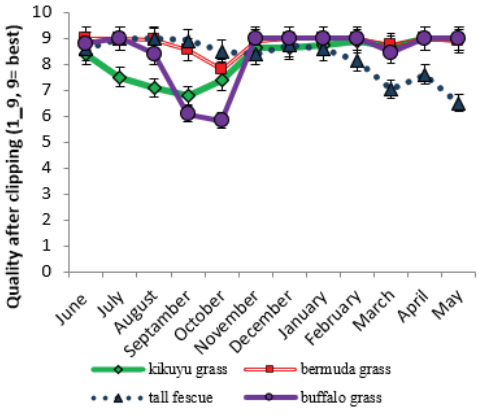


Figure 6. Monthly changes in quality after clipping in turfgrass species. Visual merit scores (1 = poorest, 9 = best) were measured according to NTEP, and during summer, autumn, winter 2015, and spring 2016. Error bars represent ± 1 standard error.

Among the studied plants in this experiment, kikuyu grass showed the highest values for plant height (21.04 cm), leaf width (0.58 mm), and fresh and dry weight (80.8 and 14.09 g, respectively). Also, bermuda grass showed the lowest plant height (12.72 cm), leaf width (0.19), and fresh and dry weight among the turfgrasses (23.22 and 8.48 g, respectively). There were significant differences in the growth of the plants in different seasons. The lowest height and width of the leaves were observed in summer, which might be due to the greater time required to establish the turf after planting. Also, the lowest clipping fresh and dry weight was achieved in the winter season (Figure 7).

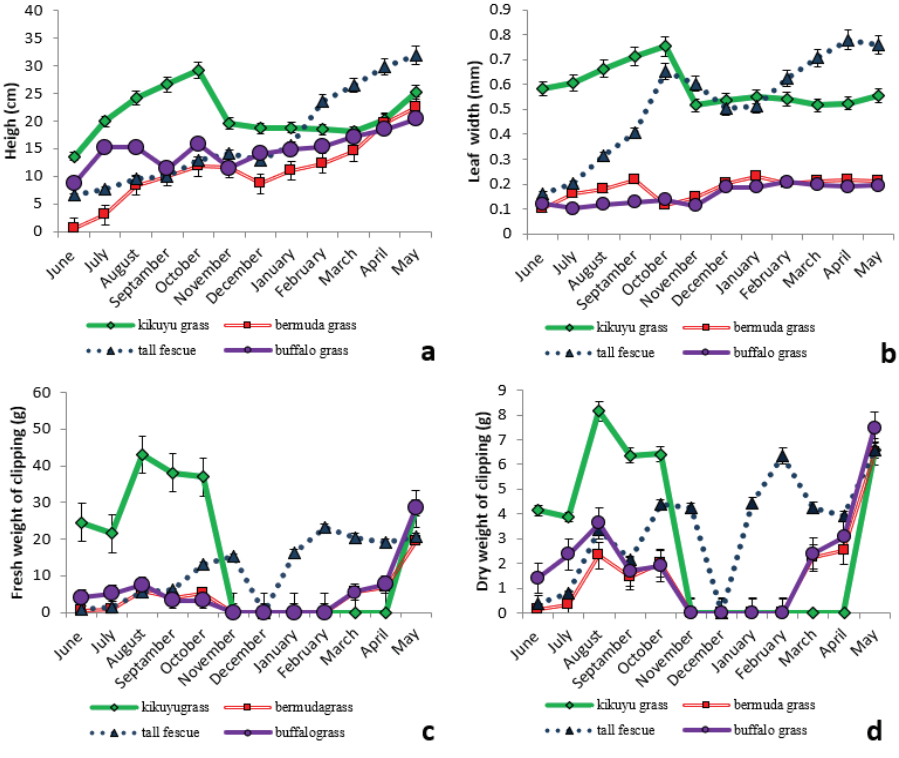


Figure 7. Monthly changes on height (**a**), leaf width (**b**), fresh weight of clipping (**c**), and dry weight of clipping (**d**) in turfgrass species. Visual merit scores (1 = poorest, 9 = best) were measured according to NTEP, and during summer, autumn, winter 2015, and spring 2016. Error bars represent ± 1 standard error.

The variance analysis on the chlorophyll a and b content showed that the interaction effect of the species and seasons was significant at a 1% probability level. However, there were no significant interactions between the species and the season's total chlorophyll in terms of chlorophyll content (Table 3). According to Figures 8 and 9, among the seasons, winter was the season when the species had the highest carotenoid content and the lowest amount of chlorophyll. Among the species, tall fescue had the highest amount of chlorophyll a, b, and total chlorophyll and the least amount of carotenoid.

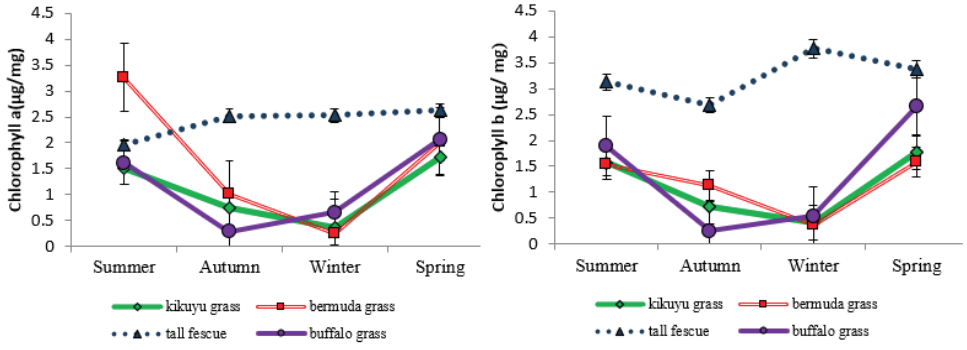


Figure 8. Interaction effect between the season and turfgrass types on chlorophyll a (**left**) and chlorophyll b (**right**). Error bars represent ± standard error.

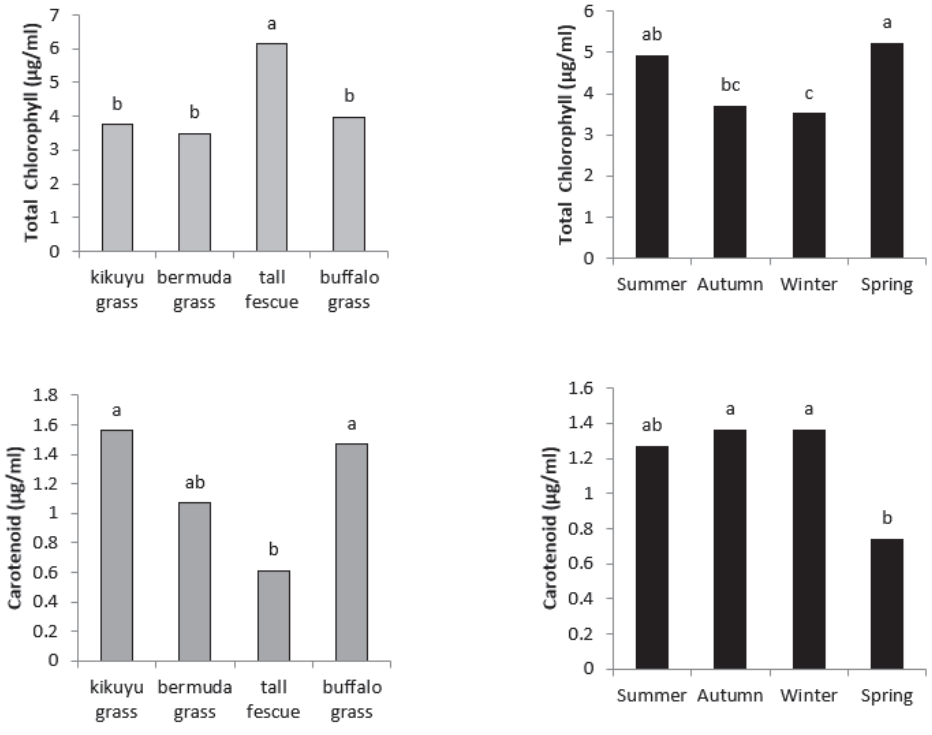


Figure 9. Differences in total chlorophyll and carotenoid contents of the examined turfgrass species in four seasons. The same letters indicate no significant differences at the probability level ($p \leq 0.05$) based on the Tukey test (a to c represents the highest and the lowest total chlorophyll and carotenoid contents, respectively).

Table 4 shows details of the qualitative assessment of the four turfgrass species studied in this study's four seasons.

Table 4. Qualitative assessment of the studied turfgrass species in four seasons.

	Color				Turf Density				Turf Texture				Uniformity of Turf				Amount of Weeds				Resistance to Cold				General Quality				Establishment of Turf				Quality after Clipping			
	s	a	w	p	s	a	w	p	s	a	w	p	s	a	w	p	s	a	w	p	s	a	w	p	s	a	w	p	s	a	w	p	s	a	w	p
Kikuyu Grass	*			*	*	*	*	*	*			*	*	*	*		*	*	*	*	*	*		*	*	*		*	*	*	*		*	*	*	*
Bermuda Grass	*			*		*		*	*	*		*				*				*	*	*		*				*	*			*	*	*	*	*
Tall Fescue	*	*	*	*			*	*	*		*						*	*		*	*	*	*	*			*	*	*			*	*	*		*
Buffalo Grass	*			*	*	*	*	*	*	*	*	*	*	*		*	*	*	*	*	*	*		*	*			*	*	*	*	*	*	*	*	*

*, means the attribute has received an acceptable grade (6 out of 9 or above based on NTEP) in the given season, (s) summer, (a) autumn, (w) winter, and (p) spring.

4. Discussion

In this study, visual quality measurements indicated the superiority of buffalo grass over the other studied grasses in a one-year study. Buffalo grass could establish quickly and produce an acceptable density, texture, and resistance to weeds during the four seasons of this experiment. However, it could not maintain an acceptable color and overall quality during winter and autumn and was not resistant to cold. It went to dormancy faster than the other species. Despite this species, kikuyu grass generated an acceptable turf density, low weed abundance score, and high quality after clipping in all four studied seasons, which could contribute to better social and ecological services of this turfgrass species [7]. Kikuyu grass did not show high-quality color, texture, and general turfgrass quality in autumn and winter. However, compared to the other warm-season grasses, it was resistant to the cold autumn weather conditions. The only problem for this species in spring was the lack of uniformity.

Based on this study's results, bermuda grass performed excellently in all the measured qualitative traits during the spring. This grass type had a good quality after clipping in all four studied seasons. This trait can be desirable for places where the lawn is constantly being clipped. However, compared to the other grass species in this experiment, it could have had a better quality in winter. Therefore, it is not recommended for the city of Mashhad, where a quality lawn is needed during the four seasons of the year. Bermuda grass survives the dormancy period using the reserves of non-structural carbohydrates and nitrogen compounds accumulated in its storage organs [39,40]. Also, the tall fescue's color and quality after clipping were high in all four study seasons. The best quality for this cool-season grass was observed during the spring season. The main problem with the quality of this grass species was the lack of uniformity, which is directly related to the aesthetic performance of the turfgrass species [22].

Color is one of the best indicators of the turfgrasses' general quality conditions [40]. Beard [38] stated that most individuals prefer dark green turfgrasses. In this study, all the visual quality measurements were the best in summer and the poorest in winter, disregarding the type of turfgrass. The turf color was lighter in winter and turned into a greener color in the spring. The highest color quality in buffalo grass was observed in spring and summer. In this study, tall fescue was darker than the three warm-season grass species during the four seasons. In a survey by Martiniello and Andrea [27], the effect of season on the color of different species, including perennial ryegrass, kentucky bluegrass, and tall fescue, was significant. Other researchers have also studied the color differences among lawn genotypes [25]. Genetic color differences among the grass species such as buffalo grass, blue grama, tall fescue, kentucky bluegrass, and agropyron and their mixtures were also reported by Bunderson et al. [41] based on the qualitative assessment scoring of 1 to 9. They also confirmed the color differences between the different grasses. In a study by Martiniello and Andrea [27] on cool-season species of perennial ryegrass, kentucky bluegrass, and tall fescue, the best coverage rate of the grass was in the spring and summer, and the lowest density was reported in winter, which corresponds with the results of our current experiment. Many researchers studied the density of different genotypes of

turfgrasses using visual assessment methods. For example, Salehi and Khosh-Khui [25] looked at mixtures of various grasses. They suggested that varieties of native fescue and bermuda grass had the least coverage. A mix of kentucky and bermuda grass had the highest coverage rate in Shiraz, Iran. In the present experiment, buffalo grass maintained a similar density during the four seasons. However, kikuyu grass showed a greater density during the three seasons of summer, autumn, and winter than the other species, while at the beginning of the spring, it could not reach its initial density. As a cool-season grass, tall fescue increased its density continuously during the four seasons of the study so that the highest density was observed in the last season of the study, spring. The density in bermuda grass decreased much more by cold than in other warm-season grasses, but at the beginning of spring, this grass increased its density like the other grass types. It seems bermuda grass reached an acceptable density and visual quality in the second year after planting. Regarding the texture of the grasses, buffalo grass showed the narrowest leaves. Although the other three types of grass were ranked statistically similarly, kikuyu grass and tall fescue had coarser leaves than bermuda grass. Further, all three warm-season types of grass had the coarsest leaves in winter, and the spring's finest leaves (Figure 7). The differences in leaf texture among the turfgrass species have also been previously reported by Saeedi Pooya [22] and Akbarzadeh [26]. An important point that should be carefully considered in turfgrass planting is not to plant a mixture of narrow and broad-leaf grasses. High density requires a high-quality lawn, as the fundamental function of grass is to cover the soil [42]. In sports field, high density is needed to cover the soil and form a cushion to reduce injury to players and provide a smooth platform for sports activities [43]. While high density is a positive turfgrass attribute for aesthetic and sports applications of lawn species, very high density negatively affects other turfgrass ecosystem services, causing low water flow, which lowers the flow's velocity, gives infiltration more time, and reduces the erosion control capacity of the turfgrass species [44].

In terms of uniformity in the grasses, the most uniformity was related to the spring, and the lowest uniformity was observed in the winter. As the planting time increased, the uniformity of buffalo grass, bermuda grass, and tall fescue increased during the four seasons of the study, and the highest uniformity among the three species was observed in the spring. Kikuyu grass had the highest uniformity in the summer, and this uniformity continued to decline and reach its lowest level in the spring. The differences in texture, density, competition between the species, color, and clipping height may affect the uniformity rate [26]. This reduction in uniformity of kikuyu grass in the spring can be related to its late growth start in the spring compared to that in the other species and can lead to weed growth and non-uniformity of the plots. Akbarzadeh [26] reported different uniformities among tall fescue, bermuda grass, and buffalo grass in Mashhad weather conditions and showed the superiority of buffalo grass compared to the other two turfgrass species in this city. Having a uniform and less weed-grown lawn will contribute to the lawn's aesthetic and low maintenance. However, it should be noted that alternatives to natural lawns, such as ground cover plant species and artificial lawns, have similar maintenance needs. Therefore, selecting the best turfgrass species should be based on multicriteria decision-making approaches by considering all lawns' aesthetic, social, recreational, functional, and ecosystem services [7].

At the beginning of the cold season in autumn in this study, the three warm-season species' reactions started with varying degrees. The most sensitive species to cold in autumn was buffalo grass, which went to dormancy at the beginning of September and began its growth earlier than the other species and showed signs of growth in winter from mid-December, indicating that its dormancy period is in autumn. This phenomenon differed in kikuyu grass and bermuda grass, which went to dormancy in winter. In contrast, tall fescue was the most resistant species in all four seasons of the year and had no dormancy period. Liu et al. [45] showed that tall fescue had higher heat and cold resistance than bermuda grass. The reason might be that tall fescue accumulates higher proline and H_2O_2 content as an antioxidant than bermuda grass after heat treatment.

According to this experiment's results, buffalo grass species, compared to other species, had a significantly higher rate of deployment and coverage during the four studied season (Table 4) The coverage rate of kikuyu grass was also acceptable, and the same was true in the experiment's first three seasons. However, kikuyu grass could not reach its initial establishment after passing the cold weather of winter, and its coverage rates were the lowest in the spring among the turfgrass types. The establishment and coverage rate of bermuda grass and tall fescue increased as the experiment passed. This study also showed that after the mixed planting of the three warm-season types of grass at the early stages of the planting, kikuyu grass was the fastest-growing species and became a dominant species compared to the other species in the plots. This species did not allow the other species to grow. The species should be selected to have the most color and growth rate similarity if an acceptable quality in the mixture of grasses is desired. In addition, none of the species should prevail over the other species and show signs of invasiveness [44]. Invasive grasses are not visually pleasant. Some are very pale and weak-looking and will grow outwards and sideways rather than straight upwards. Invasive grasses generally do not have a perfect color and will often grow more quickly than the more desirable grass types. They can quickly shadow out the desirable grasses, causing bare patches and allowing them to expand further into the available spaces. While the feature of fast grass coverage can be an essential factor in the restoration and over-seeding of the grass, particularly in sports lawns, it is neither visually aesthetic nor ecologically sound when it comes to an invasiveness feature and fast domination of one species over the other turfgrass species. Johnson [28] reported the differences in the growth rates of tall fescue and buffalo grass so that tall fescue gradually covered 100% of the plots and dominated the buffalo grass.

5. Conclusions

Turfgrasses are considered an integrated and crucial component of urban green spaces in the study area, the city of Mashhad. Therefore, the outcomes of this experiment on water-conserving turfgrass species selection are of great help to urban policymakers and landscape professionals of this region and other regions with similar climate conditions worldwide. Kikuyu grass was an invasive turfgrass species in this experiment for the studied area. It did not allow the minimal growth of other turfgrasses in the mixed cultivation. It had a very high stolon growth rate even in single species plantings, which is considered a biodiversity threat to the region's ecology. In addition to being invasive, this species did not have an optimum growth and color in the four seasons of the experiment. Therefore, it is not recommended for the region's urban landscaping. Despite the warm-season grasses in this study that all showed dormancy and periods of yellowness in cold seasons, tall fescue, as a cold-season grass species, did not show a dormancy period by being almost green in the four studied seasons. In addition, tall fescue was a native species that did not bring ecological invasiveness or threat to the region. However, it represented low visual quality attributes such as low coverage, coarse texture, and low quality after clipping. Among the studied turfgrass species in this experiment, buffalo grass (*Buchloe dactyloides*) was superior for most of the studied traits, such as turf density, color, texture, and quality after clipping and establishment in the city of Mashhad's climatic conditions. We recommend it as a suitable species for water-wise turfgrass establishment in this city. However, the species is non-native to the region. This study and our other studies (yet to be published) showed the current practice of planting this turfgrass species in controlled areas such as sports fields or urban landscape planting beds with edges together with applying turfgrass management actions such as regular mowing has represented this species as non-invasive with favorable ecosystems services. However, further research may require quantifying its effect on the long-term biodiversity, ecology, and ecosystem services it can bring to the studied city and to other urban environments worldwide.

Author Contributions: Conceptualization, F.K. and M.R.G.; Methodology, F.K. and S.M.R.K.K.; Formal analysis, F.K. and S.M.R.K.K.; Investigation, F.K. and S.M.R.K.K.; Resources, F.K.; Writing—original draft preparation, S.M.R.K.K.; Writing, review and editing, F.K. and M.R.G.; Supervision, F.K.; Project administration, F.K. and M.R.G.; Funding acquisition, F.K. All authors have read and agreed to the published version of the manuscript.

Funding: We thank Ferdowsi University of Mashhad for any possible support during early stages of conducting this research work.

Data Availability Statement: The raw data is not publicly available due to the privacy and continuity of the research.

Conflicts of Interest: The authors declare no conflicts of interest.

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Review

Biology of Hemiparasitic *Rhinanthus* Species in the Context of Grassland Biodiversity

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Abstract: The aim of the present review is to compile and analyze information on biology of *Rhinanthus* species in the context of grassland biodiversity. Root hemiparasites have been relatively less studied in comparison to economically important holoparasitic weed species. *Rhinanthus* species appear to be genetically polymorphic, but also possess high phenotypic plasticity, and ecological factors are important determinants in evolution of specialization to most appropriate hosts. *Rhinanthus* individuals have a relatively short life span, and flowering is a photoperiod- or host plant-independent phenomenon. Both insect pollination and self-pollination can occur. Seeds do not form a persistent soil seed bank and have physiological dormancy broken by stratification. In general, *Rhinanthus* species have low host specificity, but there clearly are 'preferred' or 'avoided' hosts in natural conditions. In controlled conditions, interaction with most grass species result in more prominent parasite growth stimulation in comparison to that of legumes, and, especially, forbs, but there are significant gradations and exceptions. Ecological requirements of *Rhinanthus* species have been rarely studied, but it can be expected that significant tolerance against mineral nutrient heterogeneity and water shortage can be found. It seems that host plant characteristics are important determinants of the environmental resilience of *Rhinanthus*. Parasites not only obtain resources (water and minerals) from host plants but also negatively affect their physiological functions. The most intriguing and practically unexplored question is the exchange of chemical signals between the *Rhinanthus* parasite and the host plant. Extending this idea, it can be predicted that signals will also be exchanged between multiple host plants whose roots are connected through the parasite. It is highly possible that the exchange of small RNAs between plants could influence their environmental tolerance. Host selectivity forms the functional basis of changes in species diversity in grasslands, but the outcome seems to be highly dependent on other conditions, especially, soil edaphic factors. Development of new model systems is necessary to further expand our knowledge about the complex effects of parasitic plants on ecosystems.

Keywords: environmental resilience; grassland biodiversity; host plants; reproduction; *Rhinanthus*; root hemiparasitic plants

Citation: Ievinsh, G. Biology of Hemiparasitic *Rhinanthus* Species in the Context of Grassland Biodiversity. *Land* **2024**, *13*, 814. https://doi.org/10.3390/land13060814

Academic Editors: Michael Vrahnakis and Yannis (Ioannis) Kazoglou

Received: 24 April 2024
Revised: 4 June 2024
Accepted: 5 June 2024
Published: 6 June 2024

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1. Introduction

Among plant–plant interactions, which are mostly non-specific and based on different types of competition, the relationship between parasitic plants and their host plants clearly stands out. Parasitism has evolved several times in the plant world, and parasitic species are found mainly in the family Orobanchaceae, as well as in Santalaceae and Convolvulaceae. The total number of known parasitic plant species is around 4500 from about 28 families [1].

The most clearly visible morphological feature in such a relationship is the formation of a special organ, haustoria, on the parasite, with the help of which it "connects" to the host's vascular system. Depending on attachment to either xylem or phloem, the parasite obtains resources from the host in the form of water, minerals and photosynthates. In the classification of parasitic plants, three groups of features are used, which describe the dependence on the interaction for the completion of the life cycle, the place of attachment

to the host plant, and the ability to carry out photosynthesis. In contrast to holoparasites that lack chlorophyll and cannot fix CO_2, hemiparasites retain photosynthetic function at least to a certain degree. Holoparasites are always obligate, but hemiparasites can be either obligate or facultative. All types of parasitic plants attach either to stem or root of a host plant. For general information on functional diversity and parasitic plants, readers are encouraged to consult several recent comprehensive reviews [2–7].

Plants of genus *Rhinanthus* (Orobanchaceae) are typical representatives of the Rhinanthoid group, consisting of 10 hemiparasitic and one holoparisitic genera [8]. In contrast to many parasitic plants that are economically important weeds (such as *Striga, Orobanche, Phelipanche*), *Rhinanthus* species have gained attention as positive players in improving grassland biodiversity. Perhaps for these practical differences, *Rhinanthus* species have been much less studied with respect to the mechanisms of parasitism relationship formation and host plant responses. Therefore, the aim of the present review was to compile and analyze information on the biology of *Rhinanthus* species in the context of grassland biodiversity with a special emphasis on open questions and identification of knowledge gaps in respect to ecologically and practically important aspects of *Rhinanthus* biology.

2. Taxonomic, Evolutionary, and Geographic Aspects

Among about 25 to 30 recognized *Rhinanthus* species, only a few are widely distributed throughout Europe. Among them, *Rhinanthus angustifolius* C.C.Gmel. and *Rhinanthus minor* L. are the most common species, with a high rate of co-occurrence in grasslands [9]. According to the recent taxonomy, *R. angustifolius* as well as *Rhinanthus serotinus* (Schönh) Oborny, are synonyms of *Rhinanthus major* L. [10]. In the present review, all species names are used as in the original publications. *Rhinanthus alectorolophus* (Scop.) Pollich is another species of the genus native to Central and Eastern Europe relatively frequently used as a model in studies with root hemiparasites.

Interspecific hybridization is common within the genus *Rhinanthus* [11], and several morphologically and ecologically similar but genetically different endemic taxa have been described [9,12]. It seems that ecotypes of *Rhinanthus* species evolve in a rather short time span, possibly due to local environmental and even anthropogenic factors, such as different grassland mowing regimes [13]. It is interesting that genetically determined differentiation in *R. angustifolius* is combined with relatively high phenotypic plasticity [14]. No geographical genetical structure has been established for *R. angustifolius* in Europe, which possibly reflects extensive hybridization or persistence of ancestral polymorphisms [15]. In contrast, *R. minor* s.s. populations showed a clear geographical structure of genetic similarity [9]. However, several populations initially identified as belonging to *R. minor* probably belonged to a previously unidentified taxon [9].

Traditional taxonomy previously used in local flora of Sweden distinguished broadly adapted *R. angustifolius* subsp. *angustifolius* (found in grasslands of road verges and sea coast) and meadow-specialized *R. angustifolius* subsp. *grandiflorus* (adapted to mowing practices). Moreover, a common garden experiment revealed a possibility that three groups of populations exist, representing grasslands of road verges, coastal sites, and mowed meadows [14]. When 17 *R. angustifolius* populations from South Sweden were analyzed, it appeared that interspecific genetic structure was clearly related to geographical distance [16]. Consequently, previously identified subspecies or population groups appear to be irrelevant in the context of the genetic structure of *R. angustifolius*, and a broader geographical range of populations needs to be considered for conservation purposes instead of focusing on specific habitats. Similarly, genetic factors most likely had no effect on specialization of *R. serotinus* to the most beneficial host plants, but ecological factors are more important in evolution of specialization in hemiparasitic plants [17].

3. Reproductive Biology of *Rhinanthus*

The life span of individual *R. minor* plants is only about 16 weeks. The life cycle of individuals of typical *Rhinanthus* species is shown in Figure 1, taking into account the

formation of associations with different host plants. Flowering is induced at a stage with a certain node number independently from photoperiod or presence of host plant, but due to genetic differences, can occur from May to July or late July to September [18]. Hermaphroditic flowers of *Rhinanthus* species produce nectar, and are pollinated by several Hymenoptera species, mostly belonging to *Bombus*, but self-pollination also occurs [19]. After pollination, flowers develop into capsules, containing variable numbers of relatively large flat winged seeds. Capsules dehisce several weeks later, but seeds remain in capsules until mechanical disturbance leading to local dispersal.

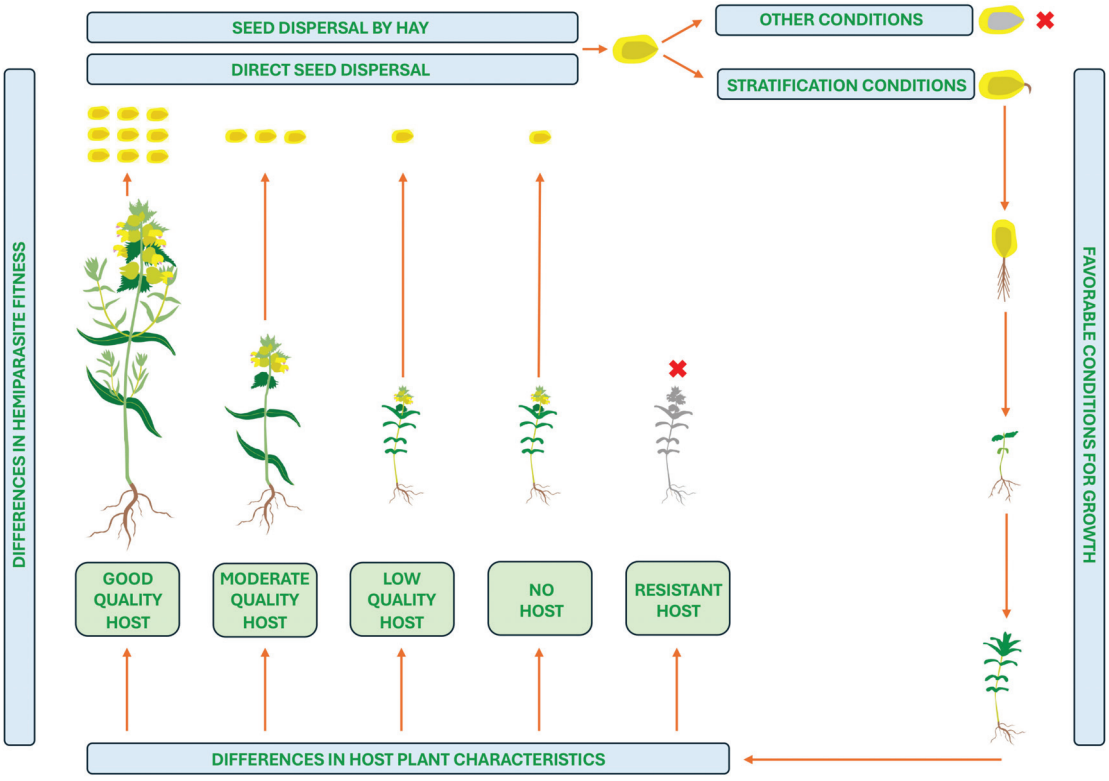


Figure 1. The life cycle of an individual of a typical *Rhinanthus* species showing dependence on host plant quality.

Unattached *R. minor* plants rarely produce more than two seed capsules per plant, but the number of capsules can exceed 20 with more than 170 seeds per plant in optimum conditions [19]. Seeds of *Rhinanthus* species lack special features for long term dispersal, and do not form a persistent seed bank in soil. At the time of dispersal, seeds of *Rhinanthus* plants have typical physiological dormancy that can be broken only by stratification. None of various seed treatments, including scarification, thermal induction, hormonal treatments (gibberellins and cytokinins), or treatment with host root extracts, can lead to an enhanced germination rate [20]. In addition, conditions of seed storage before stratification also affect germination success. However, requirements for breaking the dormancy period varies between the species within genus *Rhinanthus*, showing high species-specificity not depending on actual climatic conditions [21]. In particular, two distinct patterns can be seen within a group of five species. Species of the first group (*R. minor*, *R. angustifolius*) required a long stratification period (12 weeks and more), but species of the second group

(*R. alectorolophus*, *R. glacialis*, *R. mediterraneus*, *R. antiquus*) required only up to 12 weeks of stratification.

Seeds can germinate in the absence of any host plants when necessary conditions are met. However, it appears that response to temperature conditions after full stratification is also a species-specific trait. For the first group of *Rhinanthus* species mentioned above, transfer of seeds from stratification temperatures to 20 °C accelerated germination but decreased final germination percentage [21]. Just seven warm days in a row was a sufficient duration to accelerate germination. For the second group of species, increase in temperature did not accelerate germination while decreasing germination percentage. In fact, any duration of warmer period (1 to 7 days) slowed down germination for these *Rhinanthus* species. It is believed that the noted differences in stratification and germination patterns reflect adaptation to temperate climates with possible temperature fluctuations in spring (*R. minor*, *R. angustifolius*) or to warmer climates with relatively stable temperature conditions in spring (*R. alectorolophus*, *R. glacialis*, *R. mediterraneus*, *R. antiquus*) [21].

Different aspects of seed quality affect seedling establishment and survival, as well as future productivity of *Rhinanthus* plants. Among them, seed vigor, characterized as the time period between seed imbibition and radicle emergence, is extremely important, as high seedling mortality as well as low rate of biomass accumulation are direct consequences of low seed vigor [22]. Consequently, in addition to particular environmental factors, hemiparasite seed quality can also affect the results of interaction between parasites and host plants.

An extremely important aspect of seedling establishment in *R. alectrolophus* in field conditions is associated with the positive effect of sowing density on rate of seedling establishment, which is in striking contrast to the majority of plant species, showing existence of a prominent self-thinning mechanism at high densities [23]. As the experiment was performed on the background of potential host species, resource sharing among parasite seedlings through haustorial connections was suggested as a possible mechanism for increased survival with increasing sowing density. In contrast, degree of plant survival was not affected by sowing density, but mean plant biomass linearly decreased along with increased density.

4. Host Range and Selectivity

Analysis of a host range for parasitic plants associated with roots includes problems of a methodological nature. Such studies are usually carried out in field conditions, establishing the existence of associations between the haustoria of the parasite and the plants of a particular species, which in itself is a technically very complex process. Therefore, it is not surprising that such studies are often carried out in places with sparse vegetation, such as coastal dunes for *R. minor* [24]. Further problems are related to the interpretation of the results, because ideally the absolute data of root contacts should be related to the frequency of occurrence of the host plant in the specific location. As an alternative to direct counting of root contacts, association analysis is used to determine which plant species usually grow together with the parasite species and which do not. It is clear that such a method cannot give an accurate picture of the hosts of the parasite, but can only be useful as a preliminary approach.

By comparing frequency of roots for a particular host species available in a soil sample with number of actual haustorial contacts with that species, it was established that *R. minor* plants show a certain selectivity towards host species, as the number of haustorial contacts with roots of some species was significantly lower or higher than would be expected by chance [24]. Thus, it was concluded that some potential host species are either ‘preferred’ or ‘avoided’, but everything was complicated by the fact that certain species appeared as preferred hosts at one study site and as avoided hosts at another. It was argued that the site-specific selectivity of *Rhinanthus* species might be related either to differences in environmental conditions, variation in relative abundance of potential host plants, or genetic structure of both parasite and host populations [20]. It has also been established that

there is a positive relationship between biomass of *R. minor* and the number of haustorial contacts made for a particular parasite–host plant interaction [25].

From a total of 50 species identified as hosts of *R. minor* in Great Britain and Central Europe, 16 represented Poaceae and 11 Fabaceae [24]. However, not all species from these families were preferred by a parasite. It can also be assumed that grass species are more often parasitized, because they are more common in *Rhinanthus* habitats, in grasslands. Legume species are less frequent in these habitats, and preference for them could be related to the fact that they are supplying the soil (and parasite) with additional nitrogen as a result of bacterial symbiosis.

Until recently, only in relatively rare cases is a comparison of the suitability of several host plants for a given parasite carried out under controlled conditions [20]. Examples of various host plants with different qualities are shown in Table 1. Some recent studies have used a large number of individual species as hosts to *Rhinanthus* as well as mixtures of them. Thus, among 13 single hosts for *R. alectrolophus* (four grasses, five legumes, four forbs), the highest parasite growth stimulation was with grass species, followed by forbs and legumes [26]. However, one grass species (*Anthoxanthum odoratum*) and two legume species (*Onobrychis viciifolia* and *Anhyllis vulneraria*) were poor hosts and interaction with them did not result in any biomass increase of attached *R. alectrolophus* plants in comparison to unattached ones. Interestingly, degree of suppression of host growth was linearly related to parasite biomass.

Table 1. Examples of putative differences in host plant quality for various *Rhinanthus* species.

Species	Host Type	High Quality Host	Moderate Quality Host	Low Quality Host	Resistant Species
R. alectorolophus	Grasses	*Lolium perenne* *Poa annua*	*Cynosurus cristatus* *Dactylis glomerata* *Koeleria pyramidata*	*Anthoxanthum odoratum* *Holcus lanatus*	–
	Legumes	*Lotus corniculatus* *Trifolium pratense* *Trifolium repens*	*Medicago sativa*	*Anthyllis vulneraria* *Onobrychis viciifolia*	–
	Forbs	–	*Sanguisorba minor* *Taraxacum officinale*	*Chrysanthemum leucanthemum* *Hieracium pilosella* *Leucanthemum vulgare* *Myosotis arvensis*	–
R. minor	Grasses	*Cynosurus cristatus* *Dactylis glomerata* *Holcus lanatus* *Phleum bertolonii* *Poa annua*	*Anthoxanthum odoratum* *Bromus erectus* *Cynosurus cristatus* *Dactylis glomerata* *Koeleria pyramidata* *Lolium perenne*	–	–
	Legumes	*Lotus corniculatus* *Medicago lupulina* *Trifolium pratense* *Viccia cracca*	*Lotus corniculatus* *Medicago sativa* *Ononis repens* *Trifolium pratense*	–	*Anthyllis vulneraria*
	Forbs		*Achillea millefolium* *Daucus carora* *Sanguisorba minor* *Urtica dioica*	*Hieracium pilosella* *Myosotis arvensis* *Papaver rhoeas* *Plantago lanceolata*	*Leucanthemum vulgare* *Plantago lanceolata*

Information compiled from Rowntree et al., 2014 [25]; Sandner, Matthies 2018 [26]; Matthies 2021 [27]; Cameron et al., 2006 [28].

In a recent study, performance of two related *Rhinathus* species (*R. minor* and *R. alectorolophus*) was compared using 25 host species in controlled conditions at two soil

nutrient availability levels [27]. Most importantly, the two closely related parasite species had similar host specificity: most nonleguminous forbs were poorer hosts in comparison to legumes and, especially, grasses. However, in contrast to the previous study, the damage caused by parasites to the hosts was not related to the degree of benefit obtained for the parasites. Some good host species, including *Trifolium repens*, *Trifolium pratense*, and *Medicago lupulina*, were not negatively affected by the parasitic interaction.

An interesting question is the ability of one individual of a parasitic plant to associate with several individuals of the same host plant species or even individuals from different species. Evidence for multiple associations comes both from field observations as well as experiments in controlled conditions. In several coastal habitats in the UK, *R. minor* plants were connected with 34 different host species, and the number of host species connected with one individual parasitic plant ranged from one to seven [24]. The most frequent number of attached host species per individual parasite was four.

Simultaneous attachment to several host species in controlled conditions has been shown for a root hemiparasite *Castilleja wightii*, which has characteristics similar to those of *Rhinanthus* species [29]. It was evident that growth and reproductive performance of the parasite was significantly improved by parasitizing two different host species simultaneously. Other studies also showed an ability of *Rhinanthus* species to form multiple interspecies associations in controlled conditions [25]. Such a feature raises the question of whether signal exchange is possible not only between the parasite and the host plant, but also between two host plants through the parasite. These aspects are further discussed in Section 9.

In general, better suitability of legume species as hosts for parasites usually has been attributed to symbiotic nitrogen fixation and, consequently, better supply of nitrogen [30], and the suitability of grasses has been associated with low defense responses [31]. However, variation in host quality within these groups is usually relatively high, indicating that additional mechanisms are involved. Thus, among legumes, species from genera *Anthyllis* and *Onobrychis* were very poor hosts for *Rhinanthus* [26]. There is a possibility that active defense responses of low quality host plants can result in dieback of hemiparasitic plants. In a preliminary experiment, *R. major* plants established haustorial contacts with both *Lotus maritimus* and *Anthyllis vulneraria* subsp. *maritima* plants, but growth of the parasite was extremely poor in the case of *Anthyllis* (Figure 2). In contrast to unattached control plants of *R. major*, which remained alive longer, all parasites attached to *Anthyllis* plant roots died after several weeks.



Figure 2. Different growth of *Rhinanthus major* plants attached to roots of two legume species, *Lotus maritimus* (on the left) and *Anthyllis vulneraria* subsp. *maritima* (on the right).

Thus, it seems that more physiological evidence is needed on mechanisms of host plant resistance to *Rhinanthus* parasites. Possible host-associated characteristics relevant to their ability to resist *Rhinanthus* infection have been proposed, including constitutive presence or induction of a physical barrier in roots, localized host cell death at the infection point, and production of defense-related compounds [28]. Available experimental evidence in this respect is limited, but induction of cellular fragmentation and lignification has been shown for *R. minor* interaction with two non-host species, *Plantago lanceolata* and *Leucanthemum vulgare* [28].

An intriguing question concerns possible local adaptation of parasitic plants to their sympatric (locally coexistent) host plants. This possibility was tested by using *R. serotinus* and *Agrostis capillaris*, and it appeared that, in a common garden experiment, parasite performance was similar on both sympatric and allopatric hosts, suggesting no local adaptation to sympatric hosts [32]. However, effect on host biomass was highly variable, indicating genetic variation in virulence between parasite populations.

5. Establishment of Parasitic Relationship

There is no reason to doubt that, as in other root parasites, compounds secreted by host plant roots act as signals for haustoria development in *Rhinanthus* species as well. The initial step of haustoria development, prehaustorium formation, is induced by host-derived chemical compounds, collectively known as haustorium-inducing factors (HIFs) [33]. For other hemiparasite species, different phenolic compounds with a methoxyphenol moiety with hydroxy or oxo groups at position 3 and methoxy groups at position 3 or 5 are common HIFs [6]. Most likely, these HIFs are derivates of lignin monomers produced through laccase activity. In particular, dimetoxy-*p*-benzoquinone is a potent HIF in respect to many Orobanchaceae species [33]. However, so far there is no information available on the chemical nature of potential HIFs in *Rhinanthus* species.

Recognition of HIFs in parasite tissues occurs through receptor kinase complexes located in the plasma membrane of root cells and involves Ca^{2+} intake [6]. Several hours after signal recognition, cell expansion and division starts, leading to differentiation of prehaustorium structures within a few days [33]. In contrast to obligate parasite species usually developing "terminal" haustoria on radicle tips, facultative parasites, including *Rhinanthus*, develop "lateral" haustoria formed as a lateral extension of roots [31]. The haustorium is a rather complex structure, and further functional details on this subject can be found elsewhere [34]. Most importantly, direct anatomical continuity in haustoria between xylem vessel elements of the host plant with those of the parasite is formed (lumen to lumen connection), allowing for mass flow-driven uptake of solutes [31]. A comprehensive review on functional diversity of haustoria in parasitic plants needs to be consulted for further detail [35].

It appears that host quality has not been determined during the early phases of interaction, including host recognition and haustoria formation, but is a result of further resource supply and/or competition between the partners. In a growth experiment using agar plates, it was possible to perform choice tests with *R. alectorolophus* and different pairs of putative host plants [36]. A good grass host was represented by *Dactylis glomerata*, and a poor grass host by *Anthoxanthum odoratum*, while a good legume host was *Medicago sativa* and a poor legume host was *Anthyllis vulneraria*. Lateral growth of the parasite roots was measured for indication of directed growth. Within three weeks, growth of *R. alectorolophus* roots showed no differences between good or poor hosts either in the case of grasses or legumes. However, when a combination of a grass and a legume was used, there was a preferred root growth towards the legume host. The number of haustoria formed was not affected by host type, and functional xylem connections were made even with poor hosts. In comparison to other related hemiparasitic species, such as *Odontites rubra*, connective *R. serotinus* plants had relatively high host dependence, expressed as 23-fold growth stimulation after attachment to *Medicago sativa* for *R. serotinus* in comparison to that of 14-fold for *O. rubra* [37].

Attachment to a host of a hemiparasitic plant has greater significance for further parasite development as compared to seedling emergence, as it coincides with a rapid elongation phase and production of true leaves [38]. During this phase, haustorium maturation occurs, allowing for full xylem connectivity between the partners. Xylem-transported cytokinins produced either by a host plant or a parasite appear to have a significant role in establishment of the parasitic relationship [39].

Growth response of *Rhinanthus* plants after attachment to a suitable host is relatively fast. Already a week after a transplantation together with *Festuca cinerea*, *R. major* plants showed significant differences in height (Figure 3A), which were more prominent 20 days later, when attached plants started flowering (Figure 3B).

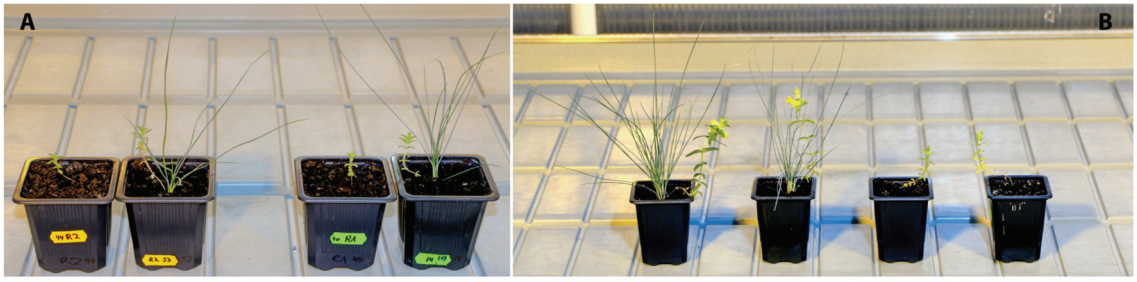


Figure 3. Differences between *Rhinanthus major* plants growing without attachment or attached to *Festuca cinerea* plants 1 week after transplantation (**A**) and 20 days later (**B**).

Host specificity of hemiparasitic Scrophulariaceae species is relatively low, but the suitability of hosts for the parasites ("host quality") can vary considerably [18]. Some hosts can even have negative consequences for parasite growth, as in the case of *Plantago lanceolata* [40]. Variation in success of *Rhinanthus* plants on different hosts seems to be associated with differences in local responses in host plant roots following haustorial contact. In contrast to legumes (high quality hosts) showing only weak and local root lignification in a response to haustoria attachment, *Plantago lanceolata* plants (low quality hosts) exhibit full anatomical isolation of xylem from haustorium by means of fragmentation of haustorial surface [31]. In the case of graminoid hosts, lignification of cell walls at the interface was evident, and host root endodermis near the penetration site had suberized lamellar layer [31].

6. Mineral Nutrition of *Rhinanthus*

Mineral nutrition of *Rhinanthus* species has not been much studied. It has been proposed, though, that significant differences need to be evident between unattached and host-attached plants due to the underdeveloped root system of the parasite [41]. However, when *R. minor* plants not attached to hosts received additional N, K, or P fertilizers, only in the case of P did significant growth stimulation occur, together with significant increase in leaf P concentration and enhanced rate of photosynthesis [41]. For unattached *R. serotinus* plants, increase in nutrient (only NPK) levels resulted in increased biomass of both shoots and roots as well as leaf length and plant height [42].

The results of studies on mineral nutrition of attached parasitic plants are more difficult to interpret. A detailed study of *R. minor* and *Lolium perenne* interaction at two P levels (limiting and optimal) revealed that parasite biomass at optimal P availability decreased by 90% in comparison to that in P-limiting conditions [43]. At the same time, biomass of parasitized *L. perenne* plants at optimum P availability was 74% higher than that in limiting conditions. Thus, from the attached parasite's point of view, it turns out that a low level of P is optimal for its growth, while a high level is inhibitory. However, it seems logical to propose that some indirect mechanism was responsible for suppression of parasite growth in high P conditions. As number of haustoria and rate of success of

initial attachment drastically decreased at optimal P availability, it was evident that changes in some morphological characteristics of host roots were related to the negative effects of increased P on *R. minor* plants [43]. However, in a similar study, high P availability suppressed growth of *R. angustifolius* attached to *L. perenne* plants but stimulated it when attached to *Deschampsia flexulosa* plants [44]. Therefore, suppression of haustoria formation and lower nutrient flux to parasite can be related to increased sink strength of host species relative to that of the parasite, where fast-growing *L. perenne* plants had increased sink strength in comparison to that of slow-growing *D. flexulosa*.

Increase in soil N availability resulted in increased shoot growth together with reduced biomass allocation to roots of host plants, but such an effect on relative root biomass was not evident for the attached hemiparasitic *R. alectorolophus*, while shoot biomass of the parasite increased [45]. The negative effect of parasite on host biomass was not affected by N availability, and these results indicate that *Rhinanthus* species can have strong effects on host plants at different soil fertility levels.

In a study using balanced fertilizer composition at two rates (low- and high-nutrient treatments), interactive effects of nutrient availability with soil moisture on performance of *R. alectrolophus* plants was shown [46]. In particular, parasite growth was stimulated by increased soil water availability at low nutrient concentration, but it was inhibited by increased soil moisture at high nutrient concentration. Moreover, when *Rhinanthus* plants were cultivated at low soil moisture but high nutrient availability and produced the largest biomass, the proportion of host-derived carbon was the lowest, indicating decreased parasitism.

7. Environmental Resilience of *Rhinanthus*

Ecological requirements of *Rhinanthus* species have not been broadly generalized, but some detailed analysis has been performed for *R. minor* [19]. In particular, it was suggested that the species has high phenotypic plasticity. Similarly, high phenotypic plasticity together with local genetic adaptation was shown to determine environmental responses of *R. angustifolius* plants [14]. Ecological optimum curves have been determined for *R. serotinus* plants as a spatial distribution of individuals along a gradient of soil edaphic factors [47]. There was no dependence of plant occurrence on macronutrient (NPK) variability, but the distribution curve in respect to ground water was skewed, with low density of individuals in dry spots and highest density close to the humid side.

In field conditions, it is frequently observed that *R. angustifolius* plants prefer relatively wetter grasslands in comparison to *R. minor* plants [18]. When effects of soil water availability on the two species were compared in experimental conditions, it appeared that *R. angustifolius* plants had the highest number of flowers per plant in wet conditions, but *R. minor* plants showed the highest number of flowers in moist conditions [48]. Both species had relatively similar drought tolerance. It has been argued that high drought tolerance of *Rhinanthus* species is a direct consequence of their parasitic growth habit due to accumulation of abscisic acid and mannitol [49]. Therefore, plants can rapidly recover even after relatively severe wilting following transient water shortage. Considering the fact that root hemiparasites rely on transpiration-driven acquisition of host water and solutes, it is logical to assume that soil water availability could play a critical role in ensuring the growth success of the parasitic plant. Water shortage increased proportion of biomass in roots for both host species and hemiparasite *R. alectorolophus* [45]. When drought stress response was assessed in *R. alectorolophus* plants, it appeared that water shortage led both to reduced survival of seedlings as well as decreased biomass of adult plants [50]. Still, at low irrigation intensity, parasites had active transpiration and an increased rate of photosynthesis.

One of the environmental factors that has a multifaceted effect on *Rhinanthus* plants is the availability of light. One such aspect is the "distance dilemma", which predicts that the reduced availability of light when situated closer to the host plant reduces the parasite's survival abilities as a result of competition for light, but closer situated parasites have better parasitism efficiency. Distance from host indeed appears to play a crucial role in

development of a *Rhinanthus* individual, but the effects are ambiguous [51]. Results from experiments in controlled conditions confirm that *Rhinanthus* plants located closer to the host received significantly less light. However, by analysis of direct root contacts in field conditions it has been established that the majority of attached *R. minor* plants were found within 10 cm of the host [24]. In addition, more sophisticated experiments in controlled conditions have revealed that the growth of already attached *R. minor* was not affected by reduced light availability [52]. In particular, when grass-attached *R. minor* plants were cultivated either in ambient irradiance intensity or in shaded conditions resulting in less than a half irradiance intensity at plant level for four weeks, there were no significant differences in parasite biomass, number of haustorial contacts per plant or whole-plant carbon and nitrogen concentration. Final height of *R. minor* plants in shaded conditions was even significantly higher in comparison to that in full light, suggesting that plants exhibited typical shade-avoidance syndrome [53]. Consequently, distance effect of parasite placement relative to host plant is not primarily associated with decreased light intensity.

Regarding global climate change, the question of a possible effect of increased atmospheric CO_2 concentration is of particular importance for hemiparasitic interactions with *Rhinanthus* species and possible consequences for grassland diversity. There is only a limited number of studies dedicated to this topic. When an experiment was performed with *R. minor* and a high quality host species *Poa pratensis* in controlled conditions, elevated CO_2 concentration increased both photosynthesis and biomass accumulation of the parasite, but transpiration rate and N concentration was unaffected [54]. At the same time, increased CO_2 led to elevated photosynthesis of host plants in the presence of parasite, while transpiration and biomass was not significantly affected. However, the total biomass of the system significantly increased at high CO_2, but not at the expense of host plant productivity. In contrast, in obligate hemiparasite species *Striga hermonthica*, photosynthesis of the parasite increased with no increase in biomass accumulation and no changes in negative effects on host plants [55].

The question of the tolerance of *Rhinanthus* species to various environmental factors cannot be separated from the question of the tolerance of potential host plants. There is reason to believe that the level of tolerance of the host plant to the given factor will have a significant effect on the degree of resistance of the parasite, but no experimental evidence is available to support this hypothesis. It would be necessary to test the responses of several various parasite–host plant combinations against a gradient of the several individual environmental factors (soil moisture, photon flux density of photosynthetically active radiation, temperature, substrate salinity) and their combinations in controlled conditions. As hemiparasitic plants, including *Rhinanthus* species, are commonly found in both relatively dry and wet coastal grasslands [56], it can be expected that they are at least partially salinity tolerant. Given the potential signal exchange between the interconnected parasites and multiple host plant species, it is possible that *Rhinanthus* parasitism affects the salt tolerance of these species, allowing less tolerant species to increase their efficiency under heterogeneous soil salinity conditions.

8. How Do *Rhinanthus* Plants Affect Host Plant Performance and Growth?

From the above analysis, it appears that the effect of *Rhinanthus* parasites on their hosts is species specific. From the perspective of the host plant, they can be more or less susceptible or even tolerant to parasite attachment. This peculiarity can lead to indirect positive effects of parasite presence in multi-species associations. Effects of *Rhinanthus* plants on their hosts can be either direct or indirect [57]. In contrast to phloem parasites that have access to host photosynthates, xylem-attached *Rhinanthus* plants exceptionally use host-derived water and minerals. It is also suggested that parasitic plants directly compete with their hosts in respect to resources from soil [57].

In fact, besides resource capture, parasitic plants can directly suppress crucial physiological functions of their host plants that makes them similar to pathogens because of use of certain chemical signals. Thus, host photosynthesis is mostly negatively affected by

Rhinanthus attachment at the level of photochemical activity [57,58]. However, no effect of *R. minor* infection on photosynthetic oxygen production in *Poa alpina* plants was evident in earlier studies [59].

The question of the harmful effects of the parasite on the host plant is more complicated than it might seem at first. *Rhinanthus* plants, as typical hemiparasites, have their own organic carbon fixation through photosynthesis and therefore rely mostly on water and minerals as resources from their hosts. Consequently, it could be assumed that under conditions of optimal availability of these resources, the effect of the parasite should be minimal. However, as discussed in the next chapter, the proportion of heterotrophically acquired carbon in the biomass of parasites can be substantial.

It is logical to assume that in natural conditions, the intensity of negative effects of *Rhinanthus* parasites on host plants will depend on the density of parasitic plants. Experiments in controlled conditions have provided support for this relationship. Thus, growth of a perennial grass *Agrostis capillaris* was more negatively affected by two simultaneously attached *R. serotinus* individuals than by a single parasite, and this effect remained also in the second growing season [60]. In addition, number of panicles per plant progressively decreased with increasing rate of parasitism. In field conditions, it has been observed that an increase in the frequency of parasitic plants is associated with a greater negative effect on growth of host plants. The existence of such a phenomenon has also been confirmed in studies under partially controlled conditions [61].

One might ask, will the negative effect of one season's annual parasite on a perennial plant have lasting effects in subsequent growing seasons? Indeed, it has been shown that *Agrostis capillaris* plants parasitized with *R. serotinus* for one season produced less biomass in the next season in comparison to control plants [60]. Such a feature could buffer the effect of the parasite against fluctuations in its numbers between seasons and ensure a continuous interseasonal effect.

In field conditions, total biomass of vascular plants in grasslands usually decreases due to the presence of *Rhinanthus* species. Indeed, while biomass of dominant species is decreased by *Rhinanthus* parasitism, low nutrient and water use efficiency results in relatively low biomass of *Rhinanthus* plants, which cannot compensate for total biomass loss [62]. A similar overall negative effect due to low resource efficiency on total productivity has also been noted in controlled conditions [37]. In practice, biomass loss can be up to 30%, and is thought to be synergistically affected by other biotic factors, such as arbuscular mycorrhizal fungi and pathogens [63].

9. How Does Contact with Host Plants Affect *Rhinanthus* Plants?

Water and solute uptake from host xylem through open xylem–xylem connections through open conductive tubes, osculae, is the main mechanism of resource transfer in *Rhinanthus* species. Therefore, possible selectivity for solute uptake initially seems doubtful. This mechanism also provides an opportunity for parasite species to exploit non-nutrient biologically active solutes of host plants—hormones, proteins and RNAs [64]. It would be logical to assume that the exchange of signals could be reciprocal and also take place in the direction from the parasite to the host, or, in the case of attachment of several hosts to the parasite, also between several hosts. Thus, both the adaptation of the parasite to the developmental stage of the host and the direct influence of the parasite on the host's physiological processes and growth are possible.

Early studies with *R. serotinus* and *Hordeum vulgare* provided information on changes in inorganic and organic constituents in different tissues of a parasite before and immediately after attachment, coinciding with fast stimulation of growth [65]. The most striking increase was evident for N, P, K, Mg and Na concentration in parasite leaves, with pronounced increase of N and P also in roots. Among organic substances, a large increase in both leaves and roots of *R. serotinus* after attachment was evident for concentration of DNA, RNA and amino acids. However, concentration of carbohydrates as well as chlorophyll content

decreased probably due to an initial decrease in photosynthesis rate, but significantly increased in later stages.

While in general xylem sap is thought to be low in organic substances, possible transfer of organic solutes from host to *Rhinanthus* plant cannot be excluded. It has been reported that around 20 to 80% of dry biomass of different hemiparasites is derived from heterotrophic carbon gain [66]. In particular, using natural abundance stable isotope profiles of ^{13}C, it was estimated that about 50% of organic carbon in *R. minor* plants in controlled conditions was derived from their host plants [67]. Experiments with closely related species *R. alectorolophus* confirmed importance of heterotrophic carbon for growth of attached *Rhinanthus* plants, where decrease in light intensity increased the fraction of heterotrophically derived carbon from 10 to 50% [68].

Attachment to a host root results in drastic changes in basic physiological features of the parasite. The characteristic physiological response of *Rhinanthus* plants after successful attachment to host roots is an increased transpiration rate and constantly open stomata. As a result, stomatal conductivity of attached hemiparasite plants is higher than that of their hosts, optimizing xylem sap extraction [69]. Unattached *R. minor* plants usually had closed stomata, whereas attached plants kept their stomata open throughout day and night [70]. If host shoots were removed but *Rhinanthus* plants remained attached to the roots, that again resulted in changes in stomatal behavior: plant stomata were open at day and closed at night. The increased transpiration rate of parasitic plants is one of the main reasons for establishing high water potential gradient between host and parasite, leading to intensive solute transfer.

R. serotinus plants attaching to host roots earlier in their life cycle had advantages both in respect of higher growth rate as well as seed production in comparison to late-attaching plants [61]. Thus, early-attaching parasite plants were about four times taller at the end of their life cycle, and produced 23 times more seeds with higher viability.

Transfer of xylem-translocated cytokinin has been shown to be an important signal in parasitic interaction of *R. minor* and *Hordeum vulgare* plants [71]. Cytokinin synthesis was extremely low in both unattached and host-attached *R. minor* plants, and decreased by 35% after attachment, but cytokinin concentration in a form of zeatin and zeatin riboside was relatively high in attached plants due to import from the host. It is suggested that cytokinins are important factors for increased size of leaves and interveinal tissues after attachment to host [71]. *Rhinanthus* plants were able to restore cytokinin biosynthesis when the parasitized host plant died due to shoot cutting [70].

One of the biochemical peculiarities of metabolism in *Rhinanthus* species and some other hemiparasites from Orobanchaceae is use of mannitol instead of sucrose as the major assimilate, leading to a high rate of abscisic acid (ABA) biosynthesis [71]. ABA, in turn, is necessary for formation and differentiation of haustoria [72]. Mannitol biosynthesis in *R. minor* shoots is increased 16-fold by parasitism to *Hordeum vulgare*, resulting in 10 times higher mannitol concentration in shoots [71]. However, no sucrose was detected in xylem of *H. vulgare*, indicating that no sucrose was transferred to the parasite. The presence of osmotically active mannitol in tissues of *Rhinanthus* plants can be crucially important for the parasitic lifestyle, including better protection against water shortage as well as diversion of nutrient flow from a host plant.

The effect of reducing the biomass of the host plant as a result of defoliation on the parasitic interaction was also studied. Thus, partial defoliation (50% of entire leaves removed) of *Agrostis capillaris* plants had no significant effect on shoot or root biomass of attached *R. serotinus* plants, but the number of flowers decreased significantly [73]. However, the effect was significant only when defoliation of the host plant was performed in the developmental phase of the parasite when the demand for resources was at its highest.

Transfer of host-derived toxins to the parasite is another intriguing possibility with a potentially large impact on defense [64]. Thus, obligate mutualistic fungal endophyte (*Neothyphodium uncinatum*) of *Lolium pratense* plants produces mycotoxins—pyrrolizidine

alkaloid loline and *N*-propionylnorloline—that are transferred to hemiparasitic *R. serotinus* plants [74]. Importantly, uptake of mycotoxins in the parasite increased its performance against a generalist aphid herbivore, *Aulacorthum solani*.

Recently, information is emerging that the transfer of small RNAs between partners could be an essential part of the life cycle of parasitic plants. Most importantly, the transfer can be bidirectional and selective [2,75]. Trafficking of phloem-mobile mRNAs in both directions during holoparasitic as well as obligate hemiparasitic interaction has been demonstrated, and the process is thought to be highly selective [75,76]. Most abundant trafficked mRNA types are from regulatory genes, involving transcription factors and calmodulin-related proteins. The question of mRNA transfer between host and parasite is rather intriguing, as any physiological response would require translation of respective mRNAs into proteins and interaction of the synthesized proteins with certain targets.

Another possibility for RNA transfer within parasite–host plant interaction is related to RNA silencing signals, aka RNA interference (RNAi) [75,77]. Non protein-coding RNAs, processed into small interfering RNA(siRNA) or micro RNA (iRNA), can participate in sequence-specific regulation of gene expression, but high gene homology is a prerequisite for this type of influence. In particular, miRNAs seem to be crucial components in regulation of interactions between parasitic plants and their hosts [78,79]. Comprehensive updates on existing knowledge of plant parasite–host signal transfer can be found in the recent scientific literature [2,79]. Updates on horizontal gene transfer in parasitic plant relationships with their hosts can be found elsewhere [80–82].

The above-mentioned examples of transfer of small RNAs during parasite–host relationship mostly refers to studies with species of obligate parasites from genera *Cuscuta*, *Orobanche*, *Phelipanche*, and *Striga* [2]. The question of transduction of such signals in xylem hemiparasites is still unresolved. One important aspect in this respect is the specificity of the relationship, which is high for phloem parasites and quite low for typical xylem parasites like *Rhinanthus*. Therefore, it is important to perform detailed transcriptome studies using classical model species from genus *Rhinanthus* together with functionally different host species in order to obtain detailed understanding of control of parasitic interactions leading to either "neutral" or "beneficial" effects for parasitic plants.

10. How Do *Rhinanthus* Plants Affect Plant Diversity in Grasslands?

Host selectivity of *Rhinanthus* species, described in Section 4, seems to be the main mechanism leading to changes in species diversity in grasslands. The existence of selectivity has been proven also in experiments in controlled conditions even among taxonomically related host species, such as within grasses or legumes [20]. Most importantly, growth of more-preferred hosts (as indicated by a larger number of haustorial contacts in host roots) were more depressed in comparison to that of less-preferred hosts, and this effect was especially pronounced in conditions of low soil nutrient availability [20]. Moreover, parasitism by *R. minor* also changed the relative competitive ability of grass species cultivated in binary mixtures. Consequently, plant species interactions in grasslands are significantly affected by the presence of hemiparasitic plants in a similar manner to that of herbivores and pathogens. Spatial theoretical modelling has been used to predict an outcome of competitive interactions involving *R. minor,* and it was demonstrated that nutrient availability is one of the major factors determining the outcome [83]. The complex dynamics of interactions resulting in unpredictable shifts in vegetation structure might be associated with formation of locally unstable parasite populations, where even relatively small quantitative changes can result in significant qualitatively different outcomes. Thus, alternation between parasite-rich and parasite-poor phases can result from the fact that when the parasite suppresses growth of high quality host plants, the shortage of preferred host plants will become a limiting factor for parasite abundance [83]. Interaction between the main factors leading to changes in grassland biodiversity by the presence of *Rhinanthus* species is shown in Figure 4.

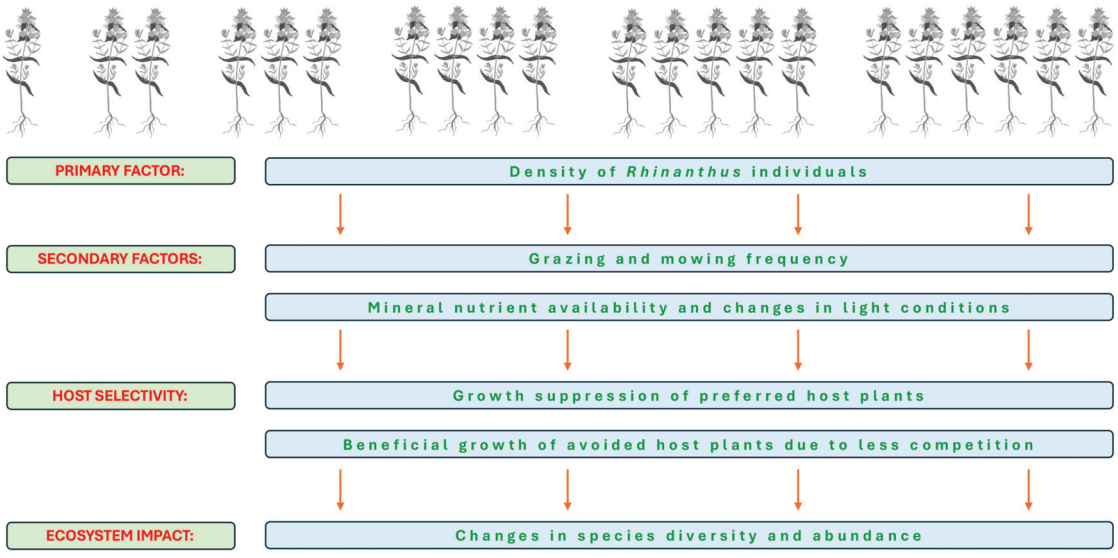


Figure 4. Interactions between the main factors leading to changes in grassland biodiversity by the presence of *Rhinanthus* species.

Practical aspects for use of *Rhinanthus* species in grassland restoration have been analyzed several times in the recent decades [84]. It was initially suggested that five criteria need to be met for the species being used as a tool in biodiversity restoration practices: (i) the species needs to be a component of natural species-rich grasslands, (ii) seeds of the species are easily available at low cost, (iii) the species reduce growth and reproduction of several competitive species, especially grasses, (iv) the species has an ability for vigorous growth and rapid spread in different types of grasslands, and (v) population size of the species can be easily managed to prevent extensive damage to vegetation [84].

From a practical point of view, it is important that successful establishment of viable populations of *R. minor* is possible only in relatively unproductive grasslands. Several studies have shown that the species cannot form a long-term population when grassland productivity exceeds 4 or 5 t ha^{-1} of dry aboveground biomass [62]. One of the possible mechanisms discussed in this respect is the negative effect of shading, as *R. minor* plants are highly sensitive to decreased light [51]. However, it is also shown that reduced light is not a problem for already attached *Rhinanthus* plants [52].

Although it is accepted that the high availability of minerals in grassland soil does not allow the effect of the parasite to manifest itself due to their low ability to compete for light, in some situations a significant effect has also been observed in soils with a high supply of nutrients. When a relatively productive ex-arable field was sowed with a standard meadow mix containing six forb and six grass species against a background of three densities of *R. minor* (0, 600, 1000 seeds m^{-2}), the immediate effect of parasite presence was evident as reduced grass biomass at both parasite densities, but forb biomass was reduced only at the higher sowing density [85]. However, no effect of parasite presence was evident in respect to species diversity. Obviously, in this particular case, as host plants also developed from seed, competition for light was not as active as it is in natural grassland conditions.

Experiments in field conditions involving removal of *R. minor* individuals gave further support to the evidence that plant species diversity in grassland communities is significantly affected by the presence of parasitic plants through both selective parasitism as well as modification of competitive ability between plants [20].

Population biology of *R. angustifolius* in grasslands in relation to vegetation structure due to management regime has been studied over the long term in several cases. As a

rule, factors leading to increased abundance of *Rhinanthus* species resulted in decrease in grass cover and increase in that of forbs [63,86,87]. Thus, grazing in autumn by cattle and sheep produced gaps in vegetation resulting in increased abundance of *R. minor* during an eight-year trial [86]. Summer cutting alone had no such effect, and cutting in autumn only had a partial influence, but mineral fertilization had a mostly negative effect. However, environmental factors have a large impact on *Rhinanthus* populations, resulting in seemingly stochastic fluctuations in number of individuals over the seasons [87]. Thus, for *R. angustifolius*, it was suggested that spring drought could be the main negative factor [87].

In addition to effects of *Rhinanthus* presence on taxonomic diversity of grassland species, it is also valuable to look at its influence on changes in dominant functional characteristics. Thus, when presence of *R. minor* was manipulated (removal vs. no removal) in an oligotrophic meadow community, it appeared that functional diversity in general was higher in populations with the parasite [88]. The presence of competitively weak species (with low clonal growth capacity and small biomass) was promoted at a community level. In particular, *Rhinanthus* positively affected species with persistent taproot, such as *Plantago lanceolata*, or monocyclic species. Surprisingly, clonal species with extensive lateral spread were especially vulnerable to presence of *R. minor*.

Development of a dense moss layer as a result of nutrient depletion-associated soil acidification could have a negative effect on *Rhinanthus* populations due to mechanical restrictions on seedling emergence and establishment [89]. However, this possibility has not been clearly demonstrated in other studies [62,90,91].

Successful attempts to use parasitic *Rhinanthus* species to improve plant diversity under natural conditions have been described. Thus, in a meadow with patches highly infested with a clonal grass *Calamagrostis epigejos*, sowing of *R. major* led to reduced biomass of *C. epigejos*, and the effect was additive to that of mowing twice in the season [92]. Besides, community composition was significantly changed by parasite introduction, with some diagnostic or constant species of the association increasing in abundance. Such a positive response was not seen in the case of mowing twice; in contrast, several important or threatened species responded in a clearly negative way. In addition, Central European species *R. alectorolophus* was successfully used to suppress *C. epigejos* in both dry meadow and industrial areas, but the effect in wet meadow was variable due to unequal establishment of *Rhinanthus* population [93]. Similar to the above study, increased mowing frequency had additional negative effects on *C. epigejos*. More experimental evidence derived from field studies is available to support the use of introduction of *R. minor* in practical management for improvement of species-poor grasslands [90,94,95].

However, effects of *Rhinanthus* introduction in grasslands does not necessarily lead to a positive immediate effect on biodiversity. Thus, sowing of *R. minor* in a species-poor calcareous grassland had a negative influence on forb richness, and the effect was directly proportional to the density of parasites [96]. Due to these contrasting results, the overall general suggestion still holds that effects of *Rhinanthus* plants on community structure are extremely variable and highly unpredictable.

No less important is the possibility of reducing the presence of invasive plants with the help of *Rhinanthus* and other root hemiparasites, which has been shown in experiments under controlled conditions [97]. Thus, biomass accumulation and shoot density was significantly suppressed for two clonal invasive species, *Solidago gigantea* and *Symphyotrichum lanceolatum*, by *Melampyrum arvense* and *R. alectorolophus*. The degree of clonal integration of the host plants was associated with the pronounced species-specificity of the interaction.

11. Ecological Complexity of Parasitic Plant Interactions and Future Perspectives

To some extent, the non-specific nature of the parasitism of *Rhinanthus* species, on the one hand, and the selectivity of action, on the other, can explain the fact that their influence in the ecosystem is quite complex. There is increasing evidence that hemiparasitic species, including *Rhinanthus*, not only directly alter plant biodiversity through parasitism, but also affect broader ecosystem functions at multiple trophic levels [98]. Among them,

plant–herbivore interactions seem to be significantly affected by the presence of parasitic *Rhinanthus* plants [25,99,100].

As discussed above, symbiotic nitrogen fixation in legume species makes them attractive as hosts to *Rhinanthus* species. However, in this case, the efficiency of N-fixing bacteria, which is largely influenced by the level of soil N availability, should also be taken into account. Since legumes are usually also characterized by the presence of mycorrhizal symbiosis, quite complex interactions can develop under natural conditions. Studies with other root hemiparasites, such as *Pedicularis* species, have shown that, in general, both rhizobial and arbuscular mycorrhizal symbiosis can alleviate negative effect of parasites on host plants [101]. On the other hand, the presence of *R. minor* significantly diminished colonization of *Lolium perenne* roots with arbuscular mycorrhizal fungi, but parasite growth drastically increased when the host plants were mycorrhizal [102].

Another interesting aspect for further exploration of biological complexity is relatedness of endophytic microbiomes of parasitic plants and their hosts. For example, it has been argued that parasitic plants are likely to be able to decrease the extent of colonization by fungal endophytes with further consequences for host plant physiology [103]. Considering the generally limited understanding of the role of endophytes in the functioning of different ecosystems, research on the relationship between hemiparasites, host plants and their endophytes could be of critical importance.

Although the emphasis in parasite resistance research has so far been on the so-called economically important parasite species [104,105], there is no doubt that root hemiparasitic species such as *Rhinanthus*, with their ability to influence the diversity of grasslands, stand out for the possibility of obtaining biologically and ecologically important information about the critical mechanisms of the interaction between parasitic plants and their hosts. The exchange of non-resource compounds with a signaling nature, especially at the level of small RNA molecules, seems to be one of the most important directions of research with *Rhinanthus* species. Expanding the models to include several host plant species with different resistance to specific environmental factors would broaden the understanding of the importance of parasitic plants in the functioning of the ecosystem and the maintenance of plant diversity.

Development of new model systems is an important aspect in any type of study of biological interactions between organisms, and this is true also for the *Rhinanthus*–host plant relationship. There is no doubt that ecologically significant results can be obtained in vegetation pot or microcosm experiments using soil or other soil-like substrates with model species native for a particular habitat. However, simpler model systems offer several advantages over vegetation pot experiments, including the need for a smaller space, as well as better repeatability and controllability. One of the underexplored directions in this respect is the use of tissue culture-based models. These could be extremely important as tools for deciphering host resistance mechanisms to root hemiparasites as well as in environmental resilience studies. So far, root cultures have been used mostly with obligate parasite species, as the ones of genus *Striga* [106,107]. However, there are also successful attempts using root and callus cultures of root hemiparasitic species, such as *Triphysaria versicolor*, to study early responses of parasitic interaction [108]. Most importantly, cultivated roots of hemiparasites retained competence for haustoria formation in the presence of host roots, exudates from host roots, or chemical inducers.

Due to the fact that some *Rhinanthus* species have been used in traditional medicine, there is an interest in revealing phytochemical composition and potential bioactive compounds in *Rhinanthus* plants [109–111]. In particular, *R. angustifolius* was characterized as a rich source of antioxidants and enzyme inhibitors [111]. In another study, methanolic extracts of *R. angustifolius* exhibited a wide-spectrum antibiotic activity [112]. Essential oil of *R. angustifolius* contains terpenes, aldehydes, alcohols, esters, acids, etc., and it has prominent antimicrobial activity [110]. Dihydroxylated derivate of methylindene, α-cubebene, 1-hexadecene, and hexadecanoic acid were identified as the major constituents of the essential oil. When compared to other grassland species, *R. minor* has an especially high

level of benzoic acid [113]. In light of an effect of host species on chemical composition of parasitic plants, and especially, due to possible transfer of secondary metabolites from host to parasite, more attention should be paid to research on the effect of different host plants on the chemical composition of *Rhinanthus* plants, especially if the host plants are active secondary-compound-producing species. This effect has not been explored with *Rhinanthus* species, but it was shown with another hemiparasite, *Castilleja miniata*, that these plants receive alkaloids from a host, *Lupinus argenteus*, leading to decreased herbivory of the parasite [114].

12. Conclusions

The performed analysis shows that *Rhinanthus* species have gained scientific interest due to their ecological importance in increasing grassland biodiversity. Although significant progress has been made in the practical use of *Rhinanthus* species in grassland improvement, this activity is often empirical in nature due to a lack of fundamental knowledge of the factors affecting the parasite–host relationship in the ecosystem. Soil edaphic factors and the level of mineral availability could be among the influences that should receive more attention in this regard.

From the functional point of view of the interaction, the most significant lack of knowledge is about the signal exchange between the *Rhinanthus* parasite and the host plant. It would be necessary to understand how differences in signal exchange between Rhinanthus and host plants of different species affect the nature of subsequent relationships, distinguishing between "high-quality" and "low-quality" hosts. A particularly interesting model system could be one involving apparently resistant host plants such as *Anthyllis vulneraria*. This could make it possible to understand whether the differences in the resistance of different host plants to *Rhinanthus* are genetically determined by a gene-to-gene mechanism.

Funding: This research received no external funding.

Conflicts of Interest: The author declare no conflicts of interest.

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Biomass of Shoots and Roots of Multicomponent Grasslands and Their Impact on Soil Carbon Accumulation in *Arenosol* Rich in Stones

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Abstract: To prevent the degradation of light-textured soils, it is advisable to use them for grasslands. These soil management systems help with the faster accumulation of soil organic carbon (SOC), thereby improving the soil's properties and reducing carbon emissions from agricultural land. In this experiment, we studied the distribution of multi-component perennial grass roots in the *Arenosol* profile and their impact on SOC sequestration in temperate climate zones. Our research aimed to identify differences in root biomass at depths of 0–15 cm, 15–30 cm, and 30–50 cm and to assess their correlation with SOC and dissolved organic carbon (DOC) in the soil. The roots, shoots, and soil samples of fertilized and unfertilized grasslands were collected at the flowering stage and after the final grass harvest two years in a row. Our findings revealed that, in sandy loam *Arenosol* rich in stones, 12.4–15.9 Mg ha^{-1} of root biomass was accumulated at 0–50 cm of soil depth. The application of NPK fertilizers did not significantly affect grass root biomass, but significantly affected shoot biomass. Most roots (84–88%) were concentrated in the 0–15 cm layer. On average, 5.10–6.62 Mg ha^{-1} of organic carbon (OC) was stored in the roots of perennial grasses within 0–50 cm of soil depth. We found that the SOC content in the 0–50 cm soil layer correlated more strongly (r = 0.62, $p < 0.001$) with C accumulated in the roots of the corresponding layer than with shoot biomass (r = 0.41, $p = 0.04$). However, a significant correlation was found between DOC and shoot biomass (r = 0.68, $p < 0.001$) and between DOC and the biomass of residues (r = 0.71, $p < 0.001$), explaining the significant increase in DOC in the 30–50 cm soil layer and indicating the leaching of mobile soil organic matter (SOM) substances from the above-ground biomass using fertilizers.

Keywords: *Arenosol*; carbon; nitrogen; leaching

Citation: Tripolskaja, L.; Toleikiene, M.; Skersiene, A.; Versuliene, A. Biomass of Shoots and Roots of Multicomponent Grasslands and Their Impact on Soil Carbon Accumulation in *Arenosol* Rich in Stones. *Land* **2024**, *13*, 1098. https://doi.org/10.3390/land13071098

Academic Editors: Michael Vrahnakis and Yannis (Ioannis) Kazoglou

Received: 3 June 2024
Revised: 17 July 2024
Accepted: 18 July 2024
Published: 20 July 2024



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1. Introduction

The sustainable development strategy encompasses various goals and varying priorities depending on each country's specific problems. However, in all countries, one of the most crucial objectives is to mitigate climate change and ensure the sustainable development of ecosystems. Soil is the most vital component of the terrestrial ecosystem, providing numerous ecosystem services such as maintaining water quality and plant productivity, regulating soil nutrient recycling and decomposition, and sequestering greenhouse gases from the atmosphere [1–3]. However, the increasing human population necessitates more food production, intensifying agricultural soil use. This can result in soil degradation, as agrotechnical practices often disrupt the ecological balance found in natural ecosystems [4–7].

Although low-productivity soils are susceptible to human impact, they are also used for agricultural production. To stabilize or improve their properties and prevent degradation, it is recommended to use forest and grassland [8,9]. These soil management systems

have a faster accumulation of SOC, enhancing the soil's physical, chemical, and microbiological properties and reducing carbon emissions from agricultural land. Reference [10] claims that root biomass and length are greater in perennial grasslands compared to annual crops, and they are an important factor in maintaining the productivity and soil quality of perennial grasslands without significant costs. Plant residues and roots contribute to the accumulation of surface OC and increases in soil quality. Agricultural soils benefit from reintroducing perennial grasses into the landscape, regaining soil organic matter and strengthening their capacity for long-term productivity and environmental resiliency [11]. The productivity of grassland is not constant; it fluctuates due to various factors, including soil quality, dominant perennial grass species, meteorological conditions, fertilization, and land use [12,13].

It is important to note that the positive effect of grassland on carbon sequestration is inconsistent across all regions [14–16]. Studies conducted in the temperate zone of Latvia on various soils did not confirm differences in carbon accumulation between arable land use and grassland. The research authors attributed these results to the dynamic changes in land use over the past decades and the significant impact of extensively cultivated arable land on carbon accumulation rates [17]. Also, scientific publications widely discuss the effects of different agricultural practices on developing grasses' above-ground and underground biomass, as well as their soil properties. In reference [18], grassland management regimes affected grass production and species composition.

Regarding mineral nitrogen fertilization, it decreases the presence of legumes and herbs. These changes in above-ground plant biomass and botanical composition also affect root distribution and morphometric parameters. Long-term grassland fertilization experiments conducted in Germany and the Netherlands found that fertilization significantly positively affected SOC stocks [19]. Annual sequestration rates ranged from 0.13 to 0.37 Mg ha^{-1} yr^{-1} depending on the fertilization. However, it has also been demonstrated that the mass of grass roots is not influenced by applied fertilization [19]. According to the research, the increase in SOC in grasslands cannot be attributed only to altered below-ground carbon inputs or decomposition rates, as the carbon stocks in the roots were unaffected by fertilization and potential litter decomposition remained unchanged. Nevertheless, fertilization resulted in lower ranges of root carbon to nitrogen ratios in the treated areas. This lower range may have increased microbial carbon use efficiency, leading to higher carbon sequestration rates per unit of carbon entering the soil.

Plants with higher root biomass have been found to have a more significant effect on SOC sequestration, but this also depends on root properties. Roots with the lowest lignin and cellulose contents and the highest nitrogen content have a higher rate of decomposition [20]. The important influence of root lignin on SOC sequestration has also been noted in publications by other authors [21,22]. Additionally, SOC sequestration is affected by soil texture, particularly the amount of clay and silt particles, which account for over 90% of the total soil content [23]. According to a study conducted in Great Britain by Ward [24], significant amounts of carbon in subsurface soil are below 30 cm, which is not accounted for in standard carbon inventories. Additional research also suggests that soil organic carbon stored in grasslands at depths below 30 cm may not be significantly affected by management changes over extended periods, thus contributing to soil carbon conservation [25].

We conducted an experiment to indicate the locations of multi-component perennial grass roots in the *Arenosol* profile and their impact on SOC sequestration. The research aimed to (1) determine differences in root mass distribution in the 0–15, 15–30, and 30–50 cm layers; (2) evaluate the influence of mineral fertilizers on root biomass; (3) measure the accumulation of OC in the roots and assess its correlation with SOC in the soil; and (4) estimate the above-ground effect of plant residues on SOC accumulation. We believe that the results of our research will be useful and help to further develop the concept of the effect of perennial grasses on soil properties, especially carbon sequestration in soil.

2. Materials and Methods

2.1. Study Object and Experimental Site

The study was carried out as a long-term experiment (since 1995) in which the influence of different land uses on *Arenosol* properties was studied. This paper examines only the data of grassland research linked to its productivity and the formation of plant root biomass in the soil profile. The experimental field is located in the LAMMC Voke Branch, Vilnius, Lithuania (54°33′49.8″ N, 25°05′12.9″ E), classified as a temperate climate zone. This region is characterized by a moderate climate, with a mean long-term (1991–2020) annual precipitation of 678 mm and an annual mean air temperature of 7.2 °C (the standard climate norm—SCN) [26]. The soil is classified as Endocalcaric Arenosol formed on fluvioglacial deposits, with the following profile: Ah-AB-B1-B2-2Cα1-2Cα2 [27]. Carbonates were identified at a depth of 80–100 cm. The soil texture characteristics are presented in Table 1. The number of coarse particles (>2000 μm) in the upper depth of 0–15 cm was 15.2–17.6% of the soil mass; at 15–30 cm, it slightly increased to 18.6–20.1%; and it significantly increased in the 30–50 cm layer.

Table 1. *Arenosol* A horizon (0–20 cm) and AB horizon texture (%) and coarse particles (>2000 μm). content.

Land Use	A Horizon (0–20 cm)			AB Horizon (28–42 cm)			Soil Coarse Particles (>2000 μm), % from Soil Mass		
	Sand 2000-63 μm	Silt 63-2 μm	Clay <2 μm	Sand 2000-63 μm	Silt 63-2 μm	Clay <2 μm	0–15 cm	15–30 cm	30–50 cm
Grassland unfertilized (GRunf)	81.0	13.7	5.3	88.0	6.1	5.9	15.2	20.1	35.1
Grassland fertilized (GRf)	83.7	11.8	4.5	90.2	3.5	6.3	17.6	18.6	22.9

At the start of the experiment (1995), in the topsoil (0–28 cm), pH_{KCl} was 6.8, plant-available phosphorus was 177 mg P_2O_5 kg^{-1}, plant-available potassium was 174 mg K_2O kg^{-1}, and OC was 9.9 g kg^{-1}. After 27 years, the soil's acidity increased to pH_{KCl} 5.9–6.1, which was associated with plant uptake and removal of calcium and magnesium from the soil with plant yield and leaching of these elements from the upper soil layer. The soil was saturated with exchangeable cations (Ca^{2+}; Mg^{2+}) because the layer of carbonate rock started at 80 cm depth. In the unfertilized site, during the research period, available phosphorus and potassium in the soil decreased accordingly by 100 mg P_2O_5 kg^{-1} and 104 K_2O kg^{-1}, while in the fertilized site, using balanced fertilization, the available phosphorus increased from 177 to 208 mg P_2O_5 kg^{-1} and the concentration of potassium changed insignificantly. In the GRf site, the accumulation of Corg significantly increased in the Ah horizon (Table 2).

Table 2. *Arenosol* Ah horizon properties.

Treatment	Years	pH_{KCl}	Available P_2O_5 mg kg^{-1}	Available K_2O mg kg^{-1}	Exchangeable Ca + Mg mekv kg^{-1*}	SOC g kg^{-1}	Bulk Density t m^{-3}
Grassland unfertilized	1995	6.8 ± 0.08	177 ± 4.9	174 ± 4.7	n.d.	9.9 ± 0.08	1.41 ± 0.02
	2022	6.2 ± 0.26	71 ± 3.7	80 ± 2.8	1225 ± 15	10.5 ± 0.16	1.37 ± 0.02
Grassland fertilized	1995	6.8 ± 0.08	177 ± 4.9	174 ± 4.7	n.d.	9.9 ± 0.08	1.45 ± 0.02
	2022	5.5 ± 0.50	208 ± 12	168 ± 12.8	924 ± 44	11.4 ± 0.12	1.32 ± 0.05

Note. In 1995, exchangeable cations were not determined.

At the grassland sites (each 200 m^2), a grass–legume mixture was grown. In 1995–2006, it included 40% alfalfa (*Medicago sativa* L.) and 4 species of grasses: 20% red fescue (*Festuca rubra* L.), 20% bromegrass (*Bromus inermis* Leyss.), 10% cock's-foot grass (*Dactylis glomerata* L.), and 10% meadowgrass (*Poa pratensis* L.). During the experiment period (1995–2023), in 2007 and 2015, they were resown to ensure the grasses' productivity. The same grass mixture was grown in 2007–2023, and only in 2007 was cock's-foot replaced by timothy (*Phleum pratense* L.). The grass was fertilized with N_{60+30} $P_{90}K_{120}$ at the fertilized site. Nitrogen (N_{60}), phosphorus (P_{90}), and potassium (K_{120}) fertilizers were applied at the beginning of grass vegetation (the last ten days of March to the first ten days of April). The grass was fertilized for the second time with nitrogen fertilizer (N_{30}) after the first grass cutting (during the first ten days of July). During the vegetation period, these grasses were cut twice or three times. The first grass-cutting was performed during the flowering phase of alfalfa (in the first ten days of July). The second cutting was performed in the first ten days of September. With a more extended and rainy vegetation period, the grass was cut three times—in the third ten days of June, the second ten days of August, and the first ten days of October. The biomass was removed from the experiment area every time.

2.2. Meteorological Conditions in 2022–2023

In Lithuania, plant vegetation begins in March and continues until November, when the air temperature drops below +5.0 °C. According to SCN, the average monthly air temperature in the summer is 16.3–18.5 °C, and the average rainfall is 65–92 mm (Table 3). The least precipitation (41–48 mm) falls in the cold period of the year (January, February, March). The average monthly air temperature in winter varies from −2.0 °C in December to −3.9 °C; there are often thaws. A flushing type of soil moisture regime characterizes Lithuania. Rainfall–runoff varies from 0.3 to 0.2 in different regions of Lithuania [28].

Table 3. Air temperature and precipitation in 2022–2023.

Month	Precipitation (mm)					Temperature (°C)				
	Year		Deviation from SCN %		SCN %	Year		Deviation from SCN ± °C		SCN
	2022	2023	2022	2023		2022	2023	2022	2023	
January	63.0	75.2	131	151	48	−1.3	−0.1	5.9	3.6	−3.7
February	47.4	32.9	113	78	42	0.4	−1.0	3.5	2.1	−3.1
Marth	1.2	43.1	3	105	41	1.4	2.5	0.7	1.8	0.7
April	50.9	32.2	118	75	43	5.5	8.1	−1.9	1.0	7.4
May	92.0	31.0	164	55	56	10.8	12.8	−2.1	−0.1	12.9
June	129.4	35.6	199	75	65	17.8	17.5	1.5	1.2	16.3
July	113.3	55.4	123	60	92	17.7	18.1	−0.8	−0.4	18.5
August	56.0	123.3	74	162	76	20.7	20.1	3.1	2.5	17.6
September	40.5	24.4	71	43	57	10.3	16.5	−2.3	3.9	12.6
October	47.9	113.2	80	189	60	9.1	7.7	2.3	0.9	6.8
November	65.0	38.1	53	97	47	2.3	1.8	0.4	−0.1	1.9
December	93.0	61.9	120	121	51	−2.4	−0.2	0.6	1.8	−2.0

In 2022, the vegetation period of the grasses was especially rainy; precipitation exceeded SCN by 118–199% in May–July. But from August to November, dry weather prevailed, and the precipitation comprised only 53–74% of the SCN. The air temperature in almost all months (except April, May, July, and September) exceeded the SCN (+0.4–5.9 °C). At the beginning of the vegetation period in 2023, the precipitation was 25–45% lower than SCN; only in August did abundant precipitation fall (162% of SCN). October was also rainy

(189% SCN). As in 2022, the average air temperature of each month exceeded the SCN (+0.9–3.9 °C), except for May, July, and November.

2.3. Plant Shoot and Root Sampling

The biomass yield of the above-ground part of the plants was determined at each harvest. The botanical composition of the grass mixture (grasses, alfalfa, herbs) was determined after the first and second harvest. Before root collection, above-ground green mass (g m^{-2}) and grass residue (g m^{-2}) were recorded at the selected location. Grass root samples were taken with cores 0.085 m in diameter and 0–0.50 m in length (volume 3925 cm^3) from three soil layers (0–0.15 m, 0.15–0.30 m and 0.30–0.50 m) at the flowering stage of the grass (end of June) and after the last grass harvest in 2022 and 2023. All samples (plant, root, and soil) were taken in three replicates from the unfertilized and NPK fertilized plots. The collected samples were tightly packed into plastic bags and stored in a freezer at +5 °C until analysis. Before analysis, soil samples with roots were carefully washed with water using 2.0 mm, 0.50 mm, and 0.25 mm sieves. Root samples were dried in an air flow oven for 48 h at 60 °C [29]. After separating the roots, a soil sample was taken for moisture determination, and the masses of coarse particles and stones were weighed. These data were used to recalculate the root mass per t ha^{-1} of dry soil [30].

2.4. Chemical Analysis

Soil chemical properties were determined as follows: the soil pH_{KCl} was measured using the ISO 10390:2005 potentiometric method (1:5); plant-available P_2O_5 and K_2O were extracted using 0.03 M ammonium lactate (Egner-Riehm-Domingo A–L method); the amount of exchangeable cations (Ca^{2+}, Mg^{2+}) was measured using atomic absorption spectrometry with an AAnalyst 200 instrument (Perkin Elmer, Waltham, MA, USA). Before the analysis, a wet digestion process with sulfuric acid was performed. The instrument utilized an air–acetylene flame and a hollow cathode lamp for the atomic absorption spectrometry measurements [31]. SOC was determined by the Nikitin modified Tyurin dichromate oxidation method using wet combustion at 160 °C. SOC measurement was performed using a spectrophotometer at a wavelength of 590 nm, with glucose as a standard [32]. The DOC analysis procedure was performed according to the methodology guided by the San++ Continuous Flow Ion Analyzer (SKALAR, The Nederlands) using $C_8H_5KO_4$ as a standard. The sieved soil was poured with 0.025 mol L^{-1} solution of K_2SO_4; the prepared extract was filtered through a 0.45 µm PTFE filter; then, the automated measurement procedure, which is based on the IR detection method following UV-catalyzed persulfate oxidation under nitrogen, was performed [33]. Soil bulk density was determined using the Core method, which involves pressing a metal ring into the soil (intact core) and determining its weight after drying [34].

The amount of C in plants (g kg^{-1}) was determined by the Nikitin-modified Tyurin dichromate oxidation method, and total N (g kg^{-1}) by the Kjeldal method, which involved a spectrophotometric measurement at 655 nm. The C:N ratio was calculated as the relationship between C (g kg^{-1}) and nitrogen content (N g kg^{-1}) in the plants' biomass.

2.5. Statistical Analysis

All data were analyzed using SAS Enterprise software, version 7.1 (SAS Institute Inc., Cary, NC, USA). The differences between the experimental treatments were tested using one-way and two-way analysis of variance (ANOVA). The significance of differences was set if the probability level was lower than or equal to 0.05. The linear regression analysis revealed the relationship between root biomass C, SOC, and DOC concentration in the soil. Duncan's post hoc test was used to carry out multiple comparisons.

3. Results

3.1. Above-Ground Biomass and C and N Accumulation of Perennial Grasses

Under the climatic conditions of Lithuania (temperate climate zone), harvesting the perennial grassland two to three times is possible. The number of cuts is usually linked to precipitation during the growing season. If there is a sufficient amount (40–70 mm per month), three cuts of above-ground biomass can be made in perennial grassland. During the research period, the average annual DM yield of grass biomass in the fertilized treatment at the GRf site was 1084 g DM m^{-2} (Table 4). Without fertilization, the grass yield was reduced by two times due to the lack of nutritional elements in the sandy soil ($p < 0.05$). The above-ground grass biomass yield did not correlate with biomass of plant roots in the 0–50 cm layer, and only shoot biomass was significantly increased by fertilization (Table 5).

Table 4. Above-ground biomass yield of perennial grasses. Average data for 2022–2023.

Treatment	Above-Ground Biomass g DM m^{-2}	Plant Residues on the Soil Surface g DM m^{-2}	All Above-Ground Biomass of Plants g DM m^{-2}	Amount of Plant Residues from Total Biomass, %
Grassland unfertilized	374 a	133 a	507 a	28.7
Grassland fertilized	892 b	193 b	1085 b	17.4

Note. Lowercase letters indicate significant differences ($p < 0.05$) between the treatments.

Table 5. Pearson correlation coefficients of root biomass and residue biomass with SOC and DOC concentrations in soil.

	SOC t ha^{-1} 0–50 cm Depth	Residue Biomass t ha^{-1}	Root Biomass t ha^{-1} 0–15 cm Depth	Root Biomass t ha^{-1} 0–50 cm Depth	Corg in Roots g kg^{-1} 0–50 cm Depth
Root biomass, t ha^{-1}, at 0–15 cm depth	r = −0.064	r = 0.120	---	r = 0.977	r = 0.946
	$p = 0.767$	$p = 0.577$	---	$p < 0.001$	$p < 0.001$
Root biomass, t ha^{-1}, at 0–50 cm depth	r = −0.054	r = 0.181	r = 0.977	---	r = 0.967
	$p = 0.804$	$p = 0.396$	$p < 0.001$	---	$p < 0.001$
Shoot biomass, t ha^{-1}	r = 0.414	r = 0.476	r = −0.082	r = −0.018	r = 0.086
	$p = 0.044$	$p = 0.019$	$p = 0.703$	$p = 0.932$	$p = 0.691$
SOC, g kg^{-1}, at 0–15 cm depth	r = 0.856	r = 0.730	r = −0.064	r = −0.054	r = 0.023
	$p < 0.001$	$p < 0.001$	$p = 0.767$	$p = 0.804$	$p = 0.914$
SOC, g kg^{-1}, at 0–50 cm depth	---	r = 0.729	r = −0.064	r = −0.054	r = 0.616
	---	$p < 0.001$	$p = 0.767$	$p = 0.804$	$p < 0.001$
DOC, g kg^{-1}, at 0–15 cm depth	r = 0.856	r = 0.714	r = 0.062	r = 0.115	r = 0.196
	$p < 0.001$	$p < 0.001$	$p = 0.775$	$p = 0.591$	$p = 0.359$

For fodder production, the above-ground part of the plants was cut, but the leaves close to the ground surface remained on the surface. They were later mineralized, replenishing the reserves of carbon and other elements in the soil. On average, after the last grass harvest (the last ten days of September), 133 g DM m^{-2} of residues in the GRunf site remained on the soil surface. At the fertilized site, it was significantly more, reaching 193 g DM m^{-2} ($p < 0.05$). However, it should be noted that relatively more grass residue (28.7% of the total above-ground biomass) remains in the GRunf site compared to the GRf (17.4%). Without fertilizers, the grasses do not grow high in the sandy soil and there are more different herbs, which results in more grass residues being found on the surface after harvesting. The remaining residues of grass contained significant concentrations of CO (385–425 g kg^{-1}) and N (3.68–5.86 g kg^{-1}), and, depending on the amount of residue, accumulated an

average of 415–1000 kg ha^{-1} C and 3.04–14.7 kg ha^{-1} N in total (Table 6). A higher C:N ratio in the residues of grasses in the unfertilized grassland was observed, while it was lower in the fertilized grassland and more favorable to microbial decomposition. After biomass mineralization, these elements can supplement C and N accumulation in the soils.

Table 6. The yield and C and N contents of remaining grass residues after the last cut.

Treatment	Yield of Remaining Residues g DM m^{-2}	$C_{org.}$		N		C:N of Remaining Grass Biomass
		g kg^{-1}	kg ha^{-1}	g kg^{-1}	kg ha^{-1}	
Grassland unfertilized	133 a	385 ± 23.8	415 a	3.68 ± 0.74	3.04 a	105
Grassland fertilized	193 b	425 ± 51.2	1000 b	5.86 ± 0.57	14.7 b	72.5

Note. Lowercase letters indicate significant differences ($p < 0.05$) between the treatments.

3.2. Root Biomass, C, and N of Perennial Grasses at Different Depths of Soil

Arenosols formed by fluvioglacial deposits are characterized by a significant amount of stone throughout the profile, which increases in the lower layers of soils. The stoniness values (coarse particles (>2000 μm) of the soil horizons in the 0–15 cm layer were, on average, 15.2–17.7%; in the 15–30 cm layer, 18.6–20.1%; and in the 30–50 cm layer, 22.9–35.2%. These data are essential for calculating the root mass in the soil profile, expressed as Mg ha^{-1}, which also affects the agronomic processes and C sequestration.

According to research data, the grasses formed a rich root system only in the upper layer of *Arenosol* (Table 7). In the upper 0–15 cm layer, root biomass reached 10.9–13.2 Mg ha^{-1}. In the 15–30 cm depth, the amount of roots drastically decreased to 1.14–1.51 Mg ha^{-1}. This value was the lowest in the 30–50 cm depth, reaching only 0.38–1.06 Mg ha^{-1} (Table 7). This shows that the main root biomass (84–88% of the total root biomass found in the 0–50 cm depth) formed in the upper 0–15 cm layer. At a depth of 15–30 cm, the amount decreased significantly to 9–10% of the total biomass, and to 3–7% at 30–50 cm of depth.

Table 7. The biomass of perennial grass roots (Mg DM ha^{-1}) at different soil depths (averaged data of 2022–2023).

Treatment	Root Biomass Mg DM ha^{-1}			
	0–50 cm	0–15 cm	15–30 cm	30–50 cm
Grassland unfertilized	12.4 ± 6.04 a	10.9 ± 5.42 aA	1.14 ± 0.70 aB	0.38 ± 0.27 aB
Grassland fertilized	15.8 ± 5.42 b	13.2 ± 5.08 bA	1.51 ± 0.73 bB	1.06 ± 0.64 bB

Note. Mean ± average deviation. Lowercase letters indicate significant differences ($p < 0.05$) between the treatments, and capital letters indicate significant differences ($p < 0.05$) between the soil depths.

The grassland consisted of four species of cereal grasses with an abundant root system, with thin roots located in the top layer of soil, as well as hybrid lucerne, with a deep tap root system. Alfalfa roots introduced into the soil sample significantly increased the mass of roots in all layers, which led to significant differences in the mass of root samples in different replicates and an increase in statistical error.

The botanical composition of the grassland was significantly impacted by the fertilization level. In the unfertilized grassland, alfalfa plants accounted for an average of 13.0% of all plants ($p < 0.05$), and a high percentage of various herbs (7.9%) was observed. Unfertilized grassland in *Arenosol* was characterized by rare herb species and a more diverse plant species composition (Table 8). In the NPK-fertilized grassland, there were significantly lower amounts of alfalfa plants (1.7%, $p < 0.05$) and forbs, but nitrogen fertilizers promoted the development of cereal grasses, especially aggressive species such as bromegrass (*Bromus inermis* Leyss), which suppressed the growth of other plants.

Table 8. Botanical composition of the grasslands.

Treatment	Composition of Plants (% of Dry Matter Yield)					
	Grasses		*Medicago sativa* L.		Herbs	
	Mean	SE	Mean	SE	Mean	SE
Grassland unfertilized	79.1 a	13.4	13.0 b	4.91	7.90 b	8.51
Grassland fertilized	98.1 b	1.56	1.70 a	1.68	0.20 a	0.27

Note. Lowercase letters indicate significant differences ($p < 0.05$) between the treatments.

After chemical analyses of the roots were performed, it was found that the amount of C varied from 402 to 422 g C $kg^{-1.}$ in the flowering stage of grasses and from 421 to 427 g C kg^{-1} at the end of the grass vegetation (Figure 1). N varied from 6.44 to 7.67 g N kg^{-1} and from 5,71 to 6.38 g N kg^{-1}, respectively (Figure 2). No significant differences in C and N content in roots, depending on fertilization and root location in the soil profile, were found. Only in the grass roots of the first cutting in the 0–15 cm layer was the amount of C significantly lower ($p < 0.05$) compared to other depths, and in the roots of the second cutting in the 0–15 cm layer, the amount of N was also significantly lower.

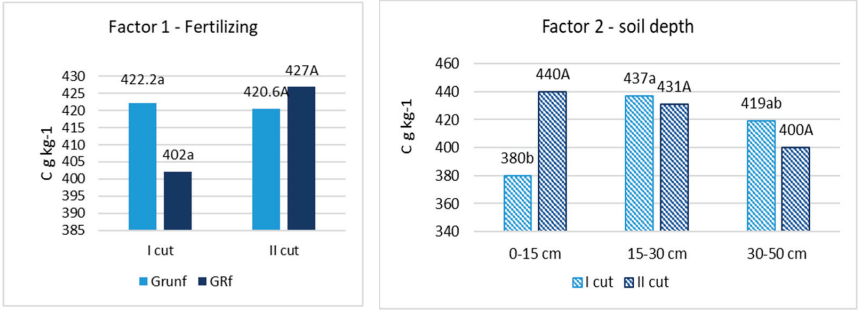


Figure 1. Concentration of C in root biomass. Note. I—plant root mass studies were carried out during the flowering stage of grasses (the third ten days of June); II—after the last grass cutting (the third ten days of September). Lowercase letters indicate significant differences ($p < 0.05$) between the treatments for the I cut, and capital letters indicate significant differences ($p < 0.05$) between the treatments for the II cut.

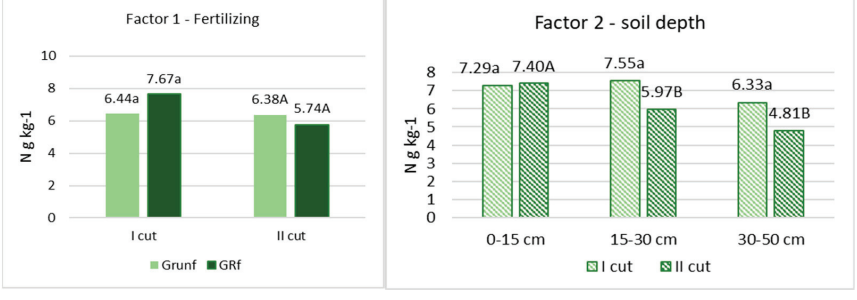


Figure 2. The concentration of N in root biomass. Note. I—plant root mass studies were carried out during the flowering stage of grasses (the third ten days of June); II—after the last grass cutting (the third ten days of September). Lowercase letters indicate significant differences ($p < 0.05$) between the treatments for the I cut, and capital letters indicate significant differences ($p < 0.05$) between the treatments for the II cut.

The accumulation of C in the roots of perennial grasses depended more on their biomass, as the differences in C concentration at various depths in the soil profile were insignificant. Most of the OC—84.9–87.1% of the total amount of OC roots in the 0–50 cm depth—was accumulated in the roots in the 0–15 cm layer (Figure 3). Below, in the 15–30 cm layer, its amount significantly decreased to 0.52–0.61 t ha^{-1}, and even less (0.13–0.45 t C ha^{-1}) was accumulated in the roots in the 30–50 cm layer. Long-term fertilization of grasses with mineral NPK fertilizers positively affected the accumulation of OC in the roots up to a depth of 30–50 cm ($p < 0.05$).

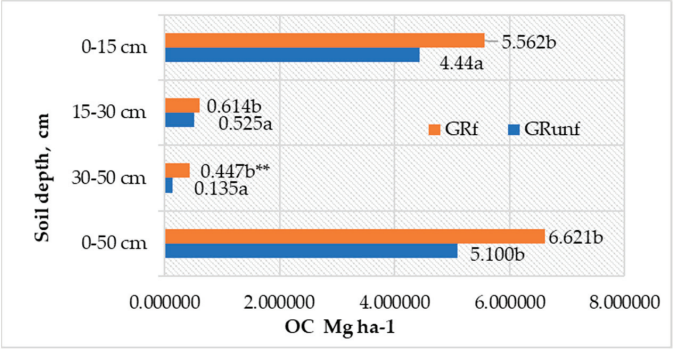


Figure 3. C accumulation in roots of perennial grasses in Arenosol profile. GRunf—grassland unfertilized; GRf—grassland fertilized. Note. Lowercase letters indicate significant differences ($p < 0.05$) between the treatments ** indicates significant differences ($p < 0.01$).

3.3. SOC Distribution in Different Arenosol Depths

A higher amount of SOC (9.31–12.32 g kg^{-1}) in the soil was accumulated at a depth of 0–15 cm (Table 9). At a depth of 15–30 cm, its amount decreased by 29.3–35.9%, and at 30–50 cm, it decreased by 75.9–80.5% (Table 9). The reduction in SOM content at different depths was significant in both the GRunf and GRf sites. Compared to unfertilized soil, mineral fertilizers significantly promoted SOC in the 0–15 cm layer ($p < 0.05$). At other depths, differences in the OC concentration were insignificant. We found that the SOC content in the 0–50 cm soil layer correlated more strongly ($r = 0.62$, $p < 0.001$) with the C accumulated in the roots of the corresponding layer than with shoot biomass ($r = 0.41$, $p = 0.04$), but it correlated weakly with the root biomass in the 0–50 cm layer. The remaining plant materials also had a specific effect on the amount of SOC, during the decomposition of which various SOM compounds were also formed.

Table 9. SOC content (g kg^{-1}) at the different soil depths.

Treatment	Soil Depth, cm					
	0–15 cm		15–30 cm		30–50 cm	
	Mean	SE	Mean	SE	Mean	SE
Grassland unfertilized	9.31 a C	1.65	7.46 a B	0.27	2.54 b A	0.16
Grassland fertilized	12.3 b C	1.18	7.56 b B	0.86	2.24 a A	0.73

Note. Lowercase letters indicate differences ($p < 0.05$) between treatments, and capital letters indicate differences ($p < 0.05$) between soil layers within the same treatment.

It was found that, at the 0–15 cm depth, the DOC content was, on average, 0.09–0.13 g kg^{-1}; at 15–30 cm, it decreased to 0.07–0.09 g kg^{-1}; and at 30–50 cm, the decrease was significant ($p < 0.05$). This corresponds to the SOC variation pattern in the *Arenosol* profile (Table 10). However, when evaluating the relative amounts of DOC and SOC (DOC/SOC), a significant increase ($p < 0.05$) in their amount was seen at the 30–50 cm depth. If the amount of

DOC in the 0–30 cm depth was 0.94–1.19% of the SOC, then at the 30–50 cm depth, it reached 1.80–2.24% ($p < 0.05$) (Figure 4). At all profile depths, the DOC content was higher in unfertilized soil compared to NPK-fertilized soil, but the increase was statistically insignificant ($p > 0.05$). A moderate correlation (r = 0.679, $p < 0.001$) was found between DOC and shoot biomass, explaining the significant increase in DOC in the 30–50 cm soil layer and indicating the leaching of mobile soil organic matter (SOM) substances from the above-ground biomass using fertilizers.

Table 10. DOC content (g kg^{-1}) at the different soil depths.

Treatment	Soil Depth, cm					
	0–15 cm		15–30 cm		30–50 cm	
	Mean	SE	Mean	SE	Mean	SE
Grassland unfertilized	0.094 a	0.017	0.070 a	0.008	0.046 b	0.001
Grassland fertilized	0.130 b	0.016	0.088 b	0.006	0.045 a	0.006

Note. Lowercase letters indicate differences ($p < 0.05$) between treatments.

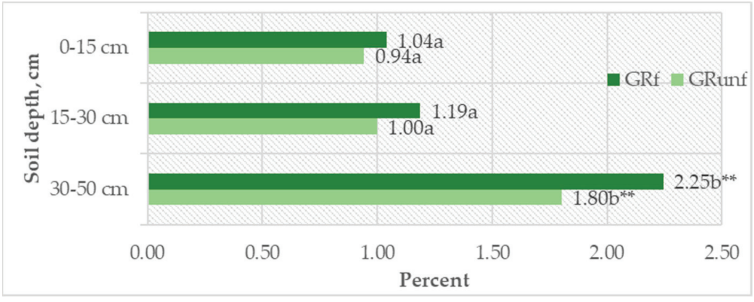


Figure 4. DOC (%) out of total SOC. GRunf—grassland unfertilized; GRf—grassland fertilized. Note. Lowercase letters indicate significant differences ($p < 0.05$) between soil layers within the same treatment, ** indicates significant differences ($p < 0.01$).

4. Discussion

Sandy soils are characterized by an insufficient amount of nutrients and organic matter [35]. However, when mineral fertilizers are used, considerable biomass quantities of perennial grasses can be grown in sandy soils, which are well-suited for fodder production [19,36–39]. According to our research, the average annual grass biomass yield in the fertilized *Arenosol* reached 892 g m^{-2}, twice as high as in the unfertilized soil. By analyzing the grass yield data of this long-term experiment [40], it was found that grass productivity is influenced not only by fertilization, but also by the meteorological conditions of the vegetation period. Under dry conditions, the productivity of non-fertilized cultivated grasses decreased by 50.2%, and that of grasses fertilized with NPK fertilizers decreased by 16.9%. In the case of excess rainfall (more than 150 mm), it decreased by 111.0% and 50.4%, respectively. The maximum productivity of cultivated grasses in *Arenosol* was obtained when the amount of precipitation in May and June was close to SKN (115.6 mm): in the fertilized grassland site, the SM yield reached 9.46 t ha^{-1}; in the unfertilized plot, 7.03 t ha^{-1}. The positive effect of mineral fertilizers on grass productivity under dry meteorological conditions has been described in other scientific publications [41,42]. Still, it should be noted that mineral nitrogen fertilizers significantly reduce plant species richness and the abundance of forbs and legumes [43–45]. The data from our experiment also confirms this. Cereal grass accounted for 98% of fertilized soil and 79% of non-fertilized soil. Especially in the fertilized grassland, the number of legume plants decreased because they were overshadowed by tall cereal grasses (*Festuca rubra* L., *Bromus inermis* Leyss). Changes in

the botanical composition of grasses also affected root biomass, as different plant species have different morphometric parameters [46–49].

After the last harvest of grass biomass, a significant amount of non-green residues remained on the soil surface (133–193 g DM m^{-2}); they were mineralized in winter and replenished the SOC accumulation. Even though the decomposition of residues occurs on the soil surface, over a certain period, the accumulation of SOC in the soil increases significantly [40]. In the fertilized GRf plot, the amount of residues after the last harvest was higher than in the unfertilized soil, but the differences were insignificant ($p > 0.05$). This difference was because the fertilized soil was dominated by tall cereal grasses, which leave less residue on the soil surface. In the unfertilized soil, grass residues averaged 29% of the annual grass biomass yield, and in fertilized soil, 17%. They accumulated 415 and 1000 kg C ha^{-1} and 3.0 and 14.7 kg ha^{-1} of N, respectively, which will be included again in the biological cycle of these elements after the mineralization of the residues. The yield of the above-ground parts of the grasses did not depend on the mass of the roots in the 0–50 cm layer or their mass in the upper 0–15 cm layer, where the concentration of nutrients was higher. The weak correlation between above-ground and root biomass could be due to differences in the botanical composition of the grasslands, as different plant species have different root systems that influence root biomass. It was found in [50] that diverse communities with species from multiple families generate 1.3 times the amount of root standing crop compared to the average root biomass from monoculture. In our research, the fertilized soil was dominated by cereal grasses (98.1%), while in the unfertilized soil, it was significantly less (79.1%), and more alfalfa and forbs were present. Alfalfa plants have a thick taproot, significantly increasing the root biomass in upper and deeper soil depths [51,52]. In sandy soil, the biomass of grass roots in the 0–50 cm depth averaged 12.39–15.78 Mg ha^{-1}. The grass root biomass in the 0–50 cm layer was higher in the GRf soil than in GRunf, but the difference was insignificant ($p >0.05$). Other researchers have described similar effects of fertilizers on root biomass [53]. The main mass of roots was in the upper 0–15 depth and accounted for an average of 84–88% of their biomass in the entire 0–50 cm layer. Below, in the 15–30 cm layer, their biomass drastically decreased and accounted for only 9–10% of the amount in the 0–50 cm layer. Even deeper (in the 30–50 cm layer), very few roots are found—3–7% of the total biomass. A similar distribution of root biomass in the soil profile has been described in many scientific publications [54–57]. Soil fertilized with mineral fertilizers, compared to unfertilized soil, increased the root biomass (+279%, $p < 0.05$), but the differences were significant only in the 30–50 cm layer. Such results were determined by the morphology of the roots of different plants, due to which the mass of the roots varied greatly in individual samples and increased the statistical errors of the reliability of the indicators. This was mainly influenced by alfalfa roots, which significantly increased the root mass in deeper layers, and rhizome plants, with thick roots in the upper layer. In a 2009 study by Titlyanova [58], scientists discovered that grasses' below-ground biomass variation is much lower than that of their above-ground biomass. The active growth of a diverse plant species' above-ground and below-ground organs may occur at different times during the season, and this growth pattern can vary from year to year.

There was 3.02–4.39 Mg ha^{-1} OC accumulated in the grass roots at the 0–50 cm depth. Fertilization with mineral fertilizers increased C accumulation in root biomass, but the difference was not statistically significant ($p > 0.05$). The main part of OC in the roots (72–87%) was accumulated in the 0–15 cm layer (2.62–3.17 Mg ha^{-1}), and in the 15–30 cm layer, its amount significantly decreased, amounting to only 0.32–0.74 Mg ha^{-1}. A global meta-analysis of 103 published studies assessed the effects of species mixtures on fine root biomass and traits. It highlighted that the community-level consequences of changes in plant diversity on fine root traits are inconsistent. Predicting these consequences requires considering the extent of changes in plant species richness, the stand age, the soil depth investigated, the background climates, and the particular fine root traits [48].

It was found that, in the sandy soil, SOC accumulation in the soil layers did not depend on the amount of OC accumulated in the roots. SOM compounds are known to be absorbed by clay and silt particles [30]. There are very few sandy soils, so the organic compounds formed in the course of root decay in sandy soils with high stone content can be leached into deeper horizons, especially in regions with excess precipitation. However, under other natural conditions, root biomass can positively affect SOC accumulation in soil [57,59]. Reference [60] found that, regardless of soil fertility, roots contributed the most (44%) to new SOC formation, while shoots (28%) and arbuscular mycorrhizal fungi (28%) exerted similar but lower contributions. Soils with higher fertility sequestered more shoot-, root-, and associated mycorrhizal fungi-derived C. Reference [61] states that plant roots control and influence SOC dynamics by providing OC to the soil, primarily in the forms of root litter and rhizodeposition. This C input results in SOC gain, particularly when plant roots promote SOC stabilization. However, roots can also destabilize SOC, exposing previously protected C to microbial decomposition and causing SOC loss. In sandy soils characterized by weak adsorption, the amount of SOC in the deeper horizons also has labile SOM substances leached from the Ah horizon. Under Lithuanian climatic conditions, leaching of SOC in sandy loam soil averages approximately 20 kg C ha^{-1} $year^{-1}$ [62,63]. DOC flux from the soil profile is negatively affected by soil clay content, with DOC export being seven times higher in clay-free *Arenosol* (55 kg C ha^{-1}) than in clay-rich *Vertisol* (8 kg C ha^{-1}). According to those studies, DOC export is not controlled by vegetation, but by physicochemical parameters such as soil mineral adsorption capacity and ecosystem water balance.

5. Conclusions

In sandy loam soil rich in stones (*Arenosol*), the yield of above-ground biomass in a multicomponent grassland was largely influenced by mineral fertilizers and meteorological conditions. NPK fertilizers' application doubled the above-ground biomass yield ($p < 0.05$). In contrast to above-ground biomass yield, mineral fertilizers did not significantly affect the grass root biomass ($p > 0.05$). Only in the 30–50 cm depth did the biomass of their roots increase significantly ($p < 0.05$) due to the use of fertilizers. However, unfertilized grassland in *Arenosol* was distinguished by its composition of more diverse plant species and the conservation of rare herb species. Meanwhile, in NPK-fertilized grassland, there was a significantly lower amount of alfalfa ($p < 0.05$), where nitrogen fertilizers promoted the development of cereal grasses and aggressive species such as bromegrass (*Bromus inermis* Leyss). The yield of the above-ground part of the grasses highly depended on the root mass in the 0–15 cm depths ($p > 0.05$).

At the 0–50 cm soil depth, 12.4–15.8 Mg ha^{-1} of root biomass was accumulated. The main amount (84–88%) was allocated in the 0–15 cm layer. The perennial grass roots of the entire investigated depth range comprised 5.10–6.62 Mg ha^{-1} OC. At the fertilized site, significantly more grass residues remained on the soil surface after the last grass harvest, reaching 193 g DM m^{-2} ($p < 0.05$). The perennial grasslands, depending on the amount of residue, accumulated an average of 415–1000 kg ha^{-1} C and 3.04–14.7 kg ha^{-1} N in total. The SOC content in the 0–50 cm soil layer moderately correlated ($r = 0.62$, $p < 0.001$) with the C accumulated in the roots of the corresponding layer, and correlated moderately with the shoot biomass ($r = 0.41$, $p = 0.04$).

According to our long-term grassland experiment data, the fertilized site provided more favorable conditions for carbon accumulation in the soil and for the growth of above-ground and below-ground grass biomass. However, with fertilization, the relative content of DOC/SOC increased, indicating the leaching of soil organic matter from the Ah horizon of the *Arenosol*. Significant correlations were found between DOC and shoot biomass ($r = 0.68$, $p < 0.001$) and between DOC and biomass of residues ($r = 0.71$, $p < 0.001$), explaining the significant increase in DOC in the 30–50 cm soil layer. The biodiversity advantage of unfertilized grassland and significantly higher above-ground biomass yield

when fertilized also need to be considered when selecting land management practices for low-productivity, vulnerable sandy soils.

Author Contributions: Conceptualization, M.T., L.T. and A.V.; methodology, M.T., L.T., A.V. and A.S.; formal analysis, L.T., M.T., A.V. and A.S., investigation, M.T., L.T. and A.V.; writing—original draft preparation, L.T. and M.T.; writing—review and editing, M.T., L.T., A.V. and A.S.; visualization, L.T. and M.T.; supervision, M.T. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the long-term program "Plant Biopotential and Quality for Multifunctional Practice and Sustainability of Agroecosystems" and was partly funded by the European Joint Program for SOIL "Towards climate-smart sustainable management of agricultural soils" (EJP SOIL) funded by the European Union Horizon 2020 research and innovation program (Grant Agreement N_862695) internal project "Are mixed species systems fostering belowground C inputs and C sequestration?" (MIXROOT-C).

Acknowledgments: This paper presents research findings obtained through the long-term research program "Biopotential and quality of plants for multifunctional use" implemented by the Lithuanian Research Centre for Agriculture and Forestry.

Conflicts of Interest: The authors declare no conflicts of interest.

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Review

Grassland Ecosystem Services: Their Economic Evaluation through a Systematic Review

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Abstract: Grasslands provide a wide range of provision, support, regulation, and cultural ecosystem services (ESs), whose valuation methods can be grouped into three categories (ecological, sociocultural, and economic). The present manuscript aims to provide an overview of academic studies on grassland ESs and of the most used economic evaluation methods. To this end, a systematic and bibliometric review was conducted using the scientific database Scopus and the VOSviewer software. The results highlighted that China and the USA were the main countries with the highest number of publications regarding ESs provided by grasslands. The number of publications began to grow starting in 2005, thanks, perhaps, to the publication of influential documents, such as the Millennium Ecosystem Assessment, and the general increase in interest in ecological policy issues. The year 2023 had the highest number of documents in absolute (646), demonstrating the timeliness, importance, and relevance of this research topic. The most studied grassland ES has been carbon storage; however, a central role was played also by biodiversity. In this context, papers that estimated grassland ESs from an economic perspective represented only 3% of all papers that Scopus has returned. More than half of these referred to the use of equivalent coefficients to calculate the ES value of different land uses/land cover categories or, at most, of 11 types of ES. All this highlights the difficulty in estimating individual ESs provided by grasslands from an economic point of view and the greater propensity to use physical, chemical, and biological indicators. Consequently, the sustainable management of grasslands requires more studies on the economic evaluation of their ES, as well as environmental aspects in the economic accounting of governments, or to implement a support system for farms in delivering various ecosystem services.

Keywords: pasturelands; economic assessment; bibliometric and systematic analysis; ecosystem functions; accounting

Citation: Pergola, M.; De Falco, E.; Cerrato, M. Grassland Ecosystem Services: Their Economic Evaluation through a Systematic Review. *Land* **2024**, *13*, 1143. https://doi.org/10.3390/land13081143

Academic Editors: Dingde Xu, Michael Vrahnakis and Yannis (Ioannis) Kazoglou

Received: 30 May 2024
Revised: 11 July 2024
Accepted: 24 July 2024
Published: 26 July 2024

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1. Introduction

According to FAO, grasslands account for 28% of the world's land area, 63% of which is concentrated in the Russian Federation, Australia, the United States of America (USA), Canada, China, Kazakhstan, and Brazil [1]. The definitions given of grassland are different depending on the aspects that are taken into consideration. Indeed, FAO [2] defined grassland as "land covered with grass and with less than 2% tree or shrub cover". The International Vegetation Classification [3] and Dixon et al. [4] identified four different grassland types: tropical grasslands, Mediterranean grasslands, temperate grasslands, and semidesert grasslands. Allen et al. [5], instead, with the aim of developing a consensus on terms and definitions to ensure clear international communication regarding grasslands, defined many grazing land terms at a global level. Among these, pastureland is defined as "land (and the vegetation growing on it) devoted to the production of introduced or indigenous forage for harvest by grazing, cutting, or both" and "is synonymous with grassland when referring to an imposed grazing-land ecosystem with a vegetation broadly interpreted to include grasses, legumes, and other forbs, and at times woody species may

be present". Among other definitions, that of rangeland is also interesting, which the authors defined as "a land on which the indigenous vegetation is predominantly grasses, grass-like plants, forbs or shrubs that are grazed or have the potential to be grazed, and which is used as a natural ecosystem to produce grazing livestock and wildlife". The latter include natural grasslands, savannas, shrublands, many deserts, steppes, tundras, alpine communities, and swamps. Table 1 reports the different types of grasslands identified by Allen et al. [5].

Table 1. The main typologies of grasslands.

Type of Grasslands	Description
Annual	The forage is established annually
Cultivated	The forage is established with domesticated species that receive periodic agricultural treatments
Permanent	With perennial or self-seeding annual forage species that may survive indefinitely or often due to limiting factors that prevent other uses (excessive slope, shallow soil depth, outcropping rockiness, stoniness)
Temporary	The vegetation is composed of annual, biennial, or perennial forage species kept for only a few years
Naturalized	The forage species are mainly introduced from other geographical places that have established themselves and have persisted for a long time in existing environmental and management conditions
Semi-natural	A managed ecosystem dominated by native or naturally occurring herbs and other herbaceous species

Referring to natural grasslands, in the world, there are 42 zonal natural grassland types [6], where the characteristic vegetation is determined by climatic and soil conditions, grazing animals, and fire. For example, in Uruguay, southern Brazil, and north-eastern Argentina, the sub-tropical climate favors the presence of "campos" [7]; in central Brazil, the tropical climate allows the presence of "cerrado" [8]; in eastern and central Argentina, the climate humid to arid ensures the presence of "pampa" (treeless grasslands on flat and fertile plains) [9]; in North America, prairies are widespread, and in south-eastern Europe, Asia, and North America are the steppes [5].

In Europe, natural grasslands cover limited areas, which according to Peeters et al. [10] are alpine and boreal tundra grasslands (beyond the tree line); rocky Pannonian grasslands (in Hungary); the Macaronesian mesophilic pasturelands of the Atlantic islands (e.g., Azores); sub-desert meadows (in Romania, Russia, and Ukraine); Mediterranean xeric grasslands (e.g., the main Mediterranean islands and Stipa grasslands in south-eastern Spain). On the contrary, in most parts of Europe, grasslands are semi-natural, which is the result of centuries or millennia of low-intensity human land use from forests as potential natural vegetation [11].

Each of these grassland types provides important provision, support, regulation, and cultural ecosystem services (ES) [12,13], namely, the benefits humans obtain from grassland functions [14]. In the past, grasslands have played an important role in people's livelihoods as areas producing fodder for animals. Today, instead, the increasing demand for animal products is being met by the production of fodder on cropland and improved grasslands [13]. Consequently, the conversion to arable land [15] and the lack of management/increased abandonment [16,17] have led to the decline of grasslands worldwide during the last century. Anyway, grass remains the most natural forage for various herbivores, providing minerals, vitamins, and other active substances supporting living processes and providing healthy meat and milk for people [18]. It is largely recognized that grasslands have not only a local importance, for the maintenance of biodiversity, pollination, and food production but also at regional (water and erosion regulation, recreation, inspiration) and global scales (climate regulation) [13]. Nowadays, grassland plants may be used for energy production needs or as natural dyes for coloring clothes, and various medical plants can be found in pastureland, especially if natural [18]. Thus, a complete picture of the ES provided by grasslands can be found in Richter et al. [12], Bengtsson et al. [13], O'Mara [19], Erb et al. [20], Veen et al. [21], Habel et al. [22], and Dengler et al. [11].

Referring to Europe, Table 2 reports the functions and the relative ecosystem services provided by the main typologies of European grasslands.

Table 2. The ESs provided by the main typologies of European grasslands.

European Area	Grasslands Type	Main Functions Provided	Ecosystem Services Category
Nordic countries (Denmark, Finland, Iceland, Norway and Sweden)	Natural	livestock production	Provisioning
	Semi-natural	grazing maintaining biodiversity maintaining landscape	Provisioning Supporting
	Cultivated	winter fodder milk in summer	Provisioning
Temperate regions (such as Ireland, the UK, France, the Benelux, Germany, Czech Republic, Slovakia, and Poland)	Permanent	feed production maintaining biodiversity soil erosion control	Provisioning Supporting Regulating
	Temporary	feed production maintaining biodiversity	Provisioning Supporting
Mediterranean basin	Natural/ Semi-natural	livestock production soil erosion control carbon sequestration biodiversity conservation maintaining landscape	Multiple

In particular, in Nordic countries, grasslands are very heterogeneous, and their importance varies greatly from country to country. In fact, natural pasturelands are very large in Iceland and play a notable function in livestock production, while they are much less relevant in other countries [23]. In temperate regions of Europe, both permanent and temporary grasslands are used, including a varying proportion of forage legumes, playing a key role in feed production (above all for cattle and sheep) and, in some areas, in the preservation of the environment [24]. Moreover, referring to soil erosion, permanent grassland is strongly recommended, and since, in this region, we are witnessing their reduction, Huyghe et al. [24] said that this important function should be valorized. Finally, in the Mediterranean basin (a global biodiversity hotspot with an extremely high number of endemic plant species) [25], grasslands and rangelands are important ecosystems that have traditionally played a significant role in the evolution of human societies [26]. In particular, here, livestock grazing (characterized by small ruminants and beef cattle belonging to local races) affects the quantity and quality of forage, vegetation dynamics, species, and landscape diversity [27]. Thus, in this context, grassland systems are not only important for livestock production but they also perform multiple ecosystem functions [25].

Although most grasslands provide the same set of ecosystem services, their values are different [18]. Ecosystem service valuation methods can be grouped into three categories: ecological, sociocultural, and economic [28]. Of these three, as noted by Richter et al. [12], the economic evaluation of ES has been recognized as an important tool for developing strategies for the sustainable management of ecosystems. To our knowledge, there are few systematic literature review studies on the economic evaluation methods of grassland ESs. Indeed, Kang et al. [29] used 61 research studies in their evaluation of grassland ecosystem services' value in China, including 564 value observations to establish a value transfer database and to construct a value model. Richter et al. [12] provided an overview of available methods for the economic valuation of ES through a review of 85 plot-scale methods to assess 29 different ES indicators for 21 provisioning, regulating, supporting, or cultural ESs. Liu et al. [30,31] provided a comprehensive evaluation of the economic value of grassland ESs: on one hand, via a meta-regression analysis of 69 studies; on the other hand, via a meta-analysis of 702 observations from 134 primary studies. Thus, the present manuscript aimed to provide a critical overview of academic studies on the ES supplied by grasslands and wanted to contribute to the research of the most commonly used economic evaluation methods.

2. Materials and Methods

According to the aim of the paper, a bibliometric and systematic review of the literature on grassland ecosystem services was carried out to highlight the geographical focus of the literature, as well as how academia approaches the study of grassland ESs and what the main economic evaluation methods are to assess such ESs. As stated by Merli et al. [32] and Denyer and Tranfield [33], systematic analysis enables one to implement a clear and reproducible process of selection, analysis, and reporting on research about a specific topic. As in Pergola et al. [34], the review process followed some phases: definition of the research question; choice of the software to use; collection of documents; systematic and bibliometric analysis of documents; selection of the documents; qualitative analysis and groupings; and evaluation of the documents (Figure 1).

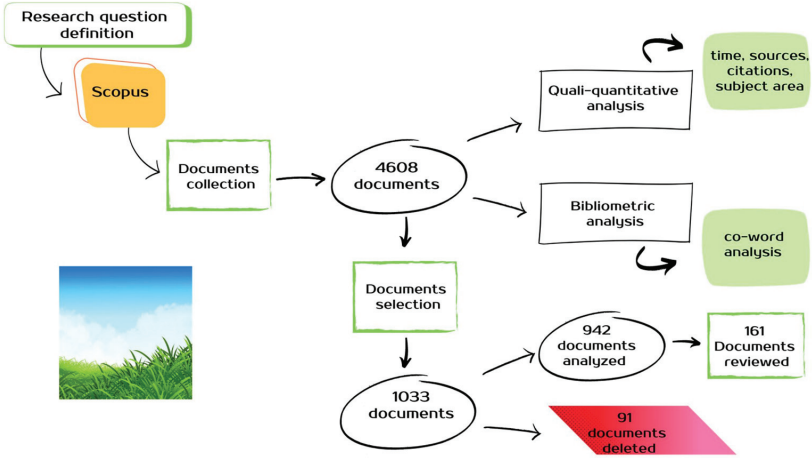


Figure 1. The review process scheme.

2.1. The Science Mapping

For the bibliometric analysis science mapping was used, a technique that examines the relationships between research components [35]. In particular, co-word analysis was used to understand the existing relationships between issues by focusing on the written content of the documents analyzed. VOSviewer software (version 1.6.20) was utilized to extract and analyze the semantic contents of titles, abstracts, and keywords of publications, correlating them to citation count data and creating network maps to visualize the results [36]. As in Muley and Medithi [37], default parameters were used for the analysis and the construction of the network maps. In particular, in the network map, the font size of words states their frequency of occurrence; if two words occurred more frequently in the analyzed documents, they were nearest to each other. Only words that had a minimum of 10 occurrences were analyzed and shown. To create the maps, two units of analysis were used ("all keywords" and "author keywords"), and the full count method was adopted, meaning that each co-occurrence link had the same weight. The default "associative strength method", with default values of attraction and repulsion, was used for the normalization of the co-occurrence matrix [35].

As detailed in Donthu et al. [35], in the network maps constructed using the VOSviewer software, each knot represented a keyword, wherein: (1) the size of the knot indicated the number of times that the keyword occurred, (2) the connection between the knots represented the co-occurrence among keywords (i.e., keywords that co-occurred or occurred together), (3) the thickness of the connection indicated the occurrence of co-occurrences among keywords (i.e., the number of times that the keywords co-occurred or occurred together), (4) the bigger the knot, the greater the occurrence of the keyword, and (5) the

thicker the connection between knot, the higher the occurrence of the co-occurrences among keywords. Each cluster was highlighted with a different color.

2.2. The Systematic Review Process

In order to conduct a reliable bibliometric and systematic analysis [38], the scientific database Scopus [39,40] was utilized for the collection of the documents, [39]. The first phase of the review process was the collection of all grassland ES publications across the scientific community. Thus, the generic expression "grasslands ecosystem services" was adopted and the research criterion "Article title, Abstract, Keywords" was employed. All types of documents were utilized as units of analysis (articles, reviews, book chapters, conference papers, books, notes, editorials, short surveys, letters, data papers, and conference reviews) and in all languages. No chronological restriction was performed, and the query on the database was carried out on 14 May 2024. From 1980, Scopus returned a total of 4608 documents. Thus, a quantitative analysis of the distribution and evolution of these documents over time, sources, number of citations, and thematic areas was made.

Subsequently, to identify papers that dealt with economic evaluation methodologies of the grassland ecosystem services, the subject areas "Social Science" and "Economics, Econometrics and Finance" were selected. In addition, the strings "grasslands ecosystem services" + "economic evaluation" and "grasslands ecosystem services" + "economic assessment" were used with the research criterion "Article title, Abstract, Keywords" to avoid the risk of leaving out some documents. Consequently, Scopus returned a total of 1033 publications, of which, after removing duplicates (91 documents), 942 papers were evaluated. From the latter, through qualitative analysis (reading of the abstracts and/or full documents), the manuscripts that presented economic methodologies for evaluating the grassland ESs were selected. Therefore, a sample of 161 papers was carefully examined in order to group them into areas of economic assessment methods.

3. Results

3.1. The Descriptive and Bibliometric Analysis of the Literature on Grasslands Ecosystem Services

The first article on grassland ecosystem services dates back to 1980 and refers to the tallgrass prairie version of the ELM Grassland Model, used to evaluate the potential impact of creating a tallgrass prairie National Park in the Flint Hills region of Kansas [41]. In particular, the model was specifically developed to study the effects of levels and types of herbivory, climatic variation, and fertilization upon pastureland ecosystems [41]. In any case, up to 2004, there were only 53 documents. As early as 2005, the number of publications referring to grassland ESs began to increase, and a steady rise in research articles has been observed since then (Figure 2). At the same time, the highest number of publications was recorded in the last five years (n = 2544), representing 55% of the total documents found on Scopus.

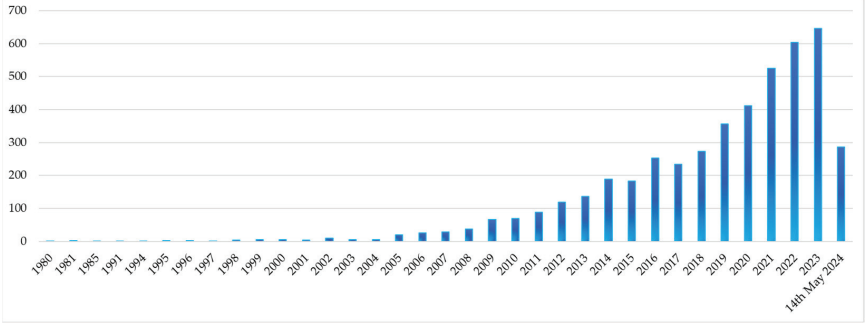


Figure 2. Trends of documents on grassland ecosystem services.

The distribution of all documents by countries shows that more than 50% of them were from China (1129), the United States (985), Germany (455), and the United Kingdom (379), as reported in Figure 3.

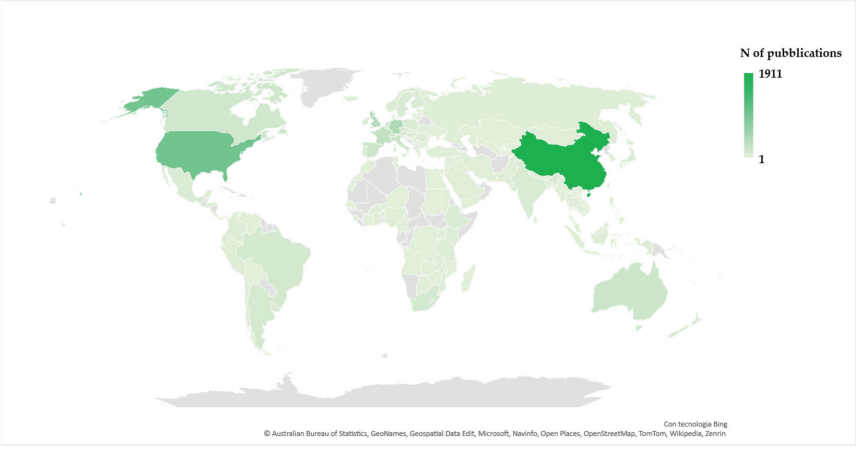


Figure 3. Distribution by countries of the publications on grassland ES (n = 4608) collected with the Scopus database in the period 1980–2024.

Most of the published manuscripts were articles (4024; 87%), followed by review papers (240; 5%), book chapters (152; 3%), and conference papers (128; 3%). About 93% of the documents (4304) were published in journals, and the top ten source types are listed in Table 3. The latter shows that the main research areas were the assessment of ecological and environmental indicators, the ecology, the environment in total (atmosphere, lithosphere, hydrosphere, biosphere, and anthroposphere), the sustainability, and everything that concerns land.

Table 3. The top ten contributor journals collected with the Scopus database in the period 1980–2024.

Source Title	Number of Documents	% of Documents
Ecological indicators	176	4%
Shengtai xuebao	175	4%
Science of the total environment	138	3%
Sustainability Switzerland	131	3%
Land	115	2%
Agriculture ecosystems and environment	110	2%
Journal of Environmental Management	95	2%
Remote sensing	71	2%
Plos one	70	2%
Journal of Applied Ecology	65	1%
Total of the top ten journals	1146	25%
Number of documents published in journals	4304	93%
Number of documents related to grassland ESs	4608	100%

In line with what has been said, the top ten cited articles dealt with the importance of biological diversity for the maintenance of ecosystems; the impact and consequences of biodiversity loss on the functioning of ecosystems, the provision of ecosystem services and human well-being; the importance of biological and microbial communities for ecosystem services and human well-being; the importance of investing in the protection and restoration of natural capital and the implementation of national policies on payments for ES to address devastating environmental crises and improve human well-being (Table 4).

Table 4. The top ten cited manuscripts collected with the Scopus database in the period 1980–2024.

Title of the Top Ten Cited Manuscripts	Journal's Name	Year of Publication	Number of Citations
Partitioning selection and complementarity in biodiversity experiments [42]	Nature	2001	2242
Quantifying the evidence for biodiversity effects on ecosystem functioning and services [43]	Ecology letters	2006	2006
Biodiversity and ecosystem stability in a decade-long grassland experiment [44]	Nature	2006	1611
Soil biodiversity and soil community composition determine ecosystem multifunctionality [45]	Proc. Natl. Acad. Sci.	2014	1495
Confronting a biome crisis: Global disparities of habitat loss and protection [46]	Ecology letters	2004	1321
Microbial diversity drives multifunctionality in terrestrial ecosystems [47]	Nature Communications	2016	1306
Incorporating plant functional diversity effects in ecosystem service assessments [48]	Proc. Natl. Acad. Sci.	2007	1250
Improvements in ecosystem services from investments in natural capital [49]	Science	2016	1228
High plant diversity is needed to maintain ecosystem services [50]	Nature	2011	1150
Ecological and socioeconomic effects of China's policies for ecosystem services [51]	Proc. Natl. Acad. Sci.	2008	1125

Referring to subject areas, those most represented were environmental science and agricultural and biological sciences (Figure 4), confirming the importance of studying grassland ESs from an environmental and agricultural management perspective. At the same time, it is interesting to note that the area of social sciences occupied the third place, absorbing 7% of the research returned by Scopus.

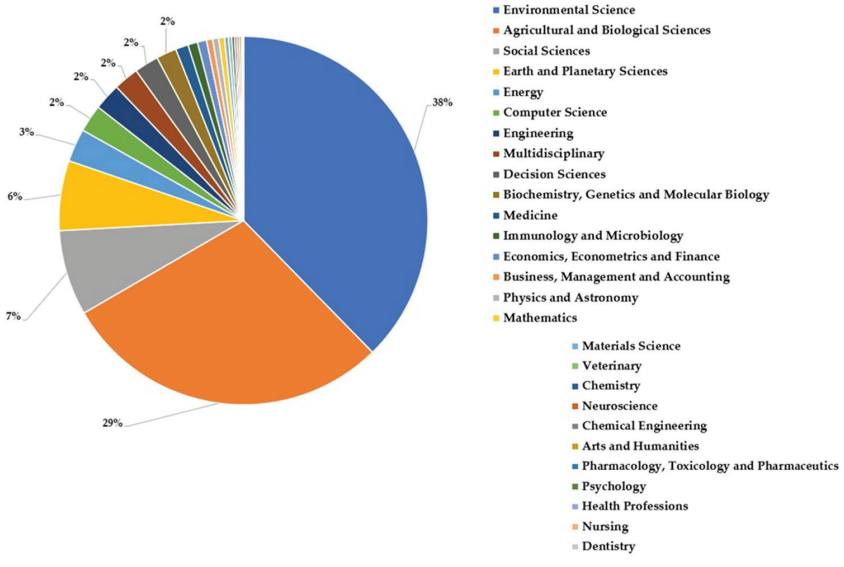


Figure 4. Distribution of documents, collected with the Scopus database in the period 1980–2024, by subject area.

The co-word analysis of all keywords of the 4608 collected documents aggregated the terms with a minimum number of occurrences of 10 into 5 clusters, easily visible in Figure 5 because they are highlighted by different colors (cluster 1 red; cluster 2 bright green; cluster 3 blue; cluster 4 light green; cluster 5 purple) (Figure 5). The top five most recurring words were grassland (occurrences: 1890), ecosystem service (occurrences: 1813), ecosystem services (occurrences: 1120), ecosystems (occurrences: 1105), and China (occurrences: 1059). The keyword "grassland" belonged to cluster 3, and the other most frequently occurring words were agricultural land, agriculture, and biodiversity. Instead, the keywords "ecosystem service", "ecosystem services", "ecosystems", and "China" belonged to cluster 1, where the other most frequently occurring words were environmental protection, forestry, land use, land use change, and sustainable development (Figure 5).

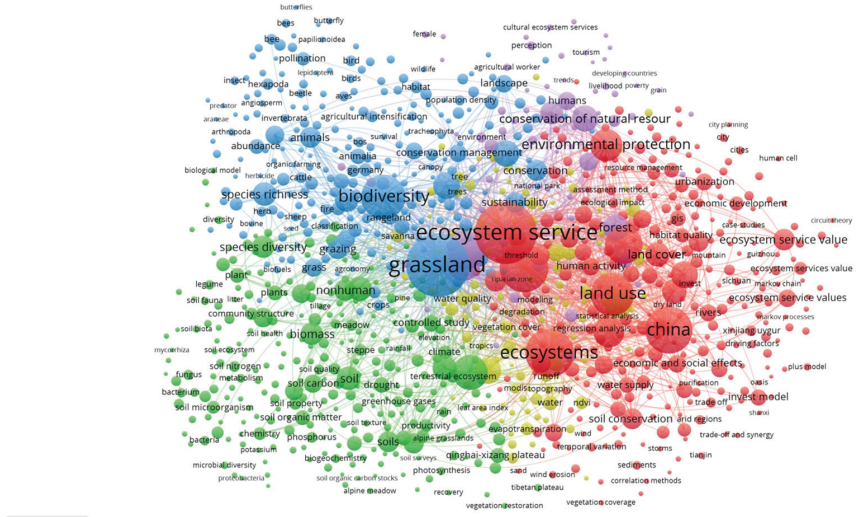


Figure 5. Co-word (all keywords co-occurrence) network map of the 4608 documents collected with the Scopus database in the period 1980–2024.

At the same time, to better outline the situation, the unit of analysis "author keywords" was used, and the corresponding density map (Figure 6) showed that the most recurring keywords were ecosystem services (825 occurrences); grassland (288 occurrences); biodiversity (274 occurrences); climate change (234 occurrences); and ecosystem service value (203 occurrences) (Figure 6).

In this analysis, 6 clusters have been identified and the terms "ecosystem services" and "ecosystem service value" belonged to cluster 1, where the other most frequently occurring words were carbon storage, ecosystem service, land use, and land use change. The keywords "grassland" belonged to cluster 6, which includes the following most frequently occurring terms: agroecology, land management, landscape, livestock, and vegetation. The keywords "biodiversity" and "climate change" belonged to cluster 2, with the words drought, ecosystem function, functional diversity, grazing, multifunctionality, soil organic carbon, and species richness.

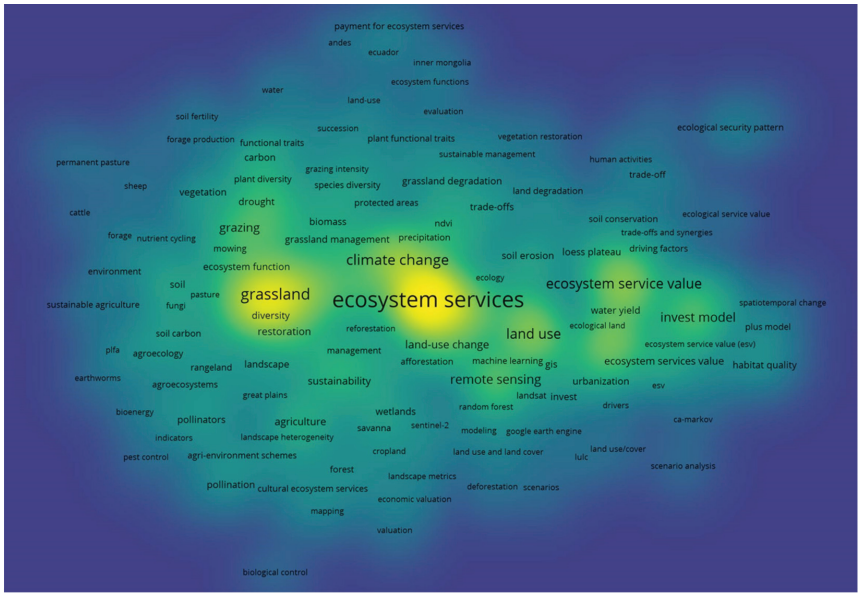


Figure 6. Density map displaying words from author keywords of the 4608 documents collected with the Scopus database in the period 1980–2024.

3.2. The Analysis of the Documents Referring to the Economic Evaluation Methodologies of the Grassland ES

To try to skim the 1033 documents that Scopus returned in reference to the economic methodologies for estimating grassland ESs, co-occurrence analysis was used. The latter, carried out considering "all keywords", was not very significant for the purpose of the research given that it identified five very varied clusters and the following five most recurring words: ecosystem service (457 occurrences), grassland (324 occurrences), China (321 occurrences), ecosystem services (258 occurrences), ecosystems (221 occurrences) (Figure 7), namely, the same ones displayed before.

At the same time, this analysis made it possible to verify the presence of terms relating to the economic evaluation of the ES. Thus, the following keywords were identified: economics (67 occurrences); economic analysis (21 occurrences); willingness to pay (14 occurrences); payment for ecosystem services (10 occurrences); and ecological economics (11 occurrences). As shown in Figure 8, which shows the links of these economic terms, only the words "economics", "ecological economics", and "willingness to pay" were simultaneously linked to the two main research terms (ecosystem services and grasslands)—among other things, the most recurring terms. On the contrary, the keyword "economic analysis" was found to be linked to ecosystem services/service, ecosystems, and land use, while "payment for ecosystem services" was found to be far from all the other keywords and not linked to any other term.

This analysis was followed by the selection of manuscripts that had the economic evaluation of ESs as their subject of study. The selected sample (161 papers) was carefully studied and divided into 15 groups of adopted methodologies, as reported in Table 5.

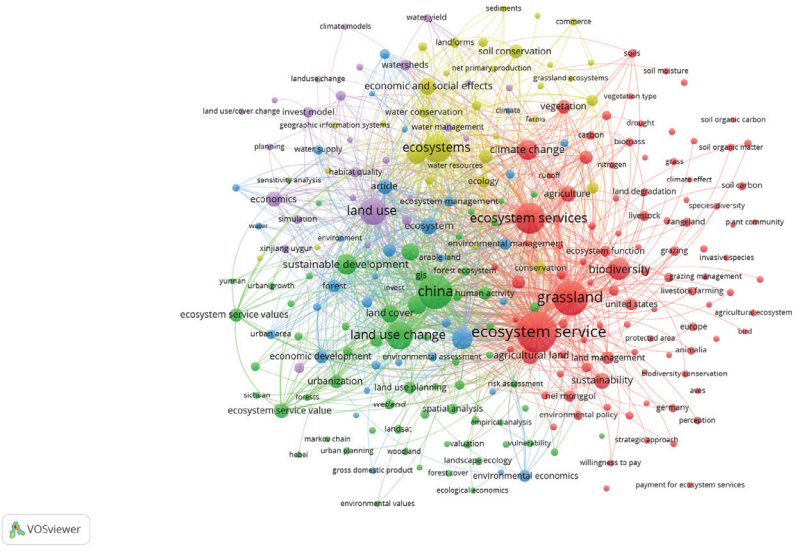


Figure 7. Co-word (all keywords co-occurrence) network map of the 1033 documents collected with the Scopus database in the period 1980–2024.

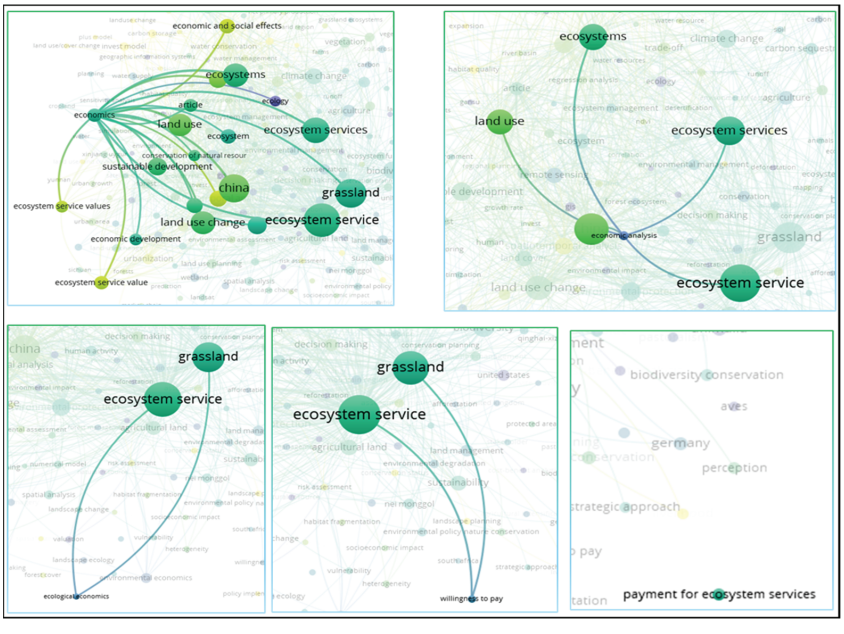


Figure 8. Co-word (all keywords co-occurrence) network map of the 1033 documents collected with the Scopus database in the period 1980–2024.: focus on the keywords "economics", "ecological economics", "willingness to pay", "economic analysis", "payment for ecosystem services".

Table 5. The groups of ES grassland economic evaluation methodologies identified, the number of publications for each group, and the relevant bibliographical references (our elaboration).

Methodologies	Number of Documents	References
Ecosystem Service Value (ESV) Assessment	91	[52–71] *
Resource Equivalency Approach	22	[72–92]
Econometric Models	11	[12,93–101]
Benefit Transfer Method	7	[102–108]
Contingent Valuation Method (CVM)	7	[109–115]
Gross Ecosystem Product (GEP)	4	[116–119]
Emergy Value Method	4	[120–122]
Focus Groups, Perception/Social Preference Method	4	[123–126]
Net Present Value	3	[127–129]
Replacement Cost Method	3	[115,130–132]
Market and Shadow Price Method	2	[133,134]
Life Cycle Assessment Approach	1	[135]
Social Cost	1	[136]
Theory of Value and Random Utility	1	[137]
Cost–Benefit Analysis	1	[138]

* For reasons of space, only publications from the last year are reported.

The most widespread economic estimation methodology for grassland ES was the ESV assessment, which, for each land-use category (per unit area) assigns an equivalent coefficient, as reported by Xie et al. [139] and Costanza et al. [14]. "Indirect" estimation methods then follow, namely, the resource equivalency approach, according to which the economic estimates of ES occur through the quantification of compensation to farmers for the loss of the environmental resource [140]. This category includes the payments for ecosystem services (PESs), the agri-environment schemes (AESs), and the eco-compensations. We define these methodologies indirectly given that the estimation of the ES in these cases occurred through the quantification of the amount for the ecological compensation received by farmers who implemented measures to increase the ES or avoid their decrease.

Econometric models, useful for carrying out meta-regression analyses when there are multiple variables to consider simultaneously, were found in only 11 papers (Table 5). At the same time, the benefit transfer method, used to estimate the economic values of ES by transferring available data from studies already completed in another location and/or context, was applied in seven manuscripts, as well as the contingent valuation method. In particular, the latter was used to estimate landowner/farmer willingness to accept (WTA) monetary compensation in exchange for implementing management practices preserving grassland ecosystems or to assess the willingness to pay (WTP) for ES.

Other economic methodologies were the estimation of ES through the computation of the gross ecosystem product (based on the calculation of the functional quantity of each ecological element and then its conversion into value quantity) [141]; the energy value method (which refers to the available energy required directly and indirectly to make a service or good [142] and measures natural capital and ESV by assessing the costs of their generation from a donor-side perspective [122]); the use of focus groups to understand the perceptions and social preferences of interviewees; and the use of the net present value (Table 5).

The use of the market and shadow price method and the replacement cost approach were less frequent (only in two research in the first and in three in the second). Finally, the life cycle assessment approach, the use of the social cost, the cost theory of value and the random utility, and the cost–benefit analysis were decidedly rare.

Referring to the type of data used for the economic evaluation of grassland ES, the analysis of the 161 selected papers showed that land use/cover change (LUCC) data were the most used data (in 37% of cases), followed by direct data acquired through structured and semi-structured interviews (SSI) (14%) (Table 6).

Table 6. Typologies of data used for the economic analysis of grassland ES.

Type of Data	Number of Documents
Land use/cover change (LUCC)	60
Data from structured and semi-structured interviews (SSIs)	22
Land cover data, net primary productivity, precipitation, and soil erosion data (LNPS)	21
Multi-source data (MS)	14
Bio-economic modeling approach (BEM)	12
Data from others research (R)	12
Land use data and socio-economic data (LSE)	9
LUCC, climatic, socioeconomic, and biophysical data (LCSEB)	7
Socio-economic data (SE)	4
Total	161

In 57% of the documents, the four categories of ES (provisioning, regulating, supporting, cultural) were estimated and, for each category, the following ecosystem services were accounted: food production, raw material production, and water supply (as regards provisioning services); gas regulation, climate regulation, environment purification, and hydrological regulation (with regards to regulating services); soil conservation, nutrient cycle maintenance, and biodiversity (as supporting services); aesthetic landscape, regarding cultural services. In 22% of the papers, the overall value of the ES offered by the grasslands was calculated (ESV), without any differentiation in the different ES offered; in 13% a single ES was estimated, and, in 8%, there were multiple ecosystem services (Figure 9).

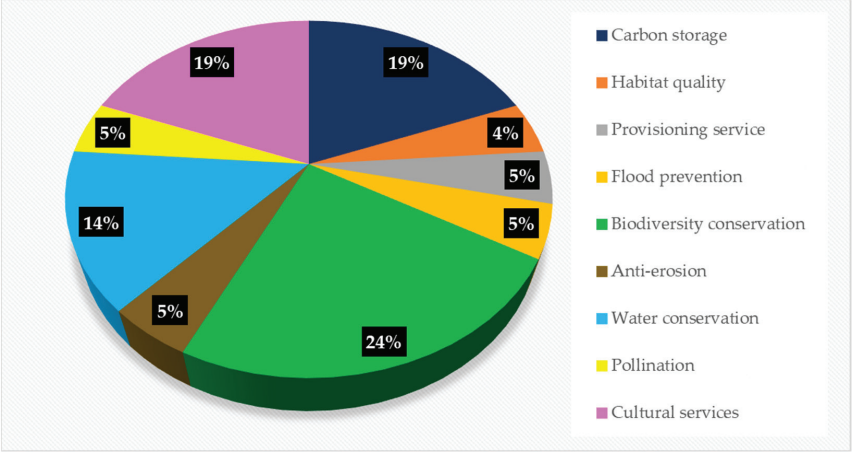


Figure 9. The individual ecosystem services estimated from an economic point of view.

Biodiversity conservation (in five papers), cultural services (in four manuscripts), carbon storage (in four documents), and water conservation (in three papers) were the most estimated single ES (Figure 9).

Among the ecosystem services that have been economically estimated in combination with other ES, carbon sequestration stands out, followed by forage provision and soil protection (Figure 10).

In conclusion, the evaluation of the 161 selected papers showed that individual ESs were evaluated from an economic point of view mainly through bio-economic modeling approaches and data coming from structured and semi-structured interviews. The economic estimate of multiple ES was carried out with data from different sources depending on the ES to be estimated, while the accounting of the ESV and the main ES falling into the four

macro categories of ES was carried out essentially through land use/cover change data more or less combined with environmental, climatic, and socio-economic data (Figure 11).

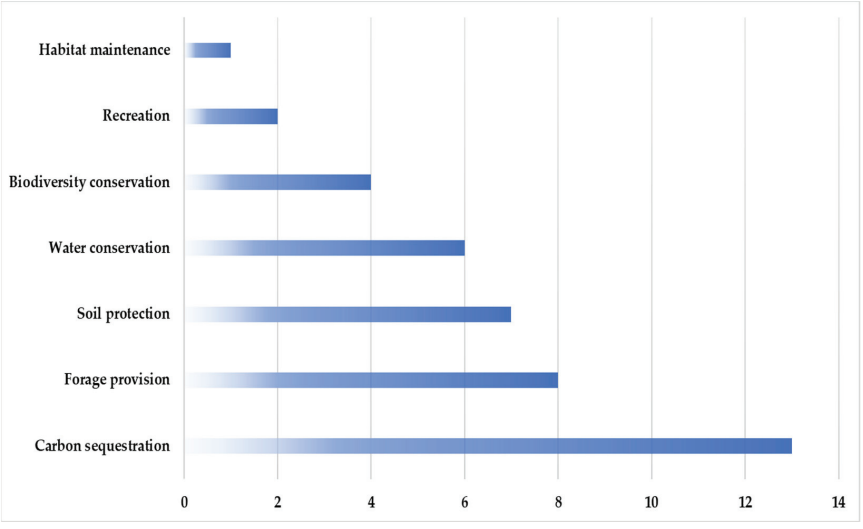


Figure 10. Multiple ecosystem services estimated from an economic point of view.

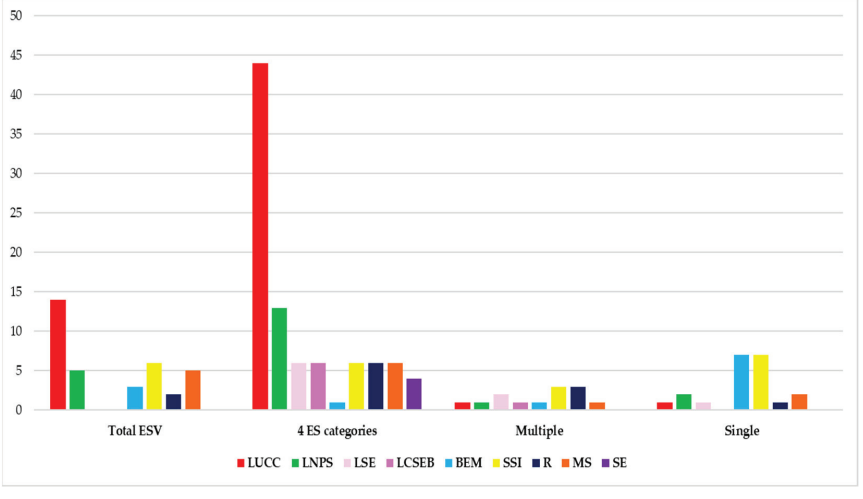


Figure 11. Types of data used to estimate the different ES grasslands from an economic point of view (ESV: ecosystem service value; 4 ES categories: provisioning, regulating, supporting, cultural services; LUCC: land use/cover change; LNPS: land cover data, net primary productivity, precipitation, and soil erosion data; LSE: land use data and socio-economic data; LCSEB: LUCC, climatic, socioeconomic, and biophysical data; BEM: bio-economic modeling approach; SSI: data from structured and semi-structured interviews; R: data from others research; MS: multi-source data; SE: Socio-economic data).

4. Discussion

4.1. Main Findings

As a first step, the present review attempted to provide a critical overview of academic studies on grassland ESs. Thus, it highlighted that China and the USA were both some of the countries with the highest percentage of grasslands (in terms of land area) and the main countries with the highest number of publications regarding ESs provided by grasslands, followed by Germany and the United Kingdom. The number of publications began to grow starting in 2005 thanks perhaps to the publication of the Millennium Ecosystem Assessment, an important document that aimed to evaluate the consequences of ecosystem change for human well-being and to establish the scientific basis for actions necessary to improve the conservation and sustainable use of ecosystems and their contribution to human well-being [143]. The increase in the number of publications on this topic is also the result of the general increase in interest in ecological policy issues that has occurred especially in the last two decades, which has led to state policies being more attentive to concerns about the condition of the environment and to an increase in scope to protect various valuable ecosystems. The main instrument of the European Union policy for the conservation of biodiversity is, undoubtedly, Natura 2000: an ecological network spread across the entire territory of the EU, established pursuant to Habitats Directive 92/43/EEC to guarantee the long-term maintenance of threatened or rare natural habitats and flora and fauna species at a community level.

At the same time, 55% of the documents found in Scopus referred to the last five years, in which 2023 represented the year with the highest number of documents ever (646). These data demonstrate the timeliness, importance, and relevance of this research topic.

The most represented subject areas of study were environmental science and agricultural and biological sciences (Figure 4), in which a central role was played by biodiversity as an important provision service. At the same time, however, among the most cited articles, in addition to those on the importance of biodiversity for the maintenance of ecosystems and on the impact and consequences of its loss for human well-being, not negligible were those that dealt with ES payments to address devastating environmental crises and improve human well-being.

The use of science mapping for bibliometric analysis has highlighted the correlation between the ecosystem services provided by grasslands with carbon storage, land use, and its change. This points out, on the one hand, that the most studied grassland ES in absolute has been the carbon storage, especially in quantitative terms, and how pasturelands act as an important soil carbon sink, and, on the other hand, how land use and its change over time can influence the provision of ES. However, the same analysis applied to the 1033 documents, selected for the subsequent screening to identify the most used methodology for the economic evaluation of grassland ESs, did not produce significant results. In our opinion, this was due to the fact that the science mapping was based on the analysis of the semantic contents of publications' titles, keywords, and abstracts, which very often were unclear (especially the latter) and did not contain the searched words. Hence, there is a need in the future to use software that analyzes entire manuscripts.

Papers that estimated grassland ESs from an economic perspective represented only 3% of all papers that Scopus returned, demonstrating that economic research, as such, is secondary to the study of environmental functions. In fact, to evaluate an ecosystem function, it is necessary to establish that this function exists and what effects its variation has. Only then it can also be valorized from an economic point of view. Moreover, more than half of these publications referred to the methodology of Costanza et al. [14], namely, the use of equivalent coefficients to calculate the ecosystem service value of different land uses/land cover categories. In this case, some studies (about 20) have estimated the total ESV provided by grasslands, highlighting the difficulty in estimating individual ecosystem services provided by pasturelands from an economic point of view (especially those that do not have a market) and the greater propensity to use physical, chemical, and biological indicators. Other research (about 70) has identified and evaluated a maximum of

11 ecosystem services in precise economic terms: food production, raw material production, water supply, gas regulation, climate regulation, environment purification, hydrological regulation, soil conservation, nutrient cycle maintenance, biodiversity, aesthetic landscape, indicating that the latter is the most important ES offered by grasslands. This was also confirmed by studies that analyzed single or multiple ESs, in which carbon storage and biodiversity conservation took precedence.

As pointed out by Liu et al. [30], the evaluation methods commonly used for valuing ecosystem services are different depending on the ES to be evaluated. For example, direct market methods are usually used for services that can be directly traded in the market, and the price is used to reflect the value of the ES. The avoided or replacement cost method uses the cost of actions taken to avoid damages as a measure of the benefits provided by an ecosystem or ES. At the same time, the travel cost method is normally used to estimate the economic use values of recreational ecosystems or sites, and the stated preference methods (the choice experiment method and the contingent valuation method) are used to assess the economic value of various ecosystems and ESs based on a hypothetical scenario. The benefit transfer method is a unit value-based method used to evaluate economic values by transferring existing benefit estimates from completed research to other sites or ES. Thus, with Table 5, which shows, for each methodology identified, the different references in which the methodology is applied, we wanted to indicate to researchers who wanted to estimate grassland ESs in economic terms where to find the most appropriate methodologies to do so.

4.2. Limitations of the Study

The present results are influenced by the use of only one database extraction (Scopus), which includes only publications with CiteScore and not many other documents where the economic valuation of grassland ESs may have been estimated.

5. Conclusions

The results of the present systematic and bibliometric literature review highlighted the importance of grassland ESs and their estimation over time, above all in quantitative, ecological, and sociocultural terms. Economic evaluation studies were few compared to the total academic publications and mainly linked to the quantification of ecosystem service values per unit area for each land-use category or, referring to grasslands, of only 11 types of ES. Consequently, the sustainable management of grasslands requires more studies on the economic evaluation of their ES. Furthermore, such research can be the basis for including environmental aspects in the economic accounting of governments or to implement a support system for farms in delivering various ecosystem services.

Author Contributions: M.P.: Conceptualization, methodology, software, validation, formal analysis, investigation, data curation, writing—original draft preparation, writing—review and editing; M.C.: visualization, writing—review and editing; E.D.F.: supervision, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: No new data were created.

Conflicts of Interest: The authors declare no conflicts of interest.

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Effect of Grazing on Plant and Soil Parameters of Steppe Pastures on Mount Aragats, Armenia

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Abstract: Steppe pastures are characteristic of the Armenian landscape and play an important role in supporting livelihoods and biodiversity conservation. The productivity and biodiversity of steppe pastures depend on grazing management, soil types, and climatic and topographical characteristics. As a whole, they form local small-scale sites. Our data on five study sites located on the southeast slope of Mt. Aragats summarized the impact of sites and grazing on canopy height; productivity; grass, legume, and forb biomass; nitrogen, phosphorus, and potassium concentrations; pH; and litter contents in the soil. Five grazed and ungrazed plots (ca. 600–800 m^2) were established at each study site. Within each plot, two permanent 40 m long transects were installed. The canopy height was recorded in ungrazed and freely grazed plots. The aboveground biomass was cut at the soil surface from May to June; grouped into grass, legumes, and forbs; dried; and weighed. Soil samples were collected in every ungrazed and freely grazed plot. The results indicated that grazing decreased the plant parameters and nitrogen and litter content across all sites, while it had no effect on the phosphorus and potassium content or the pH. It seems that plant parameters, as well as soil parameters, were more affected by the management strategy than by the topographical and climatic features of the sites, as revealed via redundancy analysis. Our results suggest that it is important to introduce livestock rotation practices for sites with respect to the sustainable management of steppe pastures. This management strategy could ensure ecosystem services, high forage quality, and soil fertility.

Keywords: steppe pastures; grazing; canopy structure; soil parameters; topography; climatic conditions

Citation: Navasardyan, M.; Sargsyan, T.; Daveyan, H.; Mezhunts, B.; Abraham, E.M. Effect of Grazing on Plant and Soil Parameters of Steppe Pastures on Mount Aragats, Armenia. *Land* **2024**, *13*, 1430. https://doi.org/10.3390/land13091430

Academic Editors: Michael Vrahnakis and Yannis (Ioannis) Kazoglou

Received: 14 July 2024
Revised: 5 August 2024
Accepted: 10 August 2024
Published: 4 September 2024

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1. Introduction

The Eurasian steppe is one of the largest vegetation types (8–13 million km^2), and it is located in different regions. Steppes are diverse ecosystems in terms of abiotic conditions and biotic composition [1]. They originated from the transformation of forest stands by herbivores [2]; as a result, steppes are characterized by a gradual development of vegetation. Climatic conditions and human activities are the main determinants that shape species composition and diversity in these ecosystems. The grasslands of steppes are usually grazed, and livestock farming comprises the traditional land use type [3].

In terms of climatic conditions, temperature, precipitation, and solar radiation are the main drivers for steppe vegetation growth [4] and the duration of the growth period [5]. The results of regional and local studies emphasized the role of annual precipitation, which explains up to 90% of the variation in the aboveground biomass of natural ecosystems [6–13]. Thus, due to global warming, biodiversity changes and the accumulation of different

elements in soils are occurring [14–18]. In meadow steppes, low temperatures and a high soil moisture index depress the growth of grass with roots accumulating in the 0–10 cm soil layer [19].

Several research studies focusing on the effects of human-induced processes on the productivity and soil structure of grasslands have been conducted over the years [20,21]. Most studies show that the most significant factor that leads to changes in the structure of grassland and soil properties is grazing by animals. There are contrasting results in the literature regarding the impact of grazing on biodiversity and soil nutrient contents. Grazing by herbivores supports the intake of mineral elements in soil and can positively modify nutrient cycling from soil to plant tissue [22,23]. Light grazing can increase grassland productivity if soil moisture and nutrients are available. Additionally, light grazing suggests increasing species richness via the succession of grassland to woodland [24,25]. In semiarid steppes with perennial grasses and forbs, continuous grazing leads to low productivity due to the substantial spreading of short plant species and decreasing species diversity [26]. Severe drought combined with grazing can negatively change species and functional component compositions in arid steppes [9].

Grazing exclusion experiments have been widely carried out in order to restore vegetation and soil properties in different steppe grasslands. Both 11-year and 31-year grazing exclusion in *Leymus chinensis* grasslands significantly improved vegetation characteristics (i.e., biomass, cover, richness, etc.) and soil organic carbon contents [26]. Sixteen years of grazing exclusion in the arid rangelands in Baluchistan (Pakistan) increased vegetation by 54–74% and soil organic carbon content by 46–54% and improved nitrogen mineralization, soil aggregation, and the water holding capacity [27]. Eight years of excluding grazing tripled the biomass production in desert steppe of the Junggar Basin region, China, and improved plant cover, especially for perennial grass; in addition, soil organic carbon (in the 0–10 cm layer) was increased by 66%, total nitrogen by 110%, and total phosphorus by 114% [28]. The contents of soil organic carbon, N, Ca, and K exhibited increasing trends as the time of grazing exclusion increased, while there was an opposite trend for Na and pH in *Stipa tenacissima* steppe [29]. Soil organic carbon and N decreased with increasing stocking rates in Inner Mongolian grasslands. Moreover, grazing exclusion enhanced soil C storage in sand grasslands in the Horqin region [30].

Armenia is a mountainous country, and steppes occupy large territories from low-altitude (500 m) to high-altitude belts (2500 m). Steppes are spread over flat areas as well as steep slopes and on thick, fine-grained and stony weathered soils [31]. Steppes are characterized by diverse vegetation composition with xerophilic and mesophilic species [32], and they play an important role in biodiversity protection and landscape preservation. In addition, steppes serve as a principal feed source for domestic animals and are therefore of economic importance for animal husbandry in the country [33]. More than 12% of the total area of pastures (about 1 million ha) is located near settlements and subjected to grazing. Due to the absence of legal regulations on grazing and the financial means for the transportation of animals to high mountain pastures, these areas are at risk of degradation. Grazing results in changes to floristic composition, an increased percentage of bare soil, and reduced productivity of steppe grasslands [34].

Despite the importance assigned to pasture management, limited information is available on the impact of grazing on vegetation and soil parameters of small-scale pasture sites. The main objective of this study was to investigate the effect of grazing and local edaphoclimatic conditions on vegetation and soil parameters in five pasture sites of Mt. Aragats in Armenia (Table 1). The following questions were addressed: (1) Is there any differentiation in productivity and floristic composition between sites? (2) Is there any differentiation in soil N, P_2O_5, K, litter content, and pH between sites? (3) How do the grazing and local edaphoclimatic conditions affect the vegetation and soil parameters of the sites? This study contributes to the identification of the main determinants of steppe pasture productivity, forage quality, and soil fertility, and it highlights the necessity for the implementation of an appropriate grazing management plan.

Table 1. Geographical, climatic, and soil characteristics of sites in the steppe zone of Mt.Aragats [https://www.arcgis.com/apps/mapviewer/index.html (accessed on 18 January 2023)]. FUP = forage utilization percentage.

Sites	Elevation, m a.s.l.	Terrain	Soil Characteristics	Precipitation, mm	Temperature, °C	FUP
NSSH1 (1–2)	1323–1425	Plain (slope up to 30°) and southeastern exposure	Loamy, slightly eroded, and very stony light chestnut soils with pH 7.8, humus 1.9%, total nitrogen 4.5%	294	11.6	64
NSSH2 (3–4)	1673–1703	Gentle slope (30–60°) and southeastern exposure	Soil dark chestnut; loamy; rubble–stony; pH 7.8, humus 2–4%, nitrogen 4–5%	384	9.7	58
AVN (5–6)	1500–1580	Northward, gentle slope	Typical chestnut; slightly eroded; moderately stony; loamy soils; with relatively low total nitrogen content (3.1%); pH 7.8, humus 2–4%	430	6.9	72
ANT (7–8)	1783–1800	Plain; northwestern	Cinnamonic, leached, clay, slightly stony, and eroded soils, which are distinguished by high humus content (5.2%), nitrogen 3.4%, and pH 7.6	477	9.1	39
ART (9–10)	1766–1824	Northern exposure, gentle slope (30–60°)	Soil chernozem-like, leached, deep-calcic, clay, non-eroded, moderately stony; nitrogen 3.7%, humus 5.5%, and pH 7.7	477	9.1	53

2. Materials and Methods

2.1. Study Area

The study was carried out in a steppe zone in the southeastern part of Mt. Aragats, Armenia (Figure 1). The area belongs to the Nerkin Sasnashen (NSSH), Avan (AVN), Antarut (ANT), and Artashavan (ART) communities of Aragatsotn Marz. It is located between 43.976266–44.386365 E/40.316728–40.38736 N and at elevations of 1300–1800 m a.s.l. (Table 1).

The study sites were located 11 and 37 km apart (Figure 1). The predominant rocks comprise mid- and upper-quaternary basaltic andesite lava, which forms many hills and mounds on the surface, with small plateaus between them. Thus, the terrain usually consists of hills with different steepness levels and flat areas. The soils in this area range from chernozem-like to dark and light chestnut types [31].

The studied steppe pastures cover an area of about 4600 ha, and according to information provided by the community administration, there are about 2800 heads of cattle of the Caucasian brown breed and 6080 heads of semi-coarse-wooled Balbas sheep. These breeds are the most adapted to the conditions of Armenia and are successfully bred for the production of milk and meat. Within these pastures, there are private plots that are fenced and protected from grazing, and they often serve as hayfields (ungrazed plots: forage utilization percentage, FUP = 0%). Additionally, pastures near villages are public and communally grazed by the entire livestock of the community from early spring to late autumn (freely grazed plots) in a continuous grazing system.

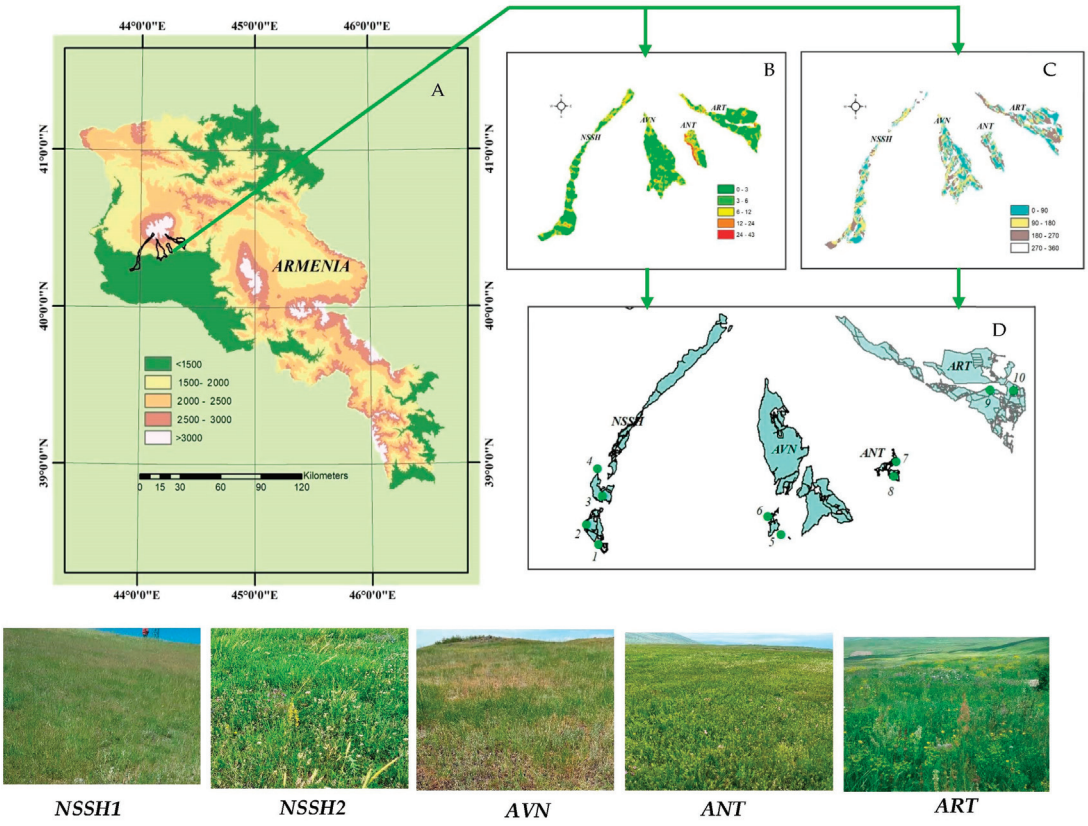


Figure 1. Location of study area on Mt. Aragats, Armenia (**A**); its geomorphological characteristics (**B**—slope ^{0}; **C**— exposure ^{0}); study sites in rangelands of NSSH, AVN, ANT, and ART communities, where 1, 3, 5, 7, and 9 are grazed plots, and 2, 4, 6, 8, and 10 are ungrazed plots (**D**).

2.2. Climate

According to the Köppen–Geiger climate classification, the climate of the area is classified as cold and semi-arid (Bsk). Based on 6-year mean meteorological data obtained from 4 Meteorological Stations of the Ministry of Environment of Armenia for the 2017–2022 period (Figure 2), during the winter, the temperature ranged from 0.5 to −4.3 °C, and during summer, it was 22.2 °C on average. The mean annual temperature (MAT) during the growing season was 10.3–23.2 °C, reaching a maximum in July. At the same time, the mean annual precipitation (MAP) was 362 mm with about 55% falling during the growing season. Precipitation is below the potential evapotranspiration (800–1000 mm) level in the study area. During the experimental years (2019–2021), precipitation and temperature, as well as their seasonal distribution, exhibited similar trends to those of the 6-year period (Figure 2). Table 1 also presents the climatic data obtained from the closest weather stations (10–15 km) of the study sites. According to these data, the MAP ranged from 227 mm in 2021 at NSSH1 to 549 mm in 2022 at AVN (growing and non-growing season precipitation was 219 and 162 mm or 58 and 42% of annual precipitation, respectively). In 2021, the MAT was the highest (12.2 °C) at site NSSH1, and in 2019 (6.7 °C), it was the lowest at site NSSH2.

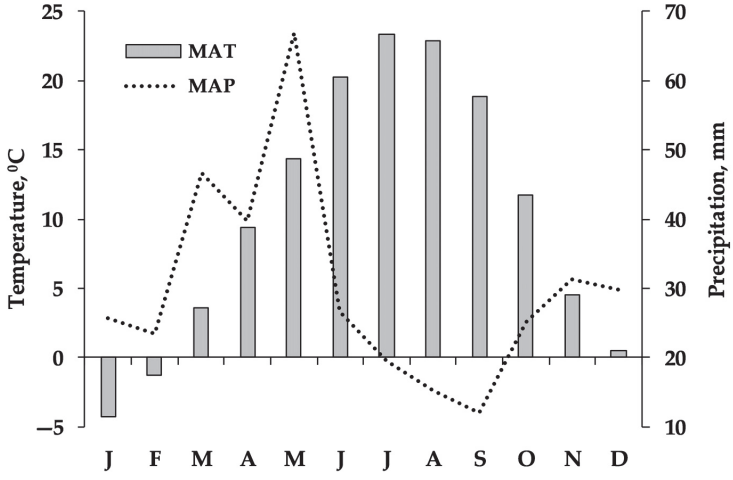


Figure 2. Monthly average temperature (°C) and monthly sum precipitation (mm) during the period 2017–2022 in the study area. The data were recorded by the Armavir, Talin, Amberd, and Eghvard Meteorological Stations of the Ministry of Environment of the Republic of Armenia.

2.3. Vegetation

The growing season for vegetation in the study area, including sod-forming grass, varies from 4 to 5 months (from March to July) [35,36]. Ephemeral species such as *Poa bulbosa* L., *Colpodium humile* (M.B.) Grisb. dominate in early spring, while perennial forbs and legumes dominate in early summer. The dominant species in this steppe zone are as follows: *Bromus fibrosus* E. Hack, *Festuca sulcata* E. Hack. Rizh., *Hordeum crinitum* Desf., *Koeleria nitidula* Vel., *Agropyrum trichophorum* Richt., *A. cristatum* (Schreb) P.B., *Aegilops cylindrica* Host., *Poa bulbosa* L., *Nepeta mussinii* Henke, *Xeranthemum squarrosum* Boiss., *Artemisia austriaca* Jack., *Thymus kotschyanus* Boiss., *Pyrethrum myriophyllum* C.A.M., *Achillea micrantha* M.B., *Medicago sativa* L., and *Onobrychis michauxii* D.C, among others.

2.4. Sampling

Five sites were established in the study area: two in NSSH and one each in AVN, ANT, and ART (Figure 1). At each site, grazed and ungrazed plots were selected (10 in total) that were about 600–800 m^2 in size. Within each plot, two permanent 40 m long transects were placed. The aboveground biomass was cut at the soil level from June to July using 8 quadrats (1 m × 1 m) at a 5 m interval along each transect (i.e., 16 quadrats per plot). The height of the canopy cover was measured with a meter ruler. The total aboveground biomass (TAGB) was grouped into 3 functional groups, namely, grass (G), forbs (F), and legumes (L), according to [37], dried in an oven at 65 °C for 48 h, and weighed.

Three soil samples were collected (400 cm^2 = 0.20 m × 0.20 m) in each experimental plot from the 0–20 cm soil layer along each transect (six samples per plot). In the laboratory, plant remains, stones, and roots were removed from the soil sample, and then they were dried at about (60 to 70 °C) for 48 h and passed through a metal sieve with 1 mm diameter holes. Then, the soil sample was averaged several times, bringing it to 100–150 g.

Hygroscopic moisture was determined by the weight difference before and after drying at 105 ± 50 °C until constant weight was reached (ISO 11465:1993) [38]. The pH of the soil solution was determined according to ISO 10390:2021 [39]. Soil suspensions for the determination of total nitrogen (N), total phosphorus (P_2O_5), and potassium (K) were obtained by boiling the sample in sulfuric acid followed by the addition of hydrogen peroxide at a temperature not exceeding 400 °C. The N content was determined using a modified Kjeldahl method (ISO 11261:1995) [40], P_2O_5 was measured using a modified spectrophotometric method in Truog's fume hood (ISO 11263:1994) [41,42], and K was measured using

a flame emission spectrometer (calibration method). For the determination of organic matter (litter) in soil, organic carbon was determined via the colorimetric Walkley–Black method [43]. The accuracy (trueness and precision) of measurement methods and results was controlled in accordance with ISO 5725:2023 [44]. N, P, and certified organic matter standard soils were used during measurements to ensure the accuracy of the data obtained.

2.5. Data Analysis

Two-way ANOVA (IBM SPSS Statistics 20), based on general linear models (GLMs), was used in order to detect the effect of grazing and sites (across years) on vegetation and soil parameters. Additionally, the effect of year and sites on the studied parameters was detected via two-way ANOVA separately in ungrazed and freely grazed areas. Tukey's Studentized range (HSD) was used to compare the difference among means at $p \leq 0.05$.

Two redundancy analyses (RDAs) [45] were carried out as follows: RDA1 for the ordination of the plant parameters and RDA2 for soil parameters to test the effect of the climatic and topographical variables of sites that differed by management strategy (Table 1) using XLStat software. Management (freely grazed vs. ungrazed) in RDAs was included in the analysis as a dummy variable.

3. Results

The effect of grazing and sites (across years) was significant for all plant parameters (Table 2). Regarding soil parameters, sites (across years) significantly affected all soil parameters, while grazing only significantly affected N and litter content (Table 2). Additionally, the interaction of grazing and sites was significant ($p < 0.05$) for G and L, as well as P_2O_5 and K contents, indicating that the effect of grazing differed among the studied sites (Table 3). No effect was observed with respect to the interaction between grazing and site on TAGB, indicating that grazing, regardless of site conditions and sampling year, plays a crucial role in plant growth and pasture productivity.

Significant differences were recorded among ungrazed and freely grazed sites for all studied plant parameters (Table 3). The highest mean values for all plant parameters were recorded in the ungrazed steppe pasture plots (Table 3). In particular, the mean values of canopy height (CH) and TAGB of ungrazed plots were 1.5 and 2.2 times higher than in freely grazed plots. Similarly, the botanical composition (G, L, and F) in ungrazed plots was 2.6, 3.2, and 1.6 times higher than in the freely grazed plots, respectively.

Table 2. Assessment of the impact of year and site on plant cover and soil (depth: 0–20 cm) parameters of ungrazed (UGR) and freely grazed (FG) pasture plots in the steppe zone of Mt. Aragats. CH: canopy height; TAGB: total aboveground biomass; G: grass; L: legumes; F: forbs.

	UGR			**FG**			**UGR/FG**		
	Year (Y)	**Site (S)**	**S/Y**	**Year (Y)**	**Site (S)**	**S/Y**	**Grazing (Gr)**	**Site (S)**	**Gr/S**
Plant									
CH, cm	ns	*	ns	ns	*	ns	*	*	ns
TAGB, g m^{-2}	ns	*	ns	*	*	*	*	*	ns
G, g m^{-2}	ns	ns	ns	ns	*	ns	*	*	*
L, g m^{-2}	ns	*	ns	ns	*	ns	*	*	*
F, g m^{-2}	ns	*	*	ns	*	ns	*	*	ns
Soil									
N, %	ns	*	*	ns	*	*	*	*	ns
P_2O_5, %	ns	*	ns	ns	*	*	ns	*	*
K, %	ns	*	ns	ns	*	ns	ns	*	*
pH	ns	*	ns	ns	*	ns	ns	*	ns
Litter, %	ns	*	ns	ns	*	*	*	*	ns

* Significant ($p \leq 0.05$); ns (not significant) $p > 0.05$.

Table 3. Plant cover and soil (depth: 0–20 cm) parameters with respect to the average of ungrazed and freely grazed sites in the steppe pastures of Mt. Aragats.

Studied Parameters	Ungrazed		Freely Grazed	
	Mean	**St.dev**	**Mean**	**St.dev**
Plant				
CH, cm	32 a *	9	21 b	12
TAGB, g m^{-2}	308 a	76	138 b	78
G, g m^{-2}	143 a	100	55 b	53
L, g m^{-2}	58 a	72	18 b	36
F, g m^{-2}	107 a	95	67 b	54
Soil				
N, %	0.46 a	0.13	0.37 b	0.11
P_2O_5, %	0.39 a	0.10	0.37 a	0.08
K, %	0.54 a	0.14	0.52 a	0.17
pH	6.40 a	0.71	6.35 a	0.73
Litter, %	6.20 a	1.25	4.95 b	1.43

* Means within each row followed by the same letter are not significantly different ($p > 0.05$). The comparison of means was carried out using Tukey's test.

Regarding soil parameters, significant differences were observed with respect to N and litter contents, which were higher in ungrazed plots (0.46% and 6.20%, respectively). The P_2O_5, K, and pH values did not change with respect to ungrazed and freely grazed sites (Table 3). The G biomass (across sites) was higher in ungrazed plots compared to freely grazed plots at all sites (Figure 3A), while in both cases, there were no significant differences among them. However, the G biomass tended to be higher in ANT for both grazed and ungrazed plots. The L biomass (across years) was significantly higher in the ungrazed plots of NSSH2 and ART sites compared to NSSH1 (Figure 3B). On the other hand, the lowest L biomass was found in the grazed plots of NSSH1 and AVN sites (3.5 and 4.6 g m^{-2}, respectively).

P_2O_5 content was similar between ungrazed and grazed plots for all sites, although it tended to be lower in grazed plots compared to ungrazed plots in NSSH1 and AVN (Figure 3C). There were no consistent trends between ungrazed and grazed plots for K content in the studied sites. Thus, K content did not differ between ungrazed and grazed plots at NSSH1, and it tended to be lower in the grazed plots at NSSH2 and AVN and higher in the grazed plots at ANT and ART compared to ungrazed ones (Figure 3D). The effect of years was studied separately in ungrazed and freely grazed plots at the studied sites. In this respect, the effect of year (across all sites) was not significant at ungrazed and freely grazed plots for all studied parameters, except the TAGB of grazed plots (Table 2). The site effect (across all years) was significant for all plant and soil parameters in both ungrazed and grazed plots, except for G biomass in ungrazed ones (Table 2).

The ungrazed plots of sites ANT, ART, and AVN (across years) had significantly higher TAGB compared to NSSH1 (Table 4). The CH was higher in the ungrazed plots of NSSH2, ANT, and ART in comparison to NSSH1 and AVN (Table 4). The TAGB increased from 270 g m^{-2} at NSSH1 to 349 g m^{-2} at ANT, and the CH increased from 25 cm at NSSH1 to 42 cm at ART. Regarding the botanical composition, small differences were recorded among the sites.

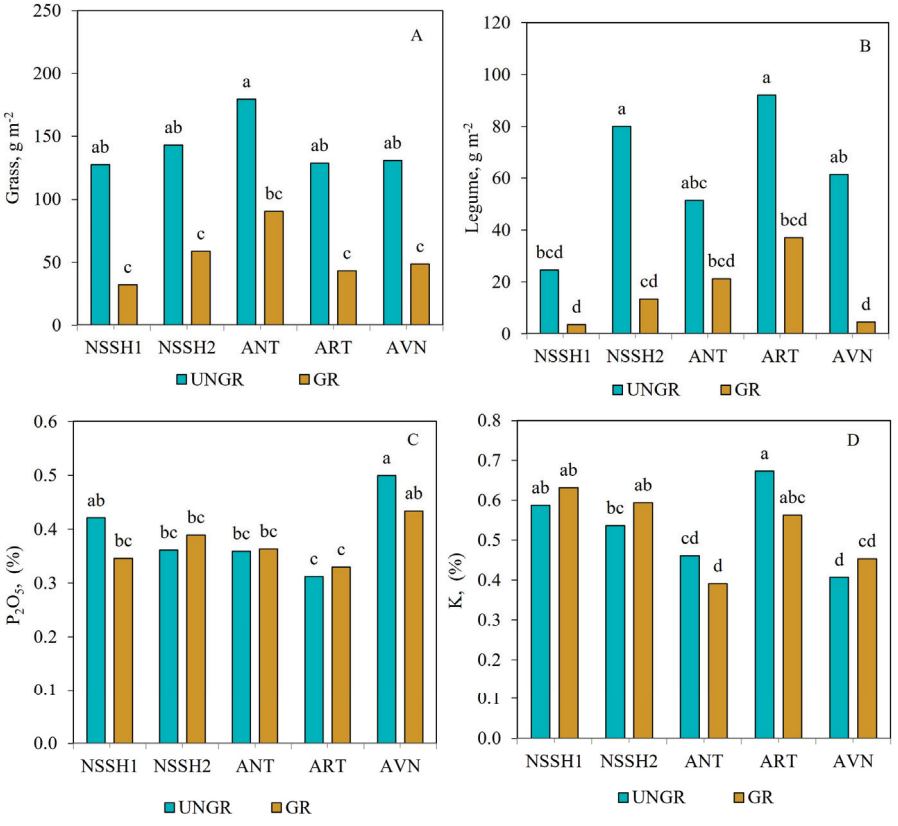


Figure 3. Effects of grazing and site interaction on G (**A**) and L biomass (**B**) and P_2O_5 (**C**) and K (**D**) contents of the steppe zone pasture plots. Columns with the same letter are not significantly different ($p > 0.05$).

Table 4. Means of plant cover and soil (depth: 0–20 cm) parameters in ungrazed plots in the steppe pastures of Mt. Aragats.

Studied Parameters	Sites				
	NSSH1	**NSSH2**	**ANT**	**ART**	**AVN**
Plant					
CH, cm	25 a *	31 b	42 b	30 b	28 a
TAGB, g m^{-2}	270 a	293 ab	349 b	311 b	309 b
G, g m^{-2}	128 a	143 ab	180 b	129 ab	130 ab
L, g m^{-2}	25 b	80 a	51 a	92 a	66 a
F, g m^{-2}	137 a	70 b	118 a	90 ab	117 a
Soil					
N, %	0.45 a	0.48 a	0.43 a	0.54 a	0.38 b
P_2O_5, %	0.42 a	0.36 ab	0.35 ab	0.31 b	0.50 a
K, %	0.59 a	0.54 a	0.46 a	0.67 ab	0.41 c
pH	5.96 a	6.11 a	6.33 a	6.79 b	7.3 b
Litter, %	5.19 a	5.78 a	5.86 ab	7.45 c	5.96 ab

* Means within each row followed by the same letter are not significantly different ($p > 0.05$). The comparison of means was carried out using Tukey's test.

The highest biomass of G (180 g m^{-2}), L (92 g m^{-2}), and F (138 g^{-2}) was recorded at ANT, ART, and NSSH1, respectively (Table 4). Only the F biomass in ANT differed between the two years of study. It was significantly higher in the second year compared to the first (Figure 4A). Regarding soil parameters, the ungrazed AVN site had significantly lower contents of N and K, while the ART site exhibited higher litter content compared to the other sites, and small differences were observed between years (Table 4 and Figure 4B).

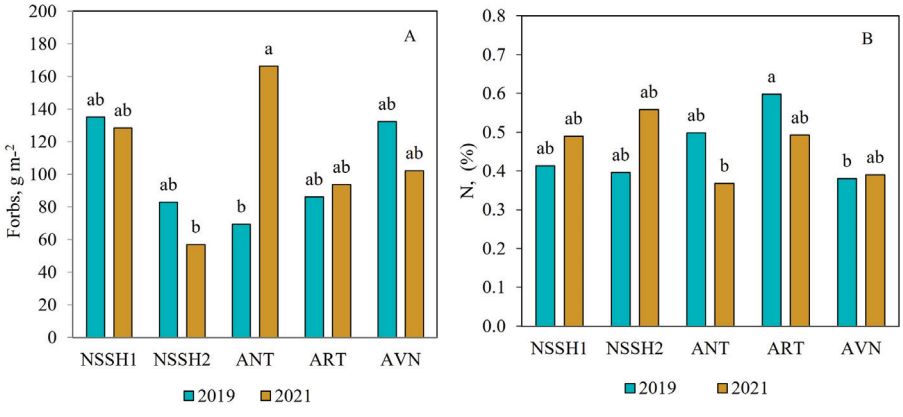


Figure 4. Effect of site and year interaction on F biomass (**A**) and N content (**B**) in ungrazed plots in the steppe pastures of Mt. Aragats. Columns with the same letter are not significantly different ($p > 0.05$).

TAGB in freely grazed plots at NSSH1 and AVN (across years) was significantly lower than that at NSSH2, ANT, and ART sites. The CH was higher at the ANT site compared to NSSH1, NSSH2, ART, and AVN. Concerning the botanical composition, the highest biomass of G and F (90 g m^{-2} 105 g m^{-2} respectively) was recorded in ANT, and the highest biomass of L was observed in ART (37 g m^{-2}). On the other hand, the lowest biomass of G and L was in NSSH1, and the lowest biomass of F was in AVN. N and litter contents were significantly higher at the ART site, P_2O_5 was higher in AVN, and K was higher in NSSH1 (Table 5).

Table 5. Means of plant cover and soil (depth: 0–20 cm) parameters in freely grazed plots in the steppe pastures of Mt. Aragats.

Studied Parameters	Sites				
	NSSH1	**NSSH2**	**ANT**	**ART**	**AVN**
Plant					
CH, cm	14 a *	18 a	38 b	18 a	19 a
TAGB, g m^{-2}	96 a	124 b	212 c	145 b	93 a
G, g m^{-2}	32 a	59 b	90 b	43 a	49 a
L, g m^{-2}	3.5 a	13.4 ab	21.3 ab	37 b	4.6 a
F, g m^{-2}	58 a	53 a	105 b	65 a	39 c
Soil					
N, %	0.34 a	0.38 a	0.36 a	0.46 b	0.33 a
P_2O_5, %	0.35 a	0.39 ab	0.36 a	0.33 a	0.43 b
K, %	0.63 a	0.59 a	0.39 b	0.56 a	0.45 a
pH	5.51 b	6.13 a	6.39 a	6.84 a	7.45 c
Litter, %	4.01 a	4.78 a	4.14 a	6.90 b	4.97 a

* Means within each row followed by the same letter are not significantly different ($p > 0.05$). The comparison of means was carried out using Tukey's test.

There were no significant differences in the TAGB among the sites in the freely grazed plots, except at ANT, where TAGB was significantly higher in the second year compared to the first ($p < 0.05$) (Figure 5A). The N and litter contents of grazed plots did not significantly differ between the two studied years at all sites, except for ART, where they were significantly higher in the first year (Figure 5B,D). Similarly, the content of P_2O_5 was significantly different during the first year compared to the second year in ANT, while there were no significant differences between the years at the other sites (Figure 5C).

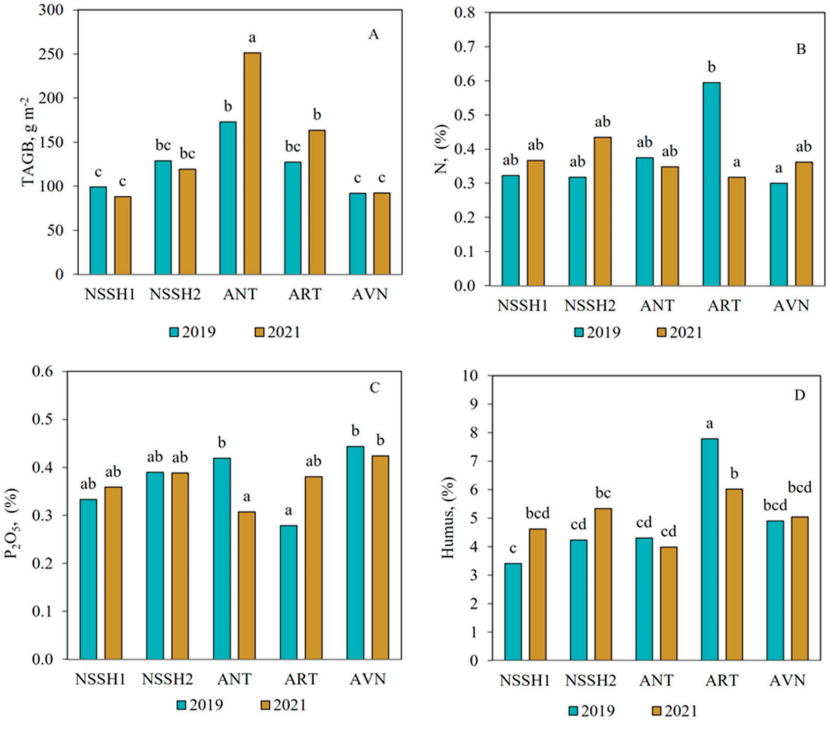


Figure 5. Effect of site and year interactions on total aboveground biomass (**A**); N, % (**B**); P_2O_5, % (**C**); and litter, % content (**D**) in freely grazed plots in steppe zone pastures. Columns with the same letter are not significantly different ($p > 0.05$).

The two obtained RDA axes explained 88% (F = 7.556, $p < 0.0001$) and 71% (F = 2.445, $p < 0.0001$) of the total variation in plant and soil parameters according to grazing management and the sites' climatic and topographical features (Figures 6 and 7). In both RDA axis management strategies, the explanatory variables FUP, MAT, MAP, and aspect best correlated with the first two axes of RDA1 and RDA2, and they explained 82% and 58% of the variance. Therefore, the management strategy and FUP explained more variance in plant parameters than the climatic and topographical features of the sites (Figure 6). The TAGB and biomass of G were mainly affected by the management strategy, and they were positively related to ungrazed fenced plots. The L biomass increased according to the elevation of sites, in accordance with the vertical belts in the fenced plots. F biomass and CH were positively related to aspect and the MAP, and these parameters increased with an increase in precipitation and relative to the southwest exposure. Ungrazed plots at NSSH1, NSSH2, and ANT greatly contributed to the F1 axes of RDA1, whereas only the grazed plots of AVN contributed to the F1 axes of RDA2.

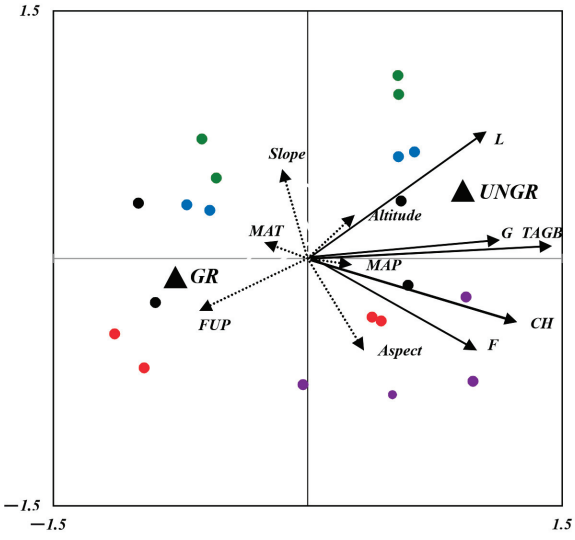


Figure 6. RDA biplot, showing the position of plant functional groups (solid arrows) and climatic and topographic variables (dashed arrows). Dummy variables are represented by solid black triangles (UNGR: ungrazed; GR: grazed). Sites are represented by red (NSSH1), blue (NSSH2), purple (ANT), green (ART), and black (AVN) points [MAT: mean annual temperature; MAP: mean annual precipitation; FUP: forage utilization percentage; CH: canopy height; G: grass; L: legumes; F: forbs; slope: slope inclination (°); aspect: slope exposure; altitude: m a.s.l.].

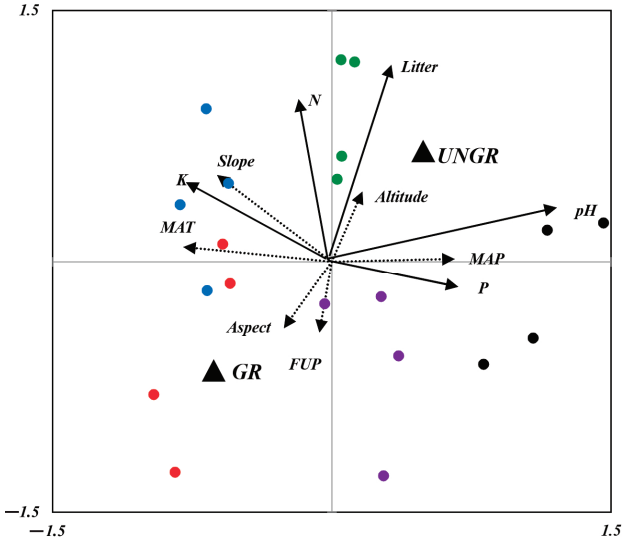


Figure 7. RDA biplot, showing the position of soil parameters (solid arrows) and climatic and topographic variables (dashed arrows). Dummy variables are represented by solid black triangles (UNGR: ungrazed; GR: grazed). Sites are represented by red (NSSH1), blue (NSSH2), purple (ANT), green (ART), and black (AVN) points [MAT: mean annual temperature; MAP: mean annual precipitation; FUP: forage utilization percentage; N: total nitrogen; P: (P_2O_5); K: potassium, pH and litter; slope: slope inclination (°); aspect: slope exposure; altitude: m a.s.l.].

Similarly to the plant parameters, the soil parameters of the pasture sites were significantly affected by grazing and the site's climatic and topographical conditions. Litter, N, and K contents were best correlated with the F1 axes of RDA2, and pH and P_2O_5 were correlated with F2 (Figure 7). All soil parameters positively correlated with the fencing of pastures. The litter and N contents of soils increased with an increase in altitude and grazing exclusion. P_2O_5 content and pH were positioned at the positive end of MAP, and K content was positioned on the positive side of the slope. With a decrease in MAP, a decreasing trend was observed with respect to P_2O_5 and pH contents. At plain sites, the K content is decreased. It is evident from RDA2 that there is no relationship between soil parameter variations and studied sites as they provide only a small contribution to the construction of the two RDA2 axes.

4. Discussion

4.1. Effect of Grazing Exclusion on Vegetation and Soil Characteristics

On ungrazed plots, all plant and soil parameters were different among the studied sites, except grass biomass. Moreover, the climatic conditions relative to different years did not influence pasture productivity. The formation of steppe vegetation is closely associated with the percentage of grass, which is evolutionarily predominant on steppes regardless of soil type and climatic conditions. However, there is a suggestion that the formation of steppes is a consequence of long-term grazing and climate change, which result in changes in vegetation cover and reduced productivity [9].

Studies on eastern European plain steppes have shown that perennial grass dominates in the northern steppes, while they are replaced by perennial forbs in the south. The MAP in the studied area increases from the north to the south: from 160 to 450 mm; in contrast, the MAT increases from 5 to 10 °C [18]. In meadow, typical, and desert steppes of the Inner Mongolia Autonomous Region, which belongs to the Eurasian steppe region, the proportions of perennial grass and forbs are 36% and 62%; 57% and 63%; and 23% and 6%, respectively. The predominance of perennial grass increased at low MAP levels and decreased at high MAP levels [46].

The results of the present study indicate that steppe pastures with cinnamonic and chernozem-like soils (sites ANT and ART) exhibit higher productivity compared to light chestnut soils (site NSSN1). At these sites, TAGB increases with an increase in MAP and a decrease in MAT, and decreases when MAP decreases and MAT increases. These changes are dependent on soil types. The difference among sites was significant for all studied soil parameters. Litter content and pH were more affected by the amount of MAP than N, P_2O_5, and K, but the effect on light chestnut (site NSSH1), chernozem-like (site ART), and typical chestnut soils (site AVN) was more evident than on dark chestnut (NSSH2 site) and cinnamonic (site ANT) soil. Many studies have examined the effects of soil nutrients on plant productivity, e.g., [47–49], and they have shown that nutrient supply is one of the major sources for promoting plant productivity and diversity in grasslands. Additionally, it has been shown that precipitation during the active period of plant growth alters the essential nutrients from soil to plants and supports communities in adapting to a severely living environment [17]. Regarding the ratio of legumes and forbs on ungrazed plots, the dominance of legumes over forbs was not observed, and they produced the same biomass only in dark chestnut and chernozem-like soils. The proportion of legumes increased with an increase in altitude, and forbs increased in the western areas. Similar results were reported by Bale et al. [50]. According to the present study, the eastern sites NSSH1 and NSSH2 are dominated mainly by graminoids, while the western site ANT is dominated mainly by forbs.

4.2. Effect of Grazing on Vegetation and Soil Characteristics

In grazed pastures, grazing is the main driver of productivity [9,22,51]. The results of the present study indicate that the vegetation and soil properties of grazed plots are mainly affected by management. However, TAGB of grazed plots differed among soil

types and study years. In all cases, the total productivity of freely grazed steppe pastures with cinnamonic and chernozem-like soils was higher (sites ANT and ART) than in plots with light and chestnut soils. The same results were obtained for canopy height and grass, legume, and forb biomass with respect to the studied pasture sites and main soil types. The content of nitrogen and litter was significantly higher in chernozem-like soils (site ART), and phosphorus and pH were significant in chestnut (site AVN) soils. Regarding the floristic composition, forbs dominate in freely grazed plots because grass and legume species are preferred by ruminants and are intensively grazed by them [46,52–54].

4.3. The Importance of Pasture Management

The comparison of protected ungrazed and freely grazed plots indicates that the plant cover height, total biomass, and floristic composition of steppe pastures are mainly affected by grazing rather than by site topography and climatic conditions. The increase in the canopy height inside the protected plots is in agreement with studies showing the positive effects of protecting this parameter [51]. The same is observed for TAGB [55], which experienced a significant increase in protected plots. The impact of grazing on vegetation composition has also been reported by other scientists [56]. From our data, it is obvious that grazing decreases the total nitrogen and litter content in the soil, which could probably be attributed to changes in vegetation composition. This effect was positively related to the altitude, as revealed via RDA2. The reduction in legumes and grass in freely grazed plots probably contributes to a decrease in nitrogen and litter, respectively. Further research is needed regarding the effect of legume reduction on soil parameters in relation to the effect of grazing on microorganisms that settle at the roots of legumes. Grazing had no effect on phosphorus, potassium, or pH. The same results were obtained on the basis of 236 site investigations conducted around the world, and the authors of [2] concluded that there is no relationship between grazing and the pH of soil. Inversely, Liu et al. [57] reported that high grazing intensities in alpine grasslands significantly increased the soil pH. Similarly, available soil P [58] and potassium [59] significantly increased in highly grazed grasslands. These inconsistencies are attributed to differences among the studied areas in terms of altitude, parent rock material, and grazing frequency and intensity. In particular, with respect to phosphorus, Zhang et al. [21] suggested that it is more dependent on the parent material and climate than grazing intensity. Additionally, this may be due to the manner in which these elements are used by plants and the degree of soil compaction, or the lack of agro-measures, i.e., applying fertilizers to natural pastures. All of these issues can be investigated in future studies.

5. Conclusions

The sustainability of steppe pasture sites is mainly influenced by grazing rather than site topography and climatic conditions. Grazing reduced the total productivity of pastures and altered the floristic composition mainly with respect to a reduction in the proportion of grass and legumes and an increase in the proportion of forbs. Grazing also reduced nitrogen and litter contents in soils. However, the major determinant of the grazing effect is the local edaphoclimatic conditions of each site. In particular, grazing had a larger impact at sites with lower precipitation and light chestnut soils compared to sites with higher precipitation and cinnamonic and chernozem-like soils. It is important to introduce management practices, particularly the rotation of livestock around sites, according to local conditions and grazing capacity. This management strategy could ensure enduring ecosystem services and high forage quality and soil fertility.

Author Contributions: Conceptualization, M.N., B.M. and E.M.A.; methodology, M.N., B.M. and E.M.A.; software, M.N., T.S. and H.D.; validation, M.N., T.S., H.D., B.M. and E.M.A.; formal analysis, M.N., T.S. and H.D.; investigation, M.N., T.S., H.D. and B.M.; resources, M.N., T.S. and B.M.; data curation, M.N., T.S. and B.M.; writing—original draft preparation, M.N., T.S. and B.M.; writing—review and editing, M.N., B.M. and E.M.A.; visualization, M.N., T.S. and H.D.; supervision, E.M.A.;

project administration, M.N.; funding acquisition, M.N. All authors have read and agreed to the published version of the manuscript.

Funding: The research was supported by the Higher Education and Science Committee of MESCS RA (research project No. 21T-4C237: Influence of different grazing regimes of pasture on key indicators of productivity and soil fertility).

Data Availability Statement: The data presented in this study are available in the figures and tables provided in the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

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Vegetation Restoration Patterns Influence the Supply and Interrelations of Grassland Ecosystem Services in Karst Desertification Control

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Abstract: An appropriate vegetation restoration pattern is crucial for maintaining and enhancing ecosystem functions and services in karst rocky desertification control areas. However, it is still unclear whether different vegetation restoration patterns will aggravate the trade-off of grassland ecosystem services in this area. This study focuses on grassland ecosystems in the karst desertification control area, comparing artificial restoration measures (*Dactylis glomerata* single-species sowing grassland, DG; *Lolium perenne* single-species sowing grassland, LP; *Lolium perenne* + *Trifolium repens* mixed-species sowing grassland, LT) with natural restoration measures (NG). Seven ecosystem services (forage yield, soil retention, soil water conservation, carbon fixation and release, soil carbon storage, soil nutrient retention, and biodiversity conservation) as well as total ecosystem services were quantified using field monitoring data. The relationships between these services were evaluated through Spearman correlation analysis. The results showed that different vegetation restoration patterns significantly influenced the provisioning, regulating, and supporting services of the grassland ecosystem ($p < 0.001$). Three types of relationships were observed (trade-off, synergy, and neutral), but the trade-off relationship was not significant ($p > 0.05$). The total ecosystem service of LT (0.79) was significantly higher than that of NG (0.21), DG (0.36), and LP (0.41), with a significant synergy observed between soil nutrient conservation, forage yield, and carbon sequestration and oxygen release ($p < 0.05$). Therefore, LT is considered the best vegetation restoration practice for the karst rocky desertification control area compared with other patterns. This study provides theoretical guidance for vegetation restoration in degraded karst ecosystems.

Keywords: grassland ecosystem service; trade-off; synergy; ecological restoration; karst desertification

Citation: Song, S.; Chen, X.; Song, Y.; Chi, Y. Vegetation Restoration Patterns Influence the Supply and Interrelations of Grassland Ecosystem Services in Karst Desertification Control. *Land* **2024**, *13*, 2023. https://doi.org/10.3390/land13122023

Academic Editors: Michael Vrahnakis and Yannis (Ioannis) Kazoglou

Received: 16 October 2024
Revised: 25 November 2024
Accepted: 25 November 2024
Published: 27 November 2024



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1. Introduction

Ecosystem services refer to the environmental conditions and benefits provided by ecosystems that support both natural processes and human well-being [1,2]. They are typically categorized into four main types: provisioning, regulating, supporting, and cultural services [3]. Studies have shown that since the 1950s, the growth of human demand has widely consumed the services provided by natural ecosystems, and about 60% are degraded or in an unsustainable use state [4], threatening ecological stability and human well-being. Balancing ecosystem services and human well-being is a key challenge for achieving harmony between humans and nature. This issue has become a central focus in research on human–environment interactions and sustainability [5,6]. With the advancement of ecological civilization and sustainable development, the study of ecosystem services has shifted from merely examining service provision to enhancing overall human well-being [7,8]. The interrelations of ecosystem services are now widely used as analytical frameworks to address interactions between natural and social systems, offering effective

approaches to exploring sustainability and human welfare [9,10]. Therefore, identifying the interrelationships of ecosystem services is essential for the scientific understanding of degradation processes and restoration mechanisms of ecosystem functions [11–13].

Karst ecosystems are a crucial component of global terrestrial ecosystems, playing an essential role in supporting human well-being and livelihoods [14]. The karst distribution area in southern China covers 1.24 million km^2, making it one of the three major concentrated karst regions in the world [15]. Due to geological constraints, there is a sharp human–environment conflict [16]. Under the framework of structure–function–habitat, it exhibits significant vulnerability characteristics [17,18], establishing it as one of the most ecologically fragile areas recognized worldwide [19]. Since the last century, the government has implemented a series of ecological protection and reconstruction projects aimed at controlling karst desertification and restoring degraded ecosystems in karst areas [20]. Over decades of efforts, the extent of karst desertification has steadily decreased [21,22], leading to significant ecological restoration. As a result, both provisioning services [23,24] and regulating services of the ecosystem have improved [25–27]. Currently, research on karst desertification control is shifting toward enhancing ecosystem services and improving human well-being [28,29]. However, effectively managing multiple ecosystem services in karst areas requires a deeper understanding of the interrelationships between these services, in addition to addressing common research issues related to service provision.

Appropriate vegetation restoration models are essential for improving the ecological environment in fragile regions and for maintaining and enhancing the functions and services of their ecosystems [30,31]. In the natural succession of vegetation, grassland serves as a transitional stage between bare land and forest communities, and it acts as a pioneer ecological community for ecological restoration [32,33]. Initiatives such as "Grain for Green" and the establishment of artificial grasslands are crucial for restoring degraded ecosystems [34,35]. Different grassland vegetation restoration patterns can significantly influence ecosystem functions and processes [36]. Research shows that artificial grassland restoration improves the provisioning [37], regulating [38], and supporting services [39,40] of karst ecosystems. However, despite these positive outcomes, the sustainability and long-term impacts of artificial grassland on ecosystem service supply remain uncertain. Additionally, the interrelations between grassland ecosystem services in karst desertification control areas are becoming increasingly complex due to natural and socioeconomic influences. Issues such as unmet expectations for service supply persist, driven by the interactions among various ecosystem services [41]. To achieve sustainable development, it is essential to analyze the interrelations among grassland ecosystem services and enhance their supply in karst desertification control areas.

Analyzing the interrelations between ecosystem services is fundamental to ecological management and a key focus in fields like ecology and geography [42–45]. Achieving the sustainable use of natural resources in fragile karst ecosystems requires understanding these relationships to minimize trade-offs and enhance synergies among services. Currently, scholars have carried out many studies on the interrelations between karst ecosystem services in the context of ecological restoration projects. Jiang et al. [46] found that the Ecological Restoration Program enhances the synergy between soil conservation and water yield in the karst plateau region. Gu et al. [47] observed that larger areas of forest and grassland reduce the area of cultivated land through a study on China's ecological conservation red lines, which in turn reduces the trade-off among water production, carbon storage, and habitat quality. Chen et al. [48] found that the Grain-for-Green Program strengthened the synergy between carbon sequestration, soil conservation, and habitat quality, but also increased the trade-off between water resources for soil conservation and vegetation restoration. Zhang et al. [49] discovered that ecological restoration programs in fragile karst areas of southern China led to forest expansion, which intensified the trade-off between provisioning and regulating services. It is not difficult to find that many studies have mapped and simulated how ecological restoration projects impact ecosystem services in karst rocky desertification control areas. However, few studies have focused on

measuring changes in grassland ecosystem services in these regions, and understanding the relationships among multiple grassland services remains limited. More importantly, it is unclear whether different vegetation restoration patterns affect these interrelations. Therefore, this study aims to address this gap by examining natural and artificial grassland ecosystems in karst rocky desertification control areas. Using field monitoring data, it investigates how vegetation restoration modes influence the supply and interrelations of grassland ecosystem services. The objectives are as follows: (1) to quantify the multiple grassland ecosystem services under different vegetation restoration modes; (2) to clarify the effect of vegetation restoration mode on grassland ecosystem service supply; (3) to analyze the interrelations of grassland ecosystem services under different vegetation restoration modes. Two hypotheses were proposed: (1) the vegetation restoration mode will affect multiple grassland ecosystem services; (2) the interrelations of grassland ecosystem services in the vegetation restoration mode will be different.

2. Materials and Methods

2.1. Study Area

The study area is located in the Liuchong River Basin of the upper reaches of the Yangtze River in Yunnan-Guizhou Plateau, within Salaxi and Yejiao Township (105°02′01″–105°08′09″ E, 27°11′36″–27°16′51″ N) in the west of Qixingguan District, Bijie City, Guizhou Province, China (Figure 1), with an average elevation of about 1800 m. The study area covers an area of 86.27 km^2, the karst area accounts for 73.94% of the study area, which belongs to the karst plateau-mountain ecological environment. The karst desertification area is 55.93 km^2, accounting for 64.83% of the study area, and different levels of karst desertification are distributed in the study area, but mainly light. The study area is subtropical plateau monsoon humid climate, with annual average temperature of about 12 °C, frost-free period of 245 days, annual mean sunshine hours of 1360 h, and annual mean precipitation of 984.4 mm. The average annual temperature in 2021 was 14 °C and the annual precipitation was 943 mm (Figure 2). The parent material of the soil is limestone and sandstone shale, and the soil types are zonal yellow earth, yellow-brown earth, and calcareous soil. The main woody species in the study area are *Pinus yunnanensis*, *Betula luminifera*, *Cyclobalanopsis glauca*, *Rhododendron simsii*, *Pyracantha fortuneana*, *Rubus glabricarpus*, *Rosa roxburghii*, *Rhus chinensis*, *Juglans regia*, etc. The herbs include *Plantago asiatica*, *Artemisia carvifolia*, *Chenopodium glaucum*, *Stellaria media*, *Polygonum hydropiper*, *Digitaria sanguinalis*, *Clinopodium chinense*, etc.

In 2012, the research team initiated a vegetation restoration project in the study area as part of the rocky desertification control efforts, considering both the management of herbivorous livestock and the broader ecological context. The restoration strategies included both natural recovery and the establishment of artificial grasslands. Prior to the restoration, the land was used for corn cultivation. After the project implementation, the land use transitioned from corn farming to either artificial grass establishment or natural grassland restoration. For the artificial grassland, forage species were chosen based on their suitability for the local climate and their potential for ecological and economic benefits. These species included *Trifolium repens*, *Lolium perenne*, *Dactylis glomerata*, etc. The establishment of artificial grassland was carried out through both single-species and mixed-species sowing. The seeding rates for *Trifolium repens*, *Dactylis glomerata*, and *Lolium perenne* were set at 2.0 g/m^2, 3.0 g/m^2, and 3.0 g/m^2, respectively.

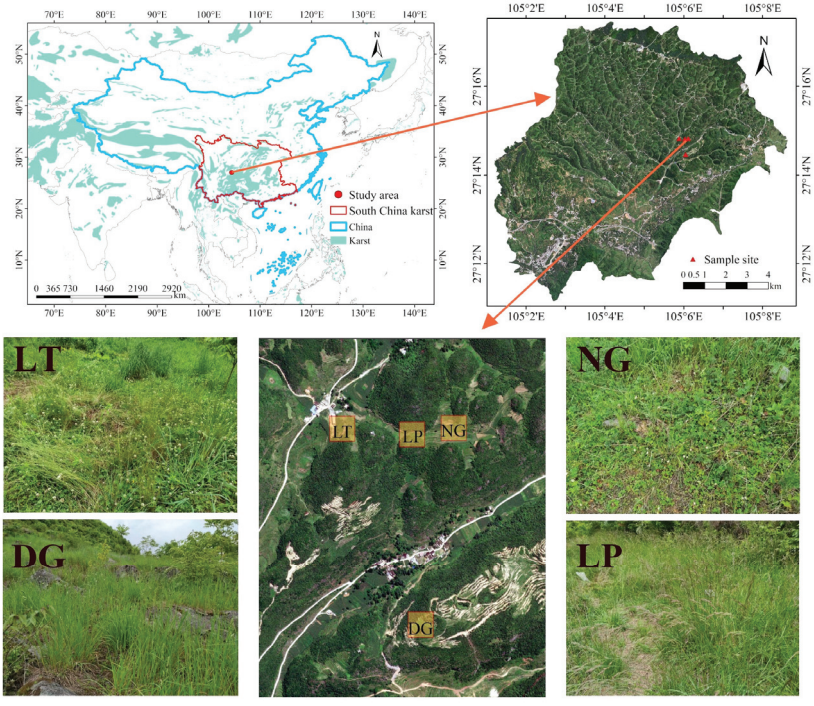


Figure 1. Location and sampling points in the study area. DG, *Dactylis glomerata* single-sowing grassland; LP, *Lolium perenne* single-sowing grassland; LT, *Lolium perenne* + *Trifolium repens* mixed-sowing grassland; NG, natural restoration grassland.

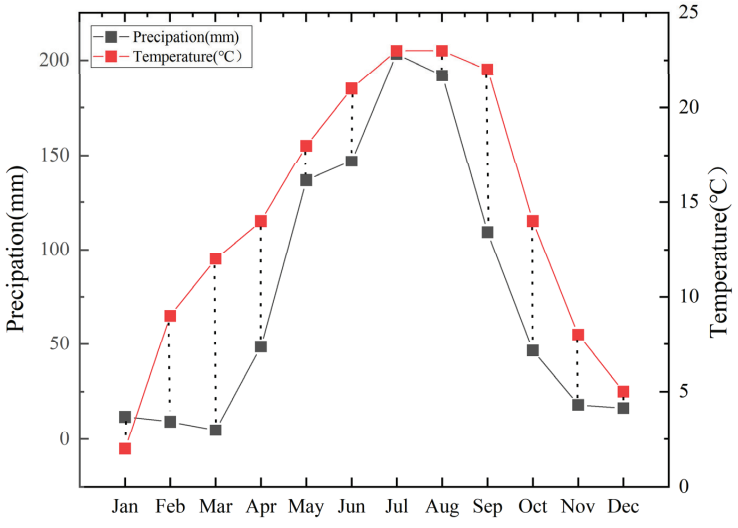


Figure 2. Monthly precipitation (mm, primary *y*-axis) and monthly temperature (°C, secondary *y*-axis) from January to December 2021. Data from small weather stations in the study area (ATMOS 41, METER, Pullman, WA, USA).

2.2. Plot Setting and Sample Collection

Three different vegetation restoration modes of grassland ecosystems were set up as experimental plots in the study area in 2012, which were *Dactylis glomerata* single-sowing grassland (DG), *Lolium perenne* single-species grassland (LP), and *Lolium perenne* + *Trifolium repens* (LT) mixed-species grassland, with natural recovery grassland (NG) as the control. Each plot consisted of six 10 × 10 m parallel subplots (a total of twenty-four sampling units). Due to the fragmented surface of the study area, the distance between plots was greater than 10 m. No agricultural activities and human disturbance were carried out in these experimental plots. Based on the ecosystem service classification framework outlined by the MEA [3] and the ecological restoration objectives for karst desertification regions, this study evaluated seven ecosystem services. These included forage yield (FY), soil retention (SR), soil water conservation (SWC), carbon fixation and release (CS), soil carbon storage (SCS), soil nutrient retention (SN), and biodiversity conservation (BI). Forage yield was classified as provisioning service. Soil retention, soil water storage, and carbon fixation and release were classified as regulating services. Soil carbon storage, soil nutrient retention, and biodiversity conservation were classified as supporting services. Due to the highly fragmented, small-scale, and dispersed nature of grassland landscapes in karst rocky desertification areas, cultural services were excluded from this analysis.

In mid-July 2021, samples were obtained from four different restoration modes to quantitative assess grassland ecosystem services. Five 1 × 1 m quadrats were established for data collection. The quadrats were randomly positioned, ensuring a 2 m spacing between them to minimize edge effects. Plant species within each quadrat were identified and counted to calculate species diversity. All vegetation within the quadrats was clipped at ground level and pooled to estimate aboveground biomass (kg/m^2). Forage with feeding values were separated from the mixture to calculate the forage yield (FY). The plant samples were oven-dried at 65 °C for 48 h until their weight stabilized, and the dry mass was recorded. After removing the litter layer from the soil surface, soil samples were collected from the surface layer (0–20 cm) at the same site with a soil auger, and five soil samples from each sampling plot were mixed to form a composite sample. The collected soil samples were divided into three portions: one for determining soil bulk density and water content, another for analyzing soil organic carbon (SOC) and total nitrogen (TN), and the third for assessing soil texture. Soil bulk density and soil water content were determined by the oven-drying method. Soil organic carbon (SOC) and total nitrogen (TN) were determined by the potassium dichromate–external heating method and sulfuric acid catalyst digestion–Kjeldahl method, respectively [50]. Soil texture was analyzed using the specific gravity method.

2.3. Ecosystem Service Calculation

Soil retention (SR, t) was calculated by using the modified general soil erosion equation (RUSLE) [51], and its formula is as follows:

$$\mathrm{SR} = \mathrm{R} \times \mathrm{K} \times \mathrm{L} \times \mathrm{S} \times (1 - \mathrm{C} - \mathrm{P}) \tag{1}$$

$$\mathrm{R} = \sum_{i=1}^{12} (0.3406 P_i - 2.6398) \tag{2}$$

$$\begin{aligned} \mathrm{K} = \left\{0.2 + 0.3e^{[-0.0256SAN(1-\frac{SIL}{100})]}\right\} \times \left(\frac{SIL}{SIL+CAL}\right)^{0.3} \times \left[1 - \frac{0.25C}{C+e^{(3.72-2.95C)}}\right] \\ \times \left[1 - \frac{0.25SN_1}{SN_1+e^{(-5.51+22.95SN1)}}\right] \end{aligned} \tag{3}$$

$$SN_1 = 1 - \frac{SAN}{100} \tag{4}$$

$$S = \begin{cases} 10.8\sin\theta + 0.03 & \theta \le 5^\circ \\ 16.8\sin\theta - 0.05 & 5^\circ < \theta \le 10^\circ \\ 21.9\sin\theta - 0.95 & \theta \ge 10 \end{cases} \quad (5)$$

$$L = \left(\frac{\lambda}{22.13}\right)^n \quad (6)$$

$$n = \begin{cases} 0.2\ if\ \theta \le 5^\circ \\ 0.3\ if\ 1^\circ < \theta \le 3^\circ \\ 0.4\ if\ 3^\circ < \theta \le 5^\circ \\ 0.5\ if\ \theta > 5^\circ \end{cases} \quad (7)$$

$$C = \begin{cases} 1 & fc = 0 \\ 0.6508 - 0.3436 lg fc & 0 < fc \le 78.3\% \\ 0 & fc > 78.3\% \end{cases} \quad (8)$$

where SR is soil retention, R is rainfall erosivity factor, K is soil erodibility factor, L is slope length factor, S is slope gradient factor, C is vegetation cover factor, and P is soil and water conservation factor. P_i is the average annual rainfall. SAN is sand content (%), SIL is powder content (%), CAL is clay content (%), and C is organic carbon content (%). θ is the slope, λ is the slope length, and fc is vegetation coverage.

Soil water content was used to express soil water conservation. According to the equations of plant photosynthesis and respiration, the grassland ecosystem plant releases 1.2 kg oxygen and fixes 1.62 kg carbon dioxide per kg dry matter produced, so the calculation of carbon fixation and oxygen release is calculated by the dry matter conversion method [52,53], and the formula as follows:

$$CS = G_c + G_o \quad (9)$$

$$G_c = A \times C_e \times 1.63 \times 0.2727 \quad (10)$$

$$G_o = A \times C_e \times 1.19 \quad (11)$$

where C_e is dry matter yield (kg/m^2), A is grassland area (m^2), G_c is carbon sequestration (kg), and G_o is oxygen release (kg).

The Shannon–Wiener index of plant species was used to express biodiversity conservation [36]. Soil total nitrogen content was used to express soil nutrient retention (%). Soil carbon storage (g/m^2) was calculated using soil depth, soil bulk density, and carbon content, and the calculation formula is as follows [54]:

$$\text{Soil carbon storage} = \text{SOC} \times \text{BD} \times \text{D} \times 10 \quad (12)$$

where SOC is soil organic carbon content (g/kg), BD is soil bulk density (g/cm^3), and D is soil depth (cm).

2.4. Data Analysis

Excel 2013 was used for preliminary statistics and analysis of the data. One-way analysis of variance and Tukey pairwise comparisons were used to analyze the effects of different vegetation restoration modes on grassland ecosystem services. Tukey pairwise comparisons were used as multiple comparisons at the $p < 0.05$ level. The data were tested for normality and homogeneity prior to one-way ANOVA in this study. One-way ANOVA, multiple comparison analysis, normality and homogeneity tests were performed in IBM SPSS (version 22.0; Armonk, NY, USA). Both Spearman and Person coefficients were used to quantify the interrelations between ecosystem services, where a positive coefficient indicates synergy interrelations and a negative coefficient indicates trade-off interrelations [55]. However, because the data in this study are not normally distributed, Spearman is more appropriate to quantify the interrelations between ecosystem services.

In order to better evaluate the impacts of different vegetation restoration modes on grassland ecosystem services, it is necessary to standardize the indicators of grassland ecosystem services. We referred to Pan et al. [50] and Fan et al. [56] whose previous studies standardized each grassland ecosystem service to make the range from 0 to 1. The formula is as follows:

$$ES_{std} = \frac{ES_i - ES_{\min(X_i)}}{ES_{\max(X_i)} - ES_{\min(X_i)}} \quad (13)$$

where ES_{std} is the standardized value of any individual ecosystem service, ES_i is the original observation value of the evaluation index i, $ES_{\max(X_i)}$ and $ES_{\min(X_i)}$ are the maximum and minimum observation value of the evaluation index i, respectively.

In order to estimate the total ecosystem services (TES) of grassland ecosystems, we used the method of Fan et al. [56] to calculate the total ecosystem services, with the formula as follows:

$$\mathrm{TES} = \frac{\sum_{i=1}^{n} ES_{\mathrm{std}}}{n} \quad (14)$$

where n is the total number of all types of ecosystem services, which is seven in this study.

3. Results

3.1. Supply of Multiple Ecosystem Services Under Different Vegetation Restoration Patterns

Table 1 shows that different vegetation restoration patterns significantly affect the supply of provisioning, regulating, and supporting services in grassland ecosystems ($p < 0.05$). Among the four different vegetation restoration types, LT provided high-level services of FY (0.50 kg/m^2), SR (0.017 t/m^2), SWC (25.46%), CS (81.26 kg), SCS (57.48 g/m^2), and SN (2.14 g/kg). NG had the lowest services of SWC (19.41%), CS (47.13 kg), SCS (35.26 g/m^2), and SN (0.79 g/kg). DG and LP provided moderate services of FY (0.35 kg/m^2 and 0.40 kg/m^2, respectively), SR (0.013 t/m^2 and 0.012 t/m^2, respectively), SWC (21.58% and 22.56%, respectively), CS (57.57 kg and 65.09 kg, respectively), SCS (44.50 g/m^2 and 46.47 g/m^2, respectively), SN (1.53 g/kg and 1.66 g/kg, respectively), and BI (0.71 and 0.67, respectively); however, there was no significant difference between DG and LP in grassland ecosystem services (except SR). Figure 3 also shows that the supply of provisioning, regulating, and supporting services of LT was the highest, while NG was the lowest, and the single-species-sowing grassland was between the LT and NG.

Table 1. One-way analysis of variance of multiple grassland ecosystem services under different vegetation restoration patterns.

	Provisioning	**Regulating**			**Supporting**		
	FY (kg/m^2)	**SR (t/m^2)**	**SWC (%)**	**CS (kg)**	**SCS (g/m^2)**	**SN (g/kg)**	**BI**
NG	0.29 ± 0.03 dc	0.011 ± 0.00 cd	19.41 ± 1.10 d	47.13 ± 4.84 d	35.26 ± 2.65 d	0.79 ± 0.05 d	1.10 ± 0.19 a
DG	0.35 ± 0.03 bc	0.013 ± 0.00 b	21.58 ± 1.50 bc	57.57 ± 2.15 bc	44.51 ± 2.12 bc	1.53 ± 0.24 bc	0.71 ± 0.20 b
LP	0.40 ± 0.05 b	0.012 ± 0.00 c	22.56 ± 1.75 b	65.09 ± 8.83 b	46.47 ± 3.12 b	1.66 ± 0.46 b	0.67 ± 0.12 bc
LT	0.50 ± 0.04 a	0.017 ± 0.00 a	25.46 ± 1.42 a	81.26 ± 7.10 a	57.48 ± 3.53 a	2.14 ± 0.28 a	1.16 ± 0.10 a
F-value	28.01 ***	128.33 ***	17.652 ***	27.588 ***	9.86 ***	21.625 ***	12.235 ***

Note: Data are mean value ± standard error. Different letters in the same column indicate significant differences at the 0.05 level between different treatments based on Tukey pairwise. *** presents significant differences at the 0.001 level. FY, forage yield; SR, soil retention; SWC, soil water conservation; CS, carbon fixation and oxygen release; SCS, soil carbon storage; SN, soil nutrient retention; BI, biodiversity conservation. DG, *Dactylis glomerata* single-sowing grassland; LP, *Lolium perenne* single-sowing grassland; LT, *Lolium perenne* + *Trifolium repens* mixed-sowing grassland; NG, natural restoration grassland.

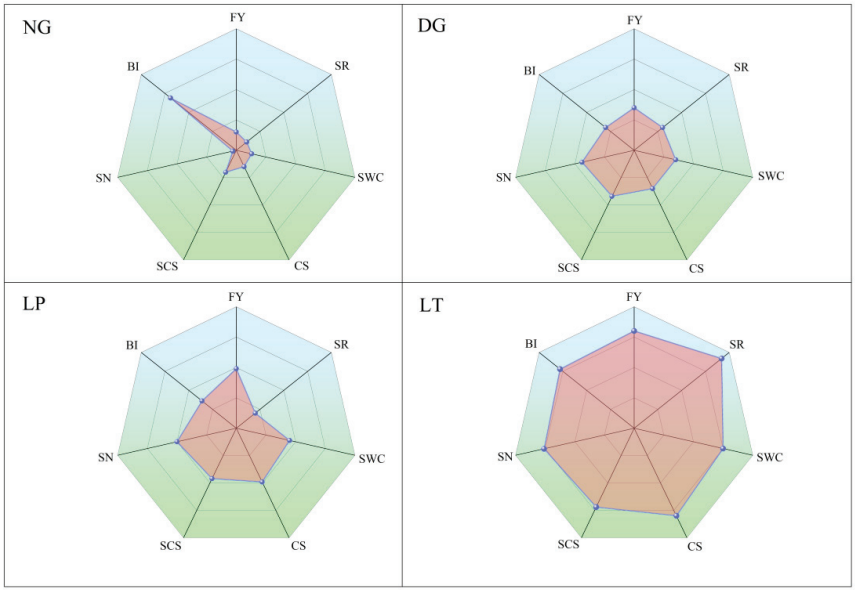


Figure 3. Radar plot of multiple ecosystem service supply under different vegetation restoration patterns. For better comparisons of each service, the data were normalized according to Equation (13). FY, forage yield; SR, soil retention; SWC, soil water conservation; CS, carbon fixation and oxygen release; SCS, soil carbon storage; SN, soil nutrient retention; BI, biodiversity conservation. DG, *Dactylis glomerata* single-sowing grassland; LP, *Lolium perenne* single-sowing grassland; LT, *Lolium perenne* + *Trifolium repens* mixed-sowing grassland; NG, natural restoration grassland.

3.2. Interrelations Among Multiple Ecosystem Services Under Different Vegetation Restoration Modes

In this study, seven individual ecosystem services were studied and their interrelations under different vegetation restoration modes were examined. Trade-off, synergy, and neutral interrelations were found in different vegetation restoration modes (Table 2). Spearman's correlation analysis of NG showed that there were no significant trade-off interrelations between FY and BI, SR and SWC, SCS and BI, CS and BI, SCS and SN, as well as SN and BI ($p > 0.05$). There was a significant synergistic relationship ($p < 0.01$) between FY and CS in all four modes ($p < 0.01$). This phenomenon may be attributed to the fact that CS is calculated by multiplying aboveground biomass by a conversion factor, with forage yield being part of the aboveground biomass. Different vegetation restoration patterns can alter the interrelations between provisioning services and regulating services, as well as between regulating services and supporting services. Compared with NG, the interaction interrelations between FY and SR, SWC, SCS in LT were changed from synergy to trade-off, the interrelations between FY and SWC, SN in DG were changed from synergy to trade-off, and the interrelations between FY and SCS, SN in LP changed from synergy to trade-off, but the trade-off was not significant. Compared with NG, the interrelations between FY and BI in DG, LP, and LT were changed from trade-off to synergy, but the synergy was not significant. Compared with DG and LP, LT significantly changed the interrelations between FY and SN from trade-off to synergy ($R = 0.886$, $p < 0.05$). In the interrelations between regulating services and supporting services, the SWC and SCS in LP exhibited a significant synergistic relationship ($R = 0.829$, $p < 0.05$) compared to other modes. Similarly, the CS and SN in LT showed a significant synergistic relationship ($R = 0.886$, $p < 0.05$), while the SCS and BI in NG demonstrated a highly significant synergistic relationship ($R = 0.943$, $p < 0.01$). However, other interrelations between regulating services

and supporting services across the vegetation restoration modes were characterized by non-significant trade-offs or non-significant synergy ($p > 0.05$).

Table 2. Interrelations among six pairs of ecosystem services under different vegetation restoration patterns.

Pairs of Ecosystem Services		NG	DG	LP	LT
FY	SR	0.314	0.086	0.6	−0.829
	SWC	0.486	−0.6	0.029	−0.257
	CS	0.986 **	0.986 **	1.000 **	0.986 **
	SCS	0.029	0.257	−0.429	−0.143
	SN	0.486	−0.143	−0.543	0.886 *
	BI	−0.086	0.771	0.029	0.086
SR	SWC	−0.429	−0.543	−0.486	0.029
	CS	0.314	0.086	0.6	−0.82
	SCS	−0.714	−0.714	−0.543	0.086
	SN	0.486	−0.143	−0.086	−0.886
	BI	−0.771	−0.257	0.771	−0.257
SWC	CS	0.486	−0.6	0.029	−0.257
	SCS	0.6	0.143	0.829 *	0.371
	SN	0.257	−0.2	0.257	−0.314
	BI	0.543	−0.2	−0.714	0.429
CS	SCS	0.029	0.257	−0.429	−0.143
	SN	0.486	−0.143	−0.543	0.886 *
	BI	−0.086	0.771	0.029	0.086
SCS	SN	−0.371	−0.371	0.543	0.086
	BI	0.943 **	0.657	−0.429	−0.257
SN	BI	−0.6	−0.429	0.029	0.086

Note: * and ** presents significant differences at the 0.05 and 0.01 level, respectively. FY, forage yield; SR, soil retention; SWC, soil water conservation; CS, carbon fixation and oxygen release; SCS, soil carbon storage; SN, soil nutrient retention; BI, biodiversity conservation. DG, *Dactylis glomerata* single-sowing grassland; LP, *Lolium perenne* single-sowing grassland; LT, *Lolium perenne* + *Trifolium repens* mixed-sowing grassland; NG, natural restoration grassland.

3.3. Total Level of Ecosystem Service Supply

The grassland ecosystem under different vegetation restoration patterns presented different provisioning, regulating, and supporting services; as a result, the total ecosystem service level provided by them was also different (Figure 4). In the four different vegetation restoration modes, the supply of TES in LT was significantly higher than that in the other three patterns ($p < 0.05$), and the TES in NG was the lowest; however, there was no significant difference between DG and LP ($p > 0.05$).

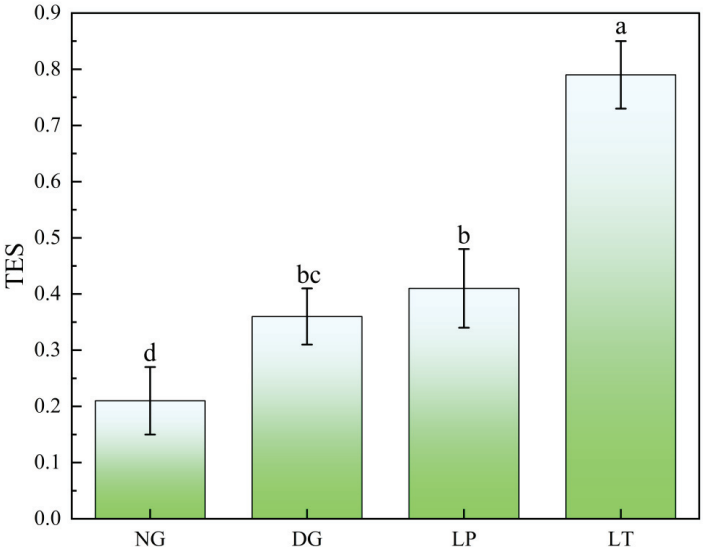


Figure 4. Total ecosystem services supply under different vegetation restoration patterns. In this study, TES refers only to provisioning, regulating, and supporting services, excluding cultural services. Different letters in the same column indicate significant differences at the 0.05 level between different treatments based on Tukey pairwise. DG, *Dactylis glomerata* single-sowing grassland; LP, *Lolium perenne* single-sowing grassland; LT, *Lolium perenne* + *Trifolium repens* mixed-sowing grassland; NG, natural restoration grassland.

4. Discussion

4.1. Effects of Different Vegetation Restoration Patterns on Grassland Multiple and Total Ecosystem Services

With the growing success of karst desertification control, improving ecosystem quality and enhancing ecosystem services have become key priorities [57]. Returning farmland to grassland and establishing artificial grasslands, as integral components of these efforts, each offer unique benefits in promoting vegetation recovery and boosting ecosystem services in karst regions [58]. Understanding how different vegetation restoration patterns affect grassland ecosystem services is essential for sustainable grassland use and the recovery of degraded ecosystems. This study examined the impact of various restoration patterns on multiple and total grassland ecosystem services. The results indicated that different restoration patterns significantly influenced the supply of these services, aligning with previous findings that restoration approaches play a critical role in shaping grassland ecosystems, particularly in degraded areas [59,60]. Additionally, the study confirmed that artificial vegetation restoration significantly improved provisioning, regulating, and supporting services. These findings are consistent with the results observed in artificial afforestation projects in northwest Yunnan, China [61].

Aboveground biomass and vegetation coverage are key indicators of vegetation restoration success, offering a clear measure of ecosystem recovery in degraded areas [62]. In this study, aboveground biomass served as an indicator of forage yield. The results showed that artificial grassland outperformed natural restoration in improving FY, consistent with findings by Wu et al. [36] and Chen et al. [63]. This improvement is likely due to the larger size and enhanced light competition of plants in artificial grasslands [64], which increases resource use efficiency and results in higher yields compared to natural restoration grasslands. Additionally, mixed-sowing grasslands showed an over-yielding effect [65,66], supporting this study's results. Regulating services, such as SR, SWC, and CS, and supporting services like SCS and SN, followed similar trends. Soil, as the carrier

of nutrient cycling in a grassland ecosystem, plays a very important role in grassland ecosystem services [67]. Vegetation coverage and soil organic carbon content were found to be significant factors influencing soil retention. Vegetation improves surface roughness and modifies water dynamics, reducing soil erosion and enhancing retention capacity [68]. Research indicates that artificial grassland significantly increases plant coverage and biomass, surface litter, and soil organic carbon through litter decomposition by microorganisms [69]. This process also promotes the formation and stability of soil aggregates, improves soil structure, boosts soil water content [70,71], and enhances carbon storage in aboveground vegetation, contributing to carbon sequestration [72]. Mixed-sowing grasslands further amplify these benefits by improving coverage, biomass, and soil organic carbon, consistent with findings by Yang et al. [73], Liu et al. [74], Peng et al. [75], and Bai et al. [76]. Soil total nutrients represent the nutrient pool and are critical for evaluating ecosystem functional restoration [77]. Studies show that vegetation restoration can improve soil nutrients in a short time [78]. Soil nitrogen is particularly important for ecosystem structure and function [79]. This study found that artificial grasslands significantly increased TN content, with the highest levels observed in LT grasslands. This increase is attributed to nitrogen-fixing rhizobia in leguminous forage, which enhance soil nitrogen accumulation, which was consistent with the research results of Li et al. [80] and Song et al. [40]. Despite the benefits of artificial grasslands, including improved forage yield and soil nutrients, challenges remain, such as low species diversity and stability [81]. These limitations explain why biodiversity conservation in DG and LP grasslands was lower than in NG. Therefore, under the interaction of these factors, LT has the highest TES and NG has the lowest. This study focused on provisioning, regulating, and supporting services in karst rocky desertification areas but did not address cultural services. This omission may have affected the TES assessment. Future research should include cultural services to enhance TES evaluation accuracy.

4.2. Effects of Different Vegetation Restoration Patterns on the Interrelations Between Grassland Ecosystem Services

Exploring the interrelations between different ecosystem services is crucial for regional ecological management, decision-making, and improving human well-being [82]. Previous studies have identified three types of interrelations between ecosystem services: trade-off, synergy, and neutral [45,55]. This study analyzed the correlation between different grassland ecosystem services under different vegetation restoration patterns and confirmed the presence of all three interrelation types in the karst rocky desertification control area. This provides empirical evidence that was previously lacking. Our findings also revealed that trade-off and synergy exist between provisioning, regulating, and supporting services under different vegetation restoration patterns. This results were consistent with research on meadows in the Qinghai-Tibet Plateau [36], but inconsistent with those on Inner Mongolia grasslands [56], likely due to variations in grassland establishment and utilization methods. Ecosystem structure and processes determine the supply of ecosystem services, which will further affect the trade-off and synergy between them [83]. The MEA report [3] that increasing provisioning services often reduces the supply of regulating and supporting services. Similarly, Bryan et al. [84] and Zhang et al. [49] found that ecological restoration programs exacerbate trade-offs between provisioning and regulating services. This was consistent with our findings. Compared to natural restoration, artificial grassland establishment exhibited trade-offs between forage yield and soil retention, soil water conservation, soil carbon storage, and soil nutrient conservation service, respectively, although it was not significant. Notably, significant synergies were observed between soil nutrient conservation and forage yield in LT, consistent with Yang et al. [85]. This suggests that artificial grasslands modify the relationships between ecosystem services through interactions among vegetation, soil, and microorganisms [86].

Previous studies have shown that ecological restoration projects in karst areas often reduce the supply of ecosystem services at regional and watershed scales [29,87–89]. However, grasslands have mostly been studied in the context of land use, with little targeted

research focusing on their specific contributions. As a key component of rocky desertification control projects, returning farmland to grassland and establishing artificial grasslands require collaboration between land managers and decision-makers to sustain and enhance ecosystem service levels in these regions. This study quantified the grassland ecosystem services under different vegetation patterns and provided empirical evidence for changes in the interrelations among provisioning, regulating, and supporting services through field monitoring. While trade-offs were observed between certain grassland ecosystem services under different restoration patterns, these trade-offs were not statistically significant. Based on the results of TES, we found that LT can provide the highest level of ecosystem services, and it can significantly promote synergy interrelations between FY and SN. This synergy not only improves ecosystem functions but also provides direct economic benefits, making LT more appealing to local residents. Effective grassland management and ecological restoration require a comprehensive consideration of total ecosystem services and their interactions. Based on these findings, we recommend adopting mixed sowing of gramineous and leguminous forages as a restoration strategy to enhance ecosystem service capacity in karst rocky desertification areas. Notably, long-term monitoring of vegetation restoration impacts is essential to identify significant trade-offs and adjust ecological management policies in a timely manner. Promoting synergies among ecosystem services will support degraded ecosystem restoration, sustainable development, and improvements in local well-being.

5. Conclusions

This study explored grassland ecosystems in karst desertification areas, focusing on how different vegetation restoration patterns influence ecosystem service supply and their interactions. It provided empirical evidence of trade-off, synergy, and neutral interrelations among ecosystem services in these areas. Different vegetation restoration patterns had different impacts on individual grassland ecosystem services, and significantly influenced provisioning, regulating, and supporting services. Among all patterns, LT delivered the highest amount of total ecosystem services. Although there was a trade-off between individual services under different vegetation restoration patterns, it was not significant. To address this, future research should prioritize long-term monitoring of ecosystem services. This will support timely ecological management decisions aimed at enhancing grassland ecosystem functions and ensuring the sustainable recovery of degraded ecosystems in karst desertification areas. This study suggested that mixed sowing of gramineous and leguminous forage delivers the highest amount of ecosystem services. This highlights its potential as an effective method for improving degraded ecosystem services and achieving sustainable restoration.

Author Contributions: Conceptualization, X.C. and Y.C.; methodology, Y.C.; software, Y.C. and S.S.; validation, Y.C., S.S.; formal analysis, Y.C.; investigation, S.S.; resources, X.C.; data curation, S.S.; writing—original draft preparation, Y.C. and S.S.; writing—review and editing, Y.C. and S.S.; visualization, S.S.; supervision, Y.S.; project administration, Y.S.; funding acquisition, Y.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Guizhou Provincial Science and Technology Projects (No. [2024]Youth 354); the Science and Technology Innovation Talent Team Building Project of Guizhou Province (Qiankehepingtairencai-CXTD [2023]010); the National Technical Innovation Center for Karst Desertification Control and Green Development (Qiankehe Zhongyindi [2023]05).

Data Availability Statement: Data are contained within the article.

Acknowledgments: All the authors would like to thank Jinzhong Fang, Cheng He, Shuyu He, and Wenfang Zhang for their contributions to the data collection.

Conflicts of Interest: The authors declare no conflicts of interest.

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Forecasting of Grasslands Distribution on Mount Zireia Using Ecological Niche Modeling and Future Climatic Scenarios

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Abstract: Grassland ecosystems cover a high percentage of the terrestrial habitats of Earth and support the livelihood and well-being of at least one-fifth of the human population. Climate change and human activities are causing increasing pressure on arid and semi-arid regions. Land use/cover change significantly affects the function and distribution of grasslands, showing diverse patterns across space and time. The study investigated the spatial distribution of grasslands of Mount Zireia (Peloponnesus, Greece) using MaxEnt modeling based on CMIP6 models (CNRM-CM6 and CCMCC-ESM2) and two Shared Socioeconomic Pathways (SSP 245 and SSP 585) covering the period of 1970–2100. The results from the current (1970–2000) and several future periods (2020–2100) revealed that the MaxEnt model provided highly accurate forecasts. The grassland distribution was found to be significantly impacted by climate change, with impacts varying by period, scenario, and climate model used. In particular, the CNRM-CM6-1 model forecasts a substantial increase in grasslands at higher elevations up to 2100 m asl. The research emphasizes the importance of exploring the combined impacts of climate change and grazing intensity on land use and cover changes in mountainous grasslands.

Keywords: MaxEnt model; Shared Socioeconomic Pathways; land use/cover change; climate change

Citation: Karatassiou, M.; Stergiou, A.; Chouvardas, D.; Tarhouni, M.; Ragkos, A. Forecasting of Grasslands Distribution on Mount Zireia Using Ecological Niche Modeling and Future Climatic Scenarios. *Land* **2024**, *13*, 2126. https://doi.org/10.3390/land13122126

Academic Editor: Yongheng Gao

Received: 20 October 2024
Revised: 3 December 2024
Accepted: 6 December 2024
Published: 8 December 2024

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1. Introduction

Land use/cover change (LUCC) and climate change are the two main categories of environmental changes at both a regional and a global scale [1]. LUCC is described as changing patterns in a specific area over time and space due to different variables, endangering both biological diversity and ecological systems [2–5]. Intense human activities and land abandonment result in LUCC which in turns reduces biodiversity, leads to habitat loss and degradation, and causes landscape isolation, fragmentation, or homogenization [6–8]. In addition, the provision and values of certain ecological services have been modified as a result of LUCC [9,10]. A significant quantity of data are needed to study LUCC and to develop strategies for the sustainable management of natural resources [5,11].

The climate is one of the most important environmental factors affecting LUCC [12–15]. There is no doubt that the climate at the global scale is getting warmer, and many of the changes observed since the 1950s have not been seen for decades or centuries [16], often resulting in extreme weather phenomena and climate patterns, leading to the extinction of species and significant modifications to their habitats and niches [17,18]. Forecasts suggest that by 2100, about 51% of the current flora species will lose half of their geographic distribution [19,20]. Through land use modeling, conservation activities can be efficiently targeted to anticipate the impact of climate change on LUCC [21].

Integrating spatial and temporal models into projections of LUCC is essential for understanding historical patterns and present conditions and making informed decisions for future scenarios [22]. The state-of-the-art machine learning model MaxEnt [23] is regularly used in distribution modeling [24–26]. MaxEnt has been extensively utilized in forecasting the potential geographic range of species and their nonlinear connections between environmental factors and documented sites [27–30]. It has been increasingly used in predicting forest fires [31,32], cultural ecosystem services [1,33], and the impact of human pressures on land use [27,34]. However, its utilization in predicting land uses still needs to be improved [35]. This method is useful for forecasting the full potential geographic distribution of a species (the fundamental environmental niche), but not its actual distribution, which might be limited due to competition, barriers, or human-induced modification of the environment. This approach is also used to determine the relative importance of various environmental variables for species distribution and the relative suitability of different areas as potential habitats [23,36]. Classifications of land cover are studied in the same way as species or habitats [37].

Grassland ecosystems cover about 20–26% of the Earth's land area [38] and support the livelihood and general well-being of one-fifth of the human population [39,40]. These ecosystems provide grazing areas for livestock [38,41], habitats for wildlife [39], and contribute to the provision of ecosystem services, such as environmental protection, water storage, carbon sequestration, and in situ conservation of plant genetic resources [42–44]. Despite their crucial role and the significant ecosystem services they provide, the importance of grasslands is often overlooked [45,46].

Climate and human activities are both key factors in the development and longevity of grasslands [44]. Grazing is acknowledged as a crucial element in protecting grassland habitats, enhancing biodiversity and ecosystem function. Nonetheless, grasslands are still at great risk from multiple sources, such as climate change, land use, soil degradation, nutrient loss, fires, habitat fragmentation, and human activities [44,47–49]. Precipitation and grazing play significant roles in determining species diversity and overall ecosystem functioning in grassland ecosystems and especially overgrazing can lead to severe habitat loss and degradation [38,50,51], which is estimated from 10–20% to 70–80% [52]. Moreover, they have been exposed to increased hazards due to extensive use and deterioration [53–55]. Elevation also has a major impact on climatic conditions and land formation, which then affects the availability of resources, plant growth regulators, and species diversity [56,57]. The above dynamics have a severe effect also in the Mediterranean basin, where 10–20% of the total area is covered by grasslands distributed across various elevation zones, each playing a unique ecological role, supporting important ecosystem services, and facing distinct environmental challenges [58].

In Greece, the highest percentage of grasslands is located in the middle and high (mountainous) elevation zones (32 and 51%, respectively) [59]. Mountainous grasslands offer excellent grazing areas for transhumant livestock and play a vital role in preserving biodiversity and landscapes, as well as in mitigating climate change and regulating water resources [60,61]. Transhumance is a type of pastoralism where animals are moved periodically between different elevation zones to take advantage of seasonally available grazing resources [61]. Nonetheless, these ecosystems are particularly vulnerable to the impacts of climate change, including altered rainfall patterns and rising temperatures [62] and the cumulative risk of desertification. The importance of integrated management strategies to maintain the ecological integrity of grasslands is amplified by the complex interactions between land use (human activities) and climate over space and time [63] in the face of ongoing environmental change. To the best of our knowledge, the Maxent model has not been applied to study the impact of climate change on land use, particularly grasslands, in the Mediterranean region.

The aim of the current study was to evaluate the effects of climate change on the structure and distribution of the grasslands in a Mediterranean mountain. We selected a typical Mediterranean mountain, Mt Zireia, lying on the northeast part of the Peloponnese,

Greece, and we tested two different climate models and scenarios by utilizing the MaxEnt modeling approach. From the Shared Socioeconomic Pathways (SSPs), we tested two scenarios: the SSP245 as intermediate and SSP585 as pessimistic, and we projected forecasts up to 2100.

2. Materials and Methods

2.1. Study Area

The study area is Mount Zireia with an altitudinal range of 310 to 2374 m a.s.l. It is located in the prefecture of Korinthos, 115 km west of Athens, and includes approximately 39,761.57 ha (Figure 1). The Natura2000 network comprises more than two thirds of the study area (Natura 2000). The lakes Stymfalia (15.285 ha) to the south and Doxa (48 ha) to the west are the primary hydrological basins in the region (Figure 1) that highly affect microclimatic conditions. The climate is categorized as Mediterranean, featuring mild winters and arid, extremely hot summers, in line with Emberger's bioclimatogram and classified as Csa in the Köppen–Geiger system (http://www.en.climate-data.org, accessed on 23 May 2022). Over the last 60 years, the mean annual temperature has fluctuated from 12.59 to 15.55 °C, and the mean annual precipitation varied from 418.62 mm to 1056 mm [64]. Agriculture and livestock production are the primary economic activities in the region [60]. The traditional transhumant livestock system has been present in the study area for many years but has experienced a significant decline in recent decades.

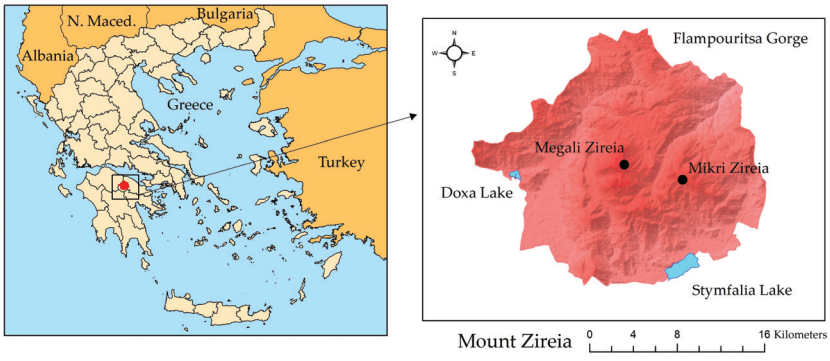


Figure 1. Geographical location of the study area.

2.2. Current Land Use/Land Cover Data

The land use/cover classification process was carried out mainly by using visual photo-interpretation techniques and digital processes in satellite images from Google Earth Pro v.7.3 software for 2017, 2019 and 2020 (georeferenced to the Hellenic Geodetic Reference System 1987-HGRS87), according to a procedure used in the study by Chouvardas et al. [8]. The above analysis was processed using ArcGIS 10.8, resulting in the creation of a digital LULC map for 2020. Among the various land use categories identified—such as agricultural areas, grasslands, open shrublands, dense shrublands, silvopastoral areas, forests, barren areas, urban areas, and lakes—this study focused specifically on the geographical distribution of grasslands. These grasslands, classified as discontinuous, covered an estimated area of 5893.51 hectares, representing 14.8% of the total study area.

Initially, a grid of 1000 m cell size was created (fishnet) in the shapefile format using ArcGIS 10.8. A total of 234 grassland distribution points were collected as dependent variables in the MaxEnt model. The samples' longitude and latitude coordinates were recorded in the Excel database and converted to CSV format for developing the MaxEnt model.

2.3. Environmental Variables for Model Fitting

Three topographic (elevation, slope, and aspect) and nineteen bioclimatic variables (bio1–bio19), reflecting seasonal changes in temperature and precipitation, with 30 s (ca.

1 km) spatial resolution were selected (Table 1) for developing an ecological niche model. The elevation in Digital Elevation Map (DEM) format was downloaded from the Jet Propulsion Laboratory of NASA (Aster GDEM v3, https://asterweb.jpl.nasa.gov/gdem.asp, accessed on 8 February 2022), while the aspect and slope were extracted from the elevation map using ArcGIS 10.8. The bioclimatic variables were obtained from the Wordclim Dataset [65] (http://www.worldclim.org, accessed on 8 February 2022). Twelve soil variables with 250 m spatial resolution and a depth of 0–5 cm except for soil organic carbon content, whose depth was set by default at 0–30 cm, were also selected and downloaded from the Soil Grids website (https://soilgrids.org, accessed on 15 January 2023) (Table 1). These layers were converted into ASCII raster format and given the same geographic projection, extent, and cell size for utilization in the MaxEnt model [19]. We included all 34 variables in our models at the same time following Feng et al.'s [66] observation that high collinearity poses a lesser issue for machine learning techniques compared to statistical models [67,68]. Furthermore, removing variables with high correlations does not improve Maxent models since the algorithm can handle redundant variables and reduce the effects of variable collinearity during model training [66]. Moreover, multicollinearity can lead to response curves that are not reliable because the impact of one factor is mixed up with its correlation to other factors, making it challenging to determine the actual effect of each predictor [69,70].

Table 1. Environmental variables for the geographic distribution of grasslands in Mount Zireia.

Type	Abbreviation	Description	Units
Bioclimatic	bio1	Mean Annual Temperature	°C
	bio2	Mean Diurnal Range (Mean of monthly (max temp-min temp))	°C
	bio3	Isothermally (Bio2/Bio7) (×100)	°C
	bio4	Temperature Seasonality (standard deviation ×100)	°C
	bio5	Max Temperature of Warmest Month	°C
	bio6	Min Temperature of Coldest Month	°C
	bio7	Temperature Annual Range (Bio5–Bio6)	°C
	bio8	Mean Temperature of Wettest Quarter	°C
	bio9	Mean Temperature of Driest Quarter	°C
	bio10	Mean Temperature of Warmest Quarter	°C
	bio11	Mean Temperature of Coldest Quarter	°C
	bio12	Annual Precipitation	mm
	bio13	Precipitation of Wettest Month	mm
	bio14	Precipitation of Driest Month	mm
	bio15	Precipitation Seasonality	mm
	bio16	Precipitation of Wettest Quarter	mm
	bio17	Precipitation of Driest Quarter	mm
	bio18	Precipitation of Warmest Quarter	mm
	bio19	Precipitation of Coldest Quarter	mm
	elevation_aster	elevation	m
Topographic	slope_aster	slope	%
	aspect_aster	aspect	°

Table 1. *Cont.*

Type	Abbreviation	Description	Units
Soil	bulk density	Bulk density of the fine earth fraction	cg/cm^3
	cationexchcap	Cation exchange capacity of the soil	mmol(c)/kg
	coarsefragm	Volumetric fraction of coarse fragments (>2 mm)	cm^3/dm^3 (vol‰)
	claycontent	Proportion of clay particles (<0.002 mm) in the fine earth fraction	g/kg
	nitrogen	Total nitrogen (N)	cg/kg
	phwater	Soil pH	pH × 10
	sand	Proportion of sand particles (>0.05 mm) in the fine earth fraction	g/kg
	silt	Proportion of silt particles (≥0.002 mm and ≤0.05 mm) in the fine earth fraction	g/kg
	soilorgcarb	Soil organic carbon content in the fine earth fraction	dg/kg
	orgcarbden	Organic carbon density	hg/m^3
	worldrbssoilg	World reference base (2008) soil groups (an international soil classification system for naming soils)	
	soilorcarbst	Organic carbon stocks	

2.4. Environmental Variables for Forecasting Model

From the Climate Model Intercomparison Project Phase 6 (CMIP6), we selected the models CNRM-CM6 [71] and CCMCC-ESM2 [72,73] to forecast the future grassland distribution on Mt Zireia. We selected four future periods (2021–2040, 2041–2060, 2061–2080, and 2081–2100) in addition to historical data (1970–2000) (http://www.worldclim.org, accessed on 8 February 2022). The CMIP6 includes various scenarios known as SSPs, representing emission scenarios based on varying socioeconomic assumptions. The SSPs are identified from SSP126 to SSP585 (https://climate-scenarios.canada.ca/?page=cmip6-overview-notes, accessed on 8 February 2022). In this study, the SSP245 and the SSP585 were selected as the intermediate scenario and as the pessimistic GHG emissions scenario, respectively, to predict the average suitable distribution areas of grasslands on Mt Zireia from 2021 to 2100 [74,75].

2.5. MaxEntropy Modeling

We used MaxEnt software (version 3.4.3) to simulate the potential current and future distribution of grasslands and identify the environmental factors that impact their distribution [23,76]. MaxEnt employs the maximum entropy algorithm and land occurrence to predict the probability of land use [77]. For model calibration and assessment, 75% of the data was utilized for training, while the remaining 25% was used to test the model's predictive capabilities for grassland distribution [78]. The automatic settings were configured for linear, quadratic, product, threshold, and hinge. The model was set up according to Tavanpour et al. [79], Saha et al. [36], and Ramasamy et al. [80] using 10,000 random background points as pseudo-absence throughout the study area and by regularizing multiplier 1 and 500 iterations with a 0.050 convergence threshold. The output of the Cloglog was utilized in the MaxEnt model to create a continuous map showing the predicted probability of presence ranging from 0 to 1. The test was conducted by excluding each variable systematically to evaluate the significance of environmental variables [78,81]. The Jackknife tests in Maxent were used to measure the dominant environmental variables contributing to grassland distribution. The software algorithm runs a maximum iteration of 500 of these

processes and 0.00001 of convergence threshold. The Receiver Operating Characteristic (ROC) shows the corresponding values for Specificity (Fractional Predicted Area on the horizontal axis) and sensitivity (Omission Rate on the vertical axis), with one point for each unique threshold [82]. The MaxEnt model's prediction accuracy is determined by the Area Under the Curve (AUC) from the ROC [23,65,83,84]. The AUC values range from 0 to 1. Fielding [85] states that the model's predictive power increases with a greater numerical value. An AUC value of less than 0.5 indicates performance poorer than chance, whereas an AUC value of more than 0.75 indicates high performance, and 0.5 suggests a forecast similar to random chance [86]. The Maxent outputs were in ASCII format, and ArcMap 10.8 was used to analyze and visualize the final forecasting maps [65]. The grassland forecasting map was classified into three classes following Coban et al. [84] based on potential suitable distribution: marginal 0.25–0.5, moderate 0.5–0.75, and high >0.75. The area of the three vegetation classes was then computed.

3. Results

3.1. Evaluations of the Model and Its Importance of Variables Under Current Climatic Conditions

The AUC values for the training and test data showed that our modeling approach's prediction accuracy for 1970–2000 was 0.864 and 0.786, respectively (Figure 2). The Jackknife test indicated that from all the independent examined environmental variables, the distribution of grasslands was mostly influenced by bio8, bio6, bio12, bio19, and elevation (Figure 3).

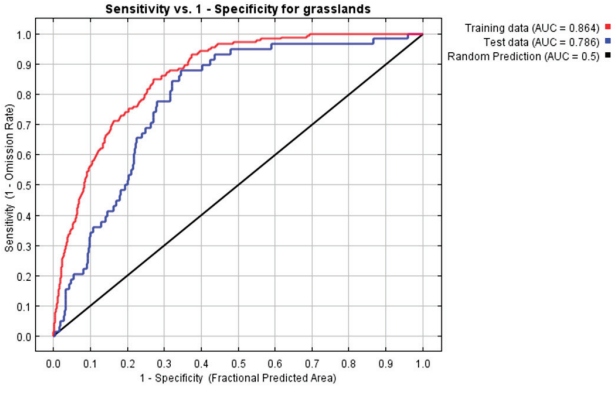


Figure 2. Area Under the Curve (AUC) value for the historical data, period 1970–2000.

3.2. Model Evaluations and Jackknife Test of Variables for Future Periods Under Different Climate Models and Scenarios

All AUC values for all future periods were >0.858 (Table 2). The climate model CCMCC-ESM2 and the pessimistic scenario (SSP585) for the period 2061–2080 demonstrated the highest AUC value (0.883), while the SSP585 from CNRM-CM6-1 appears with the lowest AUC value (0.859) for the period 2081–2100.

Table 2. The values of Area Under the Curve (AUC) for the future periods (2021–2040, 2041–2060, 2061–2080, 2081–2100) and the two CMIP6 climate models (CNRM-CM6-1 and CCMCC-ESM2) under the scenarios SSP245 and SSP585.

Future Period	CMIP6 Climatic Models			
	CNRM-CM6-1		CCMCC-ESM2	
	SSP245	SSP585	SSP245	SSP585
2021–2040	0.874	0.870	0.883	0.871
2041–2060	0.870	0.874	0.860	0.868

Table 2. *Cont.*

Future Period	CMIP6 Climatic Models			
	CNRM-CM6-1		CCMCC-ESM2	
	SSP245	SSP585	SSP245	SSP585
2061–2080	0.874	0.873	0.868	0.883
2081–2100	0.866	0.859	0.869	0.866

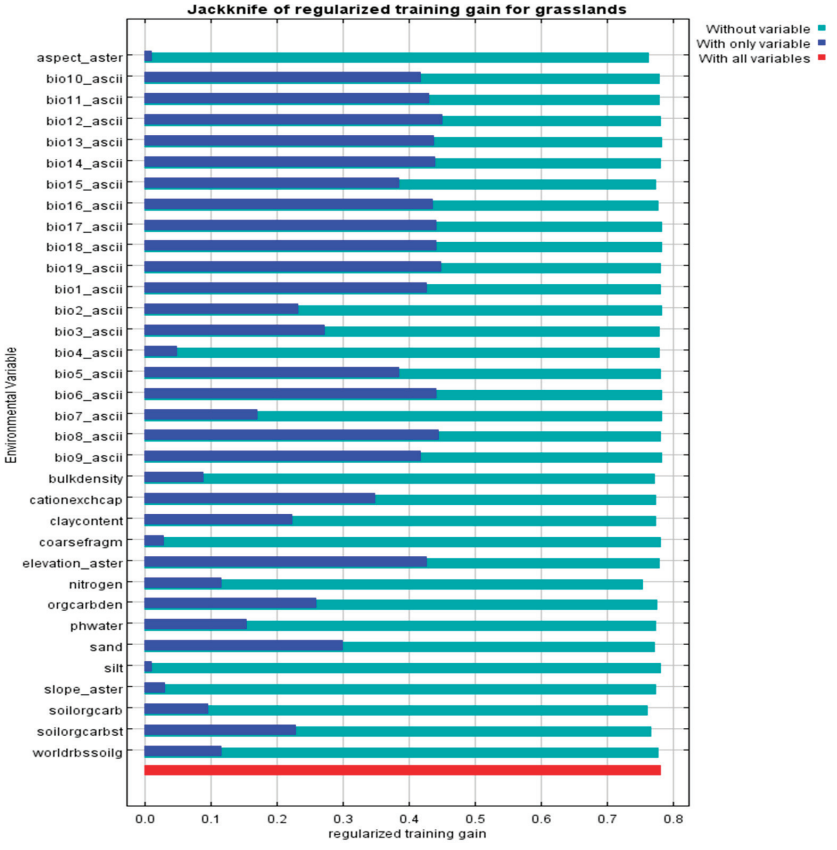


Figure 3. The relative predictive power for grasslands of the thirty study environmental variables is based on the Jackknife values of regularized training gain in the MaxEnt model.

The Jackknife test demonstrated that under the CNRM-CM6-1 model and intermediate SSP245 scenario for all future periods, the environmental variables that contributed the most to the model performance were bio12, bio6, and elevation (Figure 4). Moreover, the distribution of grasslands could be predicted for the periods 2021–2040 and 2041–2060 based on bio14, bio17, bio18, and bio19 (Figure S1a,b). The bio8 was only significant for the period 2041–2060 (Figure S1b). The Jackknife test also indicated that bio8 and bio16 were significant variables for predicting the distribution of grasslands for the years 2061–2080 (Figure S1c), while for 2081–2100, bio19 and bio14 were the most significant ones (Figure S1d).

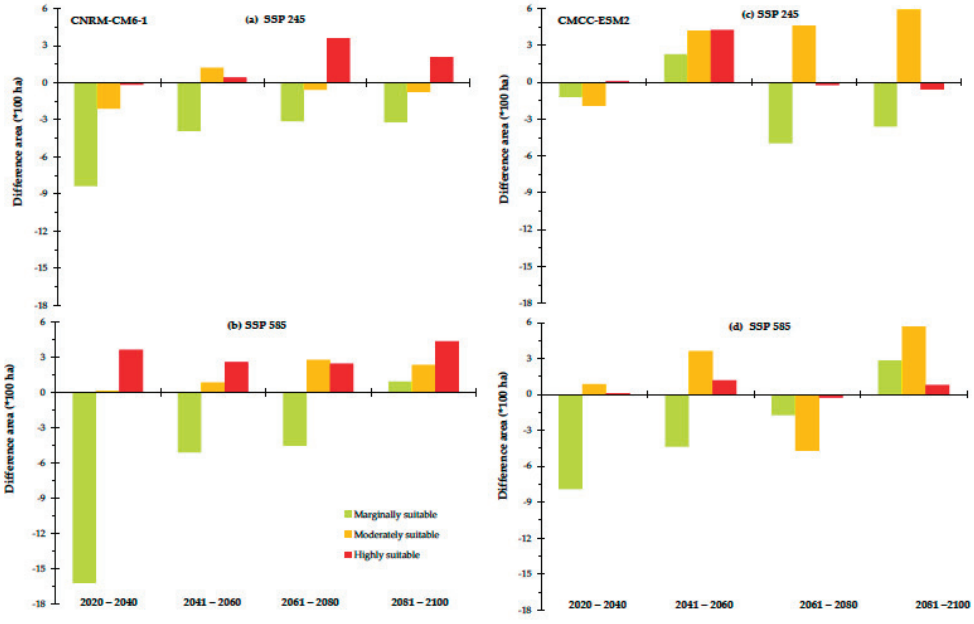


Figure 4. Potential changes in the grasslands' areas according to the suitability classes for the climate models CCNRM-CM6-1 and CMCC-ESM2 and SSP245 (**a**,**c**) and SSP585 (**b**,**d**) scenarios, respectively.

Under the CCMCC-ESM-2 climate model and the intermediate SSP245 scenario for all future periods, the Jackknife test highlighted bio12, bio6, bio8 bio14, and elevation as the environmental variables that contributed the most to model performance (Figure S2). However, for the periods 2021–2040 and 2041–2060, the distribution of grasslands could be predicted with greater accuracy based on bio17, bio18, and bio19 (Figure S2a,b). For the periods 2061–2080 and 2081–2100, the most significant variables influencing the distribution of grasslands were bio14, bio12, bio 6, and elevation (Figure S2c) as well as bio1, bio9, bio10, bio11, and bio16 were also one of the significant variables for predicting the distribution of grasslands (Figure S2d), respectively.

For all future periods under the climate model CNRM-CM6-1 and the pessimistic scenario, the Jackknife test demonstrated that the environmental variables that contributed the most to the model performance were bio14, bio6, and bio8 (Figure S3). More specifically, for the periods 2021–2040, 2041–2060, and 2061–2080, the Jackknife test showed that bio11, bio12, bio17, bio18, and bio19 were also crucial for predicting the grasslands' distribution (Figure S3a–c). The bio11 was only important for the period 2021–2040 (Figure S3a). Elevation was important in all future examined periods except for 2041–2060 (Figure S3b).

Under the climate model CCMCC-ESM2 and pessimistic (SSP585) scenario, the Jackknife test revealed that bio12 and bio19 were the dominant environmental variables in predicting the future distribution of grasslands (Figure S4). However, for the periods 2021–2040, 2041–2060, and 2061–2080, bio17 and bio18 were also very crucial predictors (Figure S4a–c) while bio16 was only significant in 2021–2040 (Figure S4a). The variables bio8, bio6, and bio14 were important for 2021–2040 and 2061–2080 (Figure S4a,c), whereas bio6 was also one of the most major relatively important variables for 2081–2100 (Figure S4d). Elevation was also an important variable for predicting the grasslands' distribution in all future periods except 2081–2100 (Figure S4d).

3.3. Current and Future Predictions of the Potential Distribution of Grasslands Using Ecological Niche Modeling

The current predictions (1970–2000) for grassland performance on Mt Zireia revealed that 7.9% of the study area showed high performance, 10.9% had moderate performance, and 19.3% displayed poor performance. Additionally, 61.9% of the entire study area was unsuitable for grasslands (Figures 4 and 5).

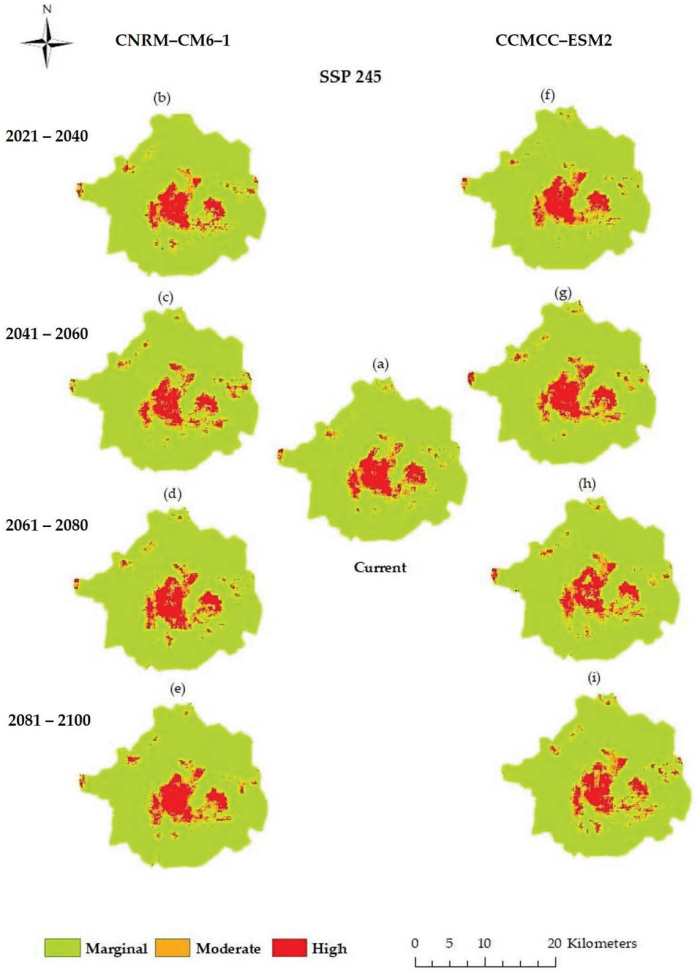


Figure 5. Potential spatial distribution of grasslands for the climate models CNRM-CM6-1 (**b**,**c**,**d**,**e**) and CCMCC-ESM-2 (**f**,**g**,**h**,**i**) under the SSP245 scenario for current (**a**) and future periods from 2020 up to 2100.

According to the predictions for the future of the CNRM-CM6-1 climatic model, there was an expansion of the high suitability area for grasslands in all periods except for 2021–2040 in the SSP245 scenario, which showed a slight decrease (Figures 4–6). Furthermore, the moderately appropriate area increased in all periods in the SSP585 scenario, as opposed to the SSP245 scenario, which increased only during 2041–2060 (Figures 4–6). The area with high suitability displayed variations across time for all upcoming years and both scenarios, with no clear pattern within the CCMCC-ESM2 climate model. The moderate suitable area increased in most periods and scenarios, except for 2021–2040 in the SSP245 scenario and 2061–2080 in the SSP585 scenario (Figures 4–6).

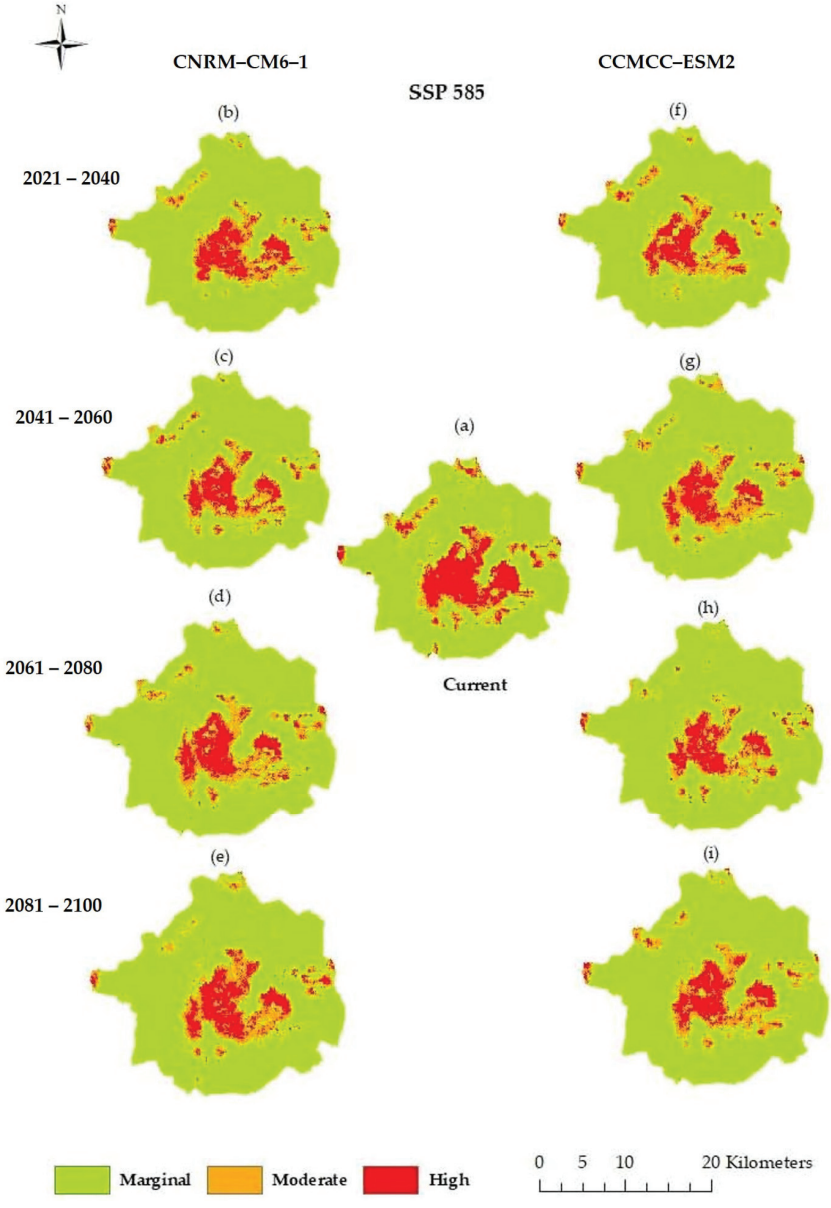


Figure 6. Potential spatial distribution of grasslands for the climate models CNRM-CM6-1 (**b**,**c**,**d**,**e**) and CCMCC-ESM-2 (**f**,**g**,**h**,**i**) under the SSP585 scenario for current (**a**) and future periods from 2020 to 2100.

Figure 7 illustrates the projected distribution of grasslands in four elevation zones based on the CNRM-CM6-1 model's predictions under SSP245 and SSP585 scenarios. The findings showed that elevations above 1200 will experience an increase in grassland area. The increase will be higher in the pessimistic scenario (SSP585).

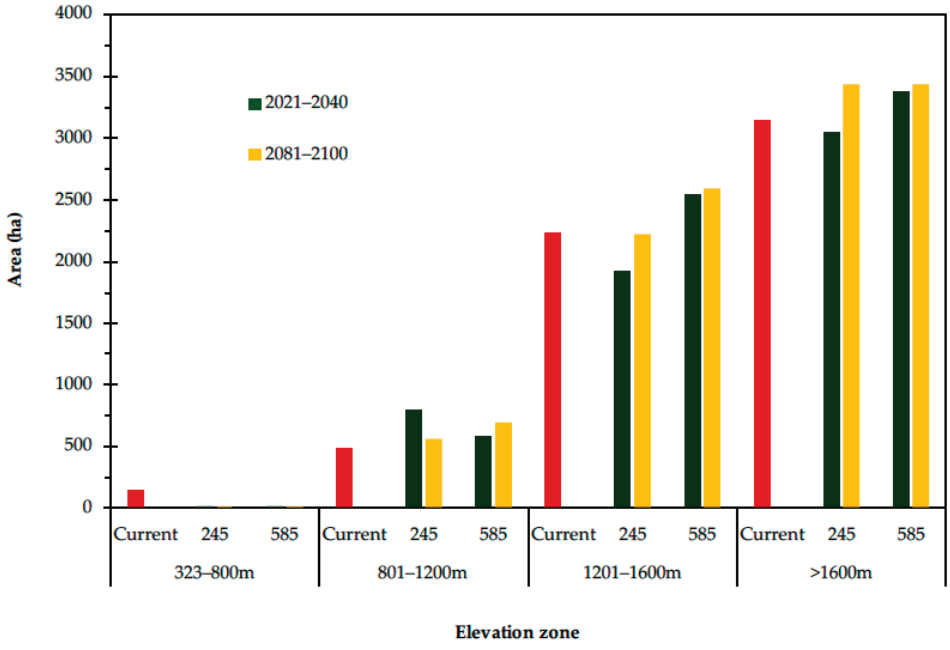


Figure 7. Forecasted current and future areas of grasslands on Mt Zireia by the CNRM-CM6-1 climate model and scenarios in the four elevation zones for current and future periods 2021–2040 and 2081–2100.

4. Discussion

The results of the present study demonstrated that (a) the MaxEnt model was highly accurate under the two examined climate models, (b) climate change strongly impacts the distribution of grasslands on Mt Zireia, (c) forecasting is differentially affected by specific periods, scenarios tested, and climatic models, and (d) the model CNRNM-CM6-1 predicts a substantial increase in grassland up to 2100, especially in elevations higher than 1200 m asl.

The accuracy of MaxEnt was high in forecasting suitable areas for grassland prevalence (AUC values from 0.858 to 0.883). It is widely accepted that AUC values higher than 0.8 demonstrate high performance of the model [68,87–90].

There is no agreement regarding the relative significance of the selection period, scenario, and climatic model as far as the prediction of the MaxEnt model is regarded [91,92]. The predictions of different models under the same scenario for the potentially suitable areas were different. The SSP245 scenario within the CNRM-CM6-1 model showed that the potential highly suitable areas for grassland areas will increase up to 2100 except for the period 2021–2040, while the CCMCC-ESM2 model did not show a clear future trend. On the other hand, in the SSP585 scenario, both models predicted similar changes in the moderately suitable grassland area, showing an increase in all future periods except for 2061–2080 in the CCMCC-ESM2 model. Our results are in agreement with Zhou et al. [65] who found different distribution trends in *Cunninghamia lanceolata* under different models and scenarios. Many studies mentioned that examining the forecasts from various models under the same scenario reveals diverse prediction outcomes [65,93]. The different SSP scenarios, based on different socio-economic patterns and carbon emissions, affected the trend in land use and cover change and were probably influenced not only by radiative forcing but also by unique local development pathways within various societies [93].

In our study, out of the examined environmental variables in the two climate models, annual precipitation (bio12), minimum temperature of the coldest month (bio6), and ele-

vation had the most important effects on the distribution of grasslands. Our results agree with the findings of Zhou et al. [65], Zhang et al. [94], and Yan et al. [76], demonstrating that temperature, precipitation, and temperature changes were the most critical environmental factors. Temperature and precipitation are significant factors for plant growth and phenology, such as the beginning and end of the growing season, timing of flowering, and physiology [95]. Changes in species phenology, as a result of climate change, have been observed to be primarily associated with temperature [95,96], while in arid and semi-arid environments, the seasonal patterns of precipitation can also have a significant impact [97]. According to Bede-Fazekas et al. [91], global climate change is causing a rearrangement of bioclimatic variables worldwide, such as precipitation and temperature timing. Researchers in recent decades have observed a rise in global net primary production because of the prolonged growing season caused by higher temperatures, particularly in mid and northern latitude areas [98]. However, in environments under water stress (arid and semi-arid), the factors influencing the timing and intensity of greenness in response to climate remain uncertain. Variables related to soil did not reveal an important role in grasslands' distribution and probably in the species that participate in their floristic composition [90]. Slope and aspect had a minimal impact on grassland distribution, as other researchers agreed that local topography can only influence microhabitat conditions in comparison to climate and disturbance regimes, which are generally the major determinants of grassland distribution [99], especially in arid and semi-arid environments [100]. On the other hand, the elevation was a significant predictor of grassland performance as is mentioned in other studies [101,102].

The influence of climate change is different in each elevation zone. These mountainous areas are identified as a "hotspot" for climate change, leading to significant impacts on mountain ecosystems, and human communities [103]. In Greece, a higher percentage of grassland occurs in the mountainous zone. However, mountainous grasslands are extensive ecosystems found across the globe, offering a range of economic and cultural benefits [46]. Our results reveal climate- and elevation-related effects, and we predicted a higher increase in mountainous grassland areas under the pessimistic scenario. This could be attributed to the change in temperature and precipitation. Considering the climate model CNRM-CM6-1, under the intermediate scenario, we predict an increase in the minimum temperature of the coldest month (bio6) by 1 °C and 2 °C for the specific periods 2020–2040 and 2081–2100, respectively. A decrease in annual precipitation (bio12) by 20 and 90 mm for the above periods, respectively, is also predicted. Under the pessimistic scenario, the changes in the above bioclimatic parameters were higher. The minimum temperature of the coldest month (bio6) increased by 1.5 °C and 4 °C, and the annual precipitation (bio12) decreased by about 20 and 170 mm for the specific periods 2020–2040 and 2081–2100, respectively (unpublished data). Especially for the mountainous zone, a 100 mm and 190 mm decrease in precipitation is predicted for the periods 2020–2040 and 2081–2100, respectively. Currier and Sala [97] revealed that precipitation impacted the beginning and end of the growing season, while temperature only impacted the start of plant growth. Changes in plant growth patterns can influence the supply of food for livestock, which has an impact on the economy of pastoral communities and especially transhumance [104,105]. The diachronically higher potential distribution of grasslands in the high elevation areas (central part of the study area) can be attributed to an observed trend in recent years called "the mountain effect" [106]. According to this trend, grasslands located at higher elevations exhibit greater resistance to change, primarily due to the traditional practice of vertical transhumance in the region [107] and the challenging soil and climatic conditions in the area [108]. These areas may provide suitable habitats for species migrating to higher altitudes in response to the impacts of climate change [109,110].

Climate change can have intricate effects on grasslands by changing plant competition, growth patterns, productivity, and plant–animal interactions, leading to a decline in forage quality [16]. Grazing is a well-established method of maintaining grasslands, especially in mountainous areas. In most grasslands, precipitation and grazing are key factors

influencing species diversity and ecosystem function [18,57,95,111]. Grazing animals, human activities, soil quality, nutrient depletion, fire, habitat fragmentation, and climate change have a significant impact on grasslands [41,44]. In most grasslands, changes in temperature along with precipitation [112] and grazing [113,114] are the main variables influencing species diversity and ecosystem function [115,116]. The grasslands on Mt Zireia are vital for sedentary and transhumant livestock as they provide necessary forage production from May to October [107]. Nevertheless, in this study, the impact of grazing was not examined. Many studies have demonstrated that grasslands can generate a range of ecosystem services sustainably, even during extreme weather events, if management adapts to changing conditions quickly and effectively [103].

Overall, our study underscores the importance of examining the combined impacts of climate change and grazing intensity on land use and cover changes, particularly in mountainous grassland ecosystems. This research lays the foundation for more in-depth analysis of how climate change affects highland ecosystems. It is crucial for farmers and other stakeholders to understand and adapt to the shifting environmental conditions, ensuring the resilience of these ecosystems. In particular, pastoral agents can play a pivotal role since they are very vulnerable to climate change dynamics, and this calls for systematic mitigation and adaptation actions to ensure their livelihoods. By integrating data and analytical tools, we can improve livestock management strategies and support informed decision-making. This approach not only aids in the sustainable management of land use but also contributes to conservation efforts, providing essential guidance for preserving these vital ecosystems in the face of ongoing climatic challenges.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/land13122126/s1, Figure S1: Jackknife test of CNRM-CM6-1 model and SSP245 scenario for periods 2021–2040 (a), 2041–2060 (b), 2061–2080 (c), and 2081–2100 (d); Figure S2: Jackknife test of CCMCC-ESM2 model and SSP245 scenario for periods 2021–2040 (a), 2041–2060 (b), 2061–2080 (c), and 2081–2100 (d); Figure S3: Jackknife test for CNRM-CM6-1 model and SSP585 scenario for periods 2021–2040 (a), 2041–2060 (b), 2061–2080 (c), and 2081–2100 (d); Figure S4: Jackknife test for CCMCC-ESM2 model and SSP585 scenario for periods 2021–2040 (a), 2041–2060 (b), 2061–2080 (c), and 2081–2100 (d).

Author Contributions: Conceptualization, M.K., A.S. and M.T.; methodology, M.K. and M.T.; software, M.K., A.S. and D.C.; validation, M.K.; formal analysis, A.S. and D.C.; investigation, M.K., A.S. and D.C., resources, M.K.; data curation, M.K., A.S. and D.C.; writing—original draft preparation, M.K. and A.S.; writing—review and editing, M.K., A.S., D.C., M.T. and A.R.; visualization, M.K.; supervision, M.K.; project administration, M.K.; funding acquisition, M.K. and A.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research is part of the project PASTINNOVA "Innovative models for sustainable future of Mediterranean pastoral systems" financed by the Partnership for Research and Innovation in the Mediterranean Area (PRIMA) program supported by the European Union under the grant agreement No 2113.

Data Availability Statement: The data presented in this study are available in the Figures and Tables provided in the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

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Assessing the Impact of Grazing and Restoration Methods on Pannonian Grasslands

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Abstract: Our study examined the impact of grazing by Hungarian Grey cattle on plant communities in grasslands restored and established through different methods. The grasslands were established and restored in 2009 using five methods: (I) naturally regenerating fallow, (II) hay-mulch addition, (III) seeded grassland following soil preparation, (IV) grazing on abandoned alfalfa fields, and (V) overseeded fallow initiated in 1989. From 2009 to 2011, all sites were uniformly mowed, after which they were grazed using free-ranging Hungarian Grey cattle starting in 2012. This project **aims** to restore traditional land use and dry grassland grazing in the region. Phytosociological recordings were conducted in 2012, the first year following grazing initiation, and again in 2020, nine years later. We assessed the botanical composition and grassland management value across sites in a 260-hectare experimental area within the Pannonian biogeographic region. The current study seeks to determine how different grassland restoration techniques have influenced the botanical composition and grassland management values of pastures and to identify the most suitable restoration methods. Statistical analyses were conducted using R to assess species count differences across four vegetation categories. The results indicate that the overseeded fallow established in 1989 and the hay-mulch addition method were the most effective techniques. Directly seeded grasslands and abandoned alfalfa fields differed most significantly from these areas. Across all types, species richness and total cover increased relative to the initial conditions in 2012, and the naturalness of the species composition also improved. Returning to traditional land use improved the ecological state of all sites. Restoration of the grazed areas proved optimal over the elapsed period, with two years of mowing followed by grazing. Grazing with Hungarian Grey cattle, a low-impact rustic breed, yielded successful grassland restoration outcomes. Among the restoration methods, direct seeding and the use of alfalfa fields proved uneconomical and ineffective in promoting the desired species composition. Natural regeneration and, particularly, the hay-mulch addition method, were the most effective for conservation and grassland management.

Keywords: traditional land use; grassland establishment; species richness; abundance; Pignatti life-form spectrum; Hungarian Grey cattle

Citation: Szentes, S.; Kevi, A.; Wagenhoffer, Z.; Saláta-Falusi, E.; Pajor, F.; Berke, J.; Turcsányi-Járdi, I.; Penksza, P.; Kunos, V.; Kende, Z.; et al. Assessing the Impact of Grazing and Restoration Methods on Pannonian Grasslands. *Land* **2024**, *13*, 2135. https://doi.org/10.3390/land13122135

Academic Editors: Michael Vrahnakis and Yannis (Ioannis) Kazoglou

Received: 30 October 2024
Revised: 30 November 2024
Accepted: 4 December 2024
Published: 9 December 2024

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1. Introduction

Grasslands constitute a significant proportion of the Earth's vegetated landscapes, making their maintenance, conservation, and proper management crucial [1–3]. In Hungary, 15% of agricultural land is grassland covering about 800,000 hectares, and 60% of them have been included in the Natura 2000 network. Many natural grasslands are global biodiversity hotspots [4–10], and the Eurasian region, known for its abundant and species-rich grasslands [11], is especially noteworthy. According to Hobohm and Bruchmann [12], 18.1% of Europe's endemic vascular plants inhabit grassland ecosystems, with climatic factors playing a key role in shaping this diversity [13–17].

Beyond preserving biodiversity, they play a pivotal role in mitigating climate change, notably through their high capacity for global carbon sequestration [18–21]. In addition to these two current areas, an extensive review by Lyon et al. [2] highlights grasslands' multifunctional roles, encompassing not only ecosystem restoration but also critical services in fodder production, erosion control, water management, and providing habitats for pollinators and wildlife. Additionally, these landscapes hold substantial cultural and social value [22].

However, many natural and semi-natural grasslands are found in areas with poor grazing potential, making them more vulnerable to climate change. Zhang et al. [23] provide evidence that drought is a significant factor in the decline of grassland biodiversity and multifunctionality. For instance, the cold spring of 2018 resulted in substantial losses among the Przewalski horse and Hungarian aurochs populations in the Hortobágy region due to limited forage availability [24]. Dry grasslands, in particular, are fragile ecosystems prone to severe wind erosion, especially when disturbed by grazing [1,25,26].

These findings suggest that managing grazing pressure in line with the principle of optimal disturbance is often essential. In the Pannonian region, grasslands have been heavily affected by the expansion of agricultural land over centuries. In Eastern Europe alone, grassland area has declined by at least 50% in the past 200 years [27]. A summary by Bíró et al. [28] shows the loss of eight natural and semi-natural grassland types in Hungary, with the least reduction occurring in saline grasslands (39%), which are unsuitable for agriculture, while other grassland types have lost 85–98% of their area. Similar reductions have been reported for steppes and forest steppes in western regions [29]. Many of these grasslands have been converted to forest, cropland, or intensive pasture or abandoned, much like in other regions of Europe [30,31].

Recently, grassland establishment and maintenance have gained renewed attention [32,33]. Restoration techniques vary widely, including direct seeding with diverse seed mixes and hay mulch addition, which can be effective [33–35]. Each method has distinct advantages; for instance, hay mulching reduces weed encroachment and enhances species richness [36,37] by inhibiting light-demanding weeds, protecting the soil from erosion and deflation, and providing a favorable microclimate for target species [38,39]. Choosing the right seed mix is also critical for conservation; ideally, native species that match the local site conditions should be selected [40,41]. Additional management practices, such as mowing, mulching, and grazing, are often required to promote restoration success. Mowing, particularly in the early stages, curbs weed growth and allows the establishment of other species, thus preserving species richness in degraded grasslands [42,43].

Grazing is especially suitable for grassland management once the grassland structure is established [44]. However, inappropriate grazing often promotes species that hinder or resist grazing due to traits, such as high silica content or dense, thorny, or toxic tissues, which animals avoid [45].

The choice of animal species, breed, and grazing season is crucial for biodiversity preservation and restoration. The size and grazing abilities of animals significantly impact vegetation. For instance, larger animals tend to avoid shrubs [46], while robust cattle breeds consume more shrub biomass [47–49]. Grazing impacts numerous plant species, particularly taller grasses [50]. Pauler et al. [48] found that herbivores' feed selection drives diversity within grazed vegetation, influenced by forage quality [51,52]. They also

observed that herbivores preferred plants with specific leaf area (SLA), high, leaf nitrogen, and phosphorus, but avoided those with leaf dry matter content (LDMC), high or defense mechanisms (e.g., thistles) [48]. In general, cattle avoid woody plants due to their toughness and low digestibility [53]. However, studies suggest that robust cattle graze more evenly and selectively across landscapes [54]. De Vries and Daleboudt [55] analyzed bite rates in different vegetative structures in Agrostis/Festuca and Lolium pastures, finding that cattle's' selectivity aligned with maximizing daily energy intake. McGeough et al. [56] reported that robust cattle are well-suited for extended grazing seasons, reducing the need for mechanical harvesting and enhancing farm efficiency.

The goal of this study was to restore traditional land use through grazing in low-biomass dry grasslands using Hungarian Grey cattle, a heritage breed adapted to the Pannonian region.

The research seeks to address the following questions:

- Is grazing with Hungarian Grey cattle indeed the most suitable method for enhancing species composition in Pannonian dry grasslands, even with the application of various grassland restoration techniques?
- How do species richness and grassland composition vary with different restoration methods in terms of ecological quality and potential utilization?
- Will the effects of different grassland establishment techniques persist, and if so, to what extent, and how will they manifest in the grassland composition during two years of mowing followed by grazing management?
- How effective and economically viable was the use of direct seeding and alfalfa sowing on the restored areas?

Our hypothesis was that grassland establishment types would continue to show substantial differences even following establishment and grazing periods. Among these, the direct-seeded area was predicted not to approach natural conditions, even after the study period. Grazing with Hungarian Grey cattle, established to restore traditional land use practices, is expected to greatly contribute to improving species composition in pastures.

2. Materials and Methods

2.1. Study Area

The research was conducted in the Páskom area (Reznek-dűlő, Túzok-rét), northeast of Zámoly, in the Zámoly Basin, Hungary (Figure 1). The soil of the area is rendzina [57]. The original vegetation of the area is slope steppe, *Salvio nemorosae-Festucetum rupicolae* Zólyomi ex Soó 1964, which usually contains many species with a Pontic and continental distribution and are generally rich in dioecious species, with a high diversity of species. The area is owned by the Pro Vértes Nature Conservation Foundation, which was plowed by the Zámoly Cooperative in the 1980s and used as cropland until 2000. Before the 1980s, this land was historically used as pasture due to its poor soil productivity. After purchasing the land, the Pro Vértes Foundation attempted restoration with small-game-friendly seed mixtures, though these attempts largely failed due to weather conditions. Considering that the Zámoly Basin's Great Bustard population inhabited the area until the early 1980s, the site was re-grassed.

The region receives an average annual precipitation of 560–600 mm, with an aridity index of 1.15–1.20 and an average annual temperature of 9.8–10 °C. The site, located at an altitude of approximately 140 m above sea level, experiences an average of 1950 h of sunshine annually and belongs to the Császár water catchment area [57]. The weather and exposure conditions of the sample areas are consistent, with northwest–southeast orientations on slightly sloping terrain (2–3%) with uniform soil.

The study area is divided into two major sections: the northern Túzok-rét, constituting the study site V, where overseeding (seeding without soil preparation, where the former vegetation remains) was conducted in 1989; and the southern Reznek-dűlő, where four parallel grassland restoration methods commenced in the 1990s.

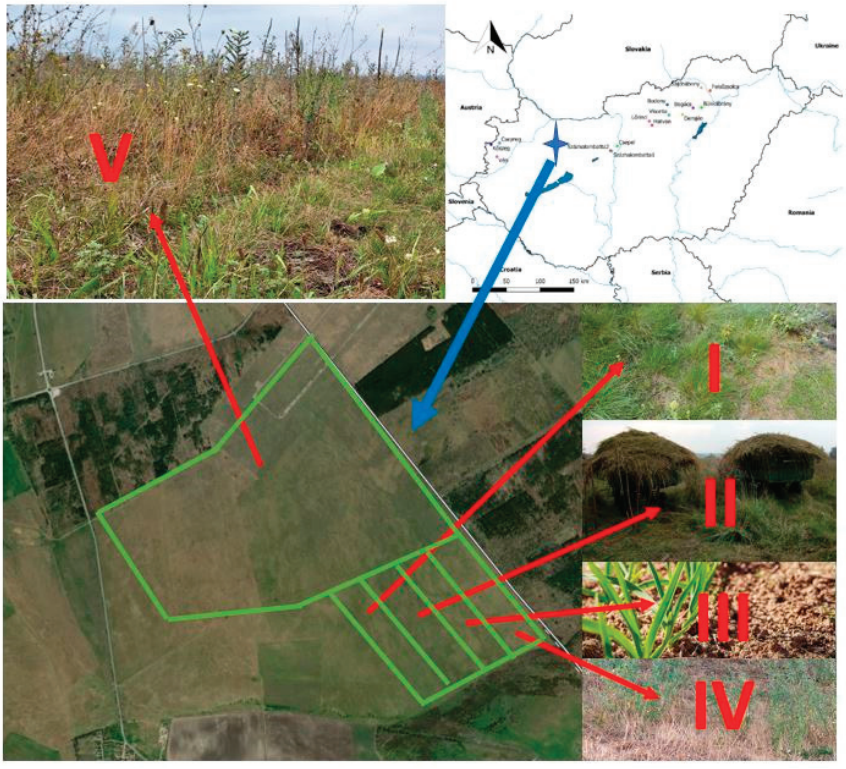


Figure 1. The location of the sample sites in Hungary (I–V: Treatments).

2.2. Sample Plots

The sample plots consisted of the following restoration treatments:

Treatment I (Spontaneously regenerating the abandoned field): Previously used as cropland until 2000, abandoned and left to spontaneous grassland development (33.18 ha).

Treatment II (Hay mulching): An abandoned former cropland, hay mulched with hay collected from a nearby natural grassland (27.79 ha).

Treatment III (Direct-seeded area): In 2009, following complete soil preparation, the area was seeded with a 60 kg/ha seed mix (*Lolium perenne* 25%, *Bromus inermis* 15%, *Festuca pratensis* 10%, *Dactylis glomerata* 10%, *Festuca rubra* 15%, *Festuca arundinacea* 25%) (18 ha).

Treatment IV (Abandoned alfalfa field): Sown with alfalfa in 2009 and subsequently abandoned (30 ha).

Treatment V (Overseeded abandoned field): An area that was overseeded with a 40 kg/ha seed mix (*Lolium perenne* 10%, *Bromus inermis* 20%, *Dactylis glomerata* 40%, *Festuca arundinacea* 30%) in 1989 and intermittently grazed or mowed from 1982 to 2009 (150.83 ha).

Following two years of mowing (2010–2011), all areas were grazed by Hungarian Grey cattle under a free-ranging system from 2012, at a moderate stocking density of 0.5–0.7 livestock units per hectare [58].

2.3. Sampling

Three times a year, in May, June and September, seven randomly selected 2 × 2 m Braun-Blanquet [59] phytosociological quadrats were sampled for each restoration treatment, recording species cover as a percentage. Quadrats were placed along the length of each treatment strip, spaced 10 m apart from the northern edge. Species nomenclature follows Király [60].

2.4. Data Analysis

Sample sites were evaluated based on species conservation value categories following Simon [61], and social behavior types (plant strategies), as described by Borhidi [62] and the following life-form categories by Pignatti [63] were applied:

Perennial Species:

H scap: species with ascending stems
H caesp: tussock-forming species
H ros: rosette-forming perennials
H rept: perennials with stolons, runners, or rhizomes
H bienn: biennial species
G bulb: geophytes with bulbs
G rhiz: rhizomatous, creeping geophytes

Annual Species:

T scap: annual species with ascending stems
T ros: rosette-forming annual species
T caesp: annual tussock species

Dwarf Shrubs:

Ch rept: creeping-stem dwarf shrubs
Ch succ: succulent dwarf shrubs

The coverage values of each grass species were calculated from the total coverage percentage per area.

The forage values [64–67] of various plots were calculated by the following formula:

$$FV = ((a \times A + b \times B + c \times C \ldots)/100) \times x$$

FV: Forage value of the vegetation plots
a, b, c: categories of forage values of species
A, B, C: coverage of species
x: total coverage of species

The grassland production was estimated by Balázs-method [66,67] using the following formula:

$$P = ((M - s) \times BM \times b)/100$$

P: yield [Kg/ha]
M: grass height [cm]
s: stubble height [cm]
BM: grass 400 [kg/ha]; alfalfa 470 [kg/ha]
b: coverage [%]

With the knowledge of the average grass height and the total coverage, the annual yield and the animal capacity of the grasslands could be estimated. The following data should be taken into account: 60 kg/day of green weight and a 210-day grazing season for cattle, 7 kg/day of green weight and a 210-day grazing season for sheep.

Statistical analyses were conducted using R version 4.2.3 (R Core Team, 2023) to assess species count differences across four vegetation categories (*Fabaceae*, Grass, Other, and Weed) commonly used in grassland surveys within five treatments (I–V) for the years 2012 and 2020 (Supplementary Material).

The data were pre-processed by removing observations with zero abundance, followed by aggregation based on treatment, year, category, and replication.

A two-way Analysis of Variance (ANOVA) was performed independently for each treatment within each year to examine differences in species counts among categories. Significant ANOVA results were followed by Tukey's Honest Significant Difference (Tukey HSD) post hoc test to conduct pairwise comparisons among categories within each treatment. In addition to the ANOVA and post hoc analyses, an Additive Main Effects and

Multiplicative Interaction (AMMI) model was applied to evaluate genotype-environment interactions, specifically to understand how different species categories responded to various treatments across years. This model allowed us to partition the variance and assess both main effects and interaction effects, providing a more nuanced view of treatment responses across species categories. A heatmap was generated using the *pheatmap* package to visualize the clustering patterns of species counts across treatments and years, aiding in the identification of categories with similar response profiles. To compare the total species number per treatment, we conducted a non-parametric Kruskal–Wallis test, due to the non-normal distribution of species counts, followed by a Bonferroni-adjusted post hoc test to control for multiple comparisons. This approach ensured a robust statistical comparison across treatments while accounting for potential Type I errors in multiple tests. The statistical and visualization analyses were conducted using various R packages: *ggplot2* for plotting, *dplyr* for data manipulation, *multcompView* for post hoc test interpretation, *readxl* for data import, *vegan* for diversity analysis, *agricolae* for non-parametric testing and multiple comparisons, *pheatmap* for generating heatmaps to explore clustering patterns in species counts, and *ggstatsplot* for enhanced visualizations with statistical overlays. Descriptive statistics, including mean and standard deviation, were calculated to summarize species richness and abundance for each category within each treatment and year. All statistical tests were conducted with a significance threshold set at $p < 0.05$ [68,69].

3. Results

A total of 134 vascular plant species were recorded across two survey periods. The total coverage values increased in all sample areas during the study period (Figure 2). In 2012, four species were present across all five study areas: *Dactylis glomerata*, *Festuca arundinacea*, *Potentilla argentea*, and *Trifolium pratense*. By 2020, these were joined by *Agrostis stolonifera*, *Agrimonia eupatoria*, *Bromus hordaceus*, *Festuca pseudovina*, *Elymus repens*, *Trifolium repens*, *Medicago lupulina*, *Achillea collina*, and *Veronica arvensis*. In 2012, Treatment V (the overseeded abandoned field) had the highest species richness, with 77 species, based on quadrat sampling. Treatment II (hay-mulched area) also exhibited notable diversity with 44 species. The overseeded grassland (V) retained the highest absolute species richness across both survey periods (77 in 2012 and 75 in 2020), though *Bromus inermis* and *Erodium cicutarium* were absent in 2020.

In 2012, both species abundance ($\chi 2 = 10.71$ $p = 0.03$ p) and species richness ($\chi 2 = 24.99$ $p < 0.000$) varied significantly across the five different pasture treatments (I. to V.), with a significant difference in abundance distributions and species count confirmed by a Kruskal–Wallis test. This finding suggests non-uniform abundance and distributions across treatments, indicating that different management strategies influenced specific species compositions (Figure 2).

Species abundance was generally low across treatments, with all treatments displaying a median abundance close to or at 1.0%, reflecting that the majority of species had relatively low abundance levels. Treatment I displayed an average abundance of 0.64% ± 1.92, with a median of 1.14%. The three most dominant species in this treatment were *Poa angustifolia* (11.43 ± 2.44%), *Festuca pseudovina* (10.0 ± 2.89%), and *Dactylis glomerata* (7.0 ± 2.83%), which together comprised a significant portion of the observed abundance distribution. Treatment II had an average species abundance of 0.64% ± 1.90, with a median of 0.88%, reflecting generally low abundance with some sporadic higher values. The most abundant species observed here were *Festuca pseudovina* (10.57 ± 1.99%), *Poa angustifolia* (9.29 ± 3.45%), and *Festuca rupicola* (6.86 ± 4.18%), contributing substantially to the observed variance in this treatment group. Treatment III exhibited a lower mean abundance of 0.51% ± 2.02, with a median of 1.0%, suggesting high variance in species abundance, though generally low values. The dominant species in this treatment included *Festuca arundinacea* (12.43 ± 2.51%), *Poa angustifolia* (9.71 ± 4.15%), and *Bromus inermis* (9.29 ± 3.45%). Treatment IV had a mean abundance of 0.57% ± 1.91, with a median of 1.14%. The dominant species in this treatment were *Agropyron repens* (9.71 ± 7.67%), *Poa*

angustifolia (7.43 ± 2.7%), and *Achillea collina* (7.14 ± 5.84%), indicating specific responses to the applied treatment conditions. Lastly, Treatment V showed the highest mean abundance at 0.67% ± 1.63, with a median of 0.71%, underscoring that while most species had low abundance, a few exhibited higher values. The most dominant species in this treatment were *Festuca pseudovina* (13.86 ± 2.04%), *Festuca rupicola* (6.14 ± 2.04%), and *Achillea pannonica* (3.71 ± 1.7%).

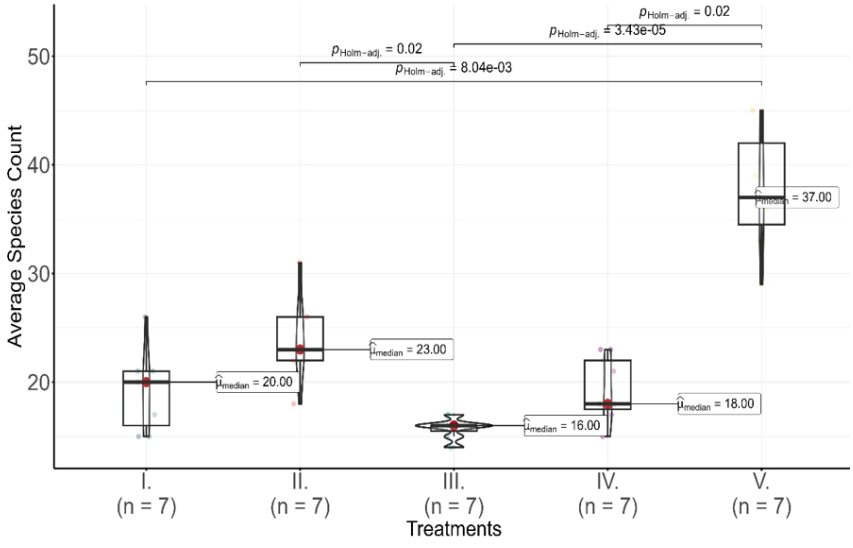


Figure 2. Average species count per treatment in 2012 across five pasture treatments (I to V), represented with violin and box plots showing the distribution of species counts. Horizontal bars indicate statistically significant pairwise differences between treatments, determined by Dunn's test with Holm adjustment at the $p = 0.05$ level. The adjusted p-values are annotated on the bars, providing a measure of significance for the observed differences in species counts. The letter n denotes the number of observed coenological quadrats sampled per treatment, with each treatment having seven observations (n = 7).

In addition to species abundance, the average species count per treatment, a measure of species richness, showed significant differences across treatments. Calculated species richness (considering only species with non-zero abundance) revealed distinct patterns: Treatment I had a mean species count of 19.29 ± 3.95, indicating moderate species diversity. Treatment II had a slightly higher mean species count of 24.00 ± 4.12, suggesting a more diverse assemblage. Treatment III had the lowest mean species count of 15.71 ± 0.95, significantly lower than the other treatments, suggesting that this treatment supported fewer species overall. Treatment IV recorded a mean species count of 19.29 ± 3.09, similar to Treatment I, while Treatment V had the highest mean species count of 37.71 ± 5.91, highlighting that this treatment promoted substantially greater species richness.

These differences in species richness are further confirmed by Dunn's pairwise test, adjusted using the Holm method, which indicated significant differences between treatments, particularly between Treatment III and Treatments I, II, IV, and V The pairwise comparisons also highlighted the significant difference between Treatment V and Treatments I, III, and IV (Holm-adjusted p-values of 0.02, 3.43×10^{-5}, and 8.04×10^{-3}, respectively), further supporting that Treatment V facilitated a higher level of species diversity compared to other treatments.

In summary, while all treatments in 2012 exhibited similar median values of species abundance, substantial variability was observed, as reflected by the standard deviations. The composition of dominant species varied across treatments, indicating that certain

species adapted to specific pasture management strategies, resulting in significant distributional differences. The differences in species richness highlight that certain treatments, particularly Treatment V, facilitated greater species diversity, whereas Treatment III supported both lower abundance and richness. This pattern indicates that distinct management approaches may selectively benefit either a few dominant species (low richness) or a broader assemblage (higher richness), influencing the overall biodiversity across treatments.

In 2020, both species abundance and species richness varied across the five pasture treatments (I to V), though without a statistically significant difference in abundance ($\chi 2 = 7.42$ $p = 0.12$), the distribution of species showed significant differences ($\chi 2 = 15.20$ $p < 0.000$) across the five treatments. This result suggests that the treatments did not produce distinct abundance just distributions, contrasting with the patterns observed in 2012 (Figure 3).

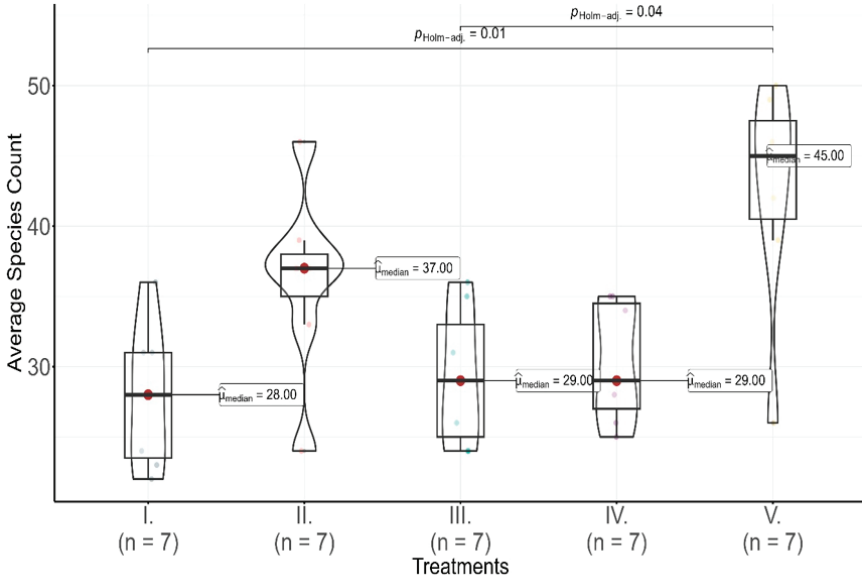


Figure 3. Average species count per treatment in 2020 across five pasture treatments (I to V), represented with violin and box plots showing the distribution of species counts. Horizontal bars indicate statistically significant pairwise differences between treatments, determined by Dunn's test with Holm adjustment at the $p = 0.05$ level. The adjusted p-values are annotated on the bars, providing a measure of significance for the observed differences in species counts. The letter n denotes the number of observed coenological quadrats sampled per treatment, with each treatment having seven observations (n = 7).

Species abundance remained generally low across treatments, with median values around 1.0%, indicating that most species were present at low abundance levels. However, notable species remained dominant within each treatment. For instance, **Treatment I** displayed a mean abundance of 0.72% ± 2.11 with a median of 1.0%. Dominant species included *Festuca pseudovina* (16.43 ± 2.44%), *Festuca rupicola* (7.86 ± 2.67%), and *Plantago lanceolata* (7.29 ± 3.5%). **Treatment II** showed a slightly higher mean abundance of 0.76% ± 2.15 and a median of 1.0%, with *Festuca pseudovina* (20.0 ± 4.08%), *Achillea collina* (5.86 ± 1.46%), and *Plantago lanceolata* (5.86 ± 2.27%) being most prevalent. **Treatment III** exhibited a mean abundance of 0.71% ± 2.02, with a median of 0.86%, where dominant species included *Plantago lanceolata* (14.57 ± 3.1%), *Festuca pseudovina* (12.71 ± 3.68%), and *Achillea collina* (5.86 ± 1.46%). **Treatment IV** had a mean abundance of 0.67% ± 1.92 with a median of 1.21% and was dominated by *Festuca pseudovina* (16.43 ± 2.44%), *Cynodon*

dactylon (8.29 ± 2.36%), and *Festuca rupicola* (6.29 ± 1.89%). Finally, **Treatment V** recorded the highest mean abundance at 0.74% ± 1.66, with a median of 0.86%, where the dominant species were *Festuca pseudovina* (13.57 ± 5.56%), *Festuca rupicola* (5.0 ± 0.0%), and *Achillea collina* (4.71 ± 1.8%).

The average species count per treatment, showed significant differences across treatments, as indicated by a Kruskal–Wallis test result ($\chi 2 = 10.8$ $p = 0.03$). **Treatment I** had a mean species count of 27.86 ± 5.15, suggesting moderate species diversity. **Treatment II** displayed a higher mean species count of 36.14 ± 6.64, indicating increased richness. **Treatment III** recorded a mean species count of 29.29 ± 4.96, aligning with moderate diversity. **Treatment IV** had a mean species count of 30.29 ± 4.31, comparable to Treatments I and III. However, **Treatment V**, showed the highest mean species count at 42.43 ± 8.18, substantially greater than the other treatments, indicating that this treatment promoted significantly higher species richness. These patterns were reinforced by Dunn's pairwise test with Holm adjustments, highlighting significant differences between Treatment V and Treatments I, III, and IV (Holm-adjusted *p*-values of 0.01, 0.04, and 0.04, respectively).

The 2020 results reveal consistent but low species abundance across treatments, with dominant species varying by treatment. In terms of species richness, Treatments II and V supported higher diversity, with Treatment V showing a particularly high mean species count, suggesting that this treatment promoted a more diverse assemblage. Contrastingly, the more moderate species counts observed in Treatments I, III, and IV highlight differences in how each pasture management strategy influenced community composition and biodiversity outcomes.

From a grassland management perspective, grass species were pivotal in this study, with five species—*Dactylis glomerata*, *Elymus repens*, *Poa angustifolia*, *Festuca rupicola*, and *Festuca arundinacea*—showing an average coverage exceeding 10%. Economically valuable legumes were not abundant, with *Lotus corniculatus* only appearing significantly in the overseeded abandoned field, averaging 3.89% cover, and *Trifolium repens* covering 8.39% in the alfalfa area. The dominance of *Medicago sativa* on the alfalfa field declined notably. Among other dicotyledons, only a few species exceeded 5% average cover: *Achillea collina*, *Achillea pannonica*, *Sanguisorba minor*, *Scabiosa ochroleuca*, and *Taraxacum officinale*, with *Sanguisorba minor* and *Scabiosa ochroleuca* confined to quadrats in the overseeded area.

The analysis of the species number across various treatments and categories in 2012 and 2020 provides insights into how different types of vegetation respond to specific management practices over time. In this study, five treatments (I, II, III, IV, and V) were analyzed with each treatment, including the four primary categories of vegetation: *Fabaceae*, Grass, Other, and Weed. Descriptive statistics were calculated for each category within each treatment to provide a detailed overview of species distribution in terms of mean values and standard deviations (Figure 4).

In 2020, the distribution of species in Treatment I showed a particularly high mean species count in the "Other" category, with an average of 15.00 ± 4.50 species. This was considerably higher than the *Fabaceae* category in the same treatment, which averaged 4.20 ± 1.35 species. Such disparities between categories were evident across other treatments in 2020 as well. For instance, in Treatment IV, the mean species number for the "Other" category was 18.50 ± 4.50, again markedly higher than *Fabaceae*, which had a mean of 4.00 ± 1.20 species. The "Grass" and "Weed" categories also demonstrated variability in species numbers across treatments in 2020, although their values generally fell between those of *Fabaceae* and Other. In general, the category "Other" consistently displayed higher species numbers across treatments, suggesting that certain treatments in 2020 may have been more conducive to supporting diverse species in this category.

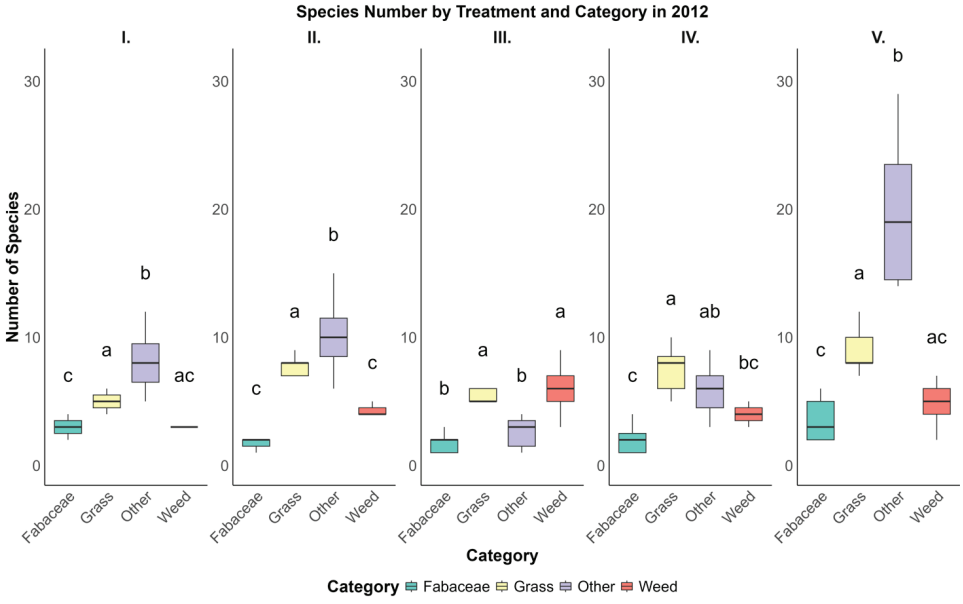


Figure 4. Species distribution across vegetation categories under five pasture treatments (I–V) in 2012, showing notable differences in species numbers across categories. The 'Grass' category demonstrated relatively high species richness in multiple treatments, while *"Fabaceae"* generally exhibited lower counts. Letters above the boxplots denote statistically significant differences between categories within each treatment based on Tukey's HSD test ($p < 0.05$).

Conversely, in 2012, the patterns were somewhat different. For example, in Treatment II, the "Grass" category exhibited a relatively high mean species count of 8.00 ± 1.63, whereas "*Fabaceae*" was again lower, with an average of 3.00 ± 1.23 species. This trend in relatively lower species counts in the *Fabaceae* category continued across other treatments in 2012, indicating a consistent pattern where *Fabaceae* was less represented in terms of species number compared to Grass and Other. Treatment III in 2012 also showed a similar distribution, with the Grass category having a mean species number of 5.00 ± 1.35, which was higher than *Fabaceae*, averaging 2.80 ± 1.15 species. These findings suggest that the *Fabaceae* category may inherently have lower species diversity or that the environmental conditions or treatment practices in 2012 were not as favorable for its proliferation.

To further investigate the effect of the treatments and categories on species numbers, a two-way ANOVA was conducted for each year. In 2020, the analysis revealed a significant effect of treatment on species number, with an F-ratio of $F(4, 24) = 15.42$ and a p-value of <0.001, indicating a statistically significant impact of treatment type on species numbers. Similarly, there was a significant effect of category on species number, $F(3, 24) = 18.33$, $p < 0.001$, confirming that the category to which a species belongs also significantly influenced the number of species observed (Figure 5). This result highlights the importance of both treatment practices and the specific type of vegetation in determining biodiversity outcomes in 2020. In 2012, the ANOVA results displayed a comparable trend. The effect of treatment on species number was again significant, with $F(4, 24) = 10.11$ and a p-value of <0.001. The effect of the categories was also significant, with $F(3, 24) = 12.75$, $p < 0.001$, suggesting that both factors—treatment and category—were crucial determinants of species number in that year as well.

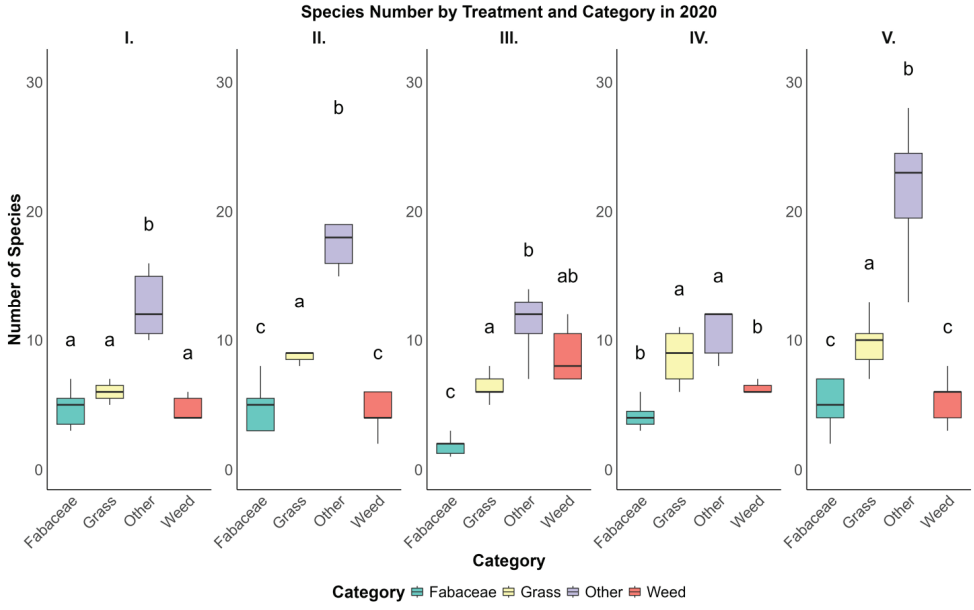


Figure 5. Species richness across different vegetation categories under five pasture treatments (I–V) in 2020. Higher species numbers were observed in the 'Other' category, especially under Treatment I and IV, indicating a favorable response to these treatments. Letters above the boxplots represent significant differences between categories within each treatment, as determined by Tukey's HSD test ($p < 0.05$).

To pinpoint specific differences between categories within each treatment, a post hoc analysis using Tukey's HSD test was employed. This analysis provided more granular insights into how individual categories differed from one another within each treatment. In 2020, the results from Tukey's test indicated that in Treatment I, the "Other" category, with a mean species number of 15.00 ± 4.50, had significantly higher species counts compared to the "Weed" category, which averaged 8.50 ± 2.50 species, with a *p*-value of 0.034. This significant difference suggests that Treatment I in 2020 was more favorable for species in the "Other" category than for those in the "Weed" category. Additionally, in Treatment IV, the "Other" category again had a notably higher mean species number (18.50 ± 4.50) than *Fabaceae* (4.00 ± 1.20), with a highly significant *p*-value of <0.01. These findings imply that certain treatments in 2020 may promote species diversity in categories such as "Other" more effectively than in others like *Fabaceae*.

The 2012 Tukey's HSD test results revealed distinct patterns as well. In Treatment I, the Grass category, with an average species number of 8.00 ± 1.63, had significantly higher counts compared to *Fabaceae*, which had a mean of 3.00 ± 1.23 species ($p = 0.049$). This result indicates that in 2012, Treatment I supported a higher diversity of Grass species relative to *Fabaceae*. Similarly, in Treatment V, the "Weed" category, with an average of 6.50 ± 2.01 species, showed a significantly higher species count compared to "*Fabaceae*" (2.20 ± 0.98), with a *p*-value of 0.021. These differences highlight the variability in species support across treatments and suggest that specific treatments in 2012 may have favored weed species over *Fabaceae*.

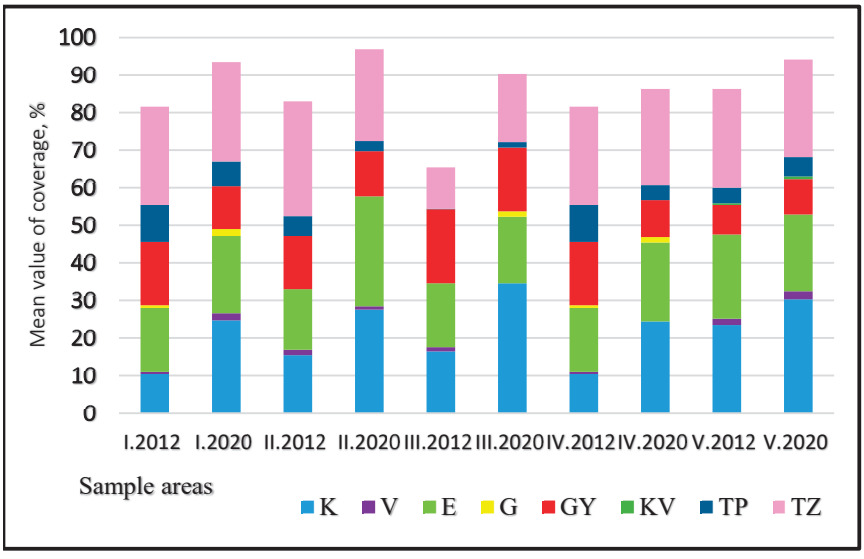
In summary, this analysis reveals that both treatment type and category significantly influence species number, with notable differences observed between 2012 and 2020. The 2020 data generally show an increase in species numbers across most treatments compared to 2012, particularly in categories such as "Other" and "Weed." This could be attributed to changes in environmental conditions or detailed treatment practices that may have

been implemented between 2012 and 2020, potentially creating more favorable conditions for a wider variety of species. On the other hand, categories like *Fabaceae* consistently exhibited lower species counts across treatments and years, suggesting that this category may either have lower inherent species diversity or may not respond as positively to the treatments applied.

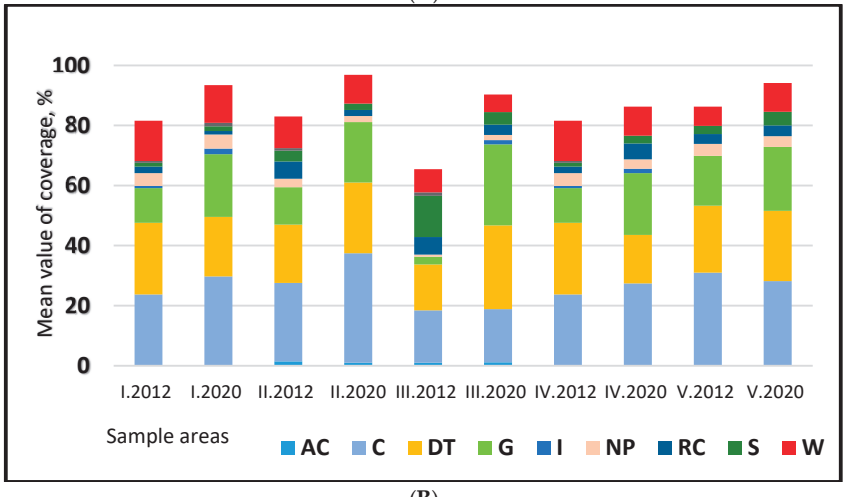
Results Based on Relative Ecological Indicators

Across all sample areas, the proportion of natural disturbance-tolerant species (TZ) was the largest. Among the community-forming species, *Festuca pseudovina* and *Poa angustifolia* were prominent, while among natural disturbance-tolerant species, *Achillea collina*, *Dactylis glomerata*, and *Lotus corniculatus* were observed (Figure 6A). In 2012, *Achillea collina*, *Dactylis glomerata*, and *Festuca arundinacea* were dominant, and the dominance of *Achillea collina* and *Dactylis glomerata* persisted into 2020. Weed species (GY) were still significant in 2012, especially in the direct-sown (III) sample area. *Stachys annua and Taraxacum officinale* were notable in sample areas I, II, and IV in 2012, but their proportions decreased across all areas by 2020. *Convolvulus arvensis* and *Cirsium arvense* also appeared in 2012 with coverage values of 1–3%. *Convolvulus arvensis* was found in every sample area in 2012, though its proportion declined by 2020. Despite being a competitive weed, *Cirsium arvense* did not achieve significant dominance by 2020. The distribution of species conservation categories similarly reflected a predominance of natural disturbance-tolerant species (DT) across the sample areas (Figure 6B). Coverage values for ruderal competitors (RC) were high even in the direct-sown area. The proportion of competitive species (C), which are natural vegetation species, increased across all areas by 2020 (e.g., *Agrostis stolonifera*, *Bromus inermis*, *Bromus erectus*, *Festuca pratensis*, *Festuca rupicola*, *Festuca pseudovina*). Additionally, the abundance of generalist species (G) increased markedly.

In terms of life forms based on Pignatti's classification, perennial grasses (H caesp) dominated all five sample areas (Figure 7). In the spontaneously grassed area (I), *Festuca rupicola*, *Poa angustifolia*, and *Dactylis glomerata* represented the largest proportions of the grass-forming species (H caesp). Among grasses, the proportion of erect-stemmed species (H scap) was also notable. In the area covered with chopped hay mulch (II), grass-forming and erect-stemmed species were predominant, with rosette-forming perennials (H ros), represented by *Taraxacum officinale* and *Plantago lanceolata*, also present in 2012. The amount of rosette-forming perennials increased across all sample areas by 2020, with the abandoned Treatment IV (alfalfa field) showing the highest proportion. Among rhizomatous geophytes (G rhiz), *Elymus repens* had particularly high coverage values. In terms of life form diversity, the overseeded old fallow Treatment V (grassland) and the area with chopped Treatment II. (hay mulch) were the most varied. In the direct-sown area (III), *Elymus repens* was the most prominent among rhizomatous geophytes, while *Bromus inermis*, *Dactylis glomerata*, *Poa angustifolia*, and *Festuca arundinacea* were notable grass species. This sample area had the lowest diversity in life forms. As an indicator of grazing pressure, creeping-stemmed perennials (H rept) and rosette-forming species (H ros) were present in significant amounts. However, by 2020 these species did not become dominant. Coverage of *Plantago lanceolata*, a rosette-forming species, increased across all sample areas by 2020, whereas *Taraxacum officinale* coverage decreased, resulting in no net increase in the category's coverage. Among creeping-stemmed species, *Trifolium repens* was the most significant, but it appeared at higher proportions only in the spontaneously grassed area (I) and the hay mulch-covered area (II) in 2020 and in the direct-sown grassland (III) in 2012.



(**A**)



(**B**)

Figure 6. Distribution of species by conservation status in different treatments I–V: ((**A**): conservation status; (**B**): social behavior types) (K: companion species, V: protected species, KV: highly protected species, E: association-forming species, GY: weed species, G: economic plants, TP: pioneer species, TZ: disturbance tolerant; AC: aggressive competitors, C: competitors, DT: interference-tolerant plants in natural habitats, G: generalists, I: introduced and emerged crops, NP: natural pioneers, RC: rudimentary competitor of native flora, S: specialists, W: weeds).

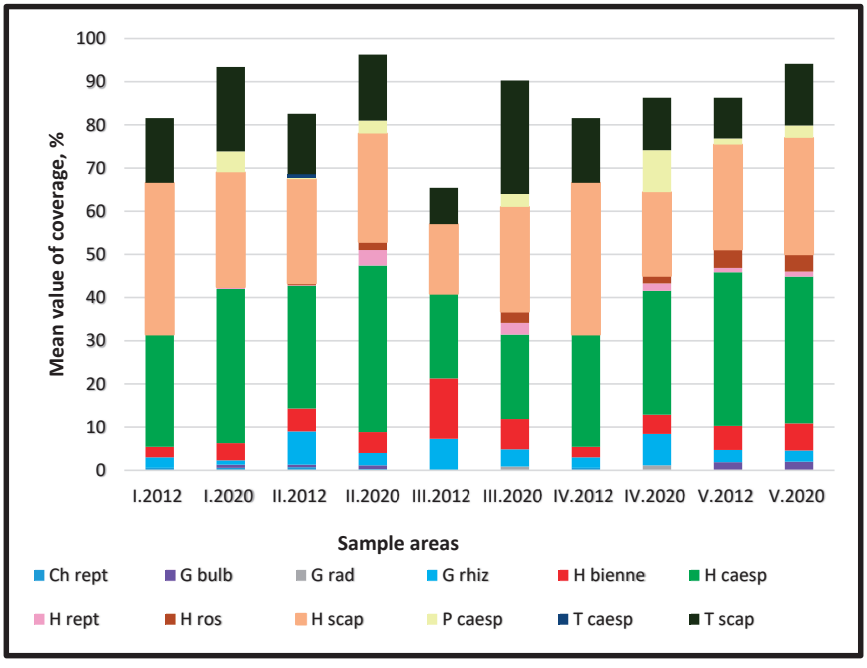


Figure 7. Distribution of species based on Pignatti's life-form types across sample areas of different treatments I–V.

Estimates based on 2020 data indicated an increase in above-ground green biomass in most sample areas compared to 2012 (Table 1). The amount of green biomass increased in most sample areas from 2012 to 2020.

Table 1. Expected green biomass by the Balázs method across sample areas (Treatments I–V; I: spontaneously grassed fallow, II: hay mulch area, III: sown grassland post-full soil preparation with two seed mixes, IV: abandoned alfalfa field undergoing transformation, V: 30-year-old oversown grassland subjected to mowing and grazing).

Sample Areas	**I**	**I**	**II**	**II**	**III**	**III**	**IV**	**IV**	**V**	**V**
study years	2012	2020	2012	2020	2012	2020	2012	2020	2012	2020
height (cm)	20	25	28	30	40	30	35	25	30	30
green mass t/ha	5.4	9.4	4.9	14.1	6.5	10.0	8.6	7.0	9.5	14.8

The quality of the fodder, forage value, which can be calculated based on the Balázs method, and is given by the product of the cover values of the rotating species and the quality values determined by Balázs, did not develop in parallel with the green biomass (Figure 8). In the area with abandoned alfalfa field treatment IV, it even decreased significantly. In the other sample areas, compared to 2012, these quality values leveled off by 2020.

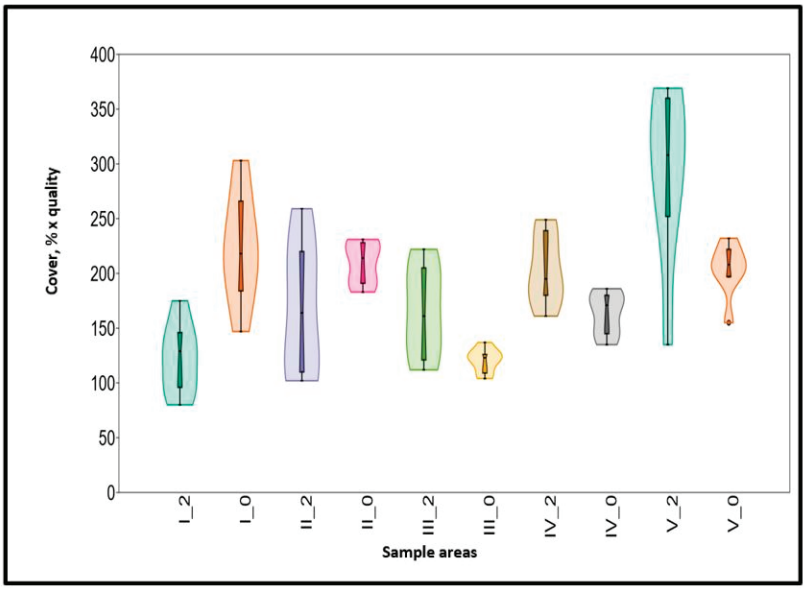


Figure 8. The development of forage values according to Balázs between 2012 (2) and 2020 (0) in all sample areas (treatment I–V).

4. Discussion

The study area, with poor soil productivity and situated in an extremely arid climate, reflects the significant impacts of recent climate change trends observed in the Carpathian Basin [70–72]. Pannonian grasslands increasingly exhibit trends similar to those in semi-desert or Mediterranean regions, where the abandonment of grasslands leads to drastic changes and vegetation degradation [73–75]. Originally classified as a dry grassland type, the study area was unsuitable for intensive agricultural use, likely hosting rocky grassland or semi-natural vegetation [76].

A detailed examination in 2012 revealed that diverse restoration techniques resulted in significant differences among the five sample areas. Only 3.7% of the species observed that year were present across all plots, consisting entirely of disturbance-tolerant species (*Dactylis glomerata, Festuca arundinacea, Potentilla argentea, Trifolium pratense*) [61,62]. By 2020, the number and proportion of shared species increased to 11%, with additional disturbance-tolerant species (*Agrimonia eupatoria, Bromus hordeaceus, Elymus repens, Trifolium repens, Achillea collina*) present. Two natural competitor species, *Agrostis stolonifera* and *Festuca pseudovina*, appeared in all sample areas, suggesting improved ecological stability, particularly due to the dominant *Festuca pseudovina* [76].

The area was mowed for two years following grassland restoration, significantly enhancing species richness across all sample sites. Studies show that mowing promotes vegetation establishment and stability, suppressing weeds and facilitating the establishment of introduced species [2,65,77,78]. Mowing also reduces litter, which can help establish target species in newly sown or overseeded areas. This practice is frequently used to control weeds, as natural species generally exhibit lower competitive capacity than introduced grasses [79–82]. Additionally, a nearby propagule source greatly benefited the sample areas.

Grazing following mowing further improved grassland structure, corroborating findings by Price et al. [3] that controlled grazing has positive effects on grassland dynamics. Conversely, vegetation can deteriorate with grazing cessation, leading to degradation. Grazing animals selectively consume green biomass, favoring certain plant groups, although this effect varies by site and climate. In fertile temperate areas, grazing often increases the abundance of grasses [82], while semi-arid regions, like savannas, experience a decrease in

grasses under grazing [83–85]. Rodríguez et al. [86] showed that grass abundance increased on sheep-grazed plots in the Pyrenees, while forbs dominated cattle-grazed plots. In the current study areas, we observed a decrease in grass species diversity but an increase in their coverage values. Broad-leaved grasses (*Dactylis glomerata*, *Elymus repens*, *Festuca arundinacea*) declined under grazing, while narrow-leaved grasses (*Festuca pseudovina*, *Festuca rupicola*) increased. This dynamic, essential for restoring natural vegetation in semi-arid climates, illustrates that robust cattle grazing reduces tall, broad-leaved grasses while promoting the spread of lower, narrow-leaved grasses, aiding in natural vegetation restoration. *Cynodon dactylon*, a creeping species common to arid environments, also increased under grazing [63].

Grazing intensity is crucial for species replacement, as higher intensity creates more bare patches, raising weed invasion risks [86,87]. Weed reduction across all sample types indicates appropriate grazing pressure without overgrazing. Grasses are dominant, contributing to both ecological structure and grassland management value. The presence of Festuca species, particularly *Festuca pseudovina* and *Festuca rupicola* (the latter characterizes *Salvinio nemorosae-Festucetosum rupicolae* association), highlights their role in supporting potential native slope steppe vegetation [76]. *Festuca arundinacea,* a Eurasian forage grass widely planted in North America [88], also plays a significant role in overseeding in the Pannonian region. Records indicate its previous use in an overseeding effort in 1989, though detailed methods and species were unavailable for this study [88,89]. *Elymus repens*' abundance and behavior are notable under grazing, as its ecological resilience and nutritional value make it valuable under arid climate conditions despite its weed classification by Borhidi [62] and Simon [61]. *Elymus repens* occurred in notable coverage in three plots in 2012 (II, III, and IV) and plays a structural role in abandoned alfalfa fields, where species richness remained low in both observations (44 and 70 species). *Poa angustifolia* became dominant in these fields, a species known for colonizing fallows [90].

An analysis of life forms following Pignatti's classification further supports that the sample areas were not overgrazed, as creeping or rhizomatous perennials (H rept) and rosette-forming species (T ros and H ros) did not dominate [63,64,91,92]. Rosette perennials (H ros) were most abundant in the overseeded alfalfa field (IV), likely due to lower cover values and increased grazing pressure. These findings align with Tóth et al. [81], who also found that spontaneously recovering grasslands achieved the best ecological states, featuring high native species richness and cover similar to those observed in this study. Although seeded grasses initially suppressed unwanted species, natural target species representative of grassland ecosystems were slower to establish.

5. Conclusions

Addressing our initial hypothesis and research questions, we observed that grassland establishment types resulted in substantial differences. Although some differences between the various grassland types persisted by the end of the study period, especially post-grazing, the magnitude of these differences diminished over time. Grazing with Hungarian Grey cattle significantly enhanced species composition across all grassland types, notably increasing the abundance of ecologically valuable species. The coverage and agricultural value of each grassland type also showed improvement in parallel.

The critical question arises: will the key to restoring semi-arid and arid Pannonian grasslands be the method of grassland establishment or grazing with robust cattle breeds? Our findings suggest that grazing with Hungarian Grey cattle offers a balanced solution, akin to mixed cattle and sheep grazing systems seen in the Pyrenees [86], which maintains a balance between grass and forb species, allowing neither to dominate excessively.

Of the seeded grass species in the sown grasslands, *Lolium perenne, Festuca pratensis*, and *Festuca rubra* were absent as early as 2012, indicating their unsuitability for dry grasslands. *Festuca pratensis* appeared only once in sample site V in 2012 but disappeared by 2020. Bromus inermis persisted with low coverage in the direct-sown grassland (III) in both 2012 and 2020. Only *Dactylis glomerata* and *Festuca arundinacea* remained from the seed

mixture, though these species were present across other sample areas as well. Therefore, the applied seed mix was uneconomical for dry grasslands. We recommend the use of *Festuca species* commonly found and structurally significant in dry grasslands (*Festuca pseudovina* and *Festuca rupicola*) alongside *Dactylis glomerata* and *Festuca arundinacea*.

Direct seeding and alfalfa-seeding grassland establishment approaches were also found to be inefficient. Instead, promoting natural grassland regeneration on fallow lands, especially when covered with hay mulch, proved the most effective restoration method and is recommended. From ecological, grassland management, and economic perspectives, these approaches offer the most viable solutions.

The restoration's success is underscored by the massive occurrence of *Orchis morio* in the overseeded fallow area (V) by 2022 (Figure 9).



Figure 9. Massive occurrence of *Orchis morio* in the overseeded fallow area.

Overall, the findings underscore the complex relationship between treatment practices and vegetation type in shaping biodiversity outcomes. These results suggest that some treatments may enhance species diversity in certain vegetation types while having limited effects on others. Future research could benefit from exploring the underlying ecological mechanisms that drive these differences, particularly examining how specific environmental conditions or management practices influence the growth and proliferation of different vegetation categories. Understanding these dynamics can inform more targeted conservation and management strategies, aimed at preserving or enhancing biodiversity across various ecosystems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/land13122135/s1.

Author Contributions: Conceptualization, K.P., S.S. and Z.W.; methodology, K.P., V.K., J.B. and S.S.; software, Z.K., P.P., F.P. and A.K.; formal analysis, Z.W. and E.S.-F.; investigation, K.P., E.S.-F., S.S. and I.T.-J.; writing—original draft preparation, K.P., V.K., E.S.-F., I.T.-J., Z.K., S.S. and J.B.; writing—review and editing, F.P., K.P. and E.S.-F.; supervision, S.S. and K.P.; funding acquisition, K.P. and Z.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by OTKA K-147342, the Strategic Research Found of the University of Veterinary Medicine Budapest (Grant No. SRF-002) and the Research Excellence Program of the Hungarian University of Agriculture and Life Sciences.

Data Availability Statement: The original contributions presented in this study are included in the Supplementary Material. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: Author Péter Penksza was employed by the company Anton Paar Hungary. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Article

Lessons Learned from the Last Moments Captured of Traditional Small-Scale Land Use in a European Fen Meadow

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Abstract: Most of Europe's semi-natural grasslands have been maintained by land use for thousands of years. Consequently, as a side effect of this type of land use, high grassland biodiversity was able to develop in those areas. Today, due to changes in land use, only fragments of the native grasslands and their biodiversity, especially in wetlands, remain. We conducted a study on changes in land use and the conservation context of a species-rich Hungarian fen meadow over 250 years. In addition to the main changes, we focused on sustainable grassland management. For our research, we built a geospatial database in which we attached great importance to georeferenced aerial photographs taken decades ago. To better understand what we can see in aerial photographs, we studied archived newspaper articles and conducted interviews. An aerial photograph taken in 1963 served as an exceptional illustration and data source for the key factors of sustainability and biodiversity. Our case study illustrates most of the major global problems affecting the European fen meadow (drainage, agricultural intensification, expansion of infrastructure networks, abandonment of farming). Based on our research, mosaic, adaptive, small-scale landscape use is necessary for the long-term sustainability of European wet grasslands and their special wildlife.

Keywords: aerial photograph; biodiversity; grassland management; land use history; nature conservation

Citation: Búzás, E.; Bódis, J. Lessons Learned from the Last Moments Captured of Traditional Small-Scale Land Use in a European Fen Meadow. *Land* **2024**, *13*, 2155. https://doi.org/10.3390/land13122155

Academic Editors: Michael Vrahnakis and Yannis (Ioannis) Kazoglou

Received: 5 November 2024
Revised: 7 December 2024
Accepted: 9 December 2024
Published: 11 December 2024



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1. Introduction

In the forest-rich landscapes of Europe, centuries of hard work by humans have turned forests, woodlands, scrubs, and marshes into treeless areas, where they can offer sufficient pasture and produce enough fodder (hay) for their animals [1]. Mowing and grazing contributed greatly to animal husbandry and thus to the livelihood of the population [2]; pastures and hay meadows were an essential part of the rural landscape [3]. This European cultural landscape has contained diverse mosaics of man-made and semi-natural habitats, with high grassland biodiversity resulting in outstanding cultural and conservation value [3,4]. Some of those anthropogenic grasslands have become biodiversity hotspots in Europe [5]. The wetlands, also deforested for animal husbandry, are valued not only because of their biodiversity but also for their ecosystem services. Their conservation and restoration are supported by EU directives and national legislation for nature protection in the various states [6]. Nevertheless, mainly due to changes in land use, only fragments of the native grasslands and their biodiversity, especially in wetlands, remain [7].

The main reason for their continued decline is that the draining and cultivation of wetlands has been going on for thousands of years [8]. Drainage of the peat soil causes a rapid mineralization process where oxidation and subsidence take place, all of which are irreversible changes [9] that lead to the disappearance of their unique vegetation and wildlife.

The other major driver of biodiversity loss is land use change [10]. During land use intensification, degradation occurred by the homogenization of grassland communities across trophic levels [11] that weakens the associations among the diversities of flora and fauna [12–14].

Lack of legal protection can also be a threat, with over 80% lost in Northern Germany since 1950 from wetlands that were not protected. They have mainly intensified and ploughed up these grasslands, which caused increasing fragmentation of floodplain meadows [15]. In Hungary, three of the Natura 2000 wet meadows habitat types (6440 Alluvial meadows of river valleys; 6410 Molinia meadows; 6510 Lowland hay meadows), have lost 90–91% of their area since 1775 [16].

The remaining nutrient-poor extensive pastures have significant botanical value, whether protected or not. Habitat types, which currently are not considered in the European Habitats Directive, should receive the same conservation attention [17]. It has highlighted the importance of existing wet meadows in the case of wildlife as well (see, e.g., wild bees: [18]; shorebirds: [19]). Their preservation has become a cultural and nature conservation objective of European importance [20]; serious restoration projects are underway to revive them [21–23]. However, the wet grassland abandonment studies mainly focus on vegetation, not any other ecosystem properties. Abandoned wet grasslands can be rehabilitated but complete restoration of their biodiversity is hardly feasible [24].

For all these reasons, managing wet grasslands rather than abandoning them is an EU-wide issue [2]. Much has been studied and experimented with to find out how best to treat them, usually using botanical approaches [25–30]. The best way to determine the best rehabilitation tools is to know as much as possible about the history of the system to be treated, including what effects and conditions have led to their current state [31]. The restoration design often applies traditional use, maintaining its elements [32].

In this paper, we presented a case study of how land use in a species-rich Hungarian fen meadow changed over 250 years to help inform and guide future conservation management. The history of use of the area is typical. Drainage, the development of transport infrastructure, and intensification and then abandonment in recent decades are all elements that are typical of European wet meadows. An aerial photograph taken in 1963 [33] has proved invaluable in the study of past land use, revealing many details of the landscape at that time. From botanical resources and interviews, we know the flora and vegetation of the area and details of the landscape use at the time. From this we are convinced that the 1963 aerial photograph [33] shows a land use practice that maintained a species-rich wet meadow complex and can therefore be of considerable help in planning future conservation management.

2. Materials and Methods

2.1. Study Site

The 120 hectare Batyk fen meadows (46°59′02.1″N 17°03′33.0″E) are located in the western part of Hungary (Europe) in the river Zala valley (Figure 1). It is not an isolated habitat but is part of the meadows along the river Zala and therefore belongs to the Alsó-Zala-völgy ('Lower Zala valley'; code: HUBF20037; area: 6561.86 ha) Sites of Community Importance (SCI) under the European Commission's Habitats Directive. The fact that it is a registered 'ex lege fen meadow' provides further legal protection at a national level. The climate is moderately cool (average annual temperature around 10 °C) and moderately humid (expected annual rainfall around 700 mm). There are Gleysols near to the river, while in local depressions, peaty Histosols prevail.

The vegetation of the Batyk fen meadows is a complex of different types of wet meadows. Alluvial meadows of river valleys (EU code: 6440) are characteristic in the vicinity of the river Zala, but most of the area is covered by Molinia meadows (EU code 6410) and lowland hay meadows (6510). In the northern part, there are remnants of alkaline mires (7230). The study site stands out from the surrounding areas for its richness of species. Its flora is well documented. In 2022, the occurrence of 40 species protected in Hungary

was documented. The largest number of individuals was *Iris sibirica* (>500,000 shoots), *Veratrum album*, and *Ophioglossum vulgatum* (both > 50,000 shoots). The population size of some typical fen species (*Carex appropinquata*, *C. davalliana*, *Parnassia palustris*) was found to be extremely low, and some previously reported fen species (*Eriophorum angustifolium*, *E. latifolium*, *Menyanthes trifoliata*, *Pedicularis palustris*, *Ranunculus lingua*) were not found [34]. The area also has a rich butterfly fauna. Butterfly species of community interest under the Habitats Directive are *Maculinea nausithous*, *M. teleius*, *Lycaena dispar*, and *Euphydryas aurinia* [35].

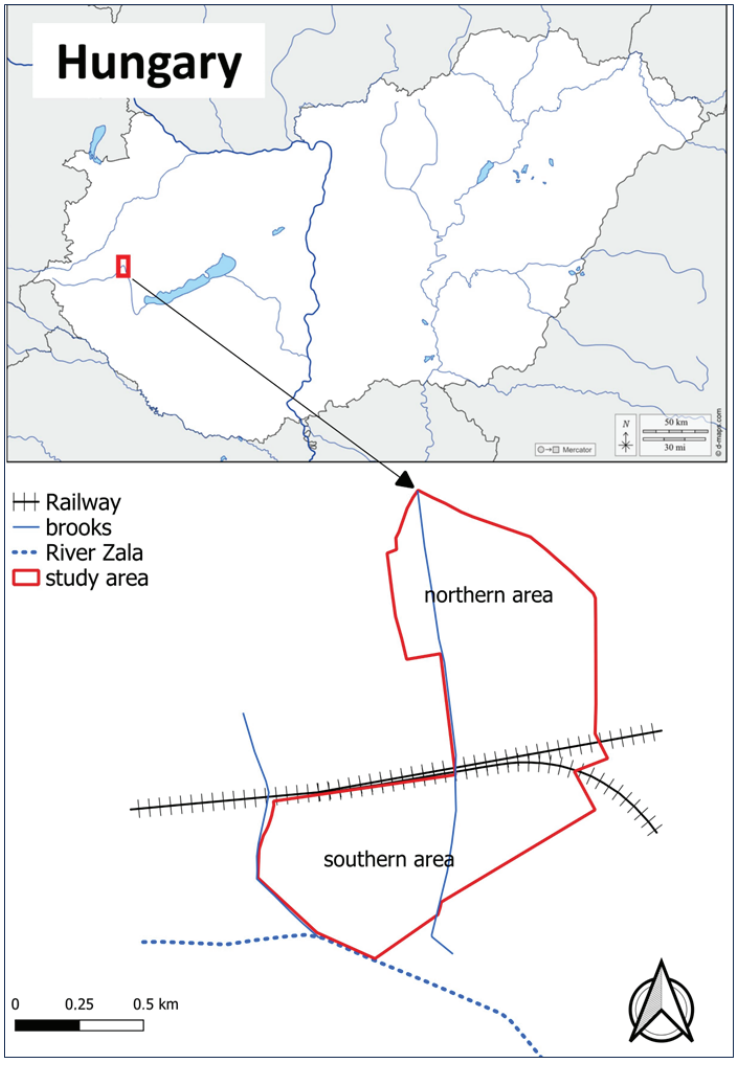


Figure 1. Study site.

2.2. Materials and Methods

For the historical analysis of the study area from a conservation perspective, we have collected different types of sources.

(1) The main sources were military maps of the Habsburg Empire, the first military survey (1784) [36], the second (1855) [37], the third (1879) [38], cadastral maps (1857) [39], WWII

Military Survey (1941) [40], aerial photos (1963–1988) [41], orthophotos (2000–2005) [42], and Google satellite images (2003–2022) [43]. All were available in a georeferenced format, or we rectified during our work with Qgis 3.16 software. We also used maps from the municipality's archives from the second half of the 20th century, but without an exact date. We interpreted the different patterns and practises of landscape change, drainage, land use, vegetation, and grassland management through maps.

(2) Using keywords, we searched for historical documents, written materials, and photographs in the most comprehensive digital databases in Hungary (Arcanum Digital Newspaper Database [44], Hungaricana—Cultural Heritage Portal [45], National Archives [46]), and the local catalogue of the Göcseji Museum [47]. The archive photographs were used to illustrate the main aspects of grassland management. The photographs were taken in the same geographical region, landscape, and similar vegetation as the study site.

(3) We have collected botanical data about the area.

(4) We conducted semi-structured interviews with four people: the mayor, a water specialist, and two older residents who have memories of the study area from their childhood. One of them is a key informant because he worked as a data collector in the local cooperative, during the communist period. The interviews lasted 1–2 h, and prior to the first interview, informed consent was obtained from all interviewees in compliance with the International Ethnobiological Society's Code of Ethics [48] and the European Union's General Data Protection Regulation [49].

We used the written documents, botanical data, and the interviews to improve the information gained from the map and orthophoto sources.

The data sources for each era differ greatly. It is therefore not possible to create and analyse standard, quantified, or quantifiable time series. It is not possible to construct a clear flowchart. We can therefore organise our data not into graphs, but only into pairs or groups of data. Comparison is therefore the most important methodological basis for our landscape research. By studying the past and present of our study area, and by comparing many maps, aerial photographs, and written sources, we draw a possible history of the area over the last 250 years. The data collected, which is considered reliable, must be interpreted and its validity established.

The information from different sources was grouped into pairs or groups of data, based on the era, event, or topic. Comparisons were made between these data and conclusions were drawn. For maps and other data sources organised in GIS systems, visual comparisons were made. For our written sources, we compared facts and findings. Visual and written sources, especially when complemented by interviews, enhance our understanding of the sources. Our aim is to organise the events and accounts of the past into a narrative story that can be described. The data sources of each period differ greatly from each other, so a pattern of understanding cannot be established. Different countries have different histories and therefore specific data sources. For Hungary, we have taken the methodological description of historical landscape ecology by Zsolt Molnár and Marianna Biró as a basis for our study [31].

3. Results and Discussion

3.1. Water Regulation

As early as 1773, it was recorded that the nobility wanted to free the river Zala valley from flooding because they could not cultivate it. The regulation of the river Zala began upstream from its mouth (Lake Balaton). The first interventions of the river took place between 1836 and 1865, but they did not meet the desired outcome, so further regulation took place between 1874 and 1894. Following the regulation, the river section of the study area was reached in 1929 [50]. The last period, between 1960 and 1970, was the time when the present riverbed was formed [Balogh; Liptai ex verbis]. The people of the village of Batyk attempted to make progress with the drainage of their meadows. In 1839, at the request of the locals, the county assembly discussed the question of draining the swampy meadows in the village area [50]. In 1843, a complaint was lodged against a local mill

because its water was backing up and flooding the surrounding meadows. At the same time, they asked for the bed of a brook to be straightened [50]. The cadastral map (1857) [39] shows that the brook (Csider brook) flowing through the study area was regulated and some drainage ditches were dug. For the study area, there is a mention from 1908 that ditches were made for drainage [51]. By 1963 [33], the surface drainage ditches had reached their final length, which was more than 10 km in the 120 ha area (Figure 2).



Figure 2. Surface drainage ditches highlighted with blue on aerial image from 1966 [52].

The water supply in the area has been drastically affected by modern infrastructure. Roads [36] bypassed the fen meadow from the north and this has remained unchanged since. This was not the case with the rail network. A railway was first built at Batyk towards the end of the 19th century [53]. Like the road, it bypassed the fen meadow from the north [40]. When this became obsolete and a more modern, stronger railway was needed, a new line was laid in 1970, cutting the fen meadow in two. Here, the railway junction was made more convenient and agricultural considerations were taken into account. The wet, marshy

land was less economically valuable, but where the old railway had been cleared, arable land could be created on its site [54]. The new railway has become the largest railway construction project in the county. First, the topsoil needed to be removed. Drainage pipes were installed at a depth of 2–3 metres, with cleaning wells every 50 metres. This was carried out over a length of 1 km, the aim being to significantly reduce the water table under the trackbed [55]. Once this was achieved, they could start building the rails. To build the railway line, 650,000 cubic metres of earth were moved [56]. The railway embankment rises 4–5 m above the fen meadow. The underground drainage (melioration) of the still wet areas of the region was started in 1973 in the hope of further agricultural use. The aim was to develop drainage on 570 ha [57]. In 1981, drainage work was carried out on 200 hectares of the wet, disused meadow at Batyk, which includes the part of our study area north of the railway line. Subsequent aerial photographs show that despite the drainage work, the area was not turned into arable field and was largely overgrown with either shrubs or trees. Inadequate design or construction could lead to drainage failure, which happened quite rarely. Only near the railway line were there small areas that had been mown for short or long periods.

The southern areas were excluded from drainage due to conservation efforts, but their water supply was also affected by the railway. In 2012, the Balaton-felvidéki National Park management tried to backfill drainage ditches in the southern areas [58]. We now know from experience that this did not solve the problem.

3.2. Changes in Landscape and Wildlife

Before the regulation of the river Zala (until the 18th century), the river flowed through swamps, marshes, and fens, sometimes forming small islands with several branches, sometimes without a clear channel [36,51]. The wetlands were so large that in 1596, the local people hid here from foreign soldiers when they were attacked [53]. These wetlands were along the river and were covered with reeds, sedges, alders, and ashes. The river Zala flooded its floodplain several times a year. Peat soil was common along the river. This peat soil was all formed in fens. At that time, several types of fens were still found in the valley. Part of this landscape was our study area, which was also a wetland with fens, swamps, and reedy areas with patchy woody vegetation (1784) [36,52]. In the mid-19th century, the northern part of the study area is depicted as treed, shrubby, wet meadow. On the map of the second military survey (1855) [37], the meadows along the river are marked in a different colour than the northern part of the study area (Figure 3). This is an important difference as the first botanical reports and the present surveys indicate that the northern areas were fens, while the southern areas were wet meadows [34,59]. Fen meadows are only preserved here, while today only wet meadows are found along the Zala.

In terms of hunting, the area was described in 1884 as follows: 'its extensive meadows are covered here and there with birch trees and small shrubs. Its southern part is bordered by the Zala river, its eastern and western parts by a crystal-clear stream flowing from a natural spring, and because of its favourable geographical position, it is the eldorado of the Common Quail (*Coturnix coturnix*) and the Grey Partridge (*Perdix perdix*) [60].

Between 1938 and 1955, 35 protected or rare plant species were recorded from the area [34]. An aerial photograph taken in 1963 [33] shows the entire area as grassland, with woody vegetation in the middle, as depicted on the map of the second military survey (1855) [37] (Figure 3). From current botanical surveys, we know that these wooded areas are alder swamps and willow carrs [34]. A few years before 1985, all the woody vegetation in the northern area was cut down [61]. A succession of scrub and woody vegetation then began, becoming dominant in the northern part. Compared to 1963 [33], the southern part has also begun to shrub, but still has the largest grassland cover. Today, the numbers and species of the most water-demanding fen specialist plant species are alarmingly low, but it remains a very valuable area. Several plant species have now been observed for the first time, but they are associated with drier grassland or forest habitats, which also indicates the drying of the area [62].

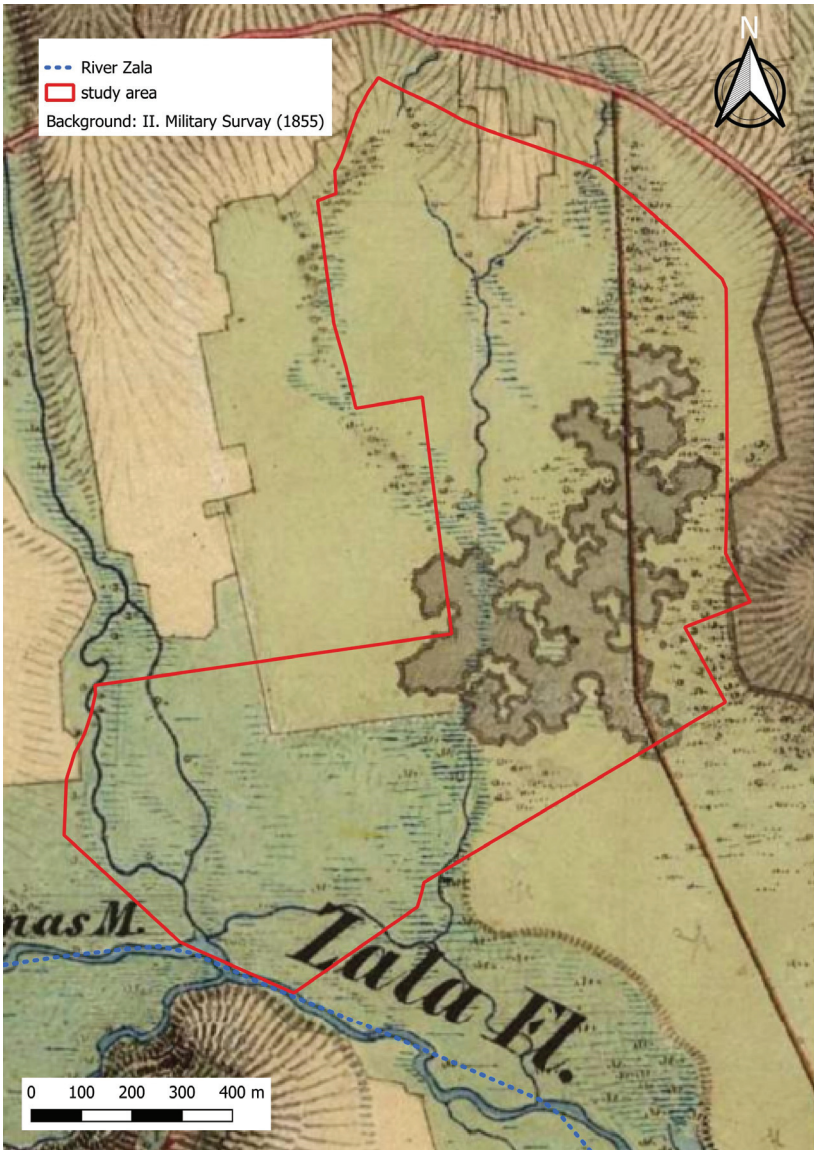


Figure 3. The study area on the map of the II Military Survey [37].

3.3. Grassland Management and Land Use

In the Middle Ages, the settlement of Batyk was not in an agriculturally favourable position as it was situated next to a large wetland. It was however advantageous for water mills and for crabbing, fishing [52], and grazing animals. According to a record from 1770, the meadow and pasture were of great value. The settlement suffered considerable damage from the frequent flooding of the river Zala, as well as from its wet and infertile soils [52]. They had a small area of grassland, which led to the renting of pasture from the neighbouring settlement in 1772 [52]. In 1854, the inhabitants of Batyk complained that their pasture had been taken away when the settlement boundaries were drawn [52].

According to the cadastral map of 1857, the study area consisted of many private meadow parcels. Each of these parcels was only a few metres wide, but long. Most of the Batyk families had land here because there was no other hay meadow in the village. Families harvested their own winter feed for their animals. The parcels were inherited by the owner's children, but they could also sell or exchange them. The village did have grassland outside the study area, but it was pasture, not mown [39]. These conditions did not change until the mid-20th century [38,40].

The first available high-resolution aerial photograph of our study area was taken on 16 September 1963 from an altitude of 1850 m. The 16,316 × 17,719 pixel image was enlarged to determine the relevant details [33]. The different stages of grazing, scything, haymaking, and hay transport can be seen in the area. At the time of the aerial photograph, the following six activities can be observed simultaneously: (a) cattle grazing; (b) scything; (c) loading hay onto carts; (d) transporting hay by ox cart; (e) haycocks and hayracks; and (f) scythed area without hayracks (already brought in) (Figures 4 and 5) Even then, the northern part was a wetter area than the southern part. Only at the end of the summer in dry weather when the heat had dried up the water could scything be performed, mostly for bedding, because there was a lot of sedge. If there was no rain, only then could they go on with the ox cart. If it was a rainy summer, the locals could not even scythe the fen meadow [Ódor ex verbis].

In the southern part, good hay could be mown for fodder, which was mown twice a year if the floods did not occur. The first time they mowed in early June, the second in August, and the grass that grew afterwards was no longer mown but grazed. The village had its own pasture, but sometimes they also grazed here, depending on how much grass had grown and the rainfall. They worked with hand tools, using scythes, rakes, and forks. The grass scythed was turned over on the third day to dry and air. On the 4th day, it was collected in haycocks. After that they made tall, pointed hayracks. The reason for the hayracks was that if it was dry but they could not bring it in and it rained, the entire hay would not become wet. The ox cart pulled up to the hayracks and they loaded as much hay as they could onto the cart. This work was carried out in a week or two. The whole family took part in this work often with the help of the neighbours. Some of them would throw the seeds that had accumulated in the manger and the hayloft back into the meadow. In the past, every plot of land was mowed, even during the communist period (1956–1989), but later many areas became overgrown. In Batyk, a farmers' cooperative was established in 1960, and from then it was no longer private land ownership. The Hungarian government forced farmers to join cooperatives with their lands and started an agricultural intensification [Hideg ex verbis]. The transition took a few years, during which time the aerial photograph was taken. Until the first tractors came along, in the late 1960s and early 1970s, they were still working with hand tools [Ódor; Hideg ex verbis]. As a supplementary nutrient, the manure was mainly applied to arable land but was also spread on meadows. Later, fertiliser was used in large quantities. The western side of the southern part is still mown and used by farmers. The eastern side is managed for nature conservation purposes only (occasional shrub and herb mulching).

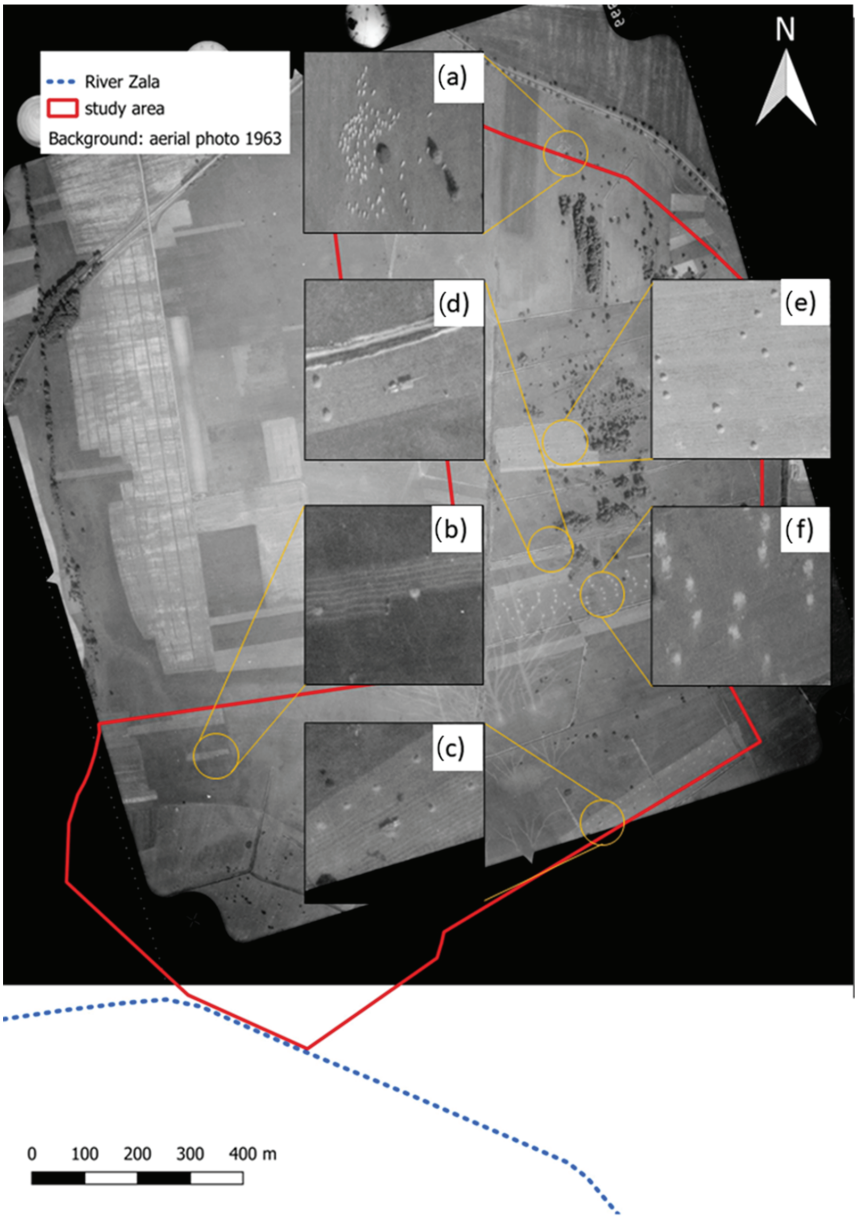


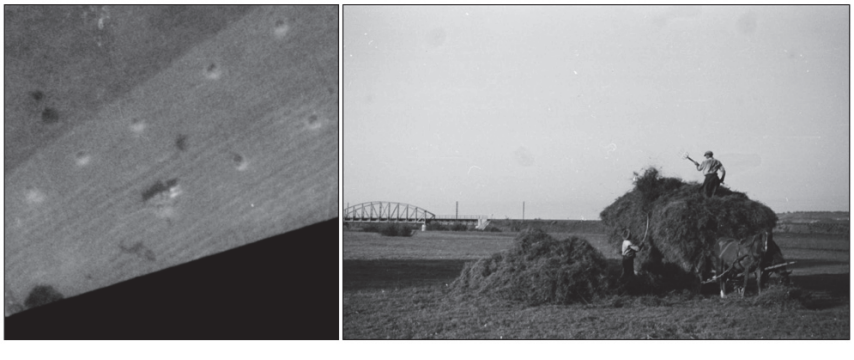
Figure 4. Observed grassland management activities from aerial photograph 1963 [33]: (**a**) cattle grazing; (**b**) scything; (**c**) loading hay onto carts; (**d**) transporting hay by ox cart; (**e**) haycocks and hayracks; and (**f**) scythed area without hayracks (already brought in).



(a)

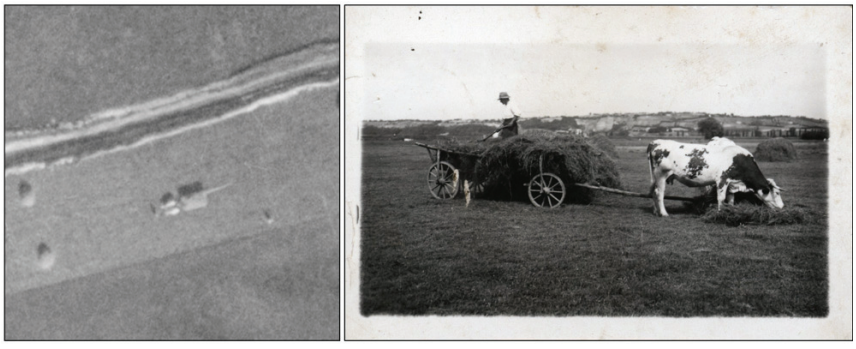


(b)



(c)

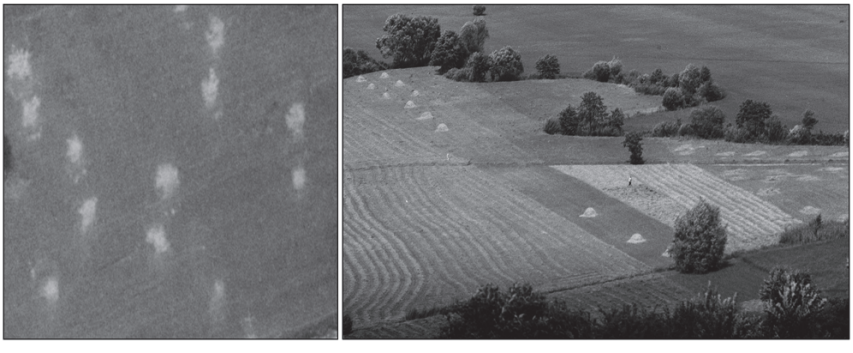
Figure 5. *Cont.*



(d)



(e)



(f)

Figure 5. The figure shows an illustration of the details extracted from Figure 4. In all cases, the image on the left is from the 1963 aerial photograph [33]. The right side of the image pair shows the same activity, illustrated with contemporary photographs from the same region on similar habitats. (**a**) [63] cattle grazing; (**b**) [64] scything; (**c**) [65] loading hay onto carts; (**d**) [66] transporting hay by ox cart; (**e**) [67] haycocks and hayracks; and (**f**) [68] scythed area without hayracks (already brought in).

Arable land was visible in the vicinity of the study area on the earliest maps, but grassland was much larger. Over time, more and more grassland was converted to arable land, until under communism in the 1980s the purpose was to convert the entire area

to arable land [69]. At that time, many meadows were ploughed up to where stands of Snakeshead Fritillary (*Fritillaria meleagris*) and Spring Snowflake (*Leucojum vernum*) were growing [Hideg ex verbis]. Most of our study area has never been ploughed, but small areas have been ploughed in the northern and southern parts. In places, ploughing was observed for shorter or longer periods at six locations during our surveys (Figure 6).



Figure 6. Observed arable fields from different times in study area [43].

3.4. Synergistic Effects of Interventions

Records from the 18th century exist about our study area, which is of importance from a nature conservation point of view, documenting the desire to regulate the river and drain the wetlands in order to make better use of the meadows, to create arable land and, in general, to improve agricultural use. River regulation and wetland drainage were carried out over a number of periods, at different stages, to varying degrees, and using techniques appropriate to the time. Until the beginning of the 19th century, the extent of water regulation depended on the skills of the local population and was therefore

limited. From the mid-19th century, state-sponsored, organised drainage companies were established, greatly increasing the scale and efficiency of water control, but still relying on manual and animal traction. In 1963, the railway had not yet affected the ecology of the area. River flooding, rainfall, and seepage not only affected the quality of natural and semi-natural vegetation, i.e., forage, but also the permeability of the area. For this reason, the timing and method of grazing or scything and the area covered could vary from year to year or even be combined. Swamp woodlands were formed along zones of seepage from the ground. These habitats can be even more persistent than grasslands, making them particularly valuable in grassland-dominated areas.

From the mid-20th century onwards, machinery increasingly improved the possibilities for water control interventions. Surface water drainage in our area reached its maximum by the 1960s at the latest, but in 1970, the groundwater level was reduced by underground drainage for the construction of the railway line. In the 1980s, underground drainage was used to convert all the surrounding wetlands and wet grassland into arable land. This technology could be used to dry out wetlands that could only be partially drained by surface drainage. In our case, the construction of the railway also involved significant drainage, which not only affected the water balance, but also the habitat fragmentation. Scattered throughout our study area, we detected areas that had been used as arable land for shorter or longer periods, but never permanently. Arable land that was abandoned for a longer period had regenerated well. The fact that the whole area was not permanently converted to arable land was partly due to poor drainage and partly due to conservation efforts at the time.

3.5. International Outlook

The history of our study area is, with minor differences, similar to the history of other wetland areas at a global or European level. The history of the area can be linked to the following international developments. The changes in wetlands through surface waterways and groundwater drainage were a global anthropogenic process that has been going on for thousands of years [8]. The main driver for changes, as we have also seen in our studied fen meadow area, is to achieve economic growth through the conversion to arable land [7,70]. The conversion to arable land is 50% responsible for the drainage of the Earth's wetlands. The peak of conversion occurred around 1950, when many countries had government support for this activity [7]. The situation was the same in Hungary in the 1970s and 1980s, where government support was extremely high [Balogh ex verbis]. In some wetlands, humans created grasslands. Some of those anthropogenic grasslands have become biodiversity hotspots in Europe [5]. But as the population grew, more and more grasslands were converted into arable land. From the first half of the 20th century, a high level of agricultural industrialisation occurred in Western European countries. This resulted in a massive decrease in the area of extensively managed land and fragmentation and decline in biodiversity. In most parts of Eastern Europe, these negative trends were not so marked until the switch to a communist economy from the 1950s to 1990s [2]. Before industrialisation and the communist economy, people used their grasslands extensively, which has partly helped preserve biodiversity [71,72]. It was the small-scale traditional management that impacted the biodiversity. It is now clear that the main cause of the biodiversity crisis is the change in traditional land use [73]. In addition to changes in farming, infrastructural interventions such as railways can also have a very negative impact on the wildlife in the area [74,75].

4. Conclusions

Humans have always shaped their environment, as far as they could, and they had different opportunities to do so in different ages. Looking at the history of our study area, we have identified the negative conservation events and highlighted the positive management that has maintained the habitats of rare species. We found a match between major local and global trends. In the European Union, at least some of the most valuable areas

have been protected [76], but identifying the most appropriate conservation managements is often difficult [22,30,77]. For conservation management, it is not enough to know the present, it is necessary to understand the history of the area [31,78]. In our case study, we have not only provided an example of a wetland conversion experiment in the second most drained country in the world [7], but we have also tried to explore and illustrate the characteristics of a land use that was not only sustainable but also contributed to the maintenance of the biodiversity of the area for centuries [34,60,67]. The aerial photograph [Figure 4] taken on 16 September 1963 proved invaluable in this respect as it was the very first aerial photograph of the area and the last to be taken before the area was prepared for intensive mechanised farming. In the aerial photograph [Figure 4], the different stages of vegetation are shown by the different shades of grey, which is a result of farming activity. A few years later, these vegetation structures were no longer visible. In that time, the traditional grassland management could follow changes in vegetation and environmental conditions, even in the short term. Scything and haymaking were carried out as quickly as possible, but the work could take several days or weeks because of the hand tools, the speed of the ox carts, and the time needed for the hay to dry. The small size of the 120 hectares meant that it was owned by many people, and the plots were often less than 10–20 m wide, but were long. Although families helped each other, work progressed at different rates in space and time. For this reason, it is possible that several haymaking and grazing operations can be seen at the same time in (Figures 4 and 5).

Humans have used and maintained grasslands but have not fundamentally altered natural processes. We are witnessing the last moment of a mosaic, an adaptive use of the landscape in space and time. This use, a mosaic in space and time, adapting to the landscape at a small scale, is the message that this image is intended to send to the future. It could be the key to sustainable agriculture and biodiversity. The aim cannot be to recreate the economic conditions and technological development of nearly 100 years ago, but the activities that sustained the landscape then can serve as inspiration for today's farmers and conservationists, so it is important to collect traditional ecological knowledge and expand research into the history of landscape use.

Author Contributions: Conceptualization, E.B. and J.B.; methodology, J.B. and E.B.; investigation, E.B.; resources, E.B.; data curation, J.B.; writing—original draft preparation, E.B.; writing—review and editing, E.B. and J.B.; visualisation, E.B.; supervision, J.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We would like to thank those who helped our research with their local knowledge, Balogh István, Hideg Józsefné Német Ilona, Liptai Gábor, Németh Klaudia, and Ódor Ferencné, and those who helped us methodologically, Magyari Máté, Saláta Dénes, and Walsh Orsolya.

Conflicts of Interest: The authors declare no conflicts of interest.

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ISBN 978-3-7258-3720-5