plants

Mediterranean Plants

Edited by

Sophia Rhizopoulou, Maria Karatassiou and Efi Levizou

Printed Edition of the Special Issue Published in *Plants*

www.mdpi.com/journal/plants

MDPI

Mediterranean Plants

Mediterranean Plants

Editors

Sophia Rhizopoulou
Maria Karatassiou
Efi Levizou

MDPI • Basel • Beijing • Wuhan • Barcelona • Belgrade • Manchester • Tokyo • Cluj • Tianjin

Editors

Sophia Rhizopoulou
National and Kapodistrian University of Athens, Panepistimiopolis
Greece

Maria Karatassiou
Aristotle University of Thessaloniki
Greece

Efi Levizou
University of Thessaly
Greece

Editorial Office
MDPI
St. Alban-Anlage 66
4052 Basel, Switzerland

This is a reprint of articles from the Special Issue published online in the open access journal *Plants* (ISSN 2223-7747) (available at: https://www.mdpi.com/journal/plants/special_issues/mediterranean_plant).

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

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

ISBN 978-3-0365-4861-6 (Hbk)
ISBN 978-3-0365-4862-3 (PDF)

Cover image courtesy of Sophia Rhizopoulou.

Contents

About the Editors

Sophia Rhizopoulou

Sophia Rhizopoulou is a Professor Emeritus at the Department of Biology, National and Kapodistrian University of Athens, Greece. She graduated in and completed her Ph.D. at the National and Kapodistrian University of Athens. She has been a visiting scientist at Kuwait University (Kuwait), Bielefeld University (Germany), and Sabanci University (Turkey), and a Consultation Board Member of IDOSI as well as the Vice President of the Scientific Sectoral Council in agricultural production, food, agrobiotechnology, and aquaculture, National Council for Research and Innovation, etc. Her research interests include ecophysiology, environmental science, plant biology and physiology, Mediterranean plants, biodiversity, historical botany, roots and water uptake, biomimetics (...art imitates nature...), etc. See more at http://publicationslist.org/sophia.rhizopoulou.

Maria Karatassiou

Maria Karatassiou is an Associate Professor at the Laboratory of Rangeland Ecology, Aristotle University of Thessaloniki, Greece. Her research interests include plant ecology and evolution, plant–water relations, plant crown architecture and hydraulic architecture, the survival of forage species under biotic or abiotic stress, etc. See more at https://www.researchgate.net/profile/Maria-Karatassiou.

Efi Levizou

Efi Levizou is an Associate Professor at the Department of Agriculture Crop Production and Rural Environment, University of Thessaly, Greece. She graduated in and completed her Ph.D. at the University of Patras, Greece. Her research interests include alternative crop nutrition systems in the context of a circular economy in addition to their effects on plants' functional characteristics and the effects of abiotic stress factors on plant physiology and production. See more at http://agr.uth.gr/en/wp-content/uploads/2022/06/Levizou-short-CV-english_June-22.pdf.

Preface to "Mediterranean Plants"

Plants grown and exposed to Mediterranean climatic conditions are a source of information of a natural heritage. Mediterranean plants have been presented as vehicles for expressing historical knowledge and environmental attributes; scientific reports have given us important insights into plant growth, structure, and function. With the current environmental and social threats, mainly posed by expanding touristic and anthropogenic activities, the importance of Mediterranean plants will once again be appreciated. In this book, the function, structure, diversity, biogeography, conservation, seasonality, and interactions of Mediterranean plants with the abiotic and biotic environment are highlighted.

Sophia Rhizopoulou, Maria Karatassiou, and Efi Levizou
Editors

plants

MDPI

Article

Seasonal Changes in the Plant Growth-Inhibitory Effects of Rosemary Leaves on Lettuce Seedlings

Kwame Sarpong Appiah [1,2,*], Richard Ansong Omari [3,4], Siaw Onwona-Agyeman [5], Christiana Adukwei Amoatey [2], John Ofosu-Anim [6], Abderrazak Smaoui [7], Abdelkarim Ben Arfa [8], Yoko Suzuki [9], Yosei Oikawa [1], Shin Okazaki [1], Keisuke Katsura [1], Hiroko Isoda [10], Kiyokazu Kawada [10,*] and Yoshiharu Fujii [1]

[1] Department of International Innovative Agricultural Science, Tokyo University of Agriculture and Technology, 3-5-8 Saiwaicho, Fuchu 183-8509, Tokyo, Japan; yosei@cc.tuat.ac.jp (Y.O.); sokazaki@cc.tuat.ac.jp (S.O.); kkatsura@go.tuat.ac.jp (K.K.); yfujii@cc.tuat.ac.jp (Y.F.)
[2] Department of Crop Science, College of Basic and Applied Science, University of Ghana, Legon, Accra P.O. Box LG 44, Ghana; camoatey@ug.edu.gh
[3] Leibniz Centre for Agricultural Landscape Research, Institute of Land Use Systems, Eberswalder Str. 84, 15374 Muencheberg, Germany; talk2jafakingonline@gmail.com
[4] Institute of Agriculture and Horticulture, Faculty of Life Science, Humboldt-University of Berlin, Albrecht-Thaer-Weg 5, 14195 Berlin, Germany
[5] Institute of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwaicho, Fuchu 183-8509, Tokyo, Japan; agyeman@cc.tuat.ac.jp
[6] School of Architecture and Science, Central University, Tema P.O. Box 2305, Ghana; jofosuanim@gmail.com
[7] Centre of Biotechnology of Borj Cédria, BP 901 Hammam-Lif, Borj Cedria 2025, Tunisia; abderrazak.smaoui@gmail.com
[8] L'Institut des Régions Arides, Route du Djorf Km 22.5, Médenine 4119, Tunisia; abdelkarim.benarfa@ira.rnrt.tn
[9] Aromatic Repos, AHOLA, A2 Soleil Jiyugaoka, 1-21-3, Jiyugaoka, Meguro 152-0035, Tokyo, Japan; yoko86252539@gmail.com
[10] School of Life and Environmental Sciences, University of Tsukuba, Tennoudai 1-1-1, Tsukuba 305-8572, Ibaraki, Japan; isoda.hiroko.ga@u.tsukuba.ac.jp
* Correspondence: ksappiah90@gmail.com (K.S.A.); kawada.kiyokazu.gu@u.tsukuba.ac.jp (K.K.)

Citation: Appiah, K.S.; Omari, R.A.; Onwona-Agyeman, S.; Amoatey, C.A.; Ofosu-Anim, J.; Smaoui, A.; Arfa, A.B.; Suzuki, Y.; Oikawa, Y.; Okazaki, S.; et al. Seasonal Changes in the Plant Growth-Inhibitory Effects of Rosemary Leaves on Lettuce Seedlings. *Plants* **2022**, *11*, 673. https://doi.org/10.3390/plants11050673

Academic Editors: Sofia Rhizopoulou, Maria Karatassiou and Efi Levizou

Received: 30 January 2022
Accepted: 24 February 2022
Published: 1 March 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Abstract: Plant biodiversity has been studied to explore allelopathic species for the sustainable management of weeds to reduce the reliance on synthetic herbicides. Rosemary (*Rosmarinus officinalis* L., syn *Salvia rosmarinus* Spenn.), was found to have plant growth-inhibitory effects, and carnosic acid was reported as an allelochemical in the plant. In this study, the effects of seasonal variation (2011–2012) on the carnosic acid concentration and phytotoxicity of rosemary leaves from two locations in Tunisia (Fahs and Matmata) were investigated. The carnosic acid concentration in rosemary leaves was determined by HPLC, and lettuce (*Lactuca sativa* L.) was used as the receptor plant in the phytotoxicity bioassay. The highest carnosic acid concentration was found in rosemary samples collected in June 2011, which also had the highest inhibitory activity. Furthermore, a significant inverse correlation ($r = -0.529$; $p < 0.01$) was found between the inhibitory activity on lettuce hypocotyl and the carnosic acid concentration in rosemary leaves. Both temperature and elevation had a significant positive correlation with carnosic acid concentration, while rainfall showed a negative correlation. The results showed that the inhibitory effects of rosemary leaf samples collected in summer was highest due to their high carnosic acid concentration. The phytotoxicity of rosemary needs to be studied over time to determine if it varies by season under field conditions.

Keywords: Mediterranean climate; elongation; allelochemicals; specific activity; phytotoxicity

1. Introduction

Interference from weeds can have a significant impact on the growth and development of field crops, resulting in substantial crop production losses [1]. The use of synthetic herbicides to minimize crop loss due to weed infestation has become the predominant

weed control strategy. However, the global increase in herbicide-resistant/tolerant weeds has triggered the need to diversify the existing weed control practices [2,3]. Subsequently, there has been a growing interest in the utilization of natural products in the management of weeds. Secondary metabolites produced by plants have no direct role in the basic processes of plant growth and development. After being released into the environment, some of these bioactive molecules (allelochemicals) influence the growth and development of other surrounding species, a phenomenon known as allelopathy [4–6]. These compounds can improve a plant's ability to compete in its local environment [7–9]. Allelochemicals interfere with various physiological processes of plants, including respiration, photosynthesis, and hormone balance, to affect the germination and growth of surrounding plants [10,11].

The phytotoxic effects of plant species have been explored to diversify existing weed management strategies for sustainable agriculture [12,13]. Plant extracts have been utilized to control pests [14], and isolated allelochemicals have the potential to be used in weed control or herbicide formulation [15]. However, allelopathy is a complex phenomenon since the production and release of plant secondary metabolites can be altered by environmental conditions. Seasonal changes in biotic and abiotic variables, such as pathogen presence [16], temperature [17], precipitation [18], and nutrient availability [19], can have a significant impact on the production and release of allelochemicals, which can contribute to seasonal fluctuations in plant phytotoxicity. Furthermore, soil bacteria can break down allelochemicals into less hazardous molecules or transform them into more toxic compounds [20,21]. Although the concentration of allelochemicals in plant tissues (flowers, leaves, stems, bark, and roots) might change during the growing season [22,23], most studies on the potential phytotoxicity of plants focus on a particular evaluation period during the season. However, understanding the potential phytotoxicity of plant species and gaining insight into the ecological interactions of plants with their environment necessitates the investigation of seasonal fluctuation [24].

Rosmarinus officinalis L. (Lamiaceae), also known as rosemary, is an evergreen shrub that grows wild in the Mediterranean region. A recent phylogenetic analysis merged the genus *Rosmarinus* with the genus *Salvia*. *Rosmarinus officinalis* is now known as *Salvia rosmarinus* [25–27]. Rosemary is an aromatic plant with needle-like leaves. The plant is now cultivated worldwide and has several reported therapeutic properties, including antidepressant [28], antiproliferative [29], and antidiabetic [30] activities. Diterpenes (such as carnosol and carnosic acid) and rosmarinic acid, both with strong antioxidant activity, have also been found in rosemary extracts [31–33]. The leaf extract of rosemary was reported to be potentially phytotoxic, and carnosic acid was reported as an allelochemical in the leaves of the plants [34]. Rosemary leaves also contain volatiles such as 1,8-cineole, which showed inhibitory effects on lettuce growth [35]. Carnosic acid has only been identified in a few plant species, all of which belong to the Lamiaceae family [36–38]. Richheimer et al. [39] reported carnosic acid concentrations in rosemary leaves between 1.7% and 3.9%. Subsequently, rosemary cultivars such as Daregal, VAU3, 4 English, Farinole, and Severn Seas were developed with higher levels of carnosic acid (4–10% on a weight basis of air-dried leaves) [40]. In addition, the concentration of carnosic acid in rosemary can also be modulated by growing conditions and the influence of genetic background. Climatic and environmental stress both affect the production of carnosic acid in rosemary [38], which further increases the importance of phytotoxic evaluation of the plant under Mediterranean climatic conditions. Although there are studies on the seasonal variation of carnosic acid concentration in rosemary, the seasonal variation in the biological activities of the plant has mainly focused on antioxidant activity [37,41].

Consequently, there is no available report on the relationship between carnosic acid concentration and the inhibitory activity of rosemary leaves. This study, therefore, aimed to investigate (i) how carnosic acid concentration in rosemary leaves changes with the season, (ii) which environmental factors play a role in this change, and (iii) whether this seasonal dependence of carnosic acid concentration is related to the inhibitory effect of leaves on lettuce seedling growth.

2. Materials and Methods

2.1. Collection of Plant Samples

Plant samples were collected from the northern (Fahs) and southern (Matmata) parts of Tunisia (Figure 1). Matmata has an annual mean temperature of 20.6 °C, while Fahs has an annual mean temperature of 18.0 °C. The annual mean precipitation at Matmata and Fahs are 204 and 451 mm·year^{-1} respectively. Fahs belongs to the Mediterranean or steppe climate zone, while Matmata belongs to the desert climate zone with a drier climate [42]. The vegetations of the collection sites are affected by the Mediterranean climate, which has less precipitation in the summer. Matmata is drier than Fahs throughout the year. The monthly mean temperature and monthly mean precipitation at the sampling locations over the sampling period are shown in Figure 2.

The meteorological data for the sampling locations were assessed using WorldClim 2.1 [43]. These collecting sites were chosen because they feature rosemary-dominated vegetation, allowing rosemary plants from various climate zones to be compared. Sampling was done four times a year by randomly selecting five sites from each of the two areas of Fahs and Matmata. A total of 40 rosemary plant samples were collected from individual rosemary plants from June, September, and November of 2011, as well as February 2012. Sampling was done while avoiding spring when nutrients are used for flower growth rather than leaves. The sampling locations at Matmata were 535–620 m above sea level, whereas those at Fahs were 300–430 m above sea level. The elevation was recorded using a GPS (Colorado 300, Garmin, Olathe, KS, USA). The samples used in this study were only those collected in the growing season, each from a single rosemary plant (Table 1).

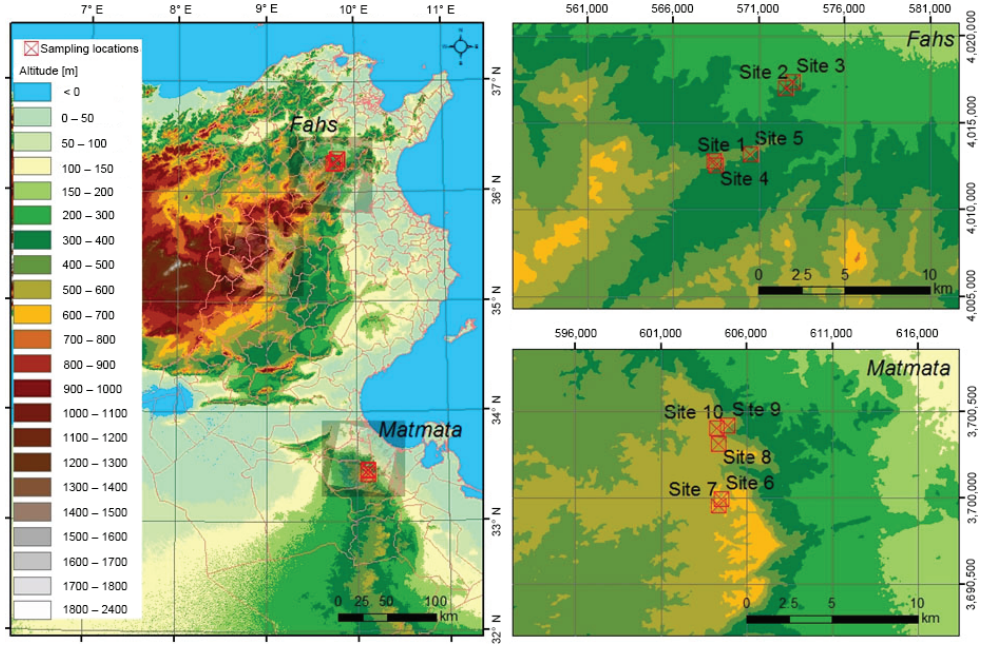


Figure 1. Map of sampling areas in Tunisia.

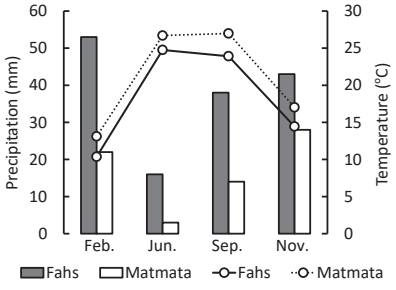


Figure 2. The monthly mean precipitation (bar graph: gray bar is Fahs; white bar is Matmata) and monthly mean temperature (line graph: the solid line is Fahs; the dotted line is Matmata) at the two sampling locations.

Table 1. Description of rosemary sampling sites (dates, areas, elevation) and specimen codes.

No.	Collection Date	Geographical Area	Elevation (m)	Sample Codes
1	June 2011	Fahs	430	UT-ARENA 00327
2	June 2011	Fahs	320	UT-ARENA 00334
3	June 2011	Fahs	300	UT-ARENA 00340
4	June 2011	Fahs	430	UT-ARENA 00349
5	June 2011	Fahs	420	UT-ARENA 00357
6	June 2011	Matmata	620	UT-ARENA 00364
7	June 2011	Matmata	585	UT-ARENA 00371
8	June 2011	Matmata	575	UT-ARENA 00379
9	June 2011	Matmata	535	UT-ARENA 00387
10	June 2011	Matmata	555	UT-ARENA 00395
11	September 2011	Fahs	430	UT-ARENA 00402
12	September 2011	Fahs	320	UT-ARENA 00411
13	September 2011	Fahs	300	UT-ARENA 00417
14	September 2011	Fahs	430	UT-ARENA 00426
15	September 2011	Fahs	420	UT-ARENA 00434
16	September 2011	Matmata	620	UT-ARENA 00442
17	September 2011	Matmata	585	UT-ARENA 00453
18	September 2011	Matmata	575	UT-ARENA 00460
19	September 2011	Matmata	535	UT-ARENA 00469
20	September 2011	Matmata	555	UT-ARENA 00478
21	November 2011	Fahs	430	UT-ARENA 00515
22	November 2011	Fahs	320	UT-ARENA 00523
23	November 2011	Fahs	300	UT-ARENA 00535
24	November 2011	Fahs	430	UT-ARENA 00543
25	November 2011	Fahs	420	UT-ARENA 00550
26	November 2011	Matmata	620	UT-ARENA 00559
27	November 2011	Matmata	585	UT-ARENA 00565
28	November 2011	Matmata	575	UT-ARENA 00574
29	November 2011	Matmata	535	UT-ARENA 00583
30	November 2011	Matmata	555	UT-ARENA 00587
31	February 2012	Fahs	430	UT-ARENA 00617
32	February 2012	Fahs	320	UT-ARENA 00622
33	February 2012	Fahs	300	UT-ARENA 00628
34	February 2012	Fahs	430	UT-ARENA 00633
35	February 2012	Fahs	420	UT-ARENA 00638
36	February 2012	Matmata	620	UT-ARENA 00647
37	February 2012	Matmata	585	UT-ARENA 00652
38	February 2012	Matmata	575	UT-ARENA 00657
39	February 2012	Matmata	535	UT-ARENA 00662
40	February 2012	Matmata	555	UT-ARENA 00667

UT-ARENA: the University of Tsukuba Alliance for Research on the Mediterranean and North Africa.

Each rosemary sample was given a unique ID (UT-ARENA management number) and stored at the Alliance for Research on the Mediterranean and North Africa's herbarium at the University of Tsukuba in Japan. Rosemary leaves were collected from the tops of the individuals that were the most exposed to the sun. The collected samples were air-dried in a well-ventilated room and then placed in a light-shielding bottle for storage in a cool and dark place.

2.2. Extraction Procedure

The crude extracts were obtained from the air-dried rosemary leaf samples. In brief, 200 mg of air-dried rosemary leaves of each sample were accurately measured and placed into a 50 mL falcon tube containing 20 mL of solvent (80% ethanol). The leaf–solvent mixture was sonicated for 30 min at room temperature, filtered through filter paper No.1 (Advantec Toyo Roshi Kaisha, Tokyo, Japan), and centrifuged using Hitachi himac CR22N (6000 rpm, 10 min); then, the supernatants were collected. The residue was re-extracted using the same procedure as above, and the supernatants were combined and used as the working solutions.

2.3. Chemicals and Reagents

Carnosic acid used in this study was purchased from Tokyo Chemical Industry (TCI, Tokyo, Japan). Formic acid and acetonitrile for analytical chromatography were purchased from Fluka, Sigma-Aldrich (Steinheim, Germany) and Fisher Scientific (Madrid, Spain), respectively. A Milli-Q system from Millipore (Bedford, MA, USA) was used to purify the water used in all the analyses.

2.4. High-Performance Liquid Chromatography (HPLC) Analysis

A total of 50 mg of ground rosemary samples (leaves) was accurately weighed, put into a 50 mL falcon tube, and extracted, as described in the extraction procedure. An aliquot of the extract after centrifugation was filtered through a 0.2 μm syringe filter before the injection of 10 μL in LC-20AD liquid chromatography (Shimadzu, Japan) for the HPLC analysis. An Inertsil ODS 2 column (250 × 4.6 mm, 5 μm particles, GL Sciences Inc, Tokyo, Japan) was used. Mobile phases A and B were water with 0.1% formic acid and acetonitrile, respectively. The column temperature was kept at 30 °C, and the flow rate of the mobile phase was set at 0.5 mL·min^{-1}. The following multistep gradient with different proportions of mobile phase B was applied: 0 min, 20% B; 10 min, 40% B; 15 min, 90% B maintained for 5 min. The initial conditions were maintained for 5 min. The analysis was monitored using an SPD-M20A detector at 210 nm. The quantification was done by comparing the peak areas of the targeted carnosic acid with the abundance of the compound in the corresponding standard used in the calibration curve. All chemical analyses were done in triplicate.

2.5. Phytotoxic Activity Bioassay

The radicle and hypocotyl elongation of *Lactuca sativa* (Great Lake 366, Takii Co., Kyoto, Japan) was evaluated in the phytotoxic activity bioassay using ethanol crude extracts of each of the 40 samples of rosemary leaves. In the phytotoxic activity bioassay, 40 samples of ethanol crude extracts of rosemary leaves were tested on the radicle and hypocotyl elongation of *Lactuca sativa*. The concentration range of the rosemary crude extracts (0.5, 1.0, 3.0, 5.0, and 10 mg DW·mL^{-1}) was adapted from a previous study [34]. In a 27 mm diameter glass Petri dish, a filter paper (27 mm, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) was inserted. A total of 0.7 mL of test solution was added to the filter paper and dried completely in vacuo. Five lettuce seedlings (pre-germinated for 20 h) were placed on the filter paper after adding 0.7 mL of 0.05% dimethyl sulfoxide (DMSO) and incubated (CN-25C, Mitsubishi Elec., Tokyo, Japan) for 52 h at 22 °C in dark conditions. The control treatments were set up with no crude extract but only 0.05% DMSO. Three replications were set for each

treatment. The radicle and hypocotyl lengths were measured after the incubation period, and elongation percentages were calculated using the following equation:

$$E = A/B \times 100 \quad (1)$$

where E is the elongation percentage, A is the average length of radicle/hypocotyl in the treatment, and B is the average length of radicle/hypocotyl in the control.

2.6. Statistical Analysis

The IBM statistics tool SPSS (SPSS Inc., Chicago, IL, USA, version 21) was used to analyze the data. Data were subjected to a two-way analysis of variance (ANOVA) to determine the significant differences among the samples collected in different months and locations. The sampling months and locations were considered as the independent factors in the analysis. Mean differences among the treatments were compared using the Tukey test at $p < 0.05$. Pearson's correlation analysis was conducted to establish significant relationships among the measured parameters.

3. Results

3.1. Variations in Carnosic Acid Concentration in Rosemary Leaves during the Growing Season

The concentration of carnosic acid in the leaves of rosemary samples collected from the two different locations (Fahs and Matmata) in Tunisia was studied over a growing season using reversed-phase high-performance liquid chromatography (RP-HPLC) (Figure 3). The equation for the calibration curve for carnosic acid was $y = 84051x + 240721$, $R^2 = 0.9994$. The limit of detection (LOD) and limit of quantification (LOQ) were determined at signal-to-noise (S/N) ratios of 3 and 10, respectively. The LOD and LOQ were 0.0150 mg·g^{-1} and 0.0455 mg·g^{-1}, respectively. This study focused primarily on carnosic acid, as it was previously found to be the major allelochemical responsible for the plant growth-inhibitory effect of rosemary leaves [34]. The results of this study showed that the accumulation of carnosic acid in rosemary leaves depended on the time of sampling.

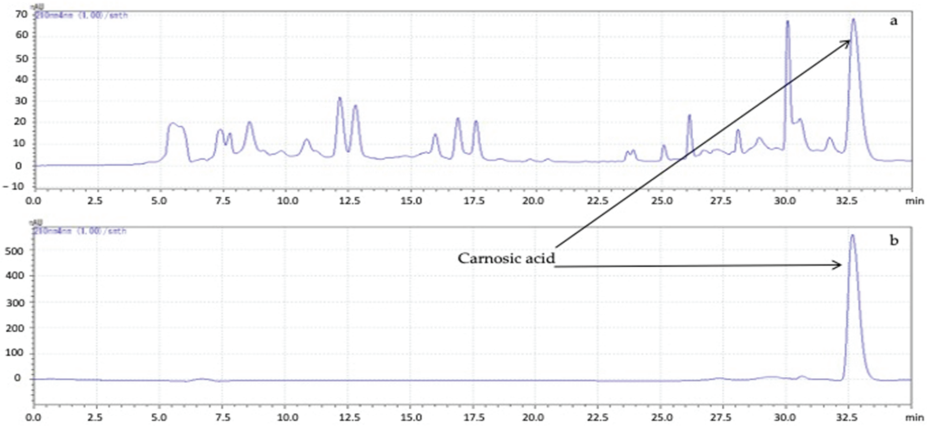


Figure 3. Chromatograph of an ethanol extract from rosemary leaves (**a**) and synthetic carnosic acid (**b**).

The carnosic acid concentration in leaves of rosemary samples collected in Tunisia during the study period varied widely between 2.9 and 28.4 mg·g^{-1} dry weight (Figure 4). The results showed that the highest average carnosic acid concentration (15.1 mg·g^{-1} dry weight) was measured in June (early summer), while the lowest concentration was measured in February (8.3 mg·g^{-1} dry weight) (Figure 5). It was observed that the concen-

tration of carnosic acid in the leaves of rosemary was higher in the samples from Matmata than in those from Fahs at all sampling times.

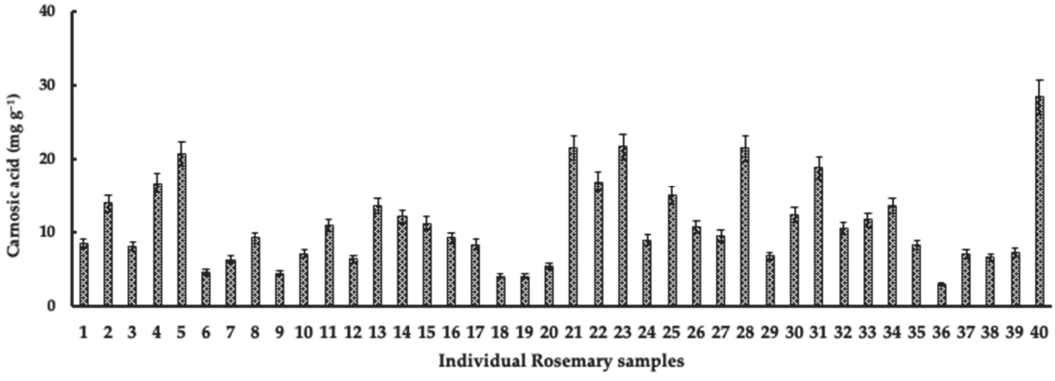


Figure 4. Distribution of carnosic acid concentration in the crude extracts of rosemary leaf samples collected from Tunisia (June 2011–February 2012). Values are the means of three replicates ± SD. CA: carnosic acid (expressed on a dry weight basis).

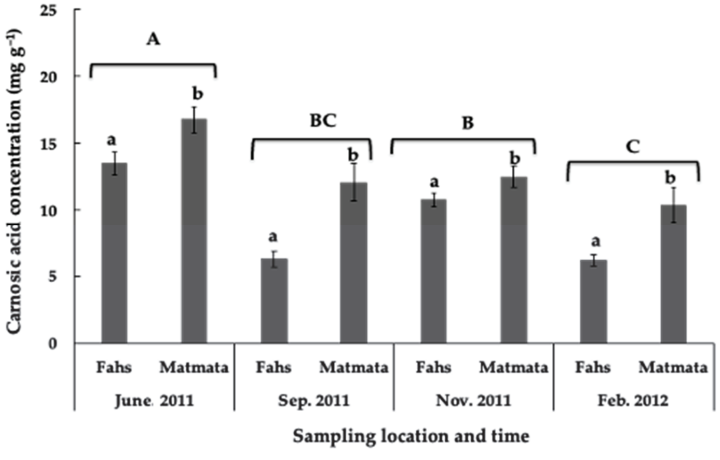


Figure 5. The effect of the sampling time and location on the concentration of carnosic acid in rosemary leaves. Data are expressed as mg·g^{-1} dry weight. Different letters (a, b, location for each month; A–C, sampling month during the season) above the error bars show treatments with significant differences throughout the season ($p < 0.05$). CA: carnosic acid.

3.2. Influence of Precipitation, Elevation, and Temperature on Carnosic Acid Concentration in Rosemary Leaves

The two sampling locations had different annual precipitation, temperature, and elevation. Matmata has a hot climate, while Fahs has a moderately hot climate. To determine which environmental factors might be related to the observed seasonal variation in carnosic acid concentration, a Pearson correlation analysis was performed based on carnosic acid concentration and environmental factors (temperature, precipitation, and altitude) during sampling (Table 2). Carnosic acid concentration showed a significant positive correlation with temperature ($r = 0.30$; $p < 0.05$) and altitude ($r = 0.33$; $p < 0.05$). However, there was a significant inverse relationship between carnosic acid concentration and precipitation at the sampling locations (Table 2). The results show that temperature and precipitation variations influence the concentration of carnosic acid in rosemary leaves during the season.

Table 2. Pearson correlation analysis for carnosic acid concentration, precipitation, elevation, and temperature.

Attribute	Elevation	Precipitation	Temperature	CA Concentration
Elevation	1.00			
Precipitation	−0.61 **	1.00		
Temperature	0.19	−0.71 **	1.00	
CA amt	0.33 *	−0.49 *	0.30 *	1.00

CA: carnosic acid. Significance level: * $p < 0.05$, ** $p < 0.01$.

3.3. Effects of Seasonality on the Plant Growth-Inhibitory Potential of Rosemary Leaves

The concentration of carnosic acid in rosemary leaves showed seasonal variation and a significant relationship with precipitation and temperature. The study also investigated whether the seasonal variation in carnosic acid concentration could influence the phytotoxic activity of rosemary during the sampling season. The phytotoxic activity assay was tested on lettuce elongation. The inhibitory effect of rosemary leaf ethanol crude extracts on lettuce radicle and hypocotyl elongation was dose-dependent. The ranges of inhibition of lettuce radicle and hypocotyl elongation were 18.3–123% and 15.6–100% (percentage of control), respectively (Table S1). Lettuce hypocotyl elongation was more sensitive to rosemary crude extract than the radicle.

The concentration of rosemary leaf extracts required for 50% growth inhibition (EC_{50} or specific activity) of lettuce elongation was determined for all the collected rosemary samples. The inhibitory effect (expressed as EC_{50}) on lettuce growth ranged from 2.1–8.6 mg DW·mL^{-1} and from 0.7–7.2 mg DW·mL^{-1} for radicle and hypocotyl, respectively (Table S1). The observed phytotoxicity of rosemary leaves on lettuce length growth showed seasonal variations. Samples collected in September and November had the lowest EC_{50} values (strong inhibition) for lettuce hypocotyl elongation (Figure 6a).

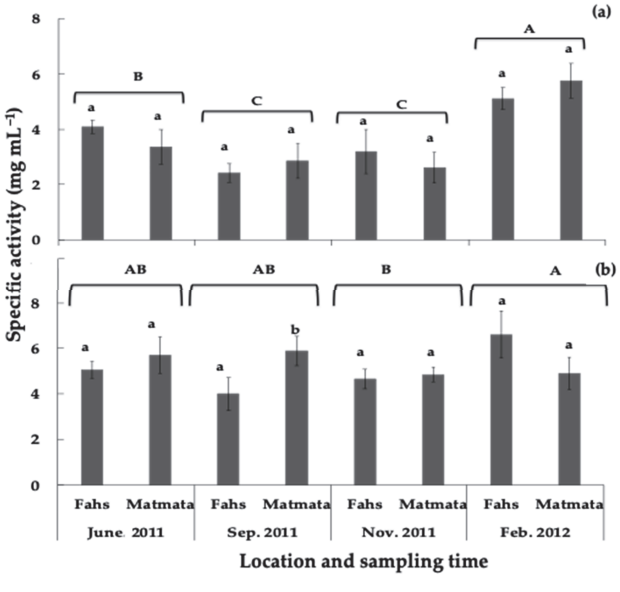


Figure 6. Effect of the sampling period and location on the growth-inhibitory activity of rosemary leaves on lettuce (**a**) hypocotyl and (**b**) radicle elongation. Different letters (a, b, location for each month; A–C, sampling month during the season) above the error bars show treatments with significant differences throughout the season ($p < 0.05$). Values are means ± SD ($n = 5$).

High EC_{50} values (low inhibition) for lettuce hypocotyl were measured in February at both locations, which coincided with the lowest carnosic acid concentration in rosemary leaves. The average specific activity of samples collected in November, September, June, and February on lettuce radicle elongation was 4.8, 5.0, 5.6, and 6.2 mg DW·mL^{-1}, respectively (Figure 6b). Except for samples collected in September, there was no significant difference in inhibitory activity between sampling locations during the season (Figure 6). The effect of sampling location, sampling period, and their interaction on lettuce carnosic acid concentration and growth elongation are shown in Table 3. Except for the effect of sampling location on hypocotyl and radicle growth, all other effects and interactions were significant. However, the seasonal variation in phytotoxicity and concentration of carnosic acid in rosemary leaves should be evaluated over 1 year in a Mediterranean climate to fully understand this relationship.

Table 3. Summary of the analysis of variance (ANOVA) for carnosic acid concentration, growth elongations, sampling location, and period.

Source of Variation	DF	CA Concentration		Hypocotyl Growth		Radicle Growth	
		MS	*p*-level	MS	*p*-level	MS	*p*-level
Location	1	167.4	<0.001 **	0.02	>0.05	0.52	>0.05
Month	3	61.9	<0.001 **	12.7	<0.001 **	1.6	<0.05 *
Location × Month	3	8.9	<0.05 *	0.9	<0.05 *	4.5	<0.001 **
Error	24	2.2		0.3		0.5	
Total	31						
R^2		0.88		0.85		0.63	

* Significant at the 0.05 level of probability. ** Significant at the 0.01 level of probability. $p > 0.05$: not significant. CA: Carnosic acid. Growth is expressed as a percentage of the control. MS: means of squares.

3.4. Correlation between Carnosic Acid Concentration and Phytotoxicity of Rosemary Leaves

To determine the relationship between carnosic acid concentration and phytotoxicity of rosemary leaf extracts, a Pearson correlation analysis was performed based on the results of carnosic acid concentration and EC_{50} (specific activity) of the leaves. The resulting graph represents a natural dose-response curve for carnosic acid in rosemary leaves. The correlation study showed a significant inverse relationship ($p < 0.01$; $r = -0.529$) between carnosic acid concentration and inhibitory effects (expressed as EC_{50} or specific activity) of rosemary leaves for hypocotyl elongation (Figure 7a). This result shows that the contribution of carnosic acid to the inhibitory effect of rosemary leaves on lettuce hypocotyl elongation is high, but low on radicle elongation. The results indicate that rosemary leaves with a high concentration of carnosic acid have great phytotoxic potential, which can be further explored. However, the degree of correlation between carnosic acid concentration and phytotoxicity of rosemary leaves indicates that other compounds may also contribute to the phytotoxicity of rosemary leaves.

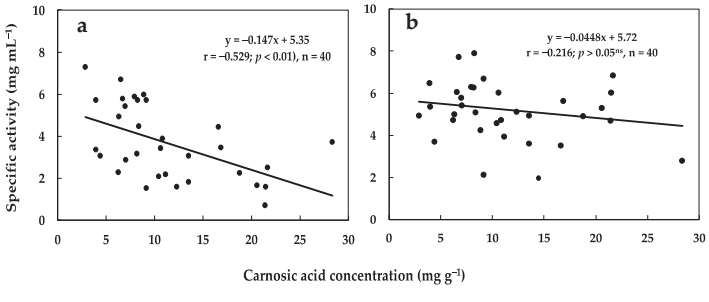


Figure 7. Relationship between carnosic acid concentration and phytotoxicity (expressed as EC_{50}) of the leaf extract of rosemary on lettuce (**a**) hypocotyl and (**b**) radicle elongations.

4. Discussion

The RP-HPLC analysis of rosemary leaves collected from the two locations in Tunisia showed that carnosic acid concentration varied throughout the season (as shown in Figure 4). Other studies reported similar variations in carnosic acid concentration in rosemary samples from different geographical zones [44,45]. The average carnosic acid concentration was highest in early summer for both sampling locations in this study. In line with the results of this study, Hidalgo et al. [45] also reported an increase in carnosic acid concentration in rosemary leaves in summer (46.2 $mg \cdot g^{-1}$ in July 1996), while the lowest values were observed in February of the same year. In Brazil, the reported carnosic acid concentration in rosemary leaves was highest in leaf samples collected in summer [46]. In contrast, Luis and Johnson [37] observed a decrease in the carnosic acid concentration of about 50% during the summer months characterized by high temperatures. The discrepancy in the concentration of carnosic acid in rosemary leaves could be due to the influence of growing conditions and other factors. The influence of environmental factors on the variation of carnosic acid concentration in rosemary leaves was reported previously [46,47]. The seasonal variations in carnosic acid concentration observed in this study may indicate that the synthesis of the compound is influenced by changes in certain climatic factors.

The results also showed a relationship between environmental conditions at the time of sampling and carnosic acid concentration in rosemary leaves. Temperature, precipitation, and elevation of sampling locations showed significant correlations with carnosic acid concentration in rosemary leaves. Similar to the results of this study, Hidalgo et al. [45] reported increasing carnosic acid concentration in rosemary leaves with increasing temperature. Lemos et al. [46] also reported the highest carnosic acid concentration in the month with the highest temperature. In contrast, Munne-Bosch et al. [48] reported a negative linear relationship between carnosic acid concentration and temperature. However, an increased amount of carnosic acid was detected during the summer with high rainfall and temperature in Brazil [46]. Borras et al. [44] reported that the observed variations in the altitude of sampling locations had significant effects on the concentration of plant metabolites (including carnosic acid) in rosemary leaves. Compared to other native Mediterranean plants, rosemary can withstand prolonged drought by avoiding damage to its photosynthetic organs [47]. Seasonal variation is associated with certain changes in soil moisture and temperature, which may lead to variations in the biosynthetic pathways of primary and secondary metabolites [17,18]. Carnosic acid was found mainly in June 2011, followed by September 2011 and November 2011. The biosynthetic pathway of terpenes could explain this observation. Terpenes are synthesized in the cytosol and plant plastids [49]. The pathway leads to the formation of sesquiterpenoids in the cytoplasm and the formation of diterpenes and tetraterpenes in the plastid. However, these processes are associated with the capture of sunlight and a photoprotective function in cell membranes [49]. Thus, according to the biosynthetic mechanisms, rosemary leaves harvested in June 2011 increased the synthesis of terpenes (including carnosic acid) in plastids at the high temperatures (26.6 °C). Moreover, carnosic acid is one of the most important antioxidants in rosemary leaves, and its concentration increases under stress conditions [46]. It should be considered that the production of carnosic acid in rosemary depends on the genetic background, plant part, and growing conditions [50], which could also explain part of the discrepancy between the results reported in different studies.

Although carnosic acid was reported as the principal allelochemical in rosemary leaves [34], other compounds found in the plant, such as ferulic, caffeic, gallic, chlorogenic, and rosmarinic acids, have been linked to phytotoxicity [51]. The antioxidative mechanism of carnosic acid in plants has been reported [52]; however, there has been no reported study on its mode of action as a plant growth inhibitor. Since other compounds contribute to the inhibitory effects of rosemary leaves, the physiological actions of some of these compounds are discussed. According to Araniti et al. [53], rosmarinic acid inhibited the main reactive oxygen species (ROS)-scavenging enzymes, resulting in high ROS levels that cause alterations in mitochondrial ultrastructure and function, leading to cell death in *Arabidopsis*

seedlings. Rudrappa et al. [54] asserted that gallic acid elevated the level of ROS in the roots of *Arabidopsis*. The activated ROS caused the root architecture of susceptible plants to be disrupted by impairing the microtubule assembly. According to dos Santos et al. [55], ferulic acid may be channelled into the phenylpropanoid pathway, where it may increase the quantity of lignin monomer in the cell wall, hardening the cell wall and inhibiting root growth. Similarly, caffeic acid channelled into the phenylpropanoid pathway increased lignin monomers that solidify the cell wall and inhibit root growth [56]. 1,8-Cineole, a significant essential oil in rosemary, decreased root growth in other plants by impeding DNA synthesis in the apical meristem of *Brassica campestris* roots [57]. Monoterpenes, which are abundant in rosemary, inhibited chlorophyll content, as well as the biosynthesis of several phenolic compounds [58].

The rosemary leaves sampled in this study showed variations in carnosic acid concentration, suggesting that the growth-inhibitory effect of the leaves may change over the season. The study further confirmed that the phytotoxicity of rosemary leaves changed during the sampling period. The changes in carnosic acid concentration and the expression of biological activities during different seasons have been reported in other studies [46,59,60]. Although the antimicrobial activity of rosemary leaves changed during the growing season [61], seasonal changes in the phytotoxicity of rosemary have not been reported. The concentration of carnosic acid in rosemary leaves showed a significant correlation ($r = -0.529$; $p < 0.01$) with growth inhibition at the hypocotyl of lettuce. Our results agree with other studies that showed that allelochemicals and growth inhibition are related in allelopathic species. Ben-Hammaouda et al. [62] reported that the phytotoxicity of sorghum hybrids had a positive correlation ($r = 0.66$) with the total concentration of phenolic compounds. Similarly, Reberg-Horton et al. [63] reported that the inhibitory effect of aqueous extracts of *Secale cereale* tissue correlated with the amount of DIMBOA extracted from the harvested tissue. In another study, the concentration of phenolic acids together with DIBOA and DIMBOA explained about 90% of the variation in growth inhibition observed in annual ryegrass [64]. Although a significant relationship was found between carnosic acid concentration and growth inhibition, the contribution of other compounds to rosemary leaf phytotoxicity should not be ignored.

5. Conclusions

The concentration of carnosic acid in rosemary leaves and the inhibitory effect of ethanolic extracts of rosemary leaves were both influenced by seasonal variations. The carnosic acid concentration in rosemary leaves peaked in early summer at both sampling locations in Tunisia and then gradually decreased until winter. Rosemary leaf phytotoxicity (expressed as EC_{50}) followed a similar pattern throughout the season and showed a significant (inverse) relationship with carnosic acid content. It is important to evaluate the seasonal variation in the inhibitory activity of rosemary leaves to avoid over-or underestimating the phytotoxicity of the plant. The efficacy of rosemary as a potential weed control agent needs further investigation under field conditions.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/plants11050673/s1: Table S1. The effects of rosemary leaf samples from different seasons and at different locations on lettuce growth elongation; Table S2. Moran's I statistic.

Author Contributions: Conceptualization, K.S.A. and Y.F.; methodology, K.S.A., R.A.O., A.S., A.B.A., J.O.-A., K.K. (Kiyokazu Kawada), and Y.F.; formal analysis, K.S.A., R.A.O., C.A.A., S.O.-A., and Y.F.; investigation, K.S.A.; resources, A.S., A.B.A., Y.S., H.I., and K.K. (Kiyokazu Kawada); data curation, K.S.A., Y.O., K.K. (Keisuke Katsura), C.A.A., Y.S., S.O., and R.A.O.; writing—original draft preparation, K.S.A.; writing—review and editing, K.S.A., R.A.O., K.K. (Kiyokazu Kawada), H.I., S.O.-A., J.O.-A., A.S.; A.B.A.; Y.S., S.O., Y.O., C.A.A., K.K. (Keisuke Katsura), and Y.F.; validation, K.K. (Kiyokazu Kawada), K.S.A., and Y.F.; visualization, K.S.A. and R.A.O.; supervision, Y.F.; project administration, K.K. (Kiyokazu Kawada), H.I., and Y.F.; funding acquisition, Y.F., K.K. (Kiyokazu Kawada), and H.I. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by a grant-in-aid for Research on Agriculture and Food Science (25029AB) from the Ministry of Agriculture, Forestry, and Fisheries of Japan. JST CREST Grant Number JPMJCR17O2, Japan, JSPS KAKENHI Grant Number 26304024, and the Technology Research Partnership for Sustainable Development (SATREPS) of JST/JICA (JPMJSA1506) also supported this work.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data included in the article with Supplementary Materials.

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

References

1. Bastiaans, L.; Kropff, M.J.; Goudriaan, J.; van Laar, H.H. Design of Weed Management Systems with a Reduced Reliance on Herbicides Poses New Challenges and Prerequisites for Modelling Crop-Weed Interactions. *Field Crops Res.* **2000**, *67*, 161–179. [CrossRef]
2. Heap, I. Global Perspective of Herbicide-Resistant Weeds. *Pest Manag. Sci.* **2014**, *70*, 1306–1315. [CrossRef] [PubMed]
3. Atwood, D.; Paisley-Jones, C. *2008–2012 Market Estimates*; The United States Environmental Protection Agency: Washington, DC, USA, 2017.
4. Rice, E.L. Allelopathy. In *Physiological Ecology*; Kozlowski, T.T., Ed.; Academic Press: New York, NY, USA, 1984.
5. Willis, R.J. What Is Allelopathy? In *The History of Allelopathy*; Springer: Dordrecht, The Netherlands, 2007; pp. 1–13.
6. Inderjit. Soil Microorganisms: An Important Determinant of Allelopathic Activity. *Plant Soil* **2005**, *274*, 227–236. [CrossRef]
7. Teoh, E.S. *Medicinal Orchids of Asia*; Springer: Cham, Switzerland, 2016; pp. 1–752. [CrossRef]
8. Wink, M. Modes of Action of Herbal Medicines, and Plant Secondary Metabolites. *Medicines* **2015**, *2*, 251–286. [CrossRef] [PubMed]
9. Wink, M. Secondary Metabolites, the Role in Plant Diversification Of. *Encycl. Evol. Biol.* **2016**, *4*, 1–9. [CrossRef]
10. Cheng, F.; Cheng, Z. Corrigendum: Research Progress on the Use of Plant Allelopathy in Agriculture and the Physiological and Ecological Mechanisms of Allelopathy. *Front. Plant Sci.* **2016**, *7*, 1697. [CrossRef]
11. Soltys, D.; Krasuska, U.; Bogatek, R.; Gniazdowsk, A. Allelochemicals as Bioherbicides—Present and Perspectives. In *Herbicides Current Research and Case Studies in Use*; IntechOpen: London, UK, 2013. [CrossRef]
12. Macías, F.A.; Mejías, F.J.R.; Molinillo, J.M.G. Recent Advances in Allelopathy for Weed Control: From Knowledge to Applications. *Pest Manag. Sci.* **2019**, *75*, 2413–2436. [CrossRef]
13. Zeng, R.H.; Mallik, A.U.; Luo, S.M. *Allelopathy in Sustainable Agriculture and Forestry*; Springer: New York, NY, USA, 2008.
14. Jabran, K.; Mahajan, G.; Sardana, V.; Chauhan, B.S. Allelopathy for Weed Control in Agricultural Systems. *Crop Prot.* **2015**, *72*, 57–65. [CrossRef]
15. Dayan, F.E.; Owens, D.K.; Duke, S.O. Rationale for a Natural Products Approach to Herbicide Discovery. *Pest Manag. Sci.* **2012**, *68*, 519–528. [CrossRef] [PubMed]
16. Heil, M.; Bostock, R.M. Induced Systemic Resistance (ISR) against Pathogens in the Context of Induced Plant Defences. *Ann. Bot.* **2002**, *89*, 503–512. [CrossRef] [PubMed]
17. Lur, H.; Hsu, C.; Wu, C.; Lee, C.; Lao, C.; Wu, Y.; Chang, S.; Wang, C. Changes in Temperature, Cultivation Timing and Grain Quality of Rice in Taiwan in Recent Years. *Crop Environ. Bioinform.* **2009**, *6*, 175–182.
18. Gray, D.E.; Pallardy, S.G.; Garrett, H.E.; Rottinghaus, G.E. Effect of Acute Drought Stress and Time of Harvest on Phytochemistry and Dry Weight of St. John's Wort Leaves and Flowers. *Planta Med.* **2003**, *69*, 1024–1030. [CrossRef]
19. Catalán, P.; Vázquez-de-Aldana, B.R.; de las Heras, P.; Fernández-Seral, A.; Pérez-Corona, M.E. Comparing the Allelopathic Potential of Exotic and Native Plant Species on Understory Plants: Are Exotic Plants Better Armed? *An. Biol.* **2013**, *35*, 65–74. [CrossRef]
20. Lou, Y.; Davis, A.S.; Yannarell, A.C. Interactions between Allelochemicals and the Microbial Community Affect Weed Suppression Following Cover Crop Residue Incorporation into Soil. *Plant Soil* **2016**, *399*, 357–371. [CrossRef]
21. Gagliardo, R.W.; Chilton, W.S. Soil Transformation of 2(3H)-Benzoxazolone of Rye into Phytotoxic 2-Amino-3H-Phenoxazin-3-One. *J. Chem. Ecol.* **1992**, *18*, 1683–1691. [CrossRef]
22. Silva, E.R.; Overbeck, G.E.; Soares, G.L.G. Phytotoxicity of Volatiles from Fresh and Dry Leaves of Two Asteraceae Shrubs: Evaluation of Seasonal Effects. *S. Afr. J. Bot.* **2014**, *93*, 14–18. [CrossRef]
23. Frizzo, C.D.; Atti-Serafini, L.; Laguna, S.E.; Cassel, E.; Lorenzo, D.; Dellacassa, E. Essential Oil Variability in *Baccharis uncinella* DC and *Baccharis dracunculifolia* DC Growing Wild in Southern Brazil, Bolivia, and Uruguay. *Flavour Fragr. J.* **2008**, *23*, 99–106. [CrossRef]
24. Anese, S.; Grisi, P.U.; de Jesus Jatobá, L.; Imatomi, M.; de Cassia Pereira, V.; Juliano Gualtieri, S.C. Seasonal Variation in Phytotoxicity of Drimys brasiliensis Miers. *Idesia* **2014**, *32*, 109–116. [CrossRef]
25. Missouri Botanical Garden. *Salvia rosmarinus*—Plant Finder. Available online: http://www.missouribotanicalgarden.org/PlantFinder/PlantFinderDetails.aspx?kempercode=b968 (accessed on 20 August 2021).

26. Royal Botanic Gardens. *Salvia rosmarinus* Spenn. | Plants of the World Online | Kew Science. Available online: http://plantsoftheworldonline.org/taxon/urn:lsid:ipni.org:names:457138-1 (accessed on 20 August 2021).
27. UniProt. Taxanomy-*Rosmarinus officinalis* (Rosemary) (*Salvia rosmarinus*). Available online: https://www.uniprot.org/taxonomy/39367 (accessed on 20 August 2021).
28. Sasaki, K.; el Omri, A.; Kondo, S.; Han, J.; Isoda, H. *Rosmarinus officinalis* Polyphenols Produce Anti-Depressant like Effect through Monoaminergic and Cholinergic Functions Modulation. *Behav. Brain Res.* **2013**, *238*, 86–94. [CrossRef] [PubMed]
29. Kontogianni, V.G.; Tomic, G.; Nikolic, I.; Nerantzaki, A.A.; Sayyad, N.; Stosic-Grujicic, S.; Stojanovic, I.; Gerothanassis, I.P.; Tzakos, A.G. Phytochemical Profile of *Rosmarinus officinalis* and *Salvia officinalis* Extracts and Correlation to their Antioxidant and Anti-Proliferative Activity. *Food Chem.* **2013**, *136*, 120–129. [CrossRef] [PubMed]
30. Bakirel, T.; Bakirel, U.; Keleş, O.Ü.; Ülgen, S.G.; Yardibi, H. In Vivo Assessment of Antidiabetic and Antioxidant Activities of Rosemary (*Rosmarinus officinalis*) in Alloxan-Diabetic Rabbits. *J. Ethnopharmacol.* **2008**, *116*, 64–73. [CrossRef]
31. Thorsen, M.A.; Hildebrandt, K.S. Quantitative Determination of Phenolic Diterpenes in Rosemary Extracts. *J. Chromatogr. A* **2003**, *995*, 119–125. [CrossRef]
32. Erkan, N.; Ayranci, G.; Ayranci, E. Antioxidant Activities of Rosemary (*Rosmarinus officinalis* L.) Extract, Blackseed (*Nigella sativa* L.) Essential Oil, Carnosic Acid, Rosmarinic Acid and Sesamol. *Food Chem.* **2008**, *110*, 76–82. [CrossRef]
33. Amaral, G.P.; Mizdal, C.R.; Stefanello, S.T.; Mendez, A.S.L.; Puntel, R.L.; de Campos, M.M.A.; Soares, F.A.A.; Fachinetto, R. Antibacterial and Antioxidant Effects of *Rosmarinus officinalis* L. Extract and Its Fractions. *J. Tradit. Complement. Med.* **2019**, *9*, 383–392. [CrossRef]
34. Appiah, K.S.; Mardani, H.K.; Omari, R.A.; Eziah, V.Y.; Ofosu-Anim, J.; Onwona-Agyeman, S.; Amoatey, C.A.; Kawada, K.; Katsura, K.; Oikawa, Y.; et al. Involvement of Carnosic Acid in the Phytotoxicity of *Rosmarinus officinalis* Leaves. *Toxins* **2018**, *10*, 498. [CrossRef] [PubMed]
35. Sekine, T.; Appiah, K.S.; Azizi, M.; Fujii, Y. Plant Growth Inhibitory Activities and Volatile Active Compounds of 53 Spices and Herbs. *Plants* **2020**, *9*, 264. [CrossRef] [PubMed]
36. Hossain, M.B.; Rai, D.K.; Brunton, N.P.; Martin-Diana, A.B.; Barry-Ryan, A.C. Characterization of Phenolic Composition in Lamiaceae Spices by LC-ESI-MS/MS. *J. Agric. Food Chem.* **2010**, *58*, 10576–10581. [CrossRef] [PubMed]
37. Luis, J.C.; Johnson, C.B. Seasonal Variations of Rosmarinic and Carnosic Acids in Rosemary Extracts. Analysis of their in Vitro Antiradical Activity. *Span. J. Agric. Res.* **2005**, *3*, 106–112. [CrossRef]
38. Achour, S.; Khelifi, E.; Attia, Y.; Ferjani, E.; Noureddine Hellal, A. Concentration of Antioxidant Polyphenols from *Thymus capitatus* Extracts by Membrane Process Technology. *J. Food Sci.* **2012**, *77*, 6. [CrossRef] [PubMed]
39. Richheimer, S.L.; Bernart, M.W.; King, G.A.; Kent, M.C.; Bailey, D.T. Antioxidant Activity of Lipid-Soluble Phenolic Diterpenes from Rosemary. *J. Am. Oil Chem. Soc.* **1996**, *73*, 507–514. [CrossRef]
40. Wellwood, C.R.L.; Cole, R.A. Relevance of Carnosic Acid Concentrations to the Selection of Rosemary, *Rosmarinus officinalis* (L.), Accessions for Optimization of Antioxidant Yield. *J. Agric. Food Chem.* **2004**, *52*, 6101–6107. [CrossRef] [PubMed]
41. del Baño, M.J.; Lorente, J.; Castillo, J.; Benavente-García, O.; del Río, J.A.; Ortuño, A.; Quirin, K.W.; Gerard, D. Phenolic Diterpenes, Flavones, and Rosmarinic Acid Distribution during the Development of Leaves, Flowers, Stems, and Roots of *Rosmarinus officinalis*. Antioxidant Activity. *J. Agric. Food Chem.* **2003**, *51*, 4247–4253. [CrossRef] [PubMed]
42. Jaouadi, S.; Lebreton, V.; Bout-Roumazeilles, V.; Siani, G.; Lakhdar, R.; Boussoffara, R.; Dezileau, L.; Kallel, N.; Mannai-Tayech, B.; Combourieu-Nebout, N. Environmental Changes, Climate and Anthropogenic Impact in South-East Tunisia during the Last 8 Kyr. *Clim. Past* **2016**, *12*, 1339–1359. [CrossRef]
43. Fick, S.E.; Hijmans, R.J. WorldClim 2: New 1-Km Spatial Resolution Climate Surfaces for Global Land Areas. *Int. J. Climatol.* **2017**, *37*, 4302–4315. [CrossRef]
44. Borrás-Linares, I.; Stojanović, Z.; Quirantes-Piné, R.; Arráez-Román, D.; Švarc-Gajić, J.; Fernández-Gutiérrez, A.; Segura-Carretero, A. *Rosmarinus officinalis* Leaves as a Natural Source of Bioactive Compounds. *Int. J. Mol. Sci.* **2014**, *15*, 20585–20606. [CrossRef]
45. Hidalgo, P.J.; Ubera, J.L.; Tena, M.T.; Valcárcel, M. Determination of the Carnosic Acid Content in Wild and Cultivated *Rosmarinus officinalis*. *J. Agric. Food Chem.* **1998**, *46*, 2624–2627. [CrossRef]
46. Lemos, M.F.; Lemos, M.F.; Endringer, D.C.; Scherer, R. Seasonality Modifies Rosemary's Composition and Biological Activity. *Ind. Crops Prod.* **2015**, *70*, 41–47. [CrossRef]
47. Munné-Bosch, S.; Nogués, S.; Alegre, L. Daily Patterns of Photosynthesis of Two Mediterranean Shrubs in Response to Water Deficit. In *Photosynthesis: Mechanisms and Effects*; Springer: Dordrecht, The Netherlands, 1998; pp. 4015–4018.
48. Munné-Bosch, S.; Alegre, L. Changes in Carotenoids, Tocopherols and Diterpenes during Drought and Recovery, and the Biological Significance of Chlorophyll Loss in *Rosmarinus officinalis* Plants. *Planta* **2000**, *210*, 925–931. [CrossRef]
49. Kasahara, H.; Hanada, A.; Kuzuyama, T.; Takagi, M.; Kamiya, Y.; Yamaguchi, S. Contribution of the Mevalonate and Methylerythritol Phosphate Pathways to the Biosynthesis of Gibberellins in *Arabidopsis*. *J. Biol. Chem.* **2002**, *277*, 45188–45194. [CrossRef]
50. Birtić, S.; Dussort, P.; Pierre, F.X.; Bily, A.C.; Roller, M. Carnosic Acid. *Phytochemistry* **2015**, *115*, 9–19. [CrossRef]
51. Ishikura, Y.; Kojima, Y.; Terrazawa, M. Effects of Phenolic Compounds on Seed Germination of Shirakamba Birch, *Betula platyphylla* var. Japonica. *Eurasian J. For. Res.* **2001**, *2*, 17–25.
52. Loussouarn, M.; Krieger-Liszkay, A.; Svilar, L.; Bily, A.; Birtić, S.; Havaux, M. Carnosic Acid and Carnosol, Two Major Antioxidants of Rosemary, Act through Different Mechanisms. *Plant Physiol.* **2017**, *175*, 1381–1394. [CrossRef] [PubMed]

53. Araniti, F.; Costas-Gil, A.; Cabeiras-Freijanes, L.; Lupini, A.; Sunseri, F.; Reigosa, M.J.; Abenavoli, M.R.; Sánchez-Moreiras, A.M. Rosmarinic Acid Induces Programmed Cell Death in *Arabidopsis* Seedlings through Reactive Oxygen Species and Mitochondrial Dysfunction. *PLoS ONE* **2018**, *13*, e0208802. [CrossRef] [PubMed]
54. Rudrappa, T.; Bonsall, J.; Gallagher, J.L.; Seliskar, D.M.; Bais, H.P. Root-Secreted Allelochemical in the Noxious Weed *Phragmites australis* Deploys a Reactive Oxygen Species Response and Microtubule Assembly Disruption to Execute Rhizotoxicity. *J. Chem. Ecol.* **2007**, *33*, 1898–1918. [CrossRef]
55. dos Santos, W.D.; Ferrarese, M.L.L.; Nakamura, C.V.; Mourão, K.S.M.; Mangolin, C.A.; Ferrarese-Filho, O. Soybean (*Glycine max*) Root Lignification Induced by Ferulic Acid. The Possible Mode of Action. *J. Chem. Ecol.* **2008**, *34*, 1230–1241. [CrossRef] [PubMed]
56. Bubna, G.A.; Lima, R.B.; Zanardo, D.Y.L.; dos Santos, W.D.; Ferrarese, M.D.L.L.; Ferrarese-Filho, O. Exogenous Caffeic Acid Inhibits the Growth and Enhances the Lignification of the Roots of Soybean (*Glycine max*). *J. Plant Physiol.* **2011**, *168*, 1627–1633. [CrossRef]
57. Koitabashi, R.; Suzuki, T.; Kawazu, T.; Sakai, A.; Kuroiwa, H.; Kuroiwa, T. 1, 8-Cineole Inhibits Root Growth and DNA Synthesis in the Root Apical Meristem of *Brassica campestris* L. *J. Plant Res.* **1997**, *110*, 1–6. [CrossRef]
58. Gouda, N.A.A.; Saad, M.M.G.; Abdelgaleil, S.A.M. PRE and POST Herbicidal Activity of Monoterpenes against Barnyard Grass (*Echinochloa crus-galli*). *Weed Sci.* **2016**, *64*, 191–200. [CrossRef]
59. Labbé, C.; Faini, F.; Calderón, D.; Molina, J.; Arredondo, S. Variations of Carnosic Acid and Carnosol Concentrations in Ethanol Extracts of Wild *Lepechinia salviae* in Spring (2008–2011). *Nat. Prod. Commun.* **2014**, *9*, 1413–1416. [CrossRef]
60. Soltanabad, M.H.; Bagherieh-Najjar, M.B.; Mianabadi, M. Seasonal Variations in Carnosic Acid Content of Rosemary Correlates with Anthocyanins and Soluble Sugars. *J. Med. Plants By-Prod.* **2018**, *7*, 163–171.
61. Celiktas, O.Y.; Kocabas, E.E.H.; Bedir, E.; Sukan, F.V.; Ozek, T.; Baser, K.H.C. Antimicrobial Activities of Methanol Extracts and Essential Oils of *Rosmarinus officinalis*, Depending on Location and Seasonal Variations. *Food Chem.* **2007**, *100*, 553–559. [CrossRef]
62. Ben-Hammouda, M.; Kremer, R.J.; Minor, H.C.; Sarwar, M. A Chemical Basis for Differential Allelopathic Potential of *Sorghum* Hybrids on Wheat. *J. Chem. Ecol.* **1995**, *21*, 6. [CrossRef] [PubMed]
63. Reberg-Horton, S.C.; Burton, J.D.; Danehower, D.A.; Ma, G.; Monks, D.W.; Murphy, J.P.; Ranells, N.N.; Williamson, J.D.; Creamer, N.G. Changes over Time in the Allelochemical Content of Ten Cultivars of Rye (*Secale cereale* L.). *J. Chem. Ecol.* **2005**, *31*, 179–193. [CrossRef] [PubMed]
64. Huang, Z.; Haig, T.; Wu, H.; An, M.; Pratley, J. Correlation between Phytotoxicity on Annual Ryegrass (*Lolium rigidum*) and Production Dynamics of Allelochemicals within Root Exudates of an Allelopathic Wheat. *J. Chem. Ecol.* **2003**, *29*, 2263–2279. [CrossRef] [PubMed]

plants

MDPI

Article

Pre-Germination Treatments at Operational Scale for Six Tree Species from the Sclerophyll Forest of Central Chile

Eduardo Cartes-Rodríguez [1], Carolina Álvarez-Maldini [2,*], Manuel Acevedo [1], Marta González-Ortega [1], Alejandro Urbina-Parra [1] and Pedro León-Lobos [3,4]

[1] Centro Tecnológico de la Planta Forestal, Instituto Forestal–Sede Biobío, Camino a Coronel Km 7.5, San Pedro de la Paz 7770223, Chile; ecartes@infor.cl (E.C.-R.); macevedo@infor.cl (M.A.); mgonzale@infor.cl (M.G.-O.); aurbina@infor.cl (A.U.-P.)
[2] Instituto Ciencias Agroalimentarias, Animales y Ambientales (ICA3), Universidad de O'Higgins, Campus Colchagua, Ruta 90 Km 3, San Fernando 2840440, Chile
[3] Grupo de Especialidad Recursos Genéticos, Centro de Investigación Regional La Platina, Instituto de Investigaciones Agropecuarias, Avenida Santa Rosa 11610, La Pintana, Santiago 8831314, Chile; pleon@inia.cl
[4] Centro de Estudios Avanzados en Zonas Áridas (CEAZA), La Serena 1720256, Chile
* Correspondence: carolina.alvarez@uoh.cl; Tel.: +56-2-3328-6050

Abstract: Sclerophyll forest in Mediterranean central Chile has been subjected to severe degradation due to anthropic disturbances and climate change and is in need of restoration. Since direct seeding is usually unsuccessful, we need to research seed propagation to produce plants for restoration. Our objective was to assess pre-germination treatments for six native woody species (*Acacia caven*, *Lithraea caustica*, *Quillaja Saponaria*, *Porlieria chilensis*, *Kageneckia angustifolia*, and *Ceratonia chilensis*) of the sclerophyll forest, considering its operational applicability and consequences for nursery plant production. Treatments were selected according to previous studies, and operational applicability in nurseries. Germination and level of seeds water imbibition were assessed. Results indicate that time for seed water imbibition is critical for germination in *A. caven*, *P. chilensis* and *K. angustifolia*, with an average germination of 90.2 ± 2.0%, 85.0 ± 4.7%, and 47.4 ± 2.3%, respectively. Gibberellin did not improve germination compared to water soaking in *Q. Saponaria*, *K. angustifolia* and *P. chilensis*. In addition, physical scarification is a suitable treatment for *L. caustica* and *C. chilensis*, instead of chemical scarification, avoiding handling toxic and corrosive compounds in nurseries. We recommend assessing seed water imbibition rates as a key factor for proper germination processes.

Keywords: Mediterranean; nursery production; seeds; water imbibition

Citation: Cartes-Rodríguez, E.; Álvarez-Maldini, C.; Acevedo, M.; González-Ortega, M.; Urbina-Parra, A.; León-Lobos, P. Pre-Germination Treatments at Operational Scale for Six Tree Species from the Sclerophyll Forest of Central Chile. *Plants* **2022**, *11*, 608. https://doi.org/10.3390/plants11050608

Academic Editors: Sofia Rhizopoulou, Maria Karatassiou and Efi Levizou

Received: 30 January 2022
Accepted: 16 February 2022
Published: 24 February 2022

1. Introduction

There are five Mediterranean-type climate regions in the world that represent less than 5% of global surface but and are catalogued as biodiversity hotspots because they harbor 20% of endemic vascular plants of the planet [1,2]. These regions are characterized by higher levels of endemism of the vascular flora, which are adapted to seasonal water deficit and high summer temperatures [3]. One of these regions is found in central Chile, between 30° S and 36° S approximately; this represents a transition between the Atacama Desert and mixed temperate forest of south Chile, with shrub formations and sclerophyll forests that dominate the landscape [1,4,5].

The Mediterranean-type climate region of Chile has been subjected to strong anthropic pressures [6] due to changes in soil use, which has reduced the coverage, structure, and composition of this ecosystem, and this has been worsened by the effect of forest and soil degradation and bushfires [7–10]. These events have increased in severity during the last decade, as Chile has been facing a mega-drought since 2010 [11–13]. In recognition of the central role of forests for carbon storage in vegetation and soil [14–16], the United Nations (UN) has declared the 2021–2030 the "Decade of Ecosystem Restoration" as an

important tool to mitigate the increasing loss of biodiversity and rise in CO_2 emissions. Altamirano et al. [17] reveal the urgent need to restore forest ecosystems at a global scale, which represents the best option for the achievement of goals described by the UN. This is even more relevant for sclerophyll forests because it requires greater restoration efforts considering its actual degradation state [18].

Chile and 114 other countries have subscribed to a series of restoration commitments [19]. In the context of the Paris Agreement and the update of the Determined Contribution at National Level in 2020, Chile is committed to afforest 70,000 hectares of native species for the formation of a permanent forest cover and to restoration processes of 1,000,000 hectares of landscape [20]. However, Bannister et al. [21] identified three bottlenecks for successful forest restoration in Chile: (1) a lack of national plan for forest landscape restoration; (2), poor quality and low supply of native plants species, which was thoroughly described by Acevedo et al. [22]; and (3) poor results in the establishment phase. Likewise, León-Lobos et al. [23] describe a fourth bottleneck focused on the low availability and seed quality of native species, which impairs plant production and the achievement of restoration goals. In addition, Mediterranean species from central Chile present several issues that negatively affect seed supply such as low density and species diversity in seed banks; besides low germination after sampling from soil [24–26], this has proven to be even more severe in landscapes dominated by *Quillaja Saponaria* Molina (Quillajaceae) and *Lithraea caustica* (Mol.) Hook. and Arn. (Anacardiaceae) [25]. Besides supply problems, seeds from the sclerophyll forest are exposed to inadequate environmental conditions during seed germination and establishment. Summer season in Mediterranean climate imposes important levels of stress, due to higher temperatures and the absence of relevant precipitations [27,28]. In the context of central Chile there is little experimental evidence that native species produce seed without dormancy at the moment of seed dispersal [26]. Evidence indicates that physical dormancy imposed by a hard and impermeable seedcoat is present in native species such as *Acacia caven* (Molina) Molina (Fabaceae) and *L. caustica* [29], which can be prompted to germinate with mechanic scarification or acid application. Likewise, the development of fleshy fruits in species such as *L. caustica* and *Porlieria chilensis* can induce dormancy due to the presence of chemical inhibitors [30,31], and in natural environments germination can be triggered by the passage through the intestinal tract of frugivore species such as native fox *Pseudalopex culpaeus* (Mol.) [32]. In these cases, the removal of the pericarp, acids or gibberellin treatment can be applied at operational levels.

Despite that seed anatomy and the environmental conditions can shed light regarding dormancy in seeds of species from the sclerophyll forest, there is a lack of information regarding specific dormancy and the identification of suitable pre-germination treatments that maximize its germination [23,33,34]. Although higher germination is a pre-requisite to unlock the following bottlenecks for restoration [21], such treatments should consider their applicability by local nurseries, that in most cases lack the proper training to, for example, handle toxic chemicals for acid stratification such as sulfuric acid [22,35].

The objective of this research is to assess pre-germination treatments for six native woody species (*A. caven*, *L. caustica*, *Q. Saponaria*, *P. chilensis* I.M. Johnst., *Kageneckia angustifolia* D. Don (Rosaceae), and *Ceratonia chilensis* (Molina) Stuntz emend. Burkart (Fabaceae) of the sclerophyll forest from central Chile, considering their operational applicability and consequences for nursery plant production.

2. Results

2.1. Seed Characterization

From the estimation of the number of seeds per kilogram, it is observed that bigger seeds belong to *A. caven* with 7,554 ± 216 seeds kg^{-1}, followed by *L. caustica* and *C. chilensis* with 23,066 ± 660 seeds kg^{-1} and 24,359 ± 543 seeds kg^{-1}, respectively, and finally *P. chilensis* with 37,169 ± 2379 seeds kg^{-1} and *Q. saponaria* with 112,413 ± 1,396 seeds kg^{-1}; seeds with the lower weight were observed in *K. angustifolia* with 136,935 ± 3855 seeds kg^{-1}.

A significant effect was observed in the time of water imbibition on the moisture content of seeds for all species (all $p < 0.0012$). The seeds initial moisture content was on average 14.9 ± 7.6%, which corresponded to seed water content immediately after storage and before soaking. After soaking in water for 24 h, significant increase was observed in this variable in all evaluated species (all $p < 0.0058$), while only for *A. caven*, *P. chilensis* and *K. angustifolia* a significant increase in moisture content was observed after 48 h of soaking (all $p < 0.0152$) (Table 1).

Table 1. Seed moisture content (w/w dry basis, g g^{-1}) initial and after soaking in water for 24 and 48 h of sclerophyll species of central Chile (mean ± s.d.; $n = 3$). Letters indicate significant differences in moisture content for each species, between the different measurement times (Tukey, $p < 0.05$).

Species	Seed Moisture Content (g g^{-1})		
	Initial	Soaking in Water 24 h	Soaking in Water 48 h
Quillaja saponaria	0.139 ± 0.042 ^{b}	0.851 ± 0.108 ^{a}	0.935 ± 0.075 ^{a}
Lithraea caustica	0.097 ± 0.023 ^{b}	0.522 ± 0.040 ^{a}	0.536 ± 0.021 ^{a}
Acacia caven	0.273 ± 0.056 ^{c}	0.627 ± 0.062 ^{b}	0.903 ± 0.098 ^{a}
Porlieria chilensis	0.182 ± 0.077 ^{c}	0.458 ± 0.065 ^{b}	0.626 ± 0.074 ^{a}
Kageneckia angustifolia	0.101 ± 0.030 ^{c}	0.867 ± 0.105 ^{b}	1.054 ± 0.089 ^{a}
Ceratonia chilensis	0.101 ± 0.027 ^{b}	0.217 ± 0.055 ^{a}	0.262 ± 0.046 ^{a}

2.2. Nurseries Survey

Surveyed nurseries represent the 41.3% ($n = 6$) of nation-wide plant production for the selected species in this study during the 2017 to 2019 seasons, where chemical and scarification treatments present higher restrictions at an operational scale. Restrictions are mainly related to technical capabilities, due to lack of knowledge about how to apply the treatments at an operational scale, and to a lesser degree due to restrictions in infrastructure and equipment (Table 2).

Table 2. Restrictions identified in surveyed nurseries for application of pre-germination treatments at operation scale in evaluated species. (^{1}): Gibberellic acid; (^{2}) Hot water; (^{3}) Sulfuric acid.

Pre-Germination Treatment	Without Restrictions (%)	Restrictions Due to Capabilities Techniques (%)	Restrictions Due to Infrastructure and/or Equipment (%)	Don't Know the Benefits (%)
Water Soaking (RT)	100	-	-	-
Plant Hormone (^{1}) *	67	16.5	16.5	-
Wet-cold Stratification	67	16.5	-	16.5
Physical Scarification (^{2})	100	-	-	-
Mechanical Scarification	50	33	17	-
Chemical Scarification (^{3})	50	33	17	-

* Gibberellic acid concentrations were not declared by the nurseries surveyed.

Among the most used pre-germination treatments in nurseries are soaking in water at room temperature and chemical scarification with sulfuric acid (Table 3) for the selected species. Soaking in water ranged between two and 72 h for most nurseries and species, while exposure to sulfuric acid varies between 30 and 180 min depending on the protocol of each nursery and species. Surveyed nurseries do not apply plant hormones as pre-germination treatments, stratification, or mechanical scarification, for the assessed species.

Table 3. Pre-germination treatments applied by nurseries at operational scale, for the species evaluated in the sclerophyll forest. ([1]): Hot water; ([2]): Sulfuric acid.

Species	Soaking in Water (%)	Physical Scarification (%) ([1])	Chemical Scarification (%) ([2])	Soaking in Coke® (%)	Direct Sowing (%)
Quillaja saponaria	83	0	0	0	17
Lithraea caustica	0	20	60	20	0
Acacia caven	0	20	60	20	0
Porlieria chilensis	100	0	0	0	0
Kageneckia angustifolia	100	0	0	0	0
Ceratonia chilensis	34	33	33	0	0

2.3. Germination Experiment

For all evaluated species, a significant effect was observed in the interaction between pre-germination and measurement time (MED × TRAT) (all $p < 0.0430$).

In *Q. saponaria* average germination at the end of the experiment reached 90.2 ± 2.0%; 30 days after sowing, no significant differences between pre-germination treatments were observed. After day 16 no significant increments in germination were observed (all $p > 0.1745$), with an average of 86.1 ± 1.2% without significant differences between pre-germination treatments (Figure 1a).

In *L. caustica*, at the end of the germination experiment (63 days after sowing), average germination reached at 45.4 ± 5.2%, and no significant differences between pre-germination treatments were observed (all $p = 1$). Chemical scarification with sulfuric acid induced a reduction in germination times of 33 and 23 days in comparison with control treatment and physical scarification, respectively. With chemical scarification, germination did not show significant increases after day 18 of sowing (38.1 ± 2.4%) (all $p > 0.2327$), while in control and physical scarification treatments, germination remained constant at day 51 (40.6 ± 6.3%) and 46 (38.4 ± 4.3%), respectively (Figure 1b).

In *A. caven* pre-germination treatments showed significant differences in germination (all $p < 0.0001$). At the end of the evaluation period (day 53 since sowing), seed soaking in water for 48 h germination was 70.1 ± 6.8%, which was significantly higher than with physical and chemical scarification that reached an average germination of 24.3 ± 6.5%, which did not show significant differences ($p = 1$) (Figure 1c). At day 25 after sowing, we did not observe significant increments in germination in the control treatment that reached an average of 61.1 ± 4.2% (all $p > 0.9071$).

Similarly, final germination of *P chilensis* seeds showed significant differences between treatments (all $p < 0.0001$). Physical scarification caused low germination (0.3 ± 0.2%) compared to control and the application of gibberellic acid. Thus, at the end of the germination period (63 days since sowing), no significant differences were observed between control treatment and gibberellic acid application at 200 mg L^{-1} ($p = 1$), with an average germination of 47.4 ± 2.3%. However, soaking in gibberellic acid decreased the germination period by 11 days compared to control treatment (Figure 1d).

In *K. angustifolia* no significant differences in germination were observed at the end of the experiment (30 days after sowing) between pre-germination treatments (all $p > 0.8581$), an average germination of 85.0 ± 4.7% was reached. At day 16 after sowing germination had not increased significatively in any treatments ($p > 0.3008$), with an average germination of 80.5 ± 6.0% (Figure 1e).

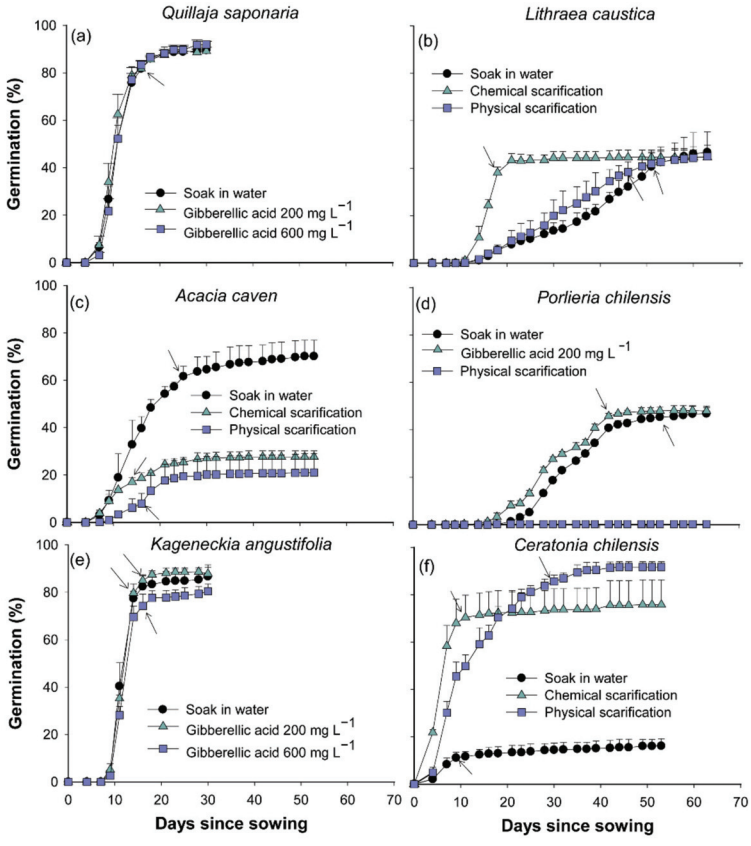


Figure 1. Germination (%) observed in *Quillaja Saponaria* (**a**), *Lithraea caustica* (**b**), *Acacia caven* (**c**), *Porlieria chilensis* (**d**), *Kageneckia angustifolia* (**e**), and *Ceratonia chilensis* (**f**) according to different pre-germination treatments. Arrows indicate the day after sowing where no further increment in germination was observed. Symbols indicate mean + s.d.

At the end of the evaluation period in *C. chilensis*, 53 days after sowing, pre-germination treatments showed a significant effect in germination (all $p < 0.0001$). Chemical and physical scarification induced a significantly higher germination (83.7 ± 11.0%) compared to the control (16.1 ± 3.0%). No significant differences were observed between chemical and physical scarification ($p = 0.5807$) (Figure 1f). In the case of chemical scarification, 11 days after sowing germination did not increase significantly (all $p > 0.690$), reaching 70.4 ± 9.4%. For physical scarification a similar pattern was observed at day 30 after sowing (all $p > 0.3874$), and 85.6 ± 2.6% of germination was observed. In the control a stable germination level of 11.1 ± 2.4% was observed at day nine after sowing (all $p > 0.8457$).

3. Discussion

3.1. Seed Characterization

A low water content after 48 h of imbibition was observed in *L. caustica*, *P. chilensis*, and *C. chilensis*, in relation to the other assessed species (Table 1), which can be attributed to physical dormancy, previously reported in *L. caustica* and *C. chilensis* [25]. In *P. chilensis*, despite that our results show higher water imbibition than the results reported by Cabello et al. [36], there is agreement that water imbibition reaches a peak after 48 h of soaking in water, indicating that seedcoat impermeability is not an impairment for ger-

mination in this species. Similar results regarding water imbibition were observed in *A. caven*, with increased water content after 48 h of soaking in water, which does not agree with results from previous research [25,37,38]. Specifically, Funes and Venier [38] indicate that low water imbibition and low germination in non-scarified seeds (0%) vs. scarified seeds (96.6 ± 1.6%) is evidence of physical dormancy imposed by an impermeable seed coat, which could act as protection against humidity and temperature fluctuations [39]. However, in the research of Funes and Venier [38], imbibition was evaluated only up to 24 h, while our results indicate that maximum imbibition was reached 48 h after soaking (Table 1). In addition, germination was assessed for five days whereas, according to our results, maximum germination is reached after 25 days (Figure 1c), indicating that while seed coat impermeability could be present it can be overcome with longer water imbibition and germination periods. Thus, longer imbibition times in *A. caven* could be linked to prevention of germination in sites with unpredictable or sporadic rainfall [40], a characteristic of its habitat in central Chile.

According to Hartmann and Kester [41], seed germination occurs with water content from 40% to 60%, which would indicate that the only seeds that did not reach the needed water content for germination are the ones from *C. chilensis*, and this could explain the low germination levels observed in the control treatment (Table 2, Figure 1f). In addition, desiccation tolerance of the species can also affect germination, which would be directly linked to the initial water content of seeds (Table 1). However, the seed storage behavior of species included in this study had not been experimentally evaluated. Most species from dryland environments, such as central Chile are likely to have desiccation tolerance seeds [42]. According to the Seed Information Database [43] seeds of these species should be desiccation tolerant (Orthodox).

3.2. Germination Experiment

High germination in *Q. saponaria* seed is in agreement with previous reports [44,45]. The absence of significant differences between pre-germination treatments (Figure 1a) indicate that *Q. saponaria* seeds do not present dormancy, which contradicts Figueroa and Jaksic [25], who indicate that the presence of an undetermined dormancy in *Q. saponaria* seeds and a physiological dormancy as proposed by Baskin and Baskin [46] according to Donoso and Cabello [47] results. However, no information regarding seed storage conditions was displayed by authors that mentioned an undetermined dormancy in this species, which could also affect germination. In addition, soaking in water for 24 h should be enough to achieve high water content in seeds (Table 1) and promote a high germination (90.2 ± 2.0%), which is a smaller imbibition time than the 72 h reported by Benedetti et al. [48]. According to nursery survey results, 83% of nurseries a proper treatment for seed germination.

In *L. caustica*, Donoso and Cabello [47] reported a germination capacity of 59% and recommended to treat seeds with sulfuric acid for at least three hours. Similar results were reported by [49] after remotion of seeds epicarp. Our results indicate that chemical scarification with sulfuric acid accelerates the germination process; however, it does not increase germination capacity in comparison to physical scarification or water soaking (Figure 1b), treatments that become in a suitable alternative to chemical scarification. However, these last alternatives extend the germination process to 46 and 51 days, respectively. These periods should be taken into consideration by nursery managers for sowing planification activities. According to our survey, at least 80% of nurseries apply a proper pre-germination treatment in this species.

In *A. caven* our results do not agree with previous reports [37,50,51], where seeds exposure to sulfuric acid before sowing promotes high germination (between 70% to 90%) as result of physical dormancy breaking of seeds [25]. However, germination observed after physical and chemical scarification treatments suggest deterioration of the seeds, questioning the existence of physical dormancy (Figure 1c). According to nursery surveys, we observed that at least 80% of nurseries applied chemical or physical scarification (Table 3)

as a substitute for chemical scarification; these treatments could be simplified by water soaking for longer periods of time, such as 48 to 72 h.

According to Cabello et al. [36], *P. chilensis* seeds do not have physical dormancy related to impermeable seed coat, which agrees with our results regarding an increased water imbibition until 48 h since sowing. Despite that Loayza et al. [52] indicated that germination in response to physical scarification depends on seed provenance, our results showed that scarification with hot water caused a decrease in germination compared to control treatment ($0.3 \pm 0.2\%$ vs. $47.4 \pm 2.3\%$, respectively) (Figure 1d), suggesting that *P. chilensis* seed coat it is not impermeable to water and imbibition in hot water should have a detrimental effect on seed viability of this species (Figure 1d). Instead, Cabello et al. [36] reported that *P. chilensis* seeds present endogenous physiological dormancy, which could be broken with 60 days of cols stratification or soaking in 400 mg L^{-1} of gibberellic acid, reaching a germination capacity of 78.2%, higher than results obtained in this research. Although our gibberellic acid treatment was 200 mg L^{-1} and no differences were observed in germination compared to control, gibberellic acid decreased germination times by 11 days relative to control treatment, indicating that physiological dormancy could be present in this species. Despite that gibberellic acid application seems the most appropriate pre-treatment for this species, 100% of nurseries that produce *P. chilensis* only apply soaking in water as pre-germination and no specific treatment to break physiological dormancy is considered (Table 3).

Results observed in *K. angustifolia* regarding germination capacity agree with Takayashiki et al. [53], although the authors indicate a soaking in water for four days as pre-treatment, while according to our results 48 h in water is enough to achieve a water content of $105.4 \pm 8.9\%$ and to promote a germination of $85.0 \pm 6.0\%$. These last results are consistent with several authors [54–56] and the surveyed nurseries that achieved germination between 70% to 80% without pre-germination treatments in direct sowing, indicating that *K. angustifolia* seeds are not dormant.

Scarification has been reported by several authors [57–60], as a method to break physical dormancy in *Prosopis* species seeds. In fact, it had been reported that passage through a digestive tract of frugivores and cattle induce germination by promoting seed coat rupture [61,62]. However, Vilela and Ravetta [60] indicated that chemical scarification for 15 min reduced germination *C. chilensis*, while physical scarification (dipped in boiling water until water reached room temperature) induced higher germination. In *Prosopis ferox*, a similar species, Ortega et al. [59] obtained higher germination after physical scarification ($93.0 \pm 0.03\%$) and chemical scarification ($91.0 \pm 0.02\%$) compare with hydrochloric acid ($14.0 \pm 0.02\%$). Our results agree with the statement that *C. chilensis* germination is promoted by chemical (with sulfuric acid) or physical scarification (Figure 1f). Physical and chemical scarification did not caused differences in germination, but affected the time when germination reached a stable value (30 vs. 9 days since sowing, respectively) (Figure 1f). Although 66% of surveyed nurseries apply a proper scarification treatment (physical or chemical, 33% each), the time needed to complete the germination process is a factor that should be considered at a large operational scale.

3.3. Operational Applicability of Pre-Germination Treatments

Among the main problems mentioned by nurseries, we highlight the lack of technical capabilities (Table 3); there is a knowledge breach regarding to preparation, application, and manipulation of some chemical products for the implementation of treatments. This lack of information could be amended through instances of training and technological transference instances. This agrees with the diagnosis reported by León-Lobos et al. [23] where lack of knowledge regarding the dormancy of breaking in seeds of several native species is identified as a bottleneck for the fulfilment of Chile restoration commitments.

In regard to chemical scarification, its operational implementation (Table 3) is limited in some nurseries by the manipulation of corrosive chemical such as sulfuric acid, and managers indicate concern regarding the risk to staff safety and chemical residue dis-

posal [35]. On the other hand, physical scarification application is limited due to technical restrictions and lack of infrastructure (Table 3), in particular the need for equipment to process large numbers of seeds at operation scale, technology that is not widely distributed in Chilean nurseries.

4. Materials and Methods

4.1. Species Selection and Locations for Seed Collection

Six tree species from the sclerophyll forest were selected between Valparaíso and Biobío regions, three were selected according to dominance and three according to the degree of ecological vulnerability (for a full description of the species see Table S1). Vulnerability was referred to the conservation state according to the Classification Regulation of the Species from the Environmental Ministry of Chile [63]. Regarding the dominance criteria *Q. saponaria*, *L. caustica* and *A. caven* were selected, while *P. chilensis*, (vulnerable), *K. angustifolia* (near threatened) and *C. chilensis* (vulnerable) were selected according to conservation criteria.

Seed collection was performed between January and March of 2020 in the populations indicated in Figure 2. Seeds were sampled from at least 10 trees for each species with a minimal distance of 15 m between each tree. Once collected, seeds were transferred to the Centro Tecnológico de la Planta Forestal from the Instituto Forestal (36°50.9′ S; 73°7.9′ W), Biobío region, Chile, for cleaning and storage at 4 °C until mid-May of 2020.

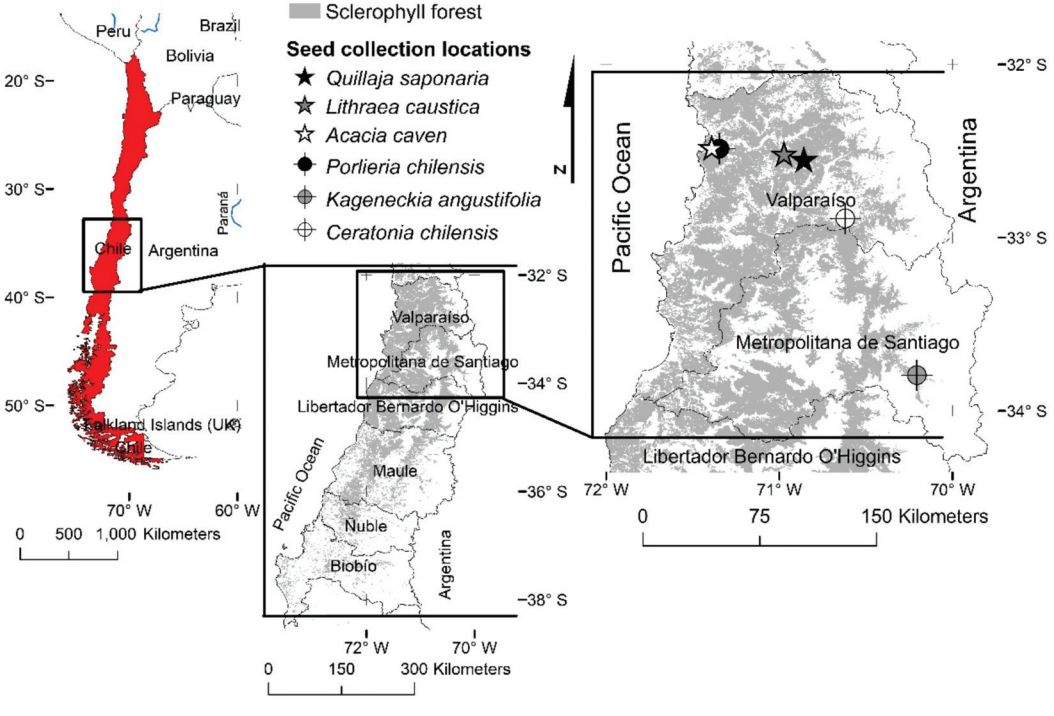


Figure 2. Distribution of sclerophyll forest (in grey) in Mediterranean central Chile and seed collection locations for *Acacia caven*, *Quillaja saponaria*, *Lithraea caustica*, *Porlieria chilensis*, *Kageneckia angustifolia* and *Ceratonia chilensis*.

4.2. Seed Characterization

As part of seed characterization prior to pre-germination treatment application, the number of seeds per kilogram, the initial water content of seeds, and the increase in water

seed imbibition was recorded, seed weight was recorded in three samples of 100 seeds (replicates) for each species to estimate the number of seeds per kilogram. Then, each sample was divided in two sub-samples of 50 seeds each, which were used to determine initial moisture content (w/w, dry basis), and after 24 and 48 h of soaking in distilled water. For this, one seed sub-sample of each species was weighted before soaking and after 24 and 48 h of soaking, while the other sub-sample was oven dried in a forced ventilation oven (Binder, model FD115, Tuttlingen, Germany) at 105 °C until constant weight. Weight of fresh and dried seeds were recorded on a 0.001 g precision scale (Quimis, Q DH-203, São Paulo, Brazil).

4.3. Pre-Germination Treatments

Three pre-germination treatments were evaluated for each species, which were selected according to available information from previous experiments [36,37,44,47,50,53,64–70] and information obtained from a survey performed to six nurseries to consider pre-germination treatments that were feasible to apply at operational scale at that were commonly applied in nurseries (Table S2). Selected nurseries for the survey produced a larger amount of the native species selected for this research between 2017 and 2019 nationally [71].

The survey identified the pre-germination treatments operationally applied for each evaluated species, and restrictions for proper treatment application linked to technical capabilities, infrastructure, or equipment. In addition, knowledge gaps regarding the benefits of the application of pre-germination treatments was assessed.

For *L. caustica*, *A. caven*, and *C. chilensis* seeds with reported physical dormancy [25] two scarification treatments were applied: (1) chemical scarification, consisted of exposure of seeds to sulfuric acid (PQM Fermont, Monterrey, Mexico) at a concentration of 97.3% for 90 min, then seeds were rinsed with distilled water and soaked in water at room temperature for 48 h; (2) physical scarification, consisted of exposure of seeds to water at 80 °C, seeds were cooled until room temperature and soaked in water for 48 h. For *Q. Saponaria* and *K. angustifolia*, pre-germination treatments consisted of the use of gibberellin to break physiological dormancy, seeds were soaked in gibberellin at 200 mg L^{-1} and 600 mg L^{-1} (GA_3, Giberplus, Anasac, Santiago, Chile) for 48 h. In the case of *P. chilensis*, treatments were the use of gibberellin at 200 mg L^{-1} and physical scarification as previously described. For all species, seed soaking in distilled water for 48 h corresponded to control treatment.

4.4. Germination Experiment

The germination experiment was performed in the Centro Tecnológico de la Planta Forestal of the Instituto Forestal, in greenhouse conditions (36 m^2). Maximum air temperature was limited to 25 °C through a forced ventilation system. A photoperiod of 12 day and 12 dark was established with six halide lamps of 400 Watts each (Philips Master HPI-Plus, Brussels, Belgium). To characterize environmental conditions during germination, air temperature (°C) and relative humidity (%) was measured with an Atmos 14 sensor (METER Group Inc., Pullman, WA, USA) and for substrate temperature (°C) a RT-1 (METER Group Inc.) sensor was used. Environmental data were recorded every 30 min with a ZL6 datalogger (METER Group Inc.). During the germination experiment an average air temperature of 19.3 ± 4.4 °C was observed, with a daily thermal oscillation of 10.2 ± 4.5 °C. Minimum and maximum daily substrate temperatures were 15.1 ± 3.0 °C and 22.4 ± 2.7 °C, respectively. Air relative humidity ranged between 35% and 84%, with an average of 63 ± 9%.

Sowing for six species was performed in June of 2020 in expanded polystyrene trays of 0.13 L (15 cm depth) and 84 cavities (336 cavities m^{-2}). Three seeds were sowed in each cavity at 1 cm depth approximately, three trays (replicates) were sowed for each germination treatment. Composted *Pinus radiata* bark was used as substrate with particles smaller than 10 mm (pH = 5.3 ± 0.01; organic matter = 76.1 ± 2.8%; total nitrogen = 0.9 ± 0.1%; C/N relation = 48.5 ± 6.4; N-NO_3 = 233.6 ± 37.3 mg kg^{-1}; N-NH_4 = 662.6 ± 56.3 mg kg^{-1}; water retention = 0.45 m^3 m^{-3}). Irrigation was performed with watering cans once a day, maintaining high humidity in the surface of the substrate.

Germination was measured three times per week (Monday, Wednesday and Friday), the number of germinated seeds was recorded out of the total sowed seeds (84 cavities $\times$ 3 seed cavities^{-1}) for each of the three trays (replicates), species and pre-germination treatment. Since the sowing, germination was monitored for 30 days in *Q. Saponaria* and *K. angustifolia*, 53 days in *A. caven* and *C. chilensis*, and 63 days in *L. caustica* and *P. chilensis*. Occurrence of germination was considered when the epicotyl emerged from the substrate surface.

4.5. Data Analysis

The average values of number of seeds per kilogram was calculated for each species from the weight of 100 seeds. The analysis of relative water content of seeds was performed through a repeated measurement analysis of one way for each species, considering the time for seed water imbibition time as factor (MED). Environmental data collection allowed the estimation of average temperature, minimum and maximum daily temperatures in air and substrate, and average relative humidity during the germination period.

For the germination, the experimental design corresponded to a completely randomized design with three pre-germination treatments (TRAT) for each species and with three replicates for each treatment. For each species, a germination analysis was performed through a repeated measures analysis of two ways for measurement time (MED) and pre-germination treatment (TRAT), assessing the main effects and interactions.

Repeated measure analysis was performed through a generalized mixed model using PROC GLIMMIX (SAS Institute, Cary, NC, USA) with selection of distribution and structure of the variance-covariance residual considering the Akaike Information Criteria (normal distribution and unstructured variance and covariance matrix for every analysis). Multiple comparison tests were performed for significant effects according to Tukey.

The time during germination with no further significant differences in the proportion of germinated seeds was evaluated performing comparison tests considering significant effects in the variance model (MED $\times$ TRAT).

Graphs development for data visualization were designed with SigmaPlot 10.0 software (Systat Software Inc., Chicago, IL, USA).

5. Conclusions

Although several nurseries apply the proper pre-germination treatment in some species such as *Q. saponaria*, *P. chilensis* and *K. angustifolia*, there is room for improvement in applied treatments in the rest of the species. For example, in *A. caven* the water imbibition time should be considered to improve germination before the application of other pre-germination treatments. In addition, in *L. caustica* and *C. chilensis* chemical scarification could be replaced by physical scarification to avoid the issues linked to manipulation of chemicals.

The evaluation of the rate of water imbibition is needed for the evaluated species before the implementation of required pre-germination treatments at an operational scale to avoid seed germination decay.

Physical scarification with hot water (80 °C) is a proper alternative to chemical scarification. However, higher germination times should be considered in sowing calendarization at a large operational scale.

Extension and technical transference instances could be helpful to reduce the breach in knowledge indicated by national nurseries. This could help to achieve an optimal implementation of pre-germination treatments and to avoid the application of incorrect treatments such as in *A. caven*, *P. chilensis*, and *C. chilensis*, which could generate high losses in seed supply. These actions tackle the bottleneck related to the lack of a proper seed supply to achieve reforestation commitments in Chile.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11050608/s1, Table S1: Selected species detailed description. Table S2: Pre-germination treatments applied by surveyed nurseries y reported by previous research for each of the evaluated species. N.R: Not reported.

Author Contributions: Conceptualization and methodology: E.C.-R., M.A. and M.G.-O. Data collection and germination measurements: A.U.-P. Data analysis: E.C.-R. Writing of the manuscript: E.C.-R., C.Á.-M., M.A., M.G.-O. and P.L.-L. Manuscript review and editing: E.C.-R., C.Á.-M. and P.L.-L. Translation: C.Á.-M. All authors have read and agreed to the published version of the manuscript.

Funding: Research was funded Corporación Nacional Forestal (CONAF), through the "Fondo de Investigación del Bosque Nativo" research grant, number 025/2019.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets generated for this study are available on request to corresponding author.

Acknowledgments: Authors would like to thank the Chilean Agricultural Ministry for supporting this research.

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

References

1. Armesto, J.J.; Arroyo, M.T.; Hinojosa, L.F. The Mediterranean Environment of Central Chile. In *The Physical Geography of South America*; Veblen, T., Young, K., Orme, A., Eds.; Oxford University Press: New York, NY, USA, 2007; pp. 184–199.
2. Myers, N.; Mittermeier, R.A.; Mittermeier, C.G.; Da Fonseca, G.A.B.; Kent, J. Biodiversity hotspots for conservation priorities. *Nature* **2000**, *403*, 853–858. [CrossRef]
3. Rundel, P.W. Mediterranean-Climate Ecosystems. *Encycl. Biodivers.* **2007**, 1–15. [CrossRef]
4. Garfias Salinas, R.; Castillo Soto, M.; Ruiz Gozalvo, F.; Vita Alonso, A.; Bown Intveen, H.; Navarro Cerrillo, R. Remanentes del Bosque Esclerófilo en la Zona Mediterránea de Chile Central: Caracterización y Distribución de Fragmentos. *Interciencia* **2018**, *43*, 655–663.
5. Salazar, A.; Baldi, G.; Hirota, M.; Syktus, J.; Mcalpine, C. Land use and land cover change impacts on the regional climate of non-Amazonian South America: A review. *Glob. Planet. Change* **2015**, *128*, 103–119. [CrossRef]
6. Mandle, L.; Bufford, J.L.; Schmidt, I.B.; Daehler, C.C. Woody exotic plant invasions and fire: Reciprocal impacts and consequences for native ecosystems. *Biol. Invasions* **2011**, *13*, 1815–1827. [CrossRef]
7. Miranda, A.; Altamirano, A.; Cayuela, L.; Lara, A.; González, M. Native forest loss in the Chilean biodiversity hotspot: Revealing the evidence. *Reg. Environ. Chang.* **2017**, *17*, 285–297. [CrossRef]
8. Schulz, J.J.; Cayuela, L.; Rey-Benayas, J.M.; Schröder, B. Factors influencing vegetation cover change in Mediterranean Central Chile (1975–2008). *Appl. Veg. Sci.* **2011**, *14*, 571–582. [CrossRef]
9. Altamirano, T. *Restauración de Los Sistemas Naturales Mediterráneos de Chile Central*; Universidad Católica: Santiago, Chile, 2008.
10. Urrutia-Jalabert, R.; González, M.E.; González-Reyes, Á.; Lara, A.; Garreaud, R. Climate variability and forest fires in central and south-central Chile. *Ecosphere* **2018**, *9*, e02171. [CrossRef]
11. Garreaud, R.D.; Boisier, J.P.; Rondanelli, R.; Montecinos, A.; Sepúlveda, H.H.; Veloso-Aguila, D. The Central Chile Mega Drought (2010–2018): A climate dynamics perspective. *Int. J. Climatol.* **2020**, *40*, 421–439. [CrossRef]
12. Boisier, J.P.; Rondanelli, R.; Garreaud, R.; Munoz, F. Anthropogenic and natural contributions to the Southeast Pacific precipitation decline and recent megadrought in central Chile. *Geophys. Res. Lett.* **2016**, *43*, 413–421. [CrossRef]
13. Garreaud, R.D.; Alvarez-Garreton, C.; Barichivich, J.; Pablo Boisier, J.; Christie, D.; Galleguillos, M.; LeQuesne, C.; McPhee, J.; Zambrano-Bigiarini, M. The 2010-2015 megadrought in central Chile: Impacts on regional hydroclimate and vegetation. *Hydrol. Earth Syst. Sci.* **2017**, *21*, 6307–6327. [CrossRef]
14. Ciccarese, L.; Mattsson, A.; Pettenella, D. Ecosystem services from forest restoration: Thinking ahead. *New For.* **2012**, *43*, 543–560. [CrossRef]
15. Lal, R. Offsetting global CO2 emissions by restoration of endangered soils and intensification of world agriculture and forestry. *Land Degrad. Dev.* **2003**, *14*, 309–322. [CrossRef]
16. Griscom, B.W.; Adams, J.; Ellis, P.W.; Houghton, R.A.; Lomax, G.; Miteva, D.A.; Schlesinger, W.H.; Shoch, D.; Siikamäki, J.V.; Smith, P.; et al. Natural climate solutions. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 11645–11650. [CrossRef]
17. Marquet, P.A.; Altamirano, A.; Arroyo, M.T.K.; Fernández, M.; Gelcich, S.; Górski, K.; Habit, E.; Lara, A.; Maass, A.; Pauchard, A.; et al. *Biodiversidad y Cambio Climático en Chile: Evidencia Científica Para la Toma de Decisiones*; Comité Científico COP25, Ed.; Ministerior de Ciencias, Tecnología, Conocimiento e Innovación: Santiago, Chile, 2019.

18. Becerra, P.; Smith-Ramírez, C.; Arellano, E. *Evaluación de Técnicas Pasivas y Activas Para la Recuperación del Bosque Esclerófilo de Chile Central*; Corporación Nacional Forestal: Santiago, Chile, 2018.
19. Sewell, A.; Van Der Esch, S.; Löwenhardt, H. *Goals and Commitments for the Restoration Decade*; PBL Publishers: The Hague, The Netherlands, 2020.
20. Cordero, C.; Vasconi, P. *Actualización de la Contribución Determinada a Nivel Nacional (NDC) 2020 de Chile*; Ministerio del Medio Ambiente: Santiago, Chile, 2020.
21. Bannister, J.R.; Vargas-Gaete, R.; Ovalle, J.F.; Acevedo, M.; Fuentes-Ramirez, A.; Donoso, P.J.; Promis, A.; Smith-Ramírez, C. Major bottlenecks for the restoration of natural forests in Chile. *Restor. Ecol.* **2018**, *26*, 1039–1044. [CrossRef]
22. Acevedo, M.; Álvarez-Maldini, C.; Kasten Dumroese, R.; Bannister, J.R.; Cartes, E.; González, M. Native Plant Production in Chile. Is It Possible to Achieve Restoration Goals by 2035? *Land* **2021**, *10*, 71. [CrossRef]
23. León-Lobos, P.; Bustamante-Sánchez, M.A.; Nelson, C.R.; Alarcón, D.; Hasbún, R.; Way, M.; Pritchard, H.W.; Armesto, J.J. Lack of adequate seed supply is a major bottleneck for effective ecosystem restoration in Chile: Friendly amendment to Bannister et al. (2018). *Restor. Ecol.* **2020**, *28*, 277–281. [CrossRef]
24. Jiménez, H.E.; Armesto, J.J. Importance of the soil seed bank of disturbed sites in Chilean matorral in early secondary succession. *J. Veg. Sci.* **1992**, *3*, 579–586. [CrossRef]
25. Figueroa, J.A.; Jaksic, F.M. Latencia y banco de semillas en plantas de la región mediterránea de Chile central. *Rev. Chil. Hist. Nat.* **2004**, *77*, 201–2015. [CrossRef]
26. Figueroa, J.A.; Teillier, S.; Jaksic, F.M. Composition, size and dynamics of the seed bank in a mediterranean shrubland of Chile. *Austral Ecol.* **2004**, *29*, 574–584. [CrossRef]
27. Rundel, P.W.; Cowling, R.M. Mediterranean-Climate Ecosystems. In *Encyclopedia of Biodiversity*, 2nd ed.; Elsevier Inc.: Amsterdam, The Netherlands, 2013; pp. 212–222. ISBN 9780123847195.
28. Ruthrof, K.X.; Bader, M.K.F.; Matusick, G.; Jakob, S.; Hardy, G.E.S.J. Promoting seedling physiological performance and early establishment in degraded Mediterranean-type ecosystems. *New For.* **2016**, *47*, 357–376. [CrossRef]
29. Cabello, A. *Estudio Anatómico y de Germinación en Litre (Lithrea Caustica (Mol.) H. et Arn.)*; Universidad de Chile: Santiago, Chile, 1979.
30. Silva, S. *Ecología Trófica y Nutricional del Zorro Culpeo (Pseudalopex Culpaeus): Restricciones Digestivas y Energéticas Asociadas a la Frugivoría y Sus Efectos Sobre la Dispersión de Semillas*; Universidad Católica de Chile: Santiago, Chile, 2001.
31. León-Lobos, P.; Kalin-Arroyo, M. Germinación de semillas de Lithrea caustica (Mol) H et A (Anacardiaceae) dispersadas por Pseudalopex sp (Canidae) en el bosque esclerófilo de Chile central. *Rev. Chil. Hist. Nat.* **1994**, *67*, 59–64.
32. Silva, S.I.; Bozinovic, F.; Jaksic, F.M. Frugivory and seed dispersal by foxes in relation to mammalian prey abundance in a semiarid thornscrub. *Austral Ecol.* **2005**, *30*, 739–746. [CrossRef]
33. Clewell, A.; Rieger, J.P. What practitioners need from restoration ecologists. *Restor. Ecol.* **1997**, *5*, 350–354. [CrossRef]
34. Broadhurst, L.M.; Jones, T.A.; Smith, F.S.; North, T.; Guja, L. Maximizing Seed Resources for Restoration in an Uncertain Future. *Bioscience* **2015**, *66*, 73–79. [CrossRef]
35. Winer, N. Germination of pretreated seed of mesquite (Prosopis chilensis) under arid conditions in northern Sudan. *For. Ecol. Manag.* **1983**, *5*, 307–312. [CrossRef]
36. Cabello, A.; Valdés, P.; Escobar, D.; Letelier, P. Efecto de la temperatura y de la aplicación de tratamientos pregerminativos sobre la germinación de semillas de Porlieria chilensis I. M. Johnst., guayacán. *Rev. Chagual* **2013**, *11*, 61–71.
37. Cabello, A.; Alvear, A. Efecto de la temperatura sobre la germinación de dos lotes de semillas de espino (Acacia caven (Mol.) Mol.). *Ciencias For.* **1991**, *7*, 3–12.
38. Funes, G.; Venier, P. Dormancy and germination in three Acacia (Fabaceae) species from central Argentina. *Seed Sci. Res.* **2006**, *16*, 77–82. [CrossRef]
39. Mohamed-Yasseen, Y.; Barringer, S.A.; Splittstoesser, W.E.; Costanza, S. The Role of Seed Coats in Seed Viability. *Bot. Rev.* **1994**, *4*, 426–439. [CrossRef]
40. Turner, S.R.; Merritt, D.J.; Baskin, C.C.; Dixon, K.W.; Baskin, J.M. Physical dormancy in seeds of six genera of Australian Rhamnaceae. *Seed Sci. Res.* **2005**, *15*, 51–58. [CrossRef]
41. Hartmann, H.; Kester, D. *Plant Propagation. Principles and Practices*, 1st ed.; Prentice-Hall: Hoboken, NJ, USA, 1983.
42. Wyse, S.V.; Dickie, J.B. Taxonomic affinity, habitat and seed mass strongly predict seed desiccation response: A boosted regression trees analysis based on 17,539 species. *Ann. Bot.* **2018**, *121*, 71–83. [CrossRef]
43. Seed Information Database: Royal Botanic Gardens, Kew. Available online: https://data.kew.org/sid/citing.html (accessed on 12 February 2022).
44. Salazar, C. *Caracterización de Semillas de Quillaja Saponaria Mol., para Distintas Procedencias de la Octava Región*; Universidad de Concepción: Concepción, Chile, 1998.
45. González Ortega, M.P.; García Rivas, E.; Quiroz Marchant, I.; Soto Guevara, H. Estándares de producción de plantas de Quillay (quillaja saponaria mol.). *Rev. Chile For.* **2011**, *353*, 43–46.
46. Baskin, C.C.; Baskin, J.M. *Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination*; Elsevier: Amsterdam, The Netherlands, 2014; pp. 1–1586. [CrossRef]
47. Donoso Zegers, C.; Cabello Lechuga, A. Antecedentes fenológicos y de germinación de especies leñosas chilenas. *Ciencias For.* **1978**, *1*, 31–41.

48. Benedetti, S.; Delard, C.; Roach, F.; González, M. *Monografía de Quillay, Quillaja Saponaria*; INFOR: Santiago, Chile, 2000.
49. Morales-Paredes, C.; Valdivia, C.E.; Sade, S. La frugivoría por cánidos nativos (*Lycalopex* spp.) y alóctonos (*Canis lupus familiaris*) reduce la germinación de semillas de litre (*Lithrea caustica*) en Chile central. *Bosque* **2015**, *36*, 481–486. [CrossRef]
50. Franco, E.T.H.; Feltrin, I.J. Quebra de dormencia de sementes de Espinilho (Acacia caven Mol..). *Ciência Rural* **1994**, *24*, 303–305. [CrossRef]
51. Plaza, Â.; Castillo, M. Germination rates of four Chilean forest trees seeds: Quillaja saponaria, Prosopis chilensis, Vachellia caven, and Caesalpinia spinosa. *F1000Research* **2018**, *7*, 1446. [CrossRef]
52. Loayza, A.P.; Rios, R.S.; Carvajal, D.E. *Estado de Conservación de Porlieria Chilensis: Evaluación a Través de Modelos Poblacionales Matriciales, Ecología y Patrones de Distribución*; Corporación Nacional Forestal Santiago: Santiago, Chile, 2015.
53. Takayashiki, M.; Williams, E.; Schenk, J.; Alvarado, M.; Greau, M. *Monografías de Especies Para la Forestación en la Zona Semiárida de Chile. Proyecto Cuencas CONAF-JICA "Control de Erosión y Forestación en Cuencas Hidrográficas de la Zona Semiárida de Chile"*; Ministerio de Agricultura Coarparación Nacional Forestal: Santiago, Chile, 1998.
54. Cavieres, L.A.; Chacón, P.; Peñaloza, A.; Molina-Montenegro, M.; Arroyo, M.T.K. Leaf litter of Kageneckia angustifolia D. Don (Rosaceae) inhibits seed germination in sclerophyllous montane woodlands of central Chile. *Plant Ecol.* **2006**, *190*, 13–22. [CrossRef]
55. Peñaloza, A.; Cavieres, L.; Arroyo, M.T.K.; Torres, C. Efecto nodriza intra-específico de Kageneckia angustifolia D. Don (Rosaceae) sobre la germinación de semillas y sobrevivencia de plántulas en el bosque esclerófilo montano de Chile central. *Rev. Chil. Hist. Nat.* **2001**, *74*, 539–548. [CrossRef]
56. Cavieres, L.A.; Peñaloza, A. Facilitation and interference at the intraspecific level: Recruitment of Kageneckia angustifolia D. Don (Rosaceae) in the montane sclerophyllous woodland of central Chile. *Perspect. Plant Ecol. Evol. Syst.* **2012**, *14*, 13–19. [CrossRef]
57. Arce, P.M.; Medina, C.; Balboa, O. Tolerancia a la salinidad en la germinación de tres especies de Prosopis (*P. alba*, *P. chilensis* y *P. tamarugo*). *Cienc. Investig. Agrar.* **1990**, *17*, 71–76. [CrossRef]
58. Arévalo, J.P. *Tratamientos Para Mejorar la Germinación de Semillas de Yerba Mate (Ilex Paraguariensis) y Algarrobo (Prosopis spp.)*; Escuela Agrícola Panamericana: Zamorano, Honduras, 1998.
59. Ortega Baes, P.; De Viana, M.L.; Suk, S. Germination in Prosopis ferox seeds: Effects of mechanical, chemical and biological scarificators. *J. Arid Environ.* **2002**, *50*, 185–189. [CrossRef]
60. Vilela, A.E.; Ravetta, D.A. The effect of seed scarification and soil-media on germination, growth, storage, and survival of seedlings of five species of Prosopis L. (Mimosaceae). *J. Arid Environ.* **2001**, *48*, 171–184. [CrossRef]
61. Cox, J.R.; De Alba-Avila, A.; Rice, R.W.; Cox, J.N. Biological and physical factors influencing Acacia constricta and Prosopis velutina establishment in the Sonoran Desert. *J. Range Environ.* **1993**, *46*, 43–48. [CrossRef]
62. Pelaez, D.V.; Boo, R.M.; Elia, O.R. Emergence and seedling survival of calden in the semiarid region of Argentina. *J. Range Manag.* **1992**, *45*, 564–566. [CrossRef]
63. Ministerio de Medio Ambiente Listado de Especies Clasificadas Desde el 1o al 16o Proceso de Clasificación RCE (Actualizado a Agosto de 2021). Available online: https://clasificacionespecies.mma.gob.cl/wp-content/uploads/2021/09/NominaDeEspeciesSegunEstadoConservacion-Chile_actualizado_16toProcesoRCE_rev04agosto2021_.xlsx (accessed on 25 January 2022).
64. Benedetti, S. *Monografía de Espino Acacia Caven (Mol.) Mol*; Instituto Forestal: Santiago, Chile, 2012.
65. Alvarado, A.; Levet, O. *Manual de Protocolos de Producción de Especies Utilizadas por el Programa de Arborización*; Corporación Nacional Forestal: Santiago, Chile, 2014.
66. Urbina, A. *Análisis de Germinación de una Procedencia de Litre (Lithraea Caustica (Mol.) Hook. et Arn.)*; Universidad de Concepción: Concepción, Chile, 2019.
67. Hoffman, A.; Kummerow, J. Estudios anatómicos sobre flor, fruto y testa de Acacia caven (Mol.) Hook, et Arm., y características de la germinación. *Phyton* **1962**, *19*, 21–25.
68. Stoehr, V. *Métodos de Reforestación con Espino (Acacia Caven) en la Zona Semiárida de Chile*; Universidad de Chile: Santiago, Chile, 1969.
69. Serrada, M. *Especies Arbóreas y Arbustivas Para las Zonas Áridas y Semiáridas de América Latina*; FAO, Red Latinoamericana de Cooperación Técnica en Sistemas Agroforestales: Santiago, Chile, 1997.
70. Hechenletiner, V.; Gardner, M.; Thomas, P.; Echeverría, C.; Escobar, B.; Brownless, P.; Martínez, A. *Plantas Amenazadas del Centro Sur de Chile: Distribución, Conservación y Propagación*, 1st ed.; Universidad Austral de Chile y Real Jardín Botánico de Edimburgo: Valdivia, Chile, 2005.
71. Corporación Nacional Forestal Listado de Viveros Forestales. Available online: https://www.conaf.cl/nuestros-bosques/bosques-en-chile/viveros/ (accessed on 25 January 2022).

plants

MDPI

Article

Documenting Greek Indigenous Germplasm of Cornelian Cherry (*Cornus mas* L.) for Sustainable Utilization: Molecular Authentication, Asexual Propagation, and Phytochemical Evaluation

Eleftherios Karapatzak [1], Nikos Krigas [1,*], Ioannis Ganopoulos [1], Katerina Papanastasi [1], Dimitris Kyrkas [2], Paraskevi Yfanti [2], Nikos Nikisianis [3], Antonis Karydas [1], Ioannis Manthos [1], Ioanna S. Kosma [4], Anastasia V. Badeka [4], Dimitrios Fotakis [5], Eleni Maloupa [1] and Giorgos Patakioutas [2,*]

[1] Institute of Plant Breeding and Genetic Resources, Hellenic Agricultural Organization Dimitra, 57001 Thessaloniki, Greece; ekarapatzak@gmail.com (E.K.); giannis.ganopoulos@gmail.com (I.G.); papanastasi@bbgk.gr (K.P.); euripidis_1999@hotmail.com (A.K.); manthosjo@yahoo.gr (I.M.); maloupa@bbgk.gr (E.M.)

[2] Department of Agriculture, University of Ioannina, 47100 Arta, Greece; dkyrkas@uoi.gr (D.K.); pyfanti@uoi.gr (P.Y.)

[3] Systada General Partnership, 55133 Thessaloniki, Greece; nnikisia@gmail.com

[4] Laboratory of Food Chemistry, Department of Chemistry, University of Ioannina, 45110 Ioannina, Greece; i.kosma@uoi.gr (I.S.K.); abadeka@uoi.gr (A.V.B.)

[5] Forest Research Institute, Hellenic Agricultural Organization Dimitra, 57006 Thessaloniki, Greece; fotakis@fri.gr

* Correspondence: nikoskrigas@gmail.com (N.K.); gpatakiu@uoi.gr (G.P.); Tel.: +30-2310-471110 (N.K.); +30-2681-050224 (G.P.)

Citation: Karapatzak, E.; Krigas, N.; Ganopoulos, I.; Papanastasi, K.; Kyrkas, D.; Yfanti, P.; Nikisianis, N.; Karydas, A.; Manthos, I.; Kosma, I.S.; et al. Documenting Greek Indigenous Germplasm of Cornelian Cherry (*Cornus mas* L.) for Sustainable Utilization: Molecular Authentication, Asexual Propagation, and Phytochemical Evaluation. *Plants* **2022**, *11*, 1345. https://doi.org/10.3390/plants11101345

Academic Editors: Sofia Rhizopoulou, Maria Karatassiou and Efi Levizou

Received: 13 April 2022
Accepted: 12 May 2022
Published: 19 May 2022

Abstract: Wild-growing Cornelian cherries (*Cornus mas* L., Cornaceae) are well-known native fruits in Greece since ancient times that are still consumed locally nowadays. Modern research has highlighted the value of Cornelian cherries as functional food with exceptional health benefits on account of the fruits' biochemical profile. However, apart from local consumption directly from wild growing individuals, Greek native *C. mas* populations have not yet been investigated or sustainably utilized. A multifaceted evaluation was conducted herein including authorized collection-documentation, taxonomic identification, and molecular authentication (DNA barcoding), asexual propagation via cuttings and phytochemical evaluation (multiple antioxidant profiling) of neglected and underutilized Greek native *C. mas* germplasm sources. Successive botanical expeditions resulted in the collection of 18 samples of genotypes from distant *C. mas* populations across different natural habitats in Greece, most of which were DNA fingerprinted for the first time. Asexual propagation trials revealed high variability in rooting frequencies among Greek genotypes with low (<25%), average (25–50%), and adequate propagation potential (>50%) using external indole-3-butyric acid (IBA) hormone application on soft- or hard-wood cuttings. The comparative phytochemical evaluation of the studied Greek genotypes showed significant potential in terms of antioxidant activity (>80% radical scavenging activity in 13 genotypes), but with variable phenolic content (47.58–355.46 mg GAE/100 g), flavonoid content (0.15–0.86 mg CE/100 g), and vitamin C content (1–59 mg AAE/100 g). The collected material is currently maintained under ex situ conservation for long-term monitoring coupled with ongoing pilot cultivation trials. The pivotal data create for the first time a framework for the sustainable utilization of Greek native C. mas germplasm as a superfood with significant agronomic potential.

Keywords: neglected and underutilized plants; phytogenetic resources; DNA barcoding; forest berries; protocols; nutraceutical potential; genotype selection; multifaceted evaluation

1. Introduction

Apart from widely used crop varieties, phytogenetic resources also include wild-growing plant species which are often neglected or underutilized, especially in biodiversity-rich areas [1]. Such germplasm resources involve a wide variety of plant species, including small fruit trees of potentially high alimentary value, relating to nutritional health benefits, but also pharmaceutical value [2–6]. Local native fruit species can be an important source of natural food products rich in antioxidants such as polyphenols with known importance for human health [7–9]. The natural heritage of Greece and its rich biodiversity at all levels offer new possibilities for evaluating and utilizing valuable wild germplasm such as small stone fruits and berries with high nutritional and medicinal (in one word nutraceutical) value [6,10]. Prior to domestication and sustainable exploitation of wild germplasm resources of interest, multifaceted research approaches are required in terms of taxonomic validation and molecular authentication of wild plant material, agronomical assessment propagation-wise, and comprehensive phytochemical evaluation aiming at the selection of promising genotypes with high potential for further applied research and future breeding. To this end, Greek native *Rosa canina* and *Sambucus nigra* germplasms constitute recently developed example cases [6,10].

Cornelian cherry (*Cornus mas* L., Cornaceae) is a rather common and wild-growing deciduous shrub or occasionally a small tree 2–4(–6) m tall in scrub and woodlands of central and southeastern Europe and western Asia, often occurring in ravines or near streams (areas with sufficient rainfall) from low (100–200 m) to intermediate and higher altitudes (800 m, occasionally up to 1700 m) [11]. In Greece, Cornelian cherry is a traditionally well-known native fruit tree that has been consumed locally for centuries and it has been known since ancient times, i.e., the Trojan horse in Homeric times was believed to be a *C. mas* woodcraft [12]. The trees bloom by the end of winter and in early spring and set fruits (ellipsoid to broadly cylindrical drupes 12–15 mm long, becoming red and cherry-like) in late summer through fall. Cornelian cherry fruits (both wild-growing and cultivated) and their food derivatives (e.g., jams, jellies, juice) contain antioxidants, phenolic compounds, iron, potassium, vitamin C, flavonoids, and many other substances of high nutritional value [13–17]. In addition, *C. mas* fruits to date have validated pharmaceutical value in terms of free radical scavenging potential among others, resulting in several health benefits including antimicrobial, antidiabetic, anti-inflammatory, anticancer, and cardioprotective activity; these assets render *C. mas* fruits as functional food products and as 'superfood' with intensifying relevant research over recent years [18–20]. Apart from the flesh of the fruits, the endocarp of *C. mas* drupes has also been demonstrated to have nutraceutical potential as a source of bioactive compounds with strong antioxidant activity [21]. Sustainable utilization efforts of *C. mas* germplasm have been attempted in eastern European countries with promising results [22–24]. In more recent studies in the same area, the phytochemical evaluation of *C. mas* domesticated germplasm (cultivars and/or hybrids) has been conducted [22]. Furthermore, *C. mas* germplasm has been characterized in terms of the nutritional value of the fruits across a wider spectrum of its distribution range [25–28] and the results showed the nutritional superiority of wild-growing germplasm [29]. However, in these studies no assessment was included regarding the native *C. mas* wild-growing genotypes occurring in Greece.

To support classical taxonomic identification and to enable the establishment of a distinct genetic identity, molecular authentication can be performed through novel DNA barcoding and bioinformatic analysis methods in selected plant materials collected from natural habitats [30]. DNA barcoding as a tool for molecular authentication of plant germplasm has been greatly developed and to date is being widely used for many medicinal plant species and phytogenetic resources [6,10,31–35]. There are also reports of DNA barcodes being successfully used in identification of ancient DNA (aDNA) of *C. mas* from fossils [36]. However, such molecular tools are still underused in authenticating native *C. mas* germplasm of different regions.

To date, wild-type populations of Cornelian cherry (*C. mas*) from several parts of the world have been successfully propagated via cuttings in the past with the use of external hormone application on hardwood and soft wood cuttings taken from mature individuals growing in the wild [37,38]. A popular hormonal substance that has been used in the past is indole-3-butyric acid (IBA) [39,40], frequently achieving high rooting rates.

In this framework, the study herein was focused on the collection and multifaceted documentation of *C. mas* plant material from geographically separated Greek populations (different genotypes) with the aim to provide: (1) molecular authentication of the population samples via DNA barcoding; (2) asexual propagation protocols for the collected genotypes via propagation trials using cuttings (dormant hardwood twigs, fresh softwood plant parts) facilitating the ex situ conservation and evaluation of the collected germplasm; and (3) comparative phytochemical evaluation of their fruits in terms of nutraceutical properties (total phenolic content, antioxidant activity, total flavonoids, and vitamin C content) to assess their potential as germplasm sources for artificial selection of genotypes and future breeding efforts. The overall work provided first-time insight into Greek native *C. mas* (Cornelian cherry) germplasm and these efforts are aimed at paving the way for its sustainable utilization as a superfood with significant agronomic potential.

2. Results

2.1. Molecular Authentication of Greek Native Cornus mas Genotypes

BLAST1 and distance-based methods were selected to validate the authentication efficiency of ITS2 for the selected Greek native *Cornus mas* genotypes. The ITS2 barcode with BLAST1 showed a high competence (97% and 100%) in sample identification at species and genus levels, respectively. The ITS2 barcode with the DISTANCE method showed almost similar identification efficiency (98%) at the species level. Thus, barcode ITS2 using the NJ tree method was proved able to distinguish Greek native *C. mas* genotypes from other genotypes of different origin, and similarly, from other *Cornus* spp. not present in Greece (Figure 1A). The neighbor-joining (NJ) phylogenetic tree obtained from DNA barcoding application with the ITS2 region discerned two main groups among the samples of *Cornus* spp. used herein (n = 21), and clearly classified the Greek native *C. mas* genotypes of this study as distinct sub-group with respect to other samples of *C. mas* of different origins that were sourced from databases (Figure 1A). The nucleotide differences in Greek genotypes are depicted in Figure 1B. The separation of the genotypes in Figure 1A presents similarity to an extent with the geographical separation of the respective habitats since genotypes GR-1-BBGK-19,195, GR-1-BBGK-19,197, GR-1-BBGK-19,198, and GR-1-BBGK-19,190 coupled with genotype GR-1-BBGK-19,196 originate from areas of the same prefecture, whereas genotype GR-1-BBGK-19,72 is both genetically and geographically further apart (Figure 1A). The same holds true for genotype GR-1-BBGK-19,502 which is geographically separated coming from the lowest altitude. The bootstrap values seem to further validate this classification scheme. Despite the fact that evolutionary relationships may also be analyzed through the neighbor-joining tree, the key function applied herein was to repeatedly evaluate bootstrap values as a measure of informed distinction of the Greek native germplasm (see distinct clades). The results of this study outlined the ITS2 gene as an efficient tool (100%) regarding the distinction of the species in concern (*C. mas*) among other *Cornus* spp. (see monophyletic clusters).

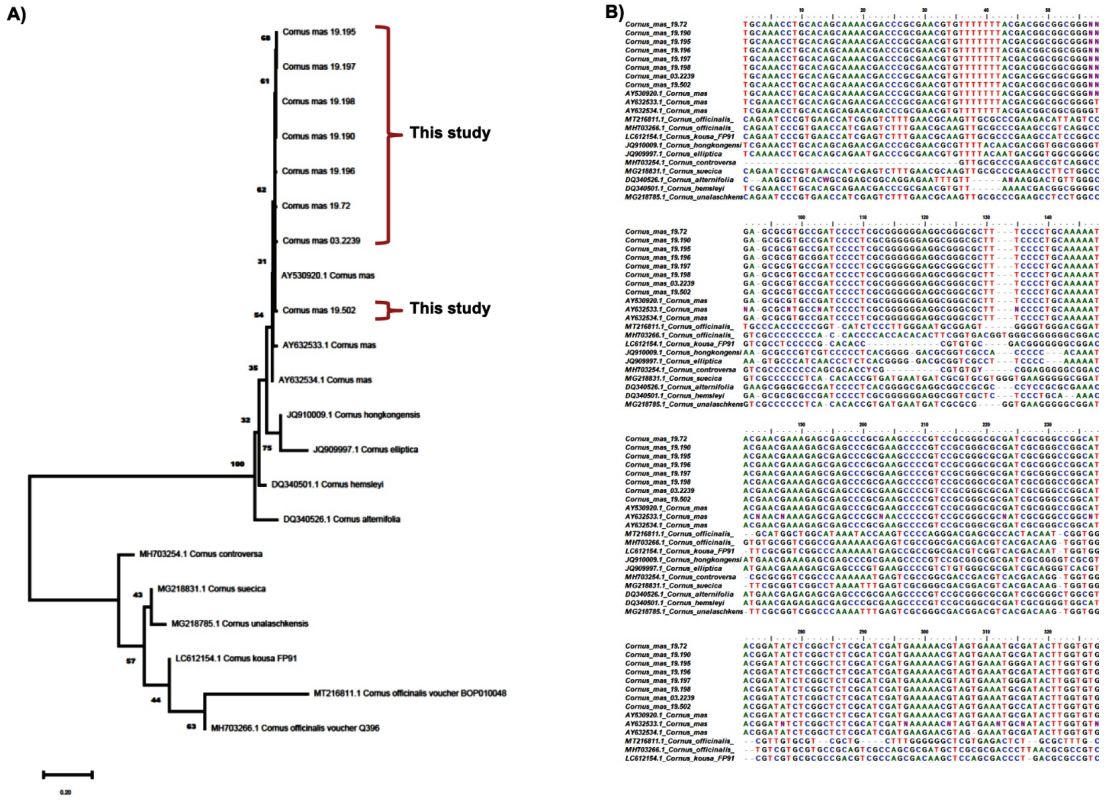


Figure 1. (**A**) Phylogenetic tree on the basis of ITS2 regions regarding the Greek native Cornus mas genotypes in contrast with other C. mas and Cornus spp. genotypes of different origin retrieved from NCBI. (**B**) Overview of the genotypes analyzed in this study with multiple sequence alignment of their ITS2 barcode region. Results from neighbor-joining (NJ) bootstrap analyses with 1000 replicates were used to assess the strength of the nodes. The node numbers indicated the bootstrap value of NJ. The distinct genotypes of this study are highlighted with blue.

2.2. Phytochemical Evaluation of the Greek Native Cornelian Cherries

The phytochemical screening of the fruits of Greek native *C. mas* samples revealed a significant level of variability between genotypes in terms of total phenolic content (TPC), vitamin C content, antioxidant activity (AA), and total flavonoids (TF) (Table 1, $p < 0.05$). No significant correlation with altitude was observed for any of the phytochemical parameters measured. Similarly, no significant correlation of the maturity index of the fruits with any of the phytochemical parameters measured was observed (Table 2, $p < 0.05$, $p < 0.01$). As expected, TPC was significantly positively correlated with TF ($p < 0.01$), while total dissolved solids (TDS) were significantly positively correlated with vitamin C content (Table 2, $p < 0.05$).

Table 1. Total phenolic content (TPC), antioxidant activity (AA), total flavonoids (TF), and vitamin C content assessed in fruit samples of wild-growing Greek genotypes of *Cornus mas*.

Population Sample	AA (%RSA)	TPC (mgGAE/100 g)	Vitamin C (mgAAE/100 g)	TF (mgCE/100 g)
GR-1-BBGK-19,72	90.99 ± 0.15 ^{g}	54.21 ± 0.1 ^{e}	1.03 ± 0.01 ^{a}	0.19 ± 0.01 ^{ab}
GR-1-BBGK-19,190A	80.48 ± 3.08 ^{d}	304.73 ± 6.56 ^{i}	21.22 ± 0.05 ^{c}	0.73 ± 0.12 ^{de}
GR-1-BBGK-19,190B	84.07 ± 0.01 ^{e}	49.29 ± 0.01 ^{cd}	33.48 ± 0.01 ^{f}	0.22 ± 0.1 ^{ab}
GR-1-BBGK-19,195	86.56 ± 0.01 ^{ef}	49.43 ± 0.01 ^{cde}	32.73 ± 0.01 ^{f}	0.15 ± 0.01 ^{a}
GR-1-BBGK-19,590	94.72 ± 0.15 ^{hi}	82.74 ± 0.31 ^{g}	1.31 ± 0.01 ^{a}	0.44 ± 0.01 ^{bc}
GR-1-BBGK-19,632	85.84 ± 0.01 ^{ef}	47.58 ± 0.01 ^{c}	28.13 ± 0.01 ^{e}	0.19 ± 0.01 ^{ab}
GR-1-BBGK-19,633A	95.52 ± 0.01 ^{hi}	52.31 ± 0.01 ^{cde}	44.69 ± 0.06 ^{j}	0.49 ± 0.01 ^{cd}
GR-1-BBGK-19,633B	90.5 ± 1.28 ^{g}	195.2 ± 0.0 ^{h}	37.18 ± 0.91 ^{g}	0.28 ± 0.06 ^{abc}
GR-1-BBGK-19,638A	55.46 ± 0.01 ^{a}	29.93 ± 0.01 ^{a}	52.33 ± 0.71 ^{k}	0.44 ± 0.01 ^{bc}
GR-1-BBGK-19,638B	94.01 ± 0.48 ^{hi}	337.14 ± 0.0 ^{j}	58.97 ± 0.9 ^{l}	0.44 ± 0.09 ^{bc}
GR-1-BBGK-19,641	95.64 ± 0.1 ^{i}	80.43 ± 0.14 ^{g}	41.33 ± 0.03 ^{i}	0.49 ± 0.03 ^{cd}
GR-1-BBGK-19,669	87.52 ± 0.1 ^{f}	355.46 ± 0.01 ^{k}	23.27 ± 0.26 ^{d}	0.86 ± 0.09 ^{e}
GR-1-BBGK-19,753	70.11 ± 0.05 ^{c}	40.56 ± 0.15 ^{b}	1.26 ± 0.02 ^{a}	0.19 ± 0.02 ^{ab}
GR-1-BBGK-19,844	95.94 ± 0.01 ^{i}	52.63 ± 0.01 ^{de}	40.05 ± 1.51 ^{hi}	0.17 ± 0.1 ^{a}
GR-1-BBGK-19,847	65.07 ± 0.01 ^{d}	40.69 ± 0.05 ^{b}	0.95 ± 0.1 ^{a}	0.11 ± 0.1 ^{a}
GR-1-BBGK-19,848	57.9 ± 0.02 ^{a}	38.82 ± 0.05 ^{b}	39.13 ± 0.1 ^{h}	0.17 ± 0.1 ^{a}
GR-1-BBGK-19,926	94.43 ± 0.02 ^{hi}	73.61 ± 0.02 ^{f}	15.96 ± 0.2 ^{b}	0.21 ± 0.2 ^{ab}

Values represent mean values ± standard deviation (S.D.) of samples analyzed in triplicate (n = 3); values within the same column that do not share the same letter are significantly different (Tukey post-hoc test, $p < 0.05$). For genotypes GR-1-BBGK-19,190, GR-1-BBGK-19,633, and GR-1-BBGK-19,638, capital letters A and B denote two consecutive years that fruits were measured.

Table 2. Pearson's correlation coefficients for the Greek *C. mas* genotypes (n = 14) between altitudes (m), four fruit nutraceutical properties assessed namely total phenolic content (TPC, mgGAE/100 g), antioxidant activity (AA, %RSA), total flavonoids (TF, mgCE/100 g), and vitamin C content (Vit C, mgAAE/100 g) as well as two complementary fruit phytochemical properties measured, i.e., maturity index (MI) expressed as the ratio of sugar content (°Brix) to malic acid content (gMA/100 g) and total dissolved solids (TDS, mg/L).

	Altitude	TDS	MI	AA	TPC	Vit C	TF
Altitude		−0.052 (0.861)	−0.375 (0.187)	−0.019 (0.949)	0.241 (0.406)	0.080 (0.787)	0.223 (0.444)
TDS			0.475 (0.086)	0.095 (0.746)	0.132 (0.654)	0.600 * (0.023)	0.172 (0.556)
MI				0.238 (0.413)	−0.184 (0.529)	0.326 (0.255)	0.060 (0.838)
AA					0.091 (0.758)	−0.295 (0.307)	0.021 (0.944)
TPC						−0.082 (0.781)	0.843 ** (0.000)
Vit C							0.188 (0.521)
TF							

Respective p-values are shown in parentheses. ** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

Antioxidant activity (AA) among all assessed genotypes ranged from 55.46 %RSA (radical scavenging activity) in GR-1-BBGK-19,638A to 95.94 %RSA in GR-1-BBGK-19,844 with most genotypes, however, presenting AA above 80% (Table 1, $p < 0.05$). TPC ranged from 29.93 mg GAE/100 g in GR-1-BBGK-19,638A to 355.46 mg GAE/100 g in GR-1-BBGK-19,669. Vitamin C content ranged from 0.95 mg AAE/100 g in GR-1-BBGK-19,847 to 58.97 mg AAE/100 g in GR-1-BBGK-19,638B. Similarly, TF ranged from 0.11 mg CE/100 g in GR-1-BBGK-19,847 to 0.86 mg CE/100 g in GR-1-BBGK-19,669. Most of the genotypes showed very high AA (>80 %RSA) except genotypes GR-1-BBGK-19,638A, GR-1-BBGK-19,753, GR-1-BBGK-19,847, and GR-1-BBGK-19,848 ($p < 0.05$). The same four genotypes also presented the lowest TPC, as expected (Table 1, $p < 0.05$). Very high values of TPC were found in three cases, namely 304.73, 337.14, and 355.46 mg GAE/100 g in genotypes GR-1-BBGK-19,190A, GR-1-BBGK-19,638B, and GR-1-BBGK-19,669, respectively. Concerning vitamin C content, a messier picture emerged with four genotypes presenting very low

values of <1.5 mg AAE/100 g including GR-1-BBGK-19,753 and GR-1-BBGK-19,847, similar to AA and TPC results but also genotypes GR-1-BBGK-19,72 and GR-1-BBGK-19,590 which performed better in TPC and AA. All other genotypes showed vitamin C content values from 10 up to 30 times higher than 1.5 mg AAE/100 g (Table 1, $p < 0.05$). Concerning TF, genotypes GR-1-BBGK-19,847 and GR-1-BBGK-19,848 along with GR-1-BBGK-19,195 were among the poorest with 0.11, 0.17, and 0.15 mg CE/100 g, respectively (Table 1, $p < 0.05$).

In addition to the above results, significant year-to-year variations were observed in genotypes that were measured for two consecutive years, especially in TPC. Particularly, genotype GR-1-BBGK-19,190 showed a drop in TPC from 304.73 mg GAE/100 g in 2019 to 49.29 mg GAE/100 g in 2020 coupled with a drop in TF from 0.73 to 0.22 mg CE/100 g (Table 1, $p < 0.05$). On the contrary, genotypes GR-1-BBGK-19,633 and GR-1-BBGK-19,638 showed an increase in TPC between 2019 and 2020 from 52.31 to 195.2 mg GAE/100 g and from 29.93 to 337.14 mg GAE/100 g, respectively, with the latter showing also a significant increase in AA (Table 1, $p < 0.05$).

2.3. Propagation of Greek Native Cornus mas Genotypes with Cuttings

A variation in rooting capacity was observed between different genotypes of *C. mas*. Rooting frequencies varied from 1.19% in genotype GR-1-BBGK-19,641 with 6000 ppm IBA/softwood cuttings in summer to 69.93% in genotype GR-1-BBGK-19,638 with 10,000 ppm IBA/softwood cuttings in late summer which was the highest rooting capacity observed ($p < 0.05$). The next best rooting capacity (58.33%) was observed in genotype GR-1-BBGK-19,753 with 4000 ppm IBA/softwood cuttings in early autumn (Table 3, $p < 0.05$). Hardwood, dormant cuttings during the winter presented generally low rooting rates which took months to be reached since cuttings remained under mist throughout winter and started to root in the following spring. The highest rooting frequency observed within this group was 20.93% ($p < 0.05$) in genotype GR-1-BBGK-19,198. For the rest of the genotypes that were tested via softwood non-dormant cuttings during the summer through early autumn, rooting was still diverse, but more cases of higher rates were found compared to genotypes tested in winter, with three and two genotypes presenting >30% and 50% rooting, respectively (Table 3, $p < 0.05$, Figure 2).

Table 3. Overview of the propagation results achieved in terms of highest rooting frequencies after experimental setups in different seasons (winter 2018, spring–late summer 2019, and early–late autumn 2019) with different types of initial material (softwood or hardwood cuttings) collected directly from wild-growing Greek native populations of *Cornus mas* (18 genotypes).

Genotype (Accession Number)	Season	Cutting Type	Hormone Treatment (ppm IBA)	Rooting (%)
GR-1-BBGK-19,72	Summer	Softwood	4000	33.30 ‡
GR-1-BBGK-19,190	Winter	Hardwood	10,000	13.29 †
GR-1-BBGK-19,195	Winter	Hardwood	10,000	1.39
GR-1-BBGK-19,196	Winter	Hardwood	10,000	8.33
GR-1-BBGK-19,197	Winter	Hardwood	10,000	2.86
GR-1-BBGK-19,198	Winter	Hardwood	10,000	20.93 †
GR-1-BBGK-19,502	Summer	Softwood	4000	18.60
GR-1-BBGK-19,590	Early autumn	Softwood	4000	16.67
GR-1-BBGK-19,632	Summer	Softwood	5000	20.41
GR-1-BBGK-19,633	Summer	Softwood	5000	2.27
GR-1-BBGK-19,638	Late summer	Softwood	10,000	69.33 ‡
GR-1-BBGK-19,641	Late summer	Softwood	6000	1.19
GR-1-BBGK-19,669	Summer	Softwood	2000	28.60
GR-1-BBGK-19,753	Late summer	Softwood	4000	58.33 ‡
GR-1-BBGK-19,844	Early autumn	Softwood	4000	33.33 ‡
GR-1-BBGK-19,847	Early autumn	Softwood	4000	31.94
GR-1-BBGK-19,848	Autumn	Hardwood	6000	8.33
GR-1-BBGK-19,926	Autumn	Hardwood	4000	10.34

The symbols † and ‡ denote the highest rooting frequencies for hardwood and softwood cuttings, respectively, following pairwise comparisons of the observed rooting frequencies via Pearson X^2 tests. All cases were tested against a control treatment with no hormone application and no rooting.

Figure 2. Softwood propagation material collected from representative wild-growing Greek native *C. mas* genotypes (**A1**: GR-1-BBGK-19,72 and **A2**: GR-1-BBGK-19,632) during botanical expeditions. Cutting preparation for genotype GR-1-BBGK-19,502 (**B1**) and GR-1-BBGK-19,72 (**B2**). Representative rooted cutting of GR-1-BBGK-19,72 (**C1**) and cutting that failed to root of GR-1-BBGK-19,72 (**C2**). Transplanted well-rooted plants under ex situ conservation of genotype GR-1-BBGK-19,72 (**D1**) and GR-1-BBGK-19,753 (**D2**). Bars in photos B1, B2, C1, and C2 represent 10 cm.

2.4. Multifaceted Evaluation of Greek Native Cornus mas Genotypes

An overview of the sustainable exploitation potential of the native *C. mas* genotypes assessed in this study is summarized in Table 4 in a multifaceted way based on the obtained molecular authentication results, the propagation success achieved, and the phytochemical profiles assessed. From the effectively authenticated genotypes, GR-1-BBGK-19,72 showed very high antioxidant activity and average propagation potential (Table 4, Figure 2) and could be prioritized for further research. However, genotypes GR-1-BBGK-19,190 and GR-1-BBGK-19,195 also showed high antioxidant activity but it is unclear from the current data whether the low propagation success observed was due to genotype effect or due to winter hardwood cuttings since these genotypes were only tested during the winter in contrast with GR-1-BBGK-19,72 (Table 1, Table 3, Table 5). As far as the rest of the genotypes are concerned, genotype GR-1-BBGK-19,638 stands out with very promising phytochemical potential and high propagation success and, as such, this genotype merits further investigation. Another genotype that showed a promising potential is GR-1-BBGK-19,753 with high propagation success and strong antioxidant activity (Table 3, Figure 2).

Table 4. Multifaceted evaluation of Greek native *Cornus mas* genotypes based on molecular authentication effectiveness (Figure 1), fruit phytochemical potential expressed as antioxidant activity (AA, %RSA: low ≤50, average 51–70, high 71–90, very high >90), total phenolic content (TPC, mg GAE/100 g: low ≤50, average 51–100, high 101–200, very high >200) and vitamin C content (mg AAE/100 g: low ≤30, average 31–50, high >50) (Table 1), and asexual propagation potential expressed as hormone-induced rooting under the most successful application (very low < 10%, low 11–30%, average 31–55%, or high >55%), (Table 3).

IPEN Accession Number	DNA Barcoding	Phytochemical Potential			Propagation Potential
		AA (%RSA)	TPC (mg GAE/100 g)	Vitamin C (mg AAE/100 g)	
GR-1-BBGK-19,72	Effective	Very high	Average	Low	Average
GR-1-BBGK-19,190	Effective	High	Very high	Average	Low
GR-1-BBGK-19,195	Effective	High	Low	Average	Very low
GR-1-BBGK-19,196	Effective	-	-	-	Very low
GR-1-BBGK-19,197	Effective	-	-	-	Very low
GR-1-BBGK-19,198	Effective	-	-	-	Low
GR-1-BBGK-19,502	Effective	-	-	-	Low
GR-1-BBGK-19,590	Easy	Very high	Average	Low	Low
GR-1-BBGK-19,632	Easy	High	Low	Low	Low
GR-1-BBGK-19,633	Easy	Very high	High	Average	Very low
GR-1-BBGK-19,638	Easy	Very high	Very high	High	High
GR-1-BBGK-19,641	Easy	Very high	Average	Average	Very low
GR-1-BBGK-19,669	Easy	High	Very high	Low	Low
GR-1-BBGK-19,753	Easy	High	Low	Low	High
GR-1-BBGK-19,844	Easy	Very high	Average	Average	Average
GR-1-BBGK-19,847	Easy	Average	Low	Low	Average
GR-1-BBGK-19,848	Easy	Average	Low	Average	Very low
GR-1-BBGK-19,926	Easy	Very high	Average	Low	Low

Table 5. IPEN accession number, location, altitude, and sampling details of the genotypes (population samples) of Greek native *Cornus mas* germplasm collected from various areas and habitats of Northern Greece in 2018–2019.

No	IPEN Accession Number	Greek Prefecture	Area	Coordinates (HGRS87/EGSA87) (Lat, Lon)	Altitude (m)	1st Sampling (Winter 2018)	2nd Sampling (Spring–Late Summer 2019)	3rd Sampling (Autumn 2019)
1	GR-1-BBGK-19,72	Central Macedonia	Pella, Aridea	40.919338, 21.900725	882	HWSC	SWSC, LS	RFS
2	GR-1-BBGK-19,190	Epirus	Preveza, Kranea	39.248017, 20.742179	513	HWSC	LS	RFS
3	GR-1-BBGK-19,195	Epirus	Ioannina, Dafni	39.43568, 21.015093	447	HWSC	LS	RFS
4	GR-1-BBGK-19,196	Epirus	Ioannina, Xirovouni	39.461535, 21.008474	1070	HWSC	SWSC, LS	

Table 5. *Cont.*

No	IPEN Accession Number	Greek Prefecture	Area	Coordinates (HGRS87/EGSA87) (Lat, Lon)	Altitude (m)	1st Sampling (Winter 2018)	2nd Sampling (Spring–Late Summer 2019)	3rd Sampling (Autumn 2019)
5	GR-1-BBGK-19,197	Epirus	Ioannina, Xirovouni	39.461535, 21.008474	1070	HWSC	LS	
6	GR-1-BBGK-19,198	Epirus	Ioannina, Xirovouni	39.461535, 21.008474	1070	HWSC	LS	
7	GR-1-BBGK-19,502	Central Macedonia	Kilkis, Goumenissa	40.92079, 22.45934	170		SWSC, LS	
8	GR-1-BBGK-19,590	Central Macedonia	Pieria, Elatochori	40.32734, 22.26310	780		SWSC	SWSC, RFS
9	GR-1-BBGK-19,632	Epirus	Ioannina, Dodoni	39.49421, 20.68520	500		SWSC, RFS	
10	GR-1-BBGK-19,633	Epirus	Ioannina, Dodoni	39.49421, 20.68520	550		SWSC, RFS	
11	GR-1-BBGK-19,638	Epirus	Ioannina, Zagori	39.86281, 20.72305	960		SWSC, RFS	
12	GR-1-BBGK-19,641	Central Macedonia	Kilkis, Pontokerasia	41.08850, 23.117975	648		SWSC, RFS	
13	GR-1-BBGK-19,669	West Macedonia	Kastoria, Mt Grammos	40.34888, 20.92475	1165		SWSC, RFS	
14	GR-1-BBGK-19,753	East Macedonia	Drama, Ahladome-lea	41.41379, 24.00165	590			SWSC, RFS
15	GR-1-BBGK-19,844	Epirus	Preveza, Anaogeio	39.37108, 20.93397	1100			SWSC, RFS
16	GR-1-BBGK-19,847	Thrace	Xanthi, Ano kalyva	41.29185, 24.62509	620			SWSC, RFS
17	GR-1-BBGK-19,848	East Macedonia	Drama, Silli	41.35046, 24.52971	750			SWSC, RFS
18	GR-1-BBGK-19,926	Central Macedonia	Pella, Notia	41.11128, 22.20423	880			SWSC, RFS

RFS: Ripe cornelian cherry fruits sample for chemical analysis; HWSC: hardwood stem cuttings; SWSC: softwood stem cuttings for propagation; LS: leaf samples for DNA analysis

3. Discussion

3.1. Molecular Authentication of Greek Native Genotypes of Cornus mas

In general, DNA barcoding may offer support to classical morphological identification of samples and insight into phylogenetic relationships of closely related species; thus it is an efficient method for the discernment of various genotypes independently from the stage of plant development [6,10]. Herein, we provide the first-ever report regarding the molecular authentication of Greek native germplasm of *Cornus mas*, with the NJ (Neighbor-Joining) tree classification obtained from the ITS2 barcoding discerning clearly the DNA fingerprints of the Greek genotypes. Undoubtedly, more genotypes from different habitats in different regions of Greece should be evaluated to further confirm this distinctiveness. Regardless, as reported herein, the ITS2 gene can be an effective marker for the identification of *Cornus* spp. and of different genotypes thereof; thus, offering to deciphering their evolutionary relationships.

3.2. Nutraceutical Potential of Greek Native Genotypes of Cornus mas

The current work presents for the first time comprehensive phytochemical data for an extended range of Greek native wild-growing *Cornus mas* genotypes assessing their potential as a functional food or superfood for ex situ conservation and future breeding efforts. The parameters assessed herein revolve around human oxidative stress and particularly plant secondary metabolites with known free radical scavenging activity such as phenolic compounds and flavonoids coupled with vitamin C content [41]. The results showed that at least five Greek genotypes with significant nutraceutical potential can be distinguished, namely GR-1-BBGK-19,72, GR-1-BBGK-19,190, GR-1-BBGK-19,195, GR-1-BBGK-19,638,

and GR-1-BBGK-19,753 (Table 4). Similarly to the current results, a wide range of TPC of the fruits was detected in studies dealing with wild-growing *C. mas* germplasm in Romania, coupled with significant variability among genotypes in other fruit phytochemical traits [42,43]. Some similarities can also be found between *C. mas* wild-growing genotypes of Serbia with those studied herein in terms of vitamin C content that also show higher sugar content due to higher fruit maturity index at the time of collection [15].

Although variable among genotypes, the phytochemical properties of the Greek germplasm are generally similar with both wild-growing and cultivated *C. mas* genotypes reported from countries at a similar latitudinal range as Greece such as Turkey [13]. However, studies on Central European genotypes of *C. mas* cultivated in higher latitudes have shown higher antioxidant activity (AA) of the fruits that ranged from 3.30 to 9.54 g AAE/1000 g, which implies an interaction of genotype with climatic conditions affecting fruit phytochemical profile [14]. Evidence regarding the effects of the climatic conditions in interaction with genotype on fruit phytochemical profile can also be seen through the significant correlation of total dissolved solids (TDS) with vitamin C content observed among genotypes herein, since TDS is physiologically connected to climatic factors [14]. In a more recent study concerning an Italian cultivated genotype of *C. mas*, total phenolic content (TPC) was found at 196.68 mgGAE/100 g [44], which is similar to some genotypes assessed herein and even lower than three of the studied genotypes, while vitamin C content was at similar ranges. Recently, the phytochemical profile of fruits was comparatively evaluated between cultivars of *C. mas*, genotypes of the Chinese endemic *Cornus officinalis* Siebold and Zucc., and hybrids thereof (*C. mas* X *C. officinalis*). *C. mas* cultivars in that study have shown a similar TPC range with the studied Greek genotypes apart from one cultivar which was higher, yet the TPC and AA of the hybrids was generally higher, indicating the significance of conservation of *C. mas* germplasm stocks for breeding programs [22]. The nutraceutical potential of native genotypes has been similarly assessed on other Greek germplasm resources such as, for example, *Rosa canina* [10] with slightly higher potential of AA and TPC detected and comparable to some extent with that of *C. mas*, but with very high content of vitamin C for *R. canina* [10].

The climatic conditions (most prominently temperature along with sunshine) during the last stages of fleshy fruit ripening in cornelian cherries may affect acid reduction and sugar increase, which in turn affects biosynthesis of secondary metabolites [45,46]. As such, the climatic conditions of the natural habitats of wild populations on one hand and the stage of fruit collection and fruits' ripening status on the other hand are considered as significant factors in terms of comparative phytochemical evaluations. However, in this study no significant correlations were observed between the fruit phytochemical properties measured and the altitude from which they were collected in the wild or their maturity index determined as the ratio of sugar content to malic acid content. This observation indicates that the variability of phytochemical properties observed among Greek native genotypes of *C. mas* may be attributed to genetic factors rather than timing of fruit harvest or altitudinal differences between collection sites. Nevertheless, further ecological profiling work in terms of correlation studies with more environmental/topoclimatic parameters and more fruit phenological traits on Greek *C. mas* wild populations is suggested.

3.3. Propagation Potential of Greek Native Genotypes of Cornus mas

A further component of the multifaceted evaluation of wild Greek *C. mas* genotypes investigated herein is the asexual propagation via cuttings which was conducted for the first time in Greek germplasm. Similarly to fruit phytochemical data, the genotypes of *C. mas* tested showed variable rooting capacity with a number of them being below the commercially accepted threshold [47]. In all cases, however, external hormone application was necessary to achieve even low rooting frequencies which are in agreement with similar studies [38,39,48]. Hardwood dormant cuttings tested in the current study remained under mist for the duration of winter and started to root the following spring giving low rooting rates (<25%) under 10,000 ppm IBA treatment. This observation concurs with similar

studies on cutting propagation of *C. mas* genotypes in other countries where softwood cuttings surpassed hardwood cuttings under the 10,000 ppm IBA treatment [38]. The poor results on hardwood cuttings indicate that it is not economically efficient to produce new plants via winter dormant cuttings [37,40], a fact that also applies for the Greek germplasm. Softwood cutting material with a small degree of lignification has known advantages in rooting quality with external hormone utilization [49], producing higher rooting rates in *Cornus alba* [50]. Even in other genera, cutting type affected by the status of mother's plant growth has been linked to rooting capacity and quality [51–53]. Consequently, since only two of the selected genotypes evaluated herein showed appreciable propagation potential (>50% rooting in genotypes GR-1-BBGK-19,638 and GR-1-BBGK-19,753) with softwood cuttings (Table 3), further research is required to explore pathways on improving rooting capacity but also to investigate the relationships between cutting type and rooting capacity and quality in more detail. Additionally, further research is proposed on the correlation of rooting factors with early plant establishment and survival in *C. mas* propagation since such a correlation has been shown to be significant in other Greek native germplasm resources such as *Sambucus nigra* [6].

Finally, asexual propagation through grafting has been attempted on European *C. mas* germplasm with positive results [54], showing that successful grafting in *C. mas* can enhance growth and development of the scion [55]. For the Greek native *C. mas* genotypes studied herein, the method of grafting a genotype with high phytochemical potential but low rooting capacity onto another genotype with improved rooting capacity may provide a good option in dealing with difficult-to-root genotypes but with valuable fruit traits. However, further research is required to systematically test this strategy.

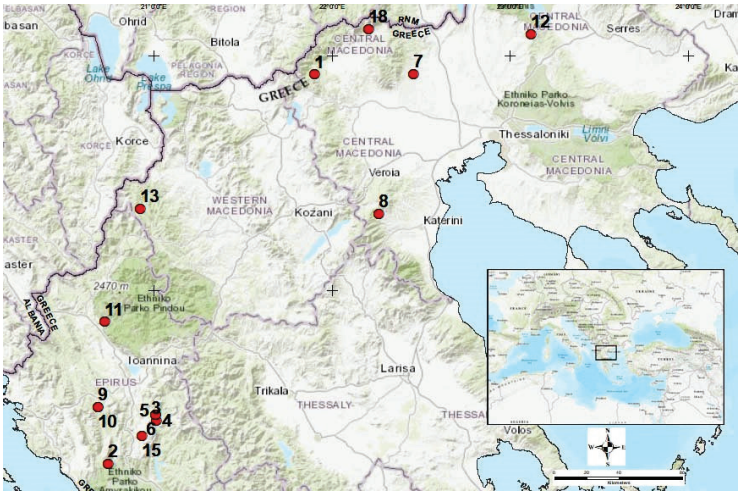
4. Materials and Methods

4.1. Collection and Documentation of Plant Material

A plethora of botanical expeditions took place covering a wide range of geographically distant and altitudinally different natural habitats of *C. mas* across Northern Greece reached at consecutive stages across two years (Table 5). Targeted and variable types of plant material were collected (Table 5): (i) leaves from 20 individuals from each genotype for DNA analysis; (ii) hardwood dormant twigs for propagation from six genotypes (winter 2018); (iii) softwood stem cuttings for propagation from 14 genotypes (14 in spring–late summer and 5 in autumn of 2019); (iv) ripe fruits from nine genotypes for phytochemical evaluation (autumn 2019). The work resulted in the documentation of 18 *C. mas* genotypes (population samples) in total from healthy wild-growing individuals (Figure 3). After each expedition, the collected material was promptly transferred to the laboratory where it was taxonomically identified [56] and was assigned with a unique IPEN (International Plant Exchange Network) accession number given by the Balkan Botanic Garden of Kroussia of the Institute of Plant Breeding and Genetic Resources (IPB&GR) of the Hellenic Agricultural Organization-Demeter (ELGO-Demeter). Plant material collection in all cases was conducted under a special permit to the IPB&GR, ELGO-Demeter (Permit 82336/879 of 18/5/2019 and 26895/1527 of 21/4/2021) issued by the Greek Ministry of Environment and Energy. The overall work was conducted under the auspices of the research project "Highlighting local traditional varieties and wild native forest fruit trees and shrubs" (acronym: EcoVariety, T1EΔK-05434).

4.2. DNA Isolation

A standardized commercial DNA extraction kit (Nucleospin Plant II, Macherey-Nagel) was used for DNA extraction following the manufacturer's instructions using approximately 30 mg of dried leaf sample from each *C. mas* genotype that was previously ground in liquid nitrogen.

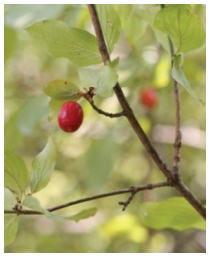


(**A**)

(**B**)

(**C**)

(**D**)



(**E**)

(**F**)

Figure 3. (**A**) Geographical distribution of the *Cornus mas* Greek native genotypes sampled for taxonomic identification, DNA barcoding, phytochemical assessment, and asexual propagation trials. (**B**) Typical habit of wild-growing individuals; morphology of flowers (**C**) leaves and fruits (**D**) in early (**E**) and full ripening (**F**).

4.3. Polymerase Chain Reaction (Pcr) Amplification and Sequence Analysis

PCR amplification was conducted according to [57] using one primer set of the nuclear ITS2 barcode region [58]. The resulting PCR products for each genotype were consecutively sequenced using an automated ABI 3730 sequencer (PE Applied Biosystems), in two directions of each fragment with a Big Dye terminator v3.1 Cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA). The sequences were aligned using the CLUSTAL W program.

4.4. Molecular Data Analysis

Following the results of the sequence analysis, the molecular authentication of the *Cornus mas* genotypes studied was conducted using three methods: (i) evaluation against the nucleotide database at NCBI using the Basic Local Alignment Search Tool (BLAST); (ii) using maximum-likelihood models for the genetic divergence method; and (iii) tree topology analysis using the Neighbor-Joining (NJ) method based on different loci in MEGAX with the K2P distance model and 1000 bootstrap replications. The generated ITS2 DNA barcoding sequences (without primers used for PCR amplification) were deposited to the NCBI-Genbank (https://www.ncbi.nlm.nih.gov/BankIt/, accessed 5 May 2022) under the accession numbers MZ35480-MZ35488.

4.5. Phylogenetic Relationships

The phylogenetic relationship of *Cornus* spp. was inferred using the Neighbor-Joining (NJ) method [59]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [60]. The evolutionary distances were estimated using the Maximum Composite Likelihood (MCL) method [61]. All ambiguous positions were removed for each sequence pair (pairwise deletion option). The phylogenetic dendrogram was constructed using the MEGA X software [62].

4.6. Propagation Trials

Depending on the propagation material obtained from each expedition, a variety of targeted experiments were conducted using different external hormone application treatments of indole-3-butyric acid (IBA) [50,63] and different cutting types taken at different seasons from varying stages of mother plant growth (Table 5). Cuttings were set for rooting in propagation trays under mist where relative humidity (RH) was maintained at >85% within a greenhouse at ambient temperature. The substrate used was peat (Klasmann, KTS 1):perlite at 1:3 *v/v*. Cuttings were attended weekly to assess their rooting capacity. The produced mother plants were kept ex situ at the laboratory's nursery in the grounds of IPB and GR under ambient conditions for plant adaptation. The plants were watered regularly and were grown in 3 L pots using a mixture of peat (Klasmann, KTS 2) and perlite (3:1, *v/v*).

4.7. Phytochemical Analysis of Cornus mas Fruits

A modified method described in [64] was used for preparation of the extracts. An appropriate volume of MeOH/H_2O (60:40) was mixed with 2–5 g of homogenized sample and was centrifuged at 4000rpm at 4°C. The collected supernatant was made up to 20 mL volume and was used for the determination of total phenolic content (TPC), total flavonoids (TF), and antioxidant activity (AA). For the determination of TPC, phenolic extract 0.20 mL along with 2.3 mL of H_2O and 0.25 mL of Folin–Ciocalteu reagent were added in a volumetric flask [64] in which, after 3 min, 0.50 mL of 20% Na_2CO_3 was added, and the volume was made up to 5 mL. The above solution was stored in a dark place for 2 h and the absorbance was measured at 725 nm against blank solution. The calculation of TPC was conducted using a standard curve of gallic acid at various concentrations giving the results expressed as gallic acid equivalents (GAE)/100 g of sample. All analyses were carried out in triplicate.

A modified method for the determination of TF was used according to [65]. In a test tube, 5 µL of the above extract were added along with 3270 µL of H_2O and 75 µL of 5% $NaNO_2$, stirred, and stored in the dark for 5 min which was then added with 150 µL of 10% $AlCl_3$-$6H_2O$, mixed, and stored in the dark for a further 6 min. Consequently, it was added with 500 µL of 1 M NaOH and the absorbance was measured at 510 nm against H_2O as blank. The calculation of TF was conducted using a standard curve of catechin at various concentrations giving results expressed as catechin equivalents (CE)/100 g of sample. All analyses were carried out in triplicate.

A modified method for the determination of AA was employed described in [66]. In a 5 mL plastic cuvette, 0.1 mL of phenolic extract was added along with 2.9 mL 0.10 mM (2,2-diphenyl-1-picrylhydrazyl (DPPH)) in MeOH, stored in the dark for 15 min and then the absorbance was measured at 517 nm against MeOH as blank. The control sample was prepared using 0.10 mM DPPH in MeOH. The percentage of radical scavenging activity (%RSA) was carried out in triplicate and was calculated using the following equation:

$$\%\text{RSA} = \frac{A_o - A_s}{A_o} * 100$$

where

A_o = absorbance of control sample.

A_s = absorbance of the sample after 15 min of incubation.

A modified method for the determination of vitamin C presented in [67] was used. A mixture of homogenized sample (2–5 g) and 5 mL of 4.5% *w/v* metaphosphoric acid (MPA) solution was stirred and centrifuged at 8000 rpm at 4°C for 20 min. The supernatant (1 mL) was taken and diluted up to 10 mL with 4.5% MPA solution and it was filtered through 0.45 µm polyethersulfone filters. The vial was covered with aluminum foil to prevent oxidation of ascorbic acid and stored at 4 °C until HPLC-DAD analysis. HPLC-DAD analysis conditions: Column (Agilent Eclipse XDB-C18) 4.6 mm × 150 mm, 5µm, elution solvent: aqueous 0.005 M H_2SO_4 solution at a flow rate of 0.5 mL/min (isocratic) and wavelength 245 nm. A standard curve of ascorbic acid at various concentrations was used for vitamin C content calculation which gave results expressed as ascorbic acid equivalents (AAE)/100 g of sample.

Sugar content of fruit samples was measured via Brix analysis (°Bx), and the maturity index (MI) of fruits was calculated as the ratio of sugar content in °Bx to malic acid (MA) content expressed as grams of MA per 100 g.

All analyses were carried out in triplicate.

4.8. Experimental Design and Statistical Analysis of Propagation and Phytochemical Data

The propagation trials followed a complete randomized design. The number of replicate cuttings per treatment varied according to the volume of material obtained from each expedition (Table 5). Targeted IBA hormone application treatments were applied ranging from 2000 to 10,000 ppm depending on the season and whether the cuttings were softwood or hardwood, respectively (Table 5). Each trial included a control with an equal number of replicate cuttings. The rooting frequencies obtained were compared through pairwise Pearson Chi-Square tests.

For the phytochemical data that were measured in triplicate, a mean coupled with its standard deviation (±S.D.) was calculated in each case and means were compared using Tukey's HSD post hoc test. In addition, the phytochemical data were subjected to Pearson's correlation coefficient analysis to determine the correlations between the different fruit phytochemical traits measured along with the differences in altitude between the natural habitats of *C. mas* that collection occurred in. All analyses were conducted using the IBM-SPSS 23.0 software.

5. Conclusions

In conclusion, the multifaceted evaluation that was conducted provides a first depiction of the sustainable exploitation potential of Greek native *Cornus mas* genotypes. All genotypes collected from the wild are under ex situ conservation at the IPB and GR for future monitoring, since juvenile–mature correlations are known in perennial crops from the literature [68,69]; such aspects should be taken into account for future long-term breeding programs. The transition from the juvenile to the mature (producing) stage followed by several phenological and developmental changes was found to vary among genotypes mainly in forest species but also in cultivated deciduous tree species with stone fruits such as apples [69]. Consequently, further work on molecular authentication, ecological profiling, and phytochemical profiling is proposed coupled with further research on propagation. In addition, the long-term monitoring of the current Greek genotypes is needed, and in fact, pilot cultivation trials of the genotypes prioritized herein have been established (ongoing process). The data obtained in the current study along with the conservation of the plant material collected can serve as a basis for future breeding efforts, creating for the first time a framework for the sustainable utilization of Greek native *C. mas* germplasm as a superfood with significant agronomic potential.

Author Contributions: Conceptualization, N.K., E.K. and I.G.; methodology, E.M., E.K., I.G., A.K., K.P., I.M., D.K., P.Y., N.N., I.S.K., A.V.B., G.P., I.G. and N.K.; software, I.G., and D.F.; validation, E.K., I.G., A.K., K.P., I.M., D.K., P.Y., N.N., I.S.K., A.V.B., G.P. and D.F.; formal analysis, E.K., I.G., I.S.K. and A.V.B.; investigation, E.K., I.G., A.K., K.P., I.M., D.K., P.Y., N.N., I.S.K., A.V.B. and G.P.; resources, E.M., I.G. and A.V.B.; data curation, N.K., E.K., K.P., I.G., N.N., I.S.K. and A.V.B.; writing—original draft preparation, E.K., I.G., D.F., A.V.B. and N.K.; writing—review and editing, E.M., E.K., I.G., A.K., K.P., D.K., P.Y., N.N., I.S.K., A.V.B., I.M., G.P., D.F. and N.K.; visualization, N.K., E.K., I.G. and D.F.; supervision, E.M., A.V.B., G.P. and N.K.; project administration, E.M. and G.P.; funding acquisition, E.M., N.N. and G.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was co-financed by the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurship and Innovation, under the call RESEARCH—CREATE—INNOVATE (project code: T1EDK-05434), entitled "Highlighting of local traditional and native wild fruit trees and shrubs".

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data supporting the results of this study are included in the manuscript and datasets are available upon request.

Acknowledgments: The authors would like to thank the staff of the Institute of Plant Breeding and Genetic Resources, Hellenic Agricultural Organization-Demeter for administrative and technical support.

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

Abbreviations

All abbreviations are explained at their first mention in the manuscript.

References

1. Krigas, N.; Tsoktouridis, G.; Anestis, I.; Khabbach, A.; Libiad, M.; Megdiche-Ksouri, W.; Ghrabi-Gammar, Z.; Lamchouri, F.; Tsiripidis, I.; Tsiafouli, M.A.; et al. Exploring the potential of neglected local endemic plants of three Mediterranean regions in the ornamental sector: Value chain feasibility and readiness timescale for their sustainable exploitation. *Sustainability* **2021**, *13*, 2539. [CrossRef]
2. Ercisli, S. A short review of the fruit germplasm resources of Turkey. *Genet. Resour.* **2004**, *51*, 419–435. [CrossRef]
3. Verma, N.; Mohanty, A.; Lal, A. Pomegranate genetic resources and germplasm conservation: A Review. *Fruit Veg. Cereal Sci. Biotechnol.* **2010**, *4*, 120–125.
4. Botu, M.; Botu, I.; Achim, G.; Preda, S.; Scutelnicu, A.; Giura, S. Conservation of fruit tree genetic resources and their use in the breeding process. *Ann. Valahia Univ. Targoviste* **2017**, *11*, 66–69. [CrossRef]

5. Manco, R.; Basile, B.; Capuozzo, C.; Scognamiglio, P.; Forlani, M.; Rao, R.; Corrado, G. Molecular and phenotypic diversity of traditional European plum (*Prunus domestica* L.) germplasm of Southern Italy. *Sustainability* **2019**, *11*, 4112. [CrossRef]
6. Karapatzak, E.; Dichala, O.; Ganopoulos, I.; Karydas, A.; Papanastasi, K.; Kyrkas, D.; Yfanti, P.; Nikisianis, N.; Fotakis, D.; Patakioutas, G.; et al. Molecular authentication, propagation trials and field establishment of Greek native genotypes of *Sambucus nigra* L. (Caprifoliaceae): Setting the basis for domestication and sustainable utilization. *Agron. J.* **2022**, *12*, 114. [CrossRef]
7. Cosmulescu, S.; Trandafir, I.; Nour, V. Phenolic acids and flavonoids profiles of extracts from edible wild fruits and their antioxidant properties. *Int. J. Food Prop.* **2017**, *20*, 3124–3134. [CrossRef]
8. Che, C.T.; Zhang, H. Plant natural products for human health. *Int. J. Mol.* **2019**, *20*, 830. [CrossRef]
9. Durazzo, A.; Lucarini, M.; Souto, E.B.; Cicala, C.; Caiazzo, E.; Izzo, A.A.; Novellino, E.; Santini, A. Polyphenols: A concise overview on the chemistry, occurrence, and human health. *Phytother. Res.* **2019**, *33*, 2221–2243. [CrossRef]
10. Maloupa, E.; Karapatzak, E.; Ganopoulos, I.; Karydas, A.; Papanastasi, K.; Kyrkas, D.; Yfanti, P.; Nikisianis, N.; Zahariadis, A.; Kosma, I.S.; et al. Molecular authentication, phytochemical evaluation and asexual propagation of wild-growing *Rosa canina* L. (Rosaceae) genotypes of Northern Greece for sustainable exploitation. *Plants* **2021**, *10*, 2634. [CrossRef]
11. Da Ronch, F.; Caudullo, G.; Houston Durrant, T.; de Rigo, D. *Cornus mas* in Europe: Distribution, habitat, usage and threats. In *The European Atlas of Forest Tree Species*; San-Miguel-Ayanz, J., de Rigo, D., Caudullo, G., Houston Durrant, T., Mauri, A., Eds.; Publication Office of the European Union: Luxembourg, 2016; p. e01ddab+. ISBN 978-92-79-36740-3.
12. Baumann, H. *The Greek Plant World in Myth, Art and Literature*; Timber Press, Incorporated: Portland, OR, USA, 1993; ISBN 978-0881922318.
13. Tural, S.; Koca, I. Physico-chemical and antioxidant properties of Cornelian cherry fruits (*Cornus mas* L.) grown in Turkey. *Sci. Hortic.* **2008**, *116*, 362–366. [CrossRef]
14. Rop, O.; Mlcek, J.; Kramarova, D.; Jurikova, T. Selected cultivars of Cornelian cherry (*Cornus mas* L.) as a new food source for human nutrition. *Afr. J. Biotechnol.* **2010**, *9*, 1205–1210. [CrossRef]
15. Bijelić, S.M.; Gološin, B.R.; Ninić Todorović, J.I.; Cerović, S.B.; Popović, B.M. Physicochemical fruit characteristics of Cornelian cherry (*Cornus mas* L.) genotypes from Serbia. *HortScience* **2011**, *46*, 849–853. [CrossRef]
16. Szczepaniak, O.M.; Kobus-Cisowska, J.; Kusek, W.; Przeor, M. Functional properties of Cornelian cherry (*Cornus mas* L.): A comprehensive review. *Eur. Food Res. Technol.* **2019**, *245*, 2071–2087. [CrossRef]
17. Tiptiri-Kourpeti, A.; Fitsiou, E.; Spyridopoulou, K.; Vasileiadis, S.; Iliopoulos, C.; Galanis, A.; Vekiari, S.; Pappa, A.; Chlichlia, K. Evaluation of antioxidant and antiproliferative properties of *Cornus mas* L. fruit juice. *Antioxidants* **2019**, *8*, 377. [CrossRef]
18. Moldovan, B.; Filip, A.; Clichici, S.; Suharoschi, R.; Bolfa, P.; David, L. Antioxidant activity of Cornelian cherry (*Cornus mas* L.) fruits extract and the in vivo evaluation of its anti-inflammatory effects. *J. Funct. Foods* **2016**, *26*, 77–87. [CrossRef]
19. Bayram, H.M.; Ozturkcan, S.A. Bioactive components and biological properties of Cornelian cherry (*Cornus mas* L.): A comprehensive review. *J. Funct. Foods* **2020**, *75*, 104252. [CrossRef]
20. Radbeh, Z.; Asefi, N.; Hamishehkar, H.; Roufegarinejad, L.; Pezeshki, A. Novel carriers ensuring enhanced anti-cancer activity of *Cornus mas* (Cornelian cherry) bioactive compounds. *Biomed. Pharmacother.* **2020**, *125*, 109906. [CrossRef]
21. Przybylska, D.; Kucharska, A.Z.; Cybulska, I.; Sozański, T.; Piórecki, N.; Fecka, I. *Cornus mas* L. stones: A valuable by-product as an ellagitannin source with high antioxidant potential. *Molecules* **2020**, *25*, 4646. [CrossRef]
22. Klymenko, S.; Kucharska, A.Z.; Sokół-Łętowska, A.; Piórecki, N.; Przybylska, D.; Grygorieva, O. Iridoids, flavonoids, and antioxidant capacity of *Cornus mas*, *C. officinalis*, and *C. mas* × *C. officinalis* fruits. *Biomolecules* **2021**, *11*, 776. [CrossRef]
23. Brindza, P.; Brindza, J.; Tóth, D.; Klimenko, S.V.; Grigorieva, O. Slovakian Cornelian cherry (*Cornus mas* L.): Potential for cultivation. *Acta Hortic.* **2007**, *760*, 433–437. [CrossRef]
24. Bijelić, S.M.; Gološin, B.; Todorović, J.N.; Cerović, S. Morphological characteristics of best Cornelian cherry (*Cornus mas* L.) genotypes selected in Serbia. *Genet. Resour. Crop Evol.* **2011**, *58*, 689–695. [CrossRef]
25. Yilmaz, K.U.; Ercisli, S.; Zengin, Y.; Sengul, M.; Kafkas, E. Preliminary characterisation of cornelian cherry (*Cornus mas* L.) genotypes for their physico-chemical properties. *Food Chem.* **2009**, *114*, 408–412. [CrossRef]
26. Hamid, H.; Hamidoghli, Y.; Hajilo, J.; Adlipour, M. Antioxidant capacity and phytochemical properties of cornelian cherry (*Cornus mas* L.) genotypes in Iran. *Sci. Hortic.* **2011**, *129*, 459–463. [CrossRef]
27. Moradi, Y.; Khadivi, A.; Salehi-Arjmand, H. Morphological and pomological characterizations of Cornelian cherry (*Cornus mas* L.) to select the superior accessions. *Sci. Hortic.* **2019**, *249*, 208–218. [CrossRef]
28. Jaćimović, V.; Božović, D.; Ercisli, S.; Bosančić, B.; Necas, T. Sustainable Cornelian cherry production in Montenegro: Importance of local genetic resources. *Sustainability* **2020**, *12*, 8651. [CrossRef]
29. Martinović, A.; Cavoski, I. The exploitation of cornelian cherry (*Cornus mas* L.) cultivars and genotypes from Montenegro as a source of natural bioactive compounds. *Food Chem.* **2020**, *318*, 126549. [CrossRef]
30. Hebert, P.D.; Cywinska, A.; Ball, S.L.; de Waard, J.R. Biological identifications through DNA barcodes. *Proc. Royal Soc. B* **2003**, *270*, 313–321. [CrossRef]
31. Tsoktouridis, G.; Krigas, N.; Sarropoulou, V.; Kampouropoulou, S.; Papanastasi, K.; Grigoriadou, K.; Menexes, G.; Maloupa, E. Micropropagation and molecular characterization of Thymus sibthorpii Benth. (Lamiaceae), an aromatic-medicinal thyme with ornamental value and conservation concern. *In Vitro Cell. Dev. Biol.-Plant* **2019**, *55*, 647–658. [CrossRef]
32. Yu, J.; Wu, X.; Liu, C.; Newmaster, S.; Ragupathy, S.; Kress, W.J. Progress in the use of DNA barcodes in the identification and classification of medicinal plants. *Ecotoxicol. Environ. Saf.* **2021**, *208*, 111691. [CrossRef]

33. Pipinis, E.; Hatzilazarou, S.; Kostas, S.; Bourgou, S.; Megdiche-Ksouri, W.; Ghrabi-Gammar, Z.; Libiad, M.; Khabbach, A.; El Haissoufi, M.; Lamchouri, F.; et al. Facilitating conservation and bridging gaps for the sustainable exploitation of the Tunisian local endemic plant *Marrubium aschersonii* (Lamiaceae). *Sustainability* **2022**, *14*, 1637. [CrossRef]
34. Hatzilazarou, S.; El Haissoufi, M.; Pipinis, E.; Kostas, S.; Libiad, M.; Khabbach, A.; Lamchouri, F.; Bourgou, S.; Megdiche-Ksouri, W.; Ghrabi-Gammar, Z.; et al. GIS-facilitated seed germination and multifaceted evaluation of the Endangered *Abies marocana* Trab. (Pinaceae) Enabling conservation and sustainable exploitation. *Plants* **2021**, *10*, 2606. [CrossRef]
35. Kostas, S.; Hatzilazarou, S.; Pipinis, E.; Bourgou, S.; Ben Haj Jilani, I.; Ben Othman, W.; Megdiche-Ksouri, W.; Ghrabi-Gammar, Z.; Libiad, M.; Khabbach, A.; et al. DNA barcoding, GIS-facilitated seed germination and pilot cultivation of *Teucrium luteum* subsp. *gabesianum* (Lamiaceae), a Tunisian local endemic with potential medicinal and ornamental value. *Biology* **2022**, *11*, 462. [CrossRef]
36. Gismondi, A.; Rolfo, M.F.; Leonardi, D.; Rickards, O.; Canini, A. Identification of ancient *Olea europaea* L. and *Cornus mas* L. seeds by DNA barcoding. *Comptes Rendus Biol.* **2012**, *335*, 472–479. [CrossRef] [PubMed]
37. Pirlak, L. Effects of different cutting times and IBA doses on the rooting rate of hardwood cuttings of cornelian cherry (*Cornus mas* L.). *Anadolu J. Aegean Agric. Res. Inst.* **2000**, *10*, 122–134.
38. Marković, M.; Grbić, M.; Djukić, M. Effects of cutting type and a method of IBA application on rooting of softwood cuttings from elite tree of cornelian cherry (*Cornus mas* L.) from Belgrade area. *Silva Balc.* **2014**, *15*, 30–37. [CrossRef]
39. Kosina, J.; Baudyšová, M. Propagation of less known fruit crops by cuttings. *Ved. Pr. Ovocn.* **2011**, *22*, 223–229.
40. Hassanpour, H.; Ali Shiri, M. Propagation of Iranian Cornelian cherry (*Cornus mas* L.) by rooted stem cuttings. *Not. Sci. Biol.* **2014**, *6*, 192–195. [CrossRef]
41. Cory, H.; Passarelli, S.; Szeto, J.; Tamez, M.; Mattei, J. The Role of polyphenols in human health and food systems: A mini-review. *Front. Nutr.* **2018**, *5*, 87. [CrossRef]
42. Cosmulescu, S.; Trandafir, I.; Cornescu, F. Antioxidant capacity, total phenols, total flavonoids and color component of Cornelian cherry (*Cornus mas* L.) wild genotypes. *Not. Bot. Horti Agrobot. Cluj-Napoca* **2018**, *47*, 390–394. [CrossRef]
43. Cosmulescu, S.; Cornescu, F. Variability in physical and chemical characteristics of Cornelian cherry fruits (*Cornus mas* L.) from Romanian Oltenia region's spontaneous flora and role of the climatic conditions. *Braz. J. Bot.* **2020**, *43*, 677–682. [CrossRef]
44. De Biaggi, M.; Donno, D.; Mellano, M.G.; Riondato, I.; Rakotoniaina, E.N.; Beccaro, G.L. *Cornus mas* (L.) fruit as a potential source of natural health-promoting compounds: Physico-chemical characterisation of bioactive components. *Plant Foods Hum. Nutr.* **2018**, *73*, 89–94. [CrossRef] [PubMed]
45. Prasanna, V.; Prabha, T.N.; Tharanathan, R.N. Fruit ripening phenomena–An overview. *Crit. Rev. Food Sci. Nutr.* **2007**, *47*, 1–19. [CrossRef] [PubMed]
46. Moretti, C.L.; Mattos, L.M.; Calbo, A.G.; Sargent, S.A. Climate changes and potential impacts on postharvest quality of fruit and vegetable crops: A review. *Food Res. Int.* **2010**, *43*, 1824–1832. [CrossRef]
47. Hartmann, H.J.; Kester, D.E.; Davies, F.I.; Geneve, R.L. *Plant Propagation: Principles and Practices*, 7th ed.; Prentice Hall: Old Tappan, NJ, USA, 2001; ISBN 9780136792352.
48. Balta, M.F.; Erol, I.U.; Özrenk, K.; Karakaya, O.; Uzun, S. Investigation on propagation with softwood cuttings of Cornelian cherry (*Cornus mas* L.) genotypes. *Türkiye Tarımsal Araştırmalar Derg.* **2019**, *6*, 136–141. [CrossRef]
49. Costa, J.M.; Heuvelink, E.; van de Pol, P. Propagation by Cuttings. In *the Reference Module in Life Sciences (Online)*; Elsevier: Amsterdam, The Netherlands, 2017. [CrossRef]
50. Pacholczak, A.; Jędrzejuk, A.; Sobczak, M. Shading and natural rooting biostimulator enhance potential for vegetative propagation of dogwood plants (*Cornus alba* L.) via stem cuttings. *S. Afr. J. Bot.* **2017**, *109*, 34–41. [CrossRef]
51. Noor Camellia, N.A.; Thohirah, L.A.; Abdullah, N.A.P.; Mohd Khidir, O. Improvement on Rooting Quality of *Jatropha curcas* using Indole Butyric Acid (IBA). *Res. J. Agric. Biol. Sci.* **2009**, *5*, 338–343.
52. Guo, X.F.; Fu, X.L.; Zang, D.K.; Ma, Y. Effect of auxin treatments, cuttings' collection date and initial characteristics on *Paeonia* 'Yang Fei Chu Yu' cutting propagation. *Sci. Hortic.* **2009**, *119*, 177–181. [CrossRef]
53. Kumar, R.; Ahmed, N.; Sharma, O.C.; Lal, S. Influence of auxins on rooting efficacy in carnation (*Dianthus caryophyllus* L.) cuttings. *J. Hortic. Sci.* **2014**, *9*, 157–160.
54. Bijelić, S.M.; Gološin, B.R.; Cerović, S.B.; Bogdanović, B.V. A comparison of grafting methods for the production of quality planting material of promising Cornelian cherry selections (*Cornus mas* L.) in Serbia. *J. Agric. Sci. Technol.* **2016**, *18*, 223–231.
55. Cornescu, F.; Achim, G.; Cosmulescu, S. Vegetative propagation of Cornelian cherry (*Cornus mas* L.) selections. *Not. Sci. Biol.* **2020**, *12*, 836–841. [CrossRef]
56. Strid, A. *Atlas of the Aegean Flora, Part 1: Text & Plates; Part 2: Maps; Englera, Volume 33*; Botanic Garden and Botanical Museum Berlin, Freie Universität Berlin: Berlin, Germany, 2016; ISBN 978-3-921800-97-3; 978-3-921800-98-0.
57. Madesis, P.; Ganopoulos, I.; Ralli, P.; Tsaftaris, A. Barcoding the major Mediterranean leguminous crops by combining universal chloroplast and nuclear DNA sequence targets. *Genet. Mol. Res.* **2012**, *11*, 2548–2558. [CrossRef]
58. Chen, S.; Yao, H.; Han, J.; Liu, C.; Song, J.; Shi, L.; Zhu, Y.; Ma, X.; Gao, T.; Pang, X.; et al. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PLoS ONE* **2010**, *5*, e8613. [CrossRef] [PubMed]
59. Saitou, N.; Nei, M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **1987**, *4*, 406–425. [PubMed]
60. Felsenstein, J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **1985**, *39*, 783–791. [CrossRef]

61. Tamura, K.; Nei, M.; Kumar, S. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Nat. Acad. Sci. USA* **2004**, *101*, 11030–11035. [CrossRef]
62. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef]
63. Bryant, P.H.; Trueman, S.J. Stem anatomy and adventitious root formation in cuttings of *Angophora*, *Corymbia* and *Eucalyptus*. *Forests* **2015**, *6*, 1227–1238. [CrossRef]
64. Vavoura, M.V.; Badeka, A.V.; Kontakos, S.; Kontominas, M.G. Characterization of four popular sweet cherry cultivars grown in Greece by volatile compound and physicochemical data analysis and sensory evaluation. *Molecules* **2015**, *20*, 1922–1940. [CrossRef]
65. Roman, I.; Stanila, A.; Stanila, S. Bioactive compounds and antioxidant activity of *Rosa canina* L. biotypes from spontaneous flora of Transylvania. *Chem. Cent. J.* **2013**, *7*, 73. [CrossRef]
66. Fattahi, S.; Jamei, R.; Hosseini Sarghein, S. Antioxidant and antiradical activities of *Rosa canina* and *Rosa pimpinellifolia* fruits from West Azerbaijan. *Iran. J. Plant Physiol.* **2012**, *2*, 523–529. [CrossRef]
67. Lee, H.S.; Coates, G.A. Vitamin C in frozen, fresh squeezed, unpasteurized, polyethylene-bottled orange juice: A storage study. *Food Chem.* **1999**, *65*, 165–168. [CrossRef]
68. Lambeth, C.C. Juvenile-Mature correlations in Pinaceae and implications for early selection. *For. Sci.* **1980**, *26*, 571–580. [CrossRef]
69. Atay, A.N. Deciphering morpho-agronomic determinants of the juvenile-mature phase change in apple progenies. *Sci. Hortic.* **2020**, *259*, 108847. [CrossRef]

plants

MDPI

Article

Hydraulic Response of Deciduous and Evergreen Broadleaved Shrubs, Grown on Olympus Mountain in Greece, to Vapour Pressure Deficit

Maria Karatassiou [1,*], Panagiota Karaiskou [1], Eleni Verykouki [2] and Sophia Rhizopoulou [3]

[1] Laboratory of Rangeland Ecology, School of Forestry and Natural Environment, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece; giotkara81@gmail.com
[2] Department of Agriculture, Crop Production and Rural Environment, University of Thessaly, Fytokou St., 38446 Volos, Greece; everykouki@uth.gr
[3] Section of Botany, Department of Biology, National and Kapodistrian University of Athens, 15784 Athens, Greece; srhizop@biol.uoa.gr
* Correspondence: karatass@for.auth.gr; Tel.: +30-2310-992302

Abstract: In this study, leaf hydraulic functionality of co-occurring evergreen and deciduous shrubs, grown on Olympus Mountain, has been compared. Four evergreen species (*Arbutus andrachne*, *Arbutus unedo*, *Quercus ilex* and *Quercus coccifera*) and four deciduous species (*Carpinus betulus*, *Cercis siliquastrum*, *Coronilla emeroides* and *Pistacia terebinthus*) were selected for this study. Predawn and midday leaf water potential, transpiration, stomatal conductance, leaf temperature and leaf hydraulic conductance were estimated during the summer period. The results demonstrate different hydraulic tactics between the deciduous and evergreen shrubs. Higher hydraulic conductance and lower stomatal conductance were obtained in deciduous plants compared to the evergreens. Additionally, positive correlations were detected between water potential and transpiration in the deciduous shrubs. The seasonal leaf hydraulic conductance declined in both deciduous and evergreens under conditions of elevated vapor pressure deficit during the summer; however, at midday, leaf water potential reached comparable low values, but the deciduous shrubs exhibited higher hydraulic conductance compared to the evergreens. It seems likely that hydraulic traits of the coexisting evergreen and deciduous plants indicate water spending and saving tactics, respectively; this may also represent a limit to drought tolerance of these species grown in a natural environment, which is expected to be affected by global warming.

Keywords: water potential; stomatal conductance; transpiration; leaf hydraulic conductance; drought

Citation: Karatassiou, M.; Karaiskou, P.; Verykouki, E.; Rhizopoulou, S. Hydraulic Response of Deciduous and Evergreen Broadleaved Shrubs, Grown on Olympus Mountain in Greece, to Vapour Pressure Deficit. *Plants* **2022**, *11*, 1013. https://doi.org/10.3390/plants11081013

Academic Editor: Daniela Trono

Received: 5 March 2022
Accepted: 5 April 2022
Published: 8 April 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

The climate in the Mediterranean region is characterized by a prolonged summer drought period, which according to the majority of climate change scenarios, is expected to become more severe in the future [1–3]. Drought is the main environmental stress directly linked to plant survival, growth, competitiveness, persistence, and productivity in Mediterranean habitats that are under an increasing risk of degradation [1,4–7]. On the other hand, plants have evolved a variety of morphological and physiological tactics [8] to withstand drought stress [9,10]; these include (a) hydraulic strategies via deep, tap root systems to extract water from deep soil layers, enabling the plants to sustain elevated water potential and xylem pressure during drought and (b) morphological and physiological adaptations at the leaf level in order to reduce water loss [11,12]. The importance of the hydraulic system in water consumption has led to the hypothesis of the functional convergence in the regulation of water use among phylogenetically diverse species [13,14]. Nonetheless, few studies have been performed to characterize the differences in leaf hydraulic conductance and hydraulic status among phylogenetically different plant species,

such as deciduous and evergreen shrubs, in Mediterranean habitats [15,16]. Leaf is a substantial organ for the transport of water throughout the plant and hence leaf hydraulic conductance is an important parameter to determine plant water status [15,17–20]. To the best of our knowledge, a comparative study among hydraulic characteristics at the leaf level of evergreen and deciduous species co-occurring in the same habitat, with respect to the impact of climatic stimuli on functionality, has not hitherto been published.

In order to comprehensively address differences between evergreen and deciduous plants concerning physiological responses to drought, the hydraulic and stomatal performance should be examined with well-established approaches. The water status for each individual, specific plant depends on the difference between transpiration (E) and water absorption (A), but it is not clear which of these factors is more important for plants' gas exchanges in response to drought stress [12]. If the soil water shortage increases, water stress will increase over time, negatively affecting many physiological and metabolic aspects of the plants [8,21,22]. Plants close the stomatal pores to regulate water loss [12,23] through transpiration when the available soil water decreases and/or an increase in the difference between the saturation (i.e., amount of water vapor that the air can hold, namely the saturation vapor pressure) and actual vapor pressure, i.e., the Vapour Pressure Deficit (VPD), is detected.

VPD has been recognized as an important parameter for plant functionality and survival, which is influenced by the so-called hydraulic failure [24–29]. Oren et al. [30] fount in drought tolerant species a regulation of water potential (Ψ) as VPD increases and recommended different sensitivity of stomatal apparatus to VPD among different functional groups. However, E could either increase or decrease in response to an increased VPD [27,31]; the first response is known as "feedback" response while the second as "feed forward" response. The transpiration rate is influenced by atmospheric conditions and, over a short time-scale, is regulated by the function of stomatal apparatus [32]. It has been argued that a declining stomatal conductance (g_s) concomitantly with increasing VPD would rather occur as a feedback response to E and water loss from the leaf, than as a direct response to humidity [33–35].

The function of the stomatal apparatus under global climate change is an essential subject in plant ecophysiological research because it affects plant growth, vegetation distribution and ecosystem function [36]. The stomatal response to VPD varies either across and within species, or within the same species [37–40]. Partial stomatal closure under elevated VPD, especially during midday, will negatively affect CO_2 assimilation rate [7,41].

The movement of water in the soil–plant–atmosphere (SPA) continuum is a function of the difference between hydrostatic pressure and hydraulic conductance along this pathway. Water flows through the SPA continuum driven by a gradient in Ψ, which depends on the water flow rate and the hydraulic conductivity of the different pathways [32]. The amount of water that will be absorbed by the roots of the plants and the amount that will move from the roots to the leaves and then to the atmosphere depends mainly on g_s, VPD and hydraulic conductance (K). The differences between plant species in Ψ, E and g_s, determine to a greater or lesser extent the plants' response to various water stresses [12,42–45]. However, the effects of rising VPD on vegetation and hydraulic dynamics remain poorly studied. It has been reported that significant higher leaf and stem hydraulic conductance [25,46,47] under increased irradiance are due to the regulation of aquaporins [46–48]. A midday decline of g_s has been obtained in many plant species and has been related with variation in midday stem water status [49,50]; this aspect supports the idea that stomatal response to VPD is substantially related to the hydraulic characteristics at both the whole plant scale and the leaf level [27,51]. There is a fundamental role of hydraulics on stomatal sensitivity to VPD [27,41], while inverse and non-linear relationship between conductance and VPD have been observed [36]. The opening and closing of the stomata seem to be controlled by complex mechanisms, which include chemical and hydraulic signals from roots, to shoots and leaves [52]. In this frame, hydraulic traits could be an important factor buffering the negative impact of drought on plant function [53].

A lot of research has been devoted to understanding how plants' hydraulic systems have evolved to accommodate survival under different environments. However, concerning old-grown, trees and shrubs the relationship between K and the response of g_s to VPD has not explicitly evaluated, in situ. Despite the above-mentioned trends, species grown under the same environmental conditions may exhibit entirely different hydraulic properties [54,55]. This interspecific variation sometimes may be ascribed to different functional types, such as deciduous and evergreen [56]. Nevertheless, variation of hydraulic traits cannot only be explained by categorization of species in functional types [56,57]. Forest and shrub communities' response to climate change are most closely related to microclimatic change and not to macroclimatic change [58,59].

In considering that the water balance (W) is expressed by the relationship W = A − E, it is very interesting to study whether: (1) variation of hydraulic conductance in Mediterranean shrubs during the dry summer period is reflected in the leaf phenology (i.e., evergreen vs. deciduous), and (2) changes of VPD and consequently of microclimatic conditions influence the physiological mechanism and the performance of evergreen vs. deciduous shrubs.

The main objective of this study was to compare the ability of co-existing deciduous and evergreen broadleaved shrubs grown on the Olympus Mountain to maintain their water status and control their stomatal conductance throughout the dry season, as well as to evaluate the response of deciduous and evergreen shrubs to enhanced vapor pressure deficit. It is likely that hydraulic responses of co-occurring deciduous and evergreen shrubs, which are to some extent linked to water exploitation and effectiveness of plants' life forms during a period of soil drying, have not been reported.

2. Results

2.1. Climatic Conditions

The seasonal pattern of VPD during the experimental period is given in Figure 1. Overall, predawn vapour pressure deficit (VPD_p) was significantly lower ($p < 0.05$) than midday vapour pressure deficit (VPD_m), except from the first measurement (mid-May) ($p \geq 0.05$). Predawn VPD_p ranged from 0.769 ± 0.019 kPa to 1.731 ± 0.054 kPa, while VPD_m ranged from 0.974 ± 0.044 kPa to 4.043 ± 0.106 kPa. The relative humidity (RH) followed a reverse course relatively to VPD during the experimental period (Figure 1). The Photosynthetic Photon Flux Density (PPFD) was maintained at a relatively high level (i.e., >1500 μmol m^{-2} s^{-1}) (Figure 2) from the end of May to the end of August; only during the first measurement (end of spring) PPFD was relatively low, approximately 800 μmol m^{-2} s^{-1}.

Data analysis revealed that only the date was a significant predictor for leaf temperature (T_l) ($p < 0.001$), whereas the shrub life form (deciduous, evergreen) and the interaction between date and shrub life form were not significant ($p \geq 0.05$). Leaf temperature (T_l) during the study period almost coincided with the midday ambient air temperature (T_m), ranging from 19.86 °C to 33.04 °C and 19.29 °C to 33.61 °C, respectively (Figure 2). Predawn air temperature (T_p) ranged from 17.80 °C to 23.21 °C. The differences between T_p and T_m were statistically significant ($p < 0.05$) throughout the experimental period, except from the first measurement (mid-May).

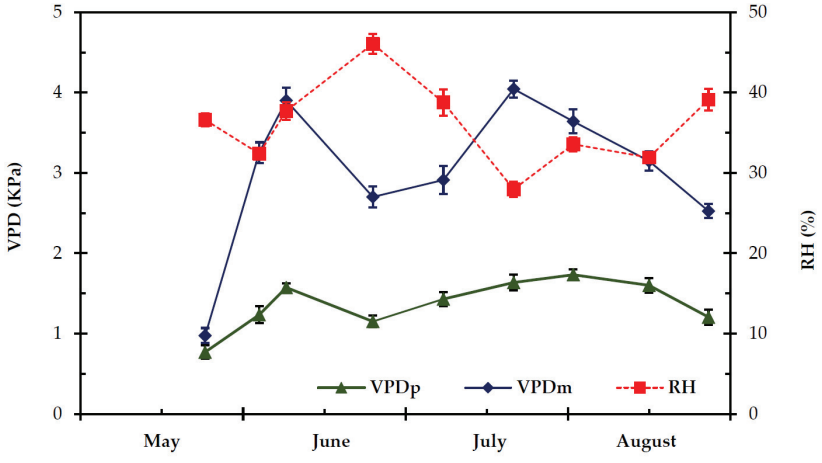


Figure 1. Seasonal predawn (VPD_p) and midday (VPD_m) vapour pressure deficit and relative humidity (RH) in the study site, during the experimental period, from 27 May to 28 August. The values are means ± SE (n = 24).

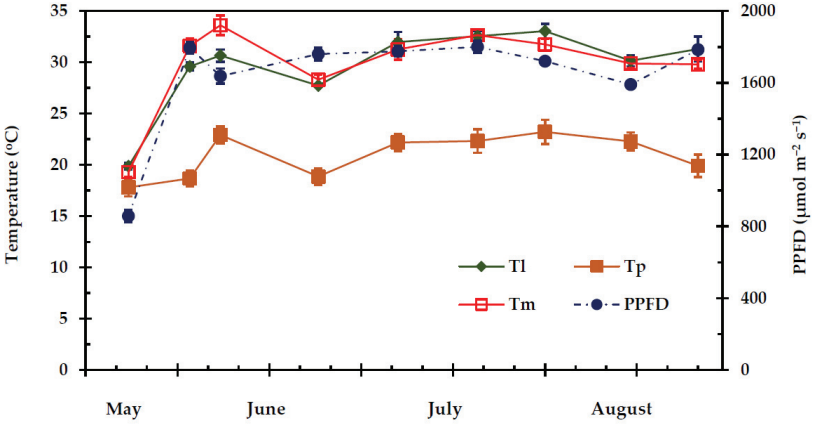


Figure 2. Seasonal Photosynthetic Photon Flux Density (PPFD), leaf temperature (T_l), morning (T_p) and midday (T_m) ambient air temperature during the experimental period from 27 May to 28 August. The values are means ± SE ($n_{Tp,Tm}$ = 24, n_{Tl} = 48).

2.2. Physiological Parameters

The principal component analysis (PCA, Figure 3) of the physiological parameters showed that the eight species are classified into the groups they belong to, deciduous and evergreen, and characterize the first principal component (Axis x), i.e., the species *Coronilla emeroides* Boiss. and Spruner, *Carpinus betulus* L., *Pistasia terebinthus* L. and *Cercis siliquastrum* L. are deciduous, while the species *Arbutus unedo* L., *Arbutus adrachnae* L., *Quercus coccifera* L. and *Quercus ilex* L. are evergreens. Additionally, variables such as predawn leaf water potential (Ψ_p), g_s and leaf hydraulic conductance (K_{Leaf}) are negatively correlated with VPD_p, VPD_m, the Julian date of the measurements and the second principal component (Axis y). Midday leaf water potential (Ψ_m) characterizes both components, while E does not characterize any of the two components. The deciduous species presented higher values (less negative) of Ψ_p, Ψ_m, g_s and K_{Leaf} relatively to evergreen species (Table 1).

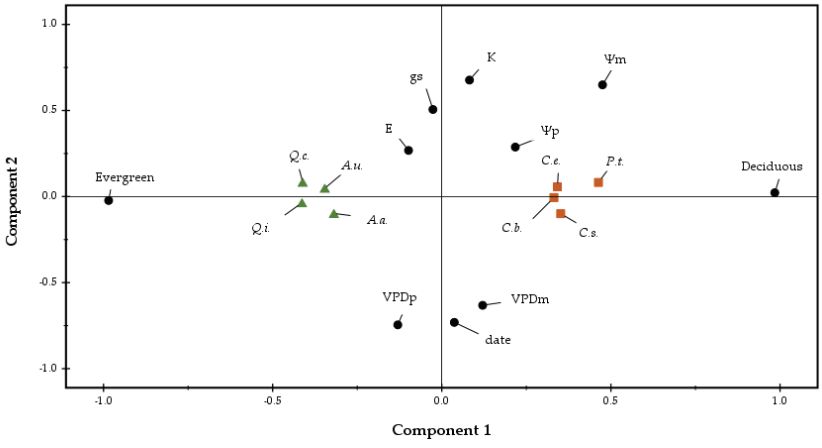


Figure 3. Principal component analysis (PCA) loading plot representing the variables and the species characterizing the two components. Black dots indicated predawn (Ψ_p) and midday (Ψ_m) leaf water potential, midday transpiration rate (E), midday stomatal conductance (g_s), midday leaf hydraulic conductance (K_{Leaf}) and midday vapour pressure deficit (VPD_m); brown squares indicated the deciduous species: *Coronilla emeroides* (*C.e.*), *Carpinus betulus* (*C.b.*), *Pistasia terebinthus* (*C.t.*). *Cercis siliquastrum* (*C.s.*), while green triangles indicated the evergreen species: *Arbutus unedo* (*A.u.*), *Arbutus adrachnae* (*A.a.*), *Quercus coccifera* (*Q.c.*) and *Quercus ilex* (*Q.i.*).

Table 1. Mean values of predawn (Ψ_p) and midday (Ψ_m) leaf water potential, midday transpiration rate (E), midday stomatal conductance (g_s), midday leaf hydraulic conductance (K_{Leaf}) and midday vapour pressure deficit (VPD_m) in deciduous and evergreen shrubs during the study period.

Parameter	Deciduous	Evergreens	*p*-Value
	Mean ± SE	**Mean ± SE**	
Ψ_p (MPa)	−0.94 ± 0.04	−1.04 ± 0.03	0.876 ^{NS}
Ψ_m (MPa)	−2.09 ± 0.06	−2.53 ± 0.05	0.001 **
E (mmol m^{-2} s^{-1})	16.12 ± 0.89	15.87 ± 0.68	0.734 ^{NS}
g_s (mmol m^{-2} s^{-1})	575.97 ± 21.00	585.5 ± 27.93	0.487 ^{NS}
K_{Leaf} (mmol MPa^{-1} m^{-2} s^{-1})	17.18 ± 1.45	13.15 ± 1.57	0.033 *
VPD_m (kPa)	2.79 ± 0.13	2.67± 0.11	0.149

* Significant for $p < 0.05$, ** significant for $p < 0.001$, NS—no significance. Mean values were compared with independent samples Student's *t*-test.

2.3. Leaf Water Potential

On one hand, data analysis revealed that the date was a significant predictor for Ψ_p and Ψ_m ($p = 0.021$ and $p < 0.001$, respectively). On the other hand, the shrub life form (deciduous, evergreen) significantly affected the Ψ_m ($p < 0.001$). The interaction between date and shrub life form was not significant concerning the two variables ($p \geq 0.05$) (Figure 4a). The estimated Ψ_m values were significantly lower in the considered evergreen shrubs in comparison to the deciduous shrubs during the experimental period ($p < 0.001$), (Figure 4b). Ψ_p ranged in deciduous shrubs from –0.87 MPa to −1.21 Mpa and in the evergreens from −0.92 Mpa to −1.14 Mpa, while Ψ_m from −1.4 Mpa to −2.59 Mpa and from −1.88 Mpa to −2.86 Mpa, respectively. The average values of the studied parameters are presented in Table 1.

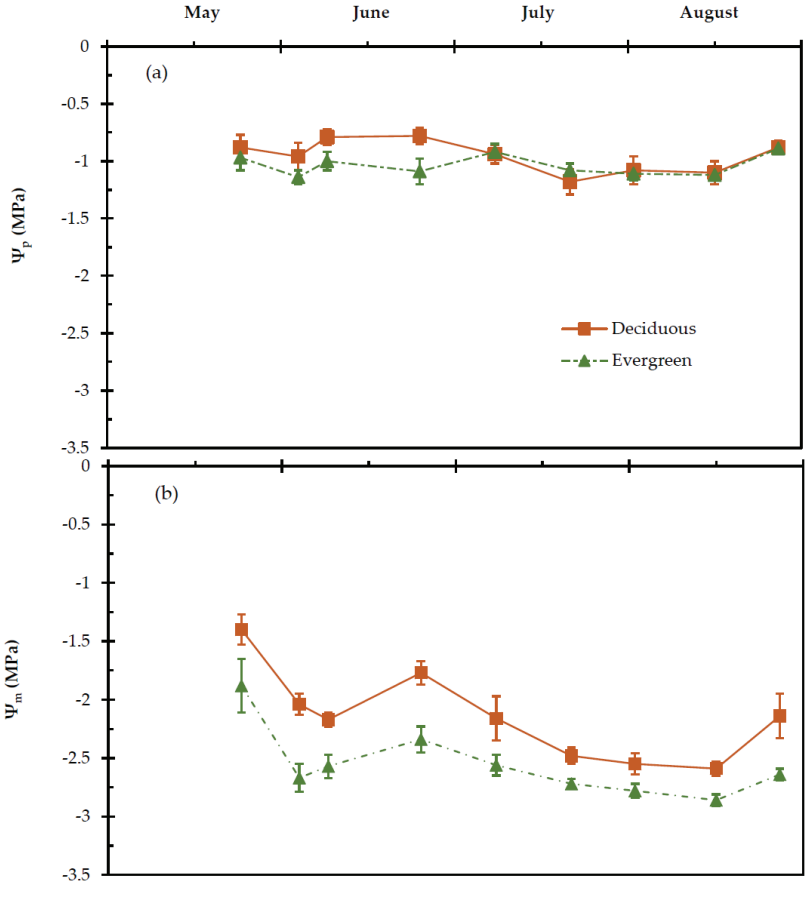


Figure 4. Seasonal (**a**) predawn (Ψ_p) and (**b**) midday (Ψ_m) leaf water potential of deciduous and evergreen shrubs during the experimental period from 27 May to 28 August. The values are means ± SE (n = 24).

2.4. Transpiration Rate, Stomatal and Leaf Hydraulic Conductance

Data analysis revealed that the date was a significant ($p < 0.001$) predictor for E, g_s and K_{Leaf}. The shrub life form (deciduous, evergreen) significantly affected variable K_{Leaf} ($p < 0.001$). The interaction between date and shrub life form was significant only for g_s ($p < 0.05$). The seasonal pattern of E (at solar noon) of evergreen and deciduous shrubs did not fluctuate substantially during the experimental period and significant difference was not observed in E between deciduous and evergreen shrubs ($p > 0.05$). The average values of E were found 16.12 ± 0.89 and 15.87 ± 0.68 mmol m^{-2} s^{-1} for deciduous and evergreen shrubs, respectively (Table 1). In particular during the dry season, E ranged from 11.71 to 20.29 mmol m^{-2} s^{-1} in the deciduous and from 13.04 to 17.02 mmol m^{-2} s^{-1} in the evergreen shrubs (Figure 5).

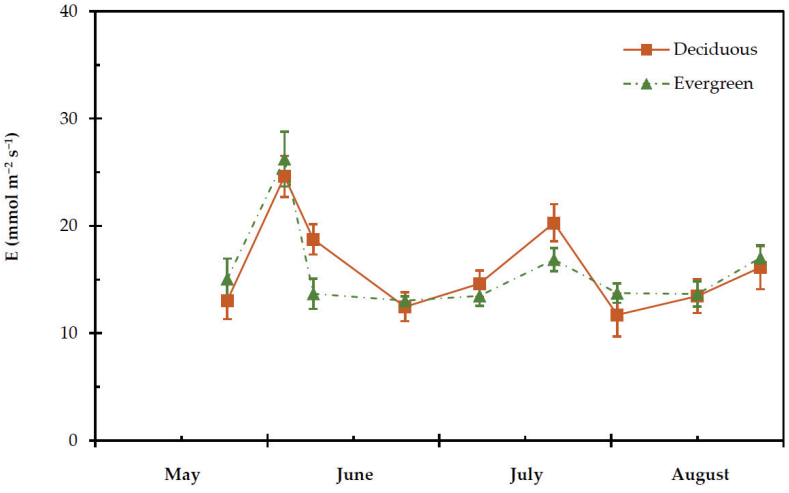


Figure 5. Seasonal transpiration rate (E) of deciduous and evergreen shrubs during the experimental period, from 27 May to 28 August. The values are means ± SE (n = 24).

Seasonal pattern of g_s was different ($p < 0.05$) between the two groups of plants. The deciduous shrubs presented significantly lower values ($p < 0.05$) of g_s in relation to the evergreens during dry period (from July until mid-August). The g_s during dry period ranged from 416.5± 22.7 to 585.25 ± 42.5 mmol m^{-2} s^{-1} in deciduous and from 413.5 ± 27.4 to 644.75 ± 43.5 mmol m^{-2} s^{-1} in evergreen shrubs (Figure 6).

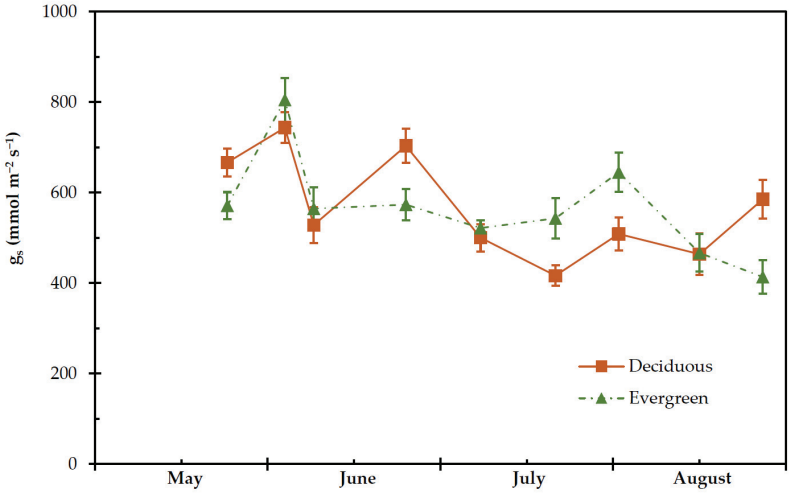


Figure 6. Seasonal stomatal conductance (g_s) of deciduous and evergreen shrubs during the experimental period, from 27 May to 28 August. The values are means ± SE (n = 24).

The K_{Leaf} was significantly higher in deciduous compared to evergreen shrubs ($p < 0.001$), (Figure 7), especially during the dry season. The mean K_{Leaf} was significantly higher in deciduous 17.18 ± 1.45 mmol Mpa^{-1} m^{-2} s^{-1} compared to evergreens 13.15 ± 1.57 mmol Mpa^{-1} m^{-2} s^{-1} ($p < 0.001$), (Figure 7, Table 1). However, the seasonal changes of K_{Leaf} in both groups of plants revealed a decrease in the K_{Leaf} when VPD_m increased during the dry period (Figure 7).

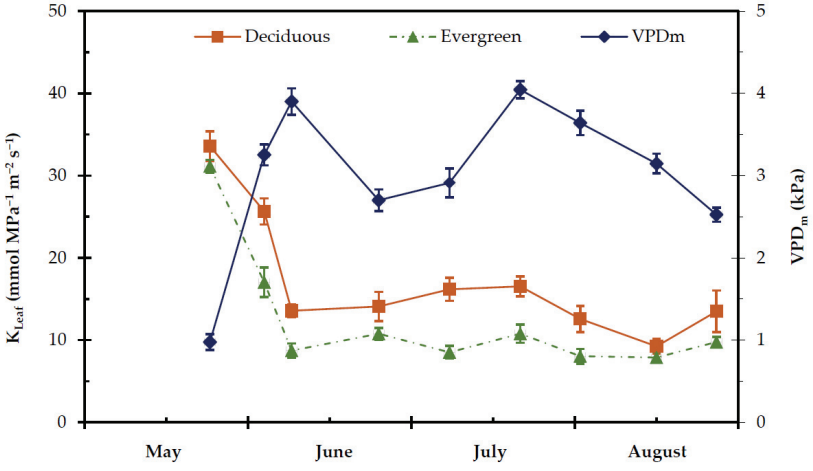


Figure 7. Seasonal midday vapour pressure deficit (VPD_m) and leaf hydraulic conductance (K_{Leaf}) of the considered deciduous and evergreen shrubs during the experimental period, from 27 May to 28 August. Values are means ± SE (n_{Kleaf} = 24, n_{VPDm} = 48).

The relationship between K_{Leaf} and Ψ_m is presented in Figure 8. It is likely that as the growing season proceeded K_{Leaf} decreased and exhibited the lowest values concomitantly with the lowest Ψ_m values. It is worth mentioning that for the same low values of Ψ_m, the deciduous shrubs exhibited higher K_{Leaf} in relations to the evergreens.

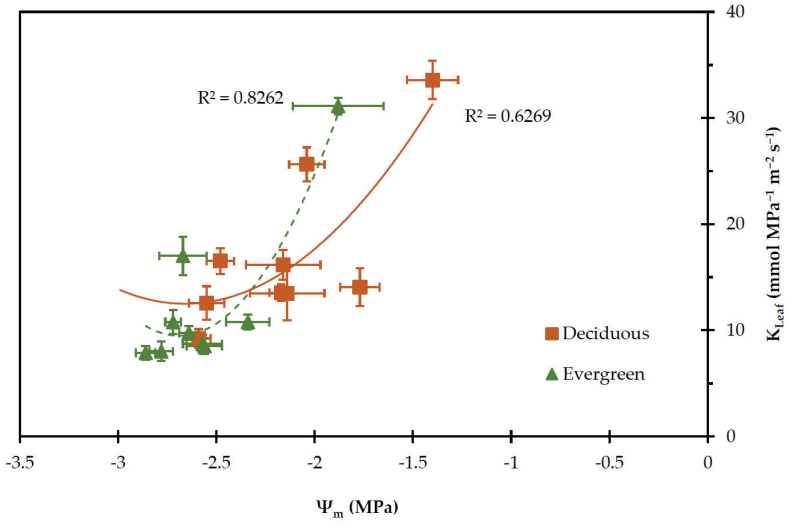


Figure 8. The relationship between midday leaf water potential (Ψ_m) and hydraulic conductance (K_{Leaf}) of deciduous and evergreen shrubs during the experimental period, from 27 May to 28 August. Values are means ± SE (n = 24). Polynomial regression analysis was used to assess the relationship between K_{Leaf} and Ψ_m.

In Table 2, the Pearson correlation between physiological parameters and VPD_m is presented. In deciduous species a significant positive correlation was detected between VPD_m and E and a negative between Ψ_m and g_s, while in evergreen shrubs VPD_m was negatively correlated with Ψ_m and K_{Leaf}.

Table 2. Pearson correlation between predawn (Ψ_p) and midday leaf water potential (Ψ_m), transpiration rate (E), stomatal conductance (g_s), leaf hydraulic conductance (K_{Leaf}) and midday Vapour Pressure Deficit (VPD_m) for deciduous (left) and evergreen (right) shrubs.

Deciduous	Ψ_p	Ψ_m	E	g_s	K_{Leaf}	VPD_m	Evergreens
Ψ_p	1	0.387 **	0.020	−0.139	−0.039	−0.108	Ψ_p
Ψ_m	0.524 **	1	−0.311 **	−0.162	0.544 **	−0.451 **	Ψ_m
E	0.234 *	−0.013	1	−0.328 **	0.135	0.147	E
g_s	0.357 **	0.620 **	−0.110	1	−0.037	0.157	g_s
K_{Leaf}	0.084	0.478 **	0.562 **	0.209	1	−0.298 **	K_{Leaf}
VPD_m	−0.088	−0.443 **	0.271 **	−0.315 **	−0.147	1	VPD_m

* Significant for $p < 0.05$, ** significant for $p < 0.001$.

3. Discussion

The results of this study demonstrate that the water status of co-occurring evergreen and deciduous broadleaved shrubs in semi-arid Mediterranean conditions under rising VPD_m is different. Additionally, it seems likely that deciduous shrubs control more efficiently their water status during the dry season, by exhibiting lower stomatal conductance and higher hydraulic conductance than the evergreens. In other words, the deciduous plants possess a more water spending behaviour than the evergreens.

The increase of air temperature accompanied by decreasing RH and increasing heat load due to incident radiation on the leaf, in combination with air speed, may elevate VPD in the atmosphere [60]. It has been reported that RH concerning future climatic scenarios will remain either constant at the global scale [61], or a negative [62] and/or positive trend between VPD and RH at a regional scale [27] will be observed. In our research, the values of PPFD were generally maintained at high levels, as it was expected concerning the characteristics of the Mediterranean climate in the experimental site. The same values in air and leaf temperature imply that broadleaved shrubs possess such leaf tissues, where the transpiration flow is not sufficient to reduce leaf temperature in relation to the ambient temperature. The increase of T_m and VPD_m under drought conditions (after June) may be linked to the physiological performance and survival of deciduous and evergreen shrubs via either reducing g_s and gas exchanges (feedforward mechanism), or increasing plant water loss (feedback mechanism) [63].

The seasonal patterns of Ψ_p and Ψ_m suggest that the co-occurring deciduous and evergreen shrubs on Olympus Mountain were able to regulate water loss and ensuring adequate hydration of leaf tissues overnight, even during the dry season, therefore Ψ_p was retained to approximately −1.0 MPa; such scale of the Ψ_p values is sufficient for the Mediterranean region (*Quercetalia ilicis* zone) where the studied species are growing [64]. Ψ_p is an essential index to study the response of plants to drought, because its value can be considered equal to soil water potential, since plants and soil reach a hydration equilibrium during the night [65–68].

The higher (less negative) Ψ_m in deciduous shrubs indicates higher values of relative water content, E and g_s in comparison to the evergreen shrubs. The Ψ_m seems to be the main factors for stomatal regulation because is directly linked to the turgor of guard cells [49,69,70]. After sunrise, the leaf water potential in Mediterranean plants decreases steadily, reaching its lower value at noon, while it begins to recover again during the afternoon [71–73]. This reflects a decline of stored water, and therefore water shortage [60]. The plants are the best indicators of soil water availability, which affect their water status [74,75]. Plants with both elevated (less negative) Ψ_m and water transport capacity can better control their leaf water status during midday, and hence they experience a smaller decline of midday g_s [49].

It is likely that the relatively higher E in deciduous species during midday is due to their elevated water status, i.e., higher Ψ_m in leaves (Figures 4a and 5); a slight variation of E values was detected between deciduous and evergreen shrubs, while the lowest values of E occurred in the two groups of shrubs during the dry season (June–August), (Figure 5).

Apparently, low E values during the dry season remained rather stable and above zero values, which indicates influx of carbon dioxide [49,76].

The deciduous shrubs exhibited higher Ψ_m and lower midday g_s than the evergreens; this may indicate that midday stomatal conductance is more related to stem rather than leaf water status, which is in accordance with earlier results [49]. In fact, g_s was negatively correlated with VPD_m (Table 2), suggesting a response to environmental conditions, which is in agreement with the published results by Auge et al. [77] from other deciduous species. This trait may help deciduous species to regulate their stomatal apparatus in order to maintained Ψ_m in a range of values that will support their water status and avoid xylem embolism under drought conditions [49,78]. Our results show that there was not any synchronization between g_s and Ψ_m in both shrub groups, which may also be due to the fact the evergreens are hypostomatic plants [79–81]. However, it is not clear which microclimate parameter, i.e., temperature and/or relative humidity, was directly linked to stomatal behaviour. It has been proposed that temperature had a greater impact on stomatal conductance and assimilation rate in the amphistomatic leaves of *Eucalyptus tetrodonta* independently of VPD [82,83]. Addington et al. [35] reported that the stomatal apparatus controls Ψ in a way that the tension on the water column created by decreasing Ψ did not cause extreme xylem cavitation. Several studies suggested that high VPD reduces g_s, consequently affecting assimilation rate and growth [84,85]. On the contrary, it has been argued that the impact of high VPD on g_s may not possess any impact on assimilation rate and growth [86]. Nevertheless, the impact of VPD on g_s and/or assimilation rate varies amongst plant species [82]. In addition, the stomatal anatomy and structure affect water loss and carbon assimilation, demonstrating the evolution and adaptations of the plants to environmental conditions [84,87].

Plants in order to grow and survive under water limited conditions evolved mechanisms to control stomatal aperture and xylem water capacity [88]. The differences in water-use strategies might be partially due to various hydraulic properties between the considered groups. The hydrodynamic status of leaf tissue expressed via leaf water potential [89] is determined by two mail functions taking place in the SPA continuum, i.e., transpiration rate and hydraulic conductance. Therefore, the favourable water status in deciduous shrubs could be attributed to the higher values of transpiration rate and/or at the highest values of the hydraulic conductance. The deciduous shrubs exhibited higher values of K_{Leaf} when compared to evergreen shrubs, especially during the dry summer period. This advantage of deciduous shrubs could be attributed to their water status and anatomical features. The positive correlation between K_{Leaf} and Ψ_m, and E in deciduous shrubs (Table 2) and the negative correlation between K_{Leaf} and VPD_m suggest that the water transport from root to the leaf plays a role to the fluctuations of leaf water status. The seasonal K_{Leaf} declined in both groups of plants when VPD increased during the summer dry period (Figure 7) especially at the end of July, when the highest value of VPD (3.99 KPa) was recorded. Probably, at a given transpiration rate in deciduous shrubs the leaf water status is maintained due to high hydraulic conductance [17,90,91]. Manzoni et al. [88] reported that, in some ecosystems, deciduous species have been found to be more hydraulically efficient than evergreen species. Choat et al. [92] suggested that deciduous species are more hydraulically efficient, but also more vulnerable to drought-induced embolism, than co-existing evergreens in a rainforest. It is well known that embolism occurs in plants under drought conditions when Ψ reaches very low values; however, the plants have the capacity to repair damage when the environmental conditions are favourable [89].

Our data are somehow in agreement with BIumler [93], who argued that although the Mediterranean climate is associated with evergreen species, some coexisting deciduous species exhibit some advantages in response to drought [92,94]. It is noteworthy that in our work deciduous and evergreen species are presented as two distinct life-history groups [91], although they probably form a continuum of variation in leaf life-history span [95].

4. Materials and Methods

4.1. Study Area and Climate

The study was conducted on Olympus Mountain (40°06′54″ N, 22°28′42″ E), which is a great, long-lived natural laboratory [96–98] in 2009, at an altitude of 554 m a.s.l., in an area located 5 Km from the town of Litochoro, 95 km south-east of Thessaloniki, in Greece. The climate of the study area is characterized as Cfa in the Köppen-Geiger system (http://www.en.climate-data.org, 7 January 2022) and as Mediterranean with cold and wet winters, dry and warm summer according to the bioclimatogram of Emberger. The mean annual rainfall in the study area ranges from 800 to 1000 mm, while substantially elevated precipitation is recorded during winter. The minimum air temperature ranges from 13 to 6 °C (during summer and winter, respectively). The maximum air temperature ranges from 4.9 to 6.7 °C during winter, and from 20 to 26 °C during summer. The warmest month is July and the coldest is December. The mean annual relative humidity (RH) ranges from 75 to 80%. Average RH values during the most humid month (December) is 85–90% and during the driest month (July) 30–50%. The monthly changes of temperature and precipitation (ombrothermic diagram) during the year of the measurements, from the nearest meteorological station of Dion (40°12′00″ N, 22°30′00″ E), are presented in Figure 9.

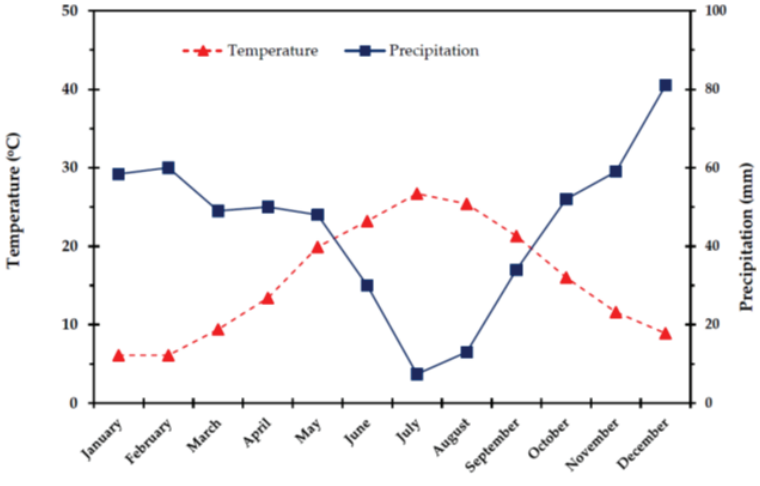


Figure 9. Ombrothermic diagram in the study area throughout a year.

The microclimatic conditions (temperature, humidity), in the study area during the experimental period was measured with the Hobo H8 Pro (Hobo H8 Pro Series 1997–2003, Onset Computer Coorporation, Bourne, MA, USA). Furthermore, on specific Julian dates and hours when plant physiological parameters were investigated, ambient air predawn and midday temperature and RH were measured using a Novasima MS1 microclimatic sensor (Novatron Scientific Ltd., Horsham, UK). Predawn (VPD_p) and midday (VPD_m) vapour pressure deficit were calculated according to Abtew and Melesse (2013). The Photosynthetic Photon Flux Density was measured with the steady-state diffusion porometer LI-1600 (LI- COR, Inc., Lincoln, NE, USA). The presented values of VPD_p, VPD_m, RH, PPFD, T_p and T_m are means of twenty-four measurements.

4.2. Plant Material

The study area is part of the Mediterranean zone of the evergreen broadleaved plants *Quercus ilex* L. and *Arbutus andrachne* L. Deciduous and evergreen shrubs and small trees such as *Acer monspesulanum* L., *Carpinus betulus* L., *Cercis siliquastrum* L., *Cotinus coggygria* Scop., *Fraxinus ornus* L., *Pistacia terebinthus* L., *Coronilla emeroides* Boiss. and

Spruner, *Quercus coccifera* L., *Arbutus unedo* L., *Phillyrea media* L., and *Juniperus oxycedrus* L. are widespread in the study area.

Four evergreen shrubs *Arbutus andrachne* (commonly known as Grecian strawberry tree), *Arbutus unedo* (strawberry tree), *Quercus ilex* (holm oak) and *Quercus coccifera* (kermes oak) and four deciduous shrubs *Carpinus betulus* (common hornbeam), *Cercis siliquastrum* (Juda's tree), *Coronilla emeroides* (scorpion senna), and *Pistacia terebinthus* (terebinth) were selected for this study. The shrubs were randomly selected for sampling. More specifically, the area of interest was equal to 20 ha. We divided this area into 20 tiles, 1 ha each, and then we randomly selected 6 tiles where each one of the eight species was randomly chosen. Concerning dendrometric parameters, the considered species possessed the same height (3–4 m) and diameter (1.5–2.0 m).

4.3. Physiological Parameters

Leaf water potential (Ψ), transpiration rate (E), stomatal conductance (g_s) and leaf temperature (T_l) were measured on the newest fully developed mature leaves of six different individuals from sun-exposed terminal branches. Using the pressure-bomb technique (PMS, Albany, OR, USA), leaf water potential was measured twice during the day, i.e., at predawn (Ψ_p) and midday (Ψ_m). Transpiration rate, g_s and T_l were measured using steady state porometer (Li1600, LI-COR Lincoln, NE, USA). Seasonal measurements of Ψ_p were obtained before sunrise, while the measurements of Ψ_m, E, and g_s were obtained on clear sunny days at around solar noon (12:00–14:30 h), approximately 10–15 days intervals. The presented values, for each of the parameters, are means of six replications per studied shrub.

Leaf hydraulic conductance (K_{Leaf}) was calculating according to Ohm's law following the formula:

$$K_{Leaf} = \frac{E}{(\Psi_{soil} - \Psi_{Leaf})} = \frac{E}{(\Psi_p - \Psi_m)} \quad (1)$$

It has been assumed that Ψ_{soil} is in equilibrium with Ψ_p and the lowest diurnal Ψ_{leaf} is equal to Ψ_m [66,67,99]. However, sometimes the first assumption may lead to overestimation of K_{plant} [100]. Additionally, values of K_{Leaf} reflect the capacity of evergreen and deciduous plants, grown under ambient conditions, for water exploitation during a period of soil drying.

4.4. Statistical Analysis

The Kolmogorov–Smirnov test was employed to evaluate data normality. To explore whether the ecophysiological response of deciduous and evergreen shrubs vary during the dry season, a two-way analysis of variance (ANOVA) was performed on the studied parameters (T_L, Ψ_p, Ψ_m, E, g_s, K_{leaf}) [81]. Student's *t*-test for independent samples was used to examine differences in T_L, E, g_s, Ψ_m, Ψ_p and K_{Leaf} between deciduous and evergreen shrubs. The climatic parameters VPD_p, VPD_m, T_p, and T_m at each sampling date were compared using also the Student's *t*-test for independent samples. Polynomial regression analysis was used to determine the relationship between K_{Leaf} and Ψ_m, between deciduous and evergreen shrubs. In addition, a Principal Component Analysis (PCA) with varimax rotation was used to assess the relationships among the measurements of interest between the eight shrubs to see whether they could be classified according to their ecophysiological response in the life form in which belong (deciduous, evergreen). Pearson correlation was used to explore links among VPD_m, E, g_s, Ψ_p, Ψ_m and K_{Leaf}. P-values lower than 0.05 were considered statistically significant. All statistical analyses were performed using the SPSS statistical package v. 27.0 (IBM Corp. in Armonk, NY, USA).

5. Conclusions

The results of this research work demonstrate different hydraulic strategies between co-occurring deciduous and evergreen shrubs grown on Olympus Mountain. The deciduous life-form presented a strategy of higher hydraulic and lower stomatal conductance, while the evergreen exhibited lower hydraulic and higher stomatal conductance. Although,

seasonal leaf hydraulic conductance declined in both groups of plants when vapor pressure deficit increased during the summer dry period, evergreen shrubs sustain a water transport to their foliage at a rate sufficient to prevent severe damage due to desiccation.

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

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable for studies not involving humans or animals.

Informed Consent Statement: Not applicable for studies not involving humans or animals.

Data Availability Statement: The data presented in this study are available in figures and tables provided in the manuscript.

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

References

1. Pachauri, R.K.; Allen, M.R.; Barros, V.R.; Broome, J.; Cramer, W.; Christ, R.; Church, J.A.; Clarke, L.; Dahe, Q.; Dasgupta, P. *Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*; IPCC: Geneva, Switzerland, 2014.
2. Moreno, M.; Simioni, G.; Limousin, J.M.; Rodriguez-Calcerrada, J.; Ruffault, J.; Cochard, H.; Torres, J.; Delzon, S.; Tournant, A.; Dumas, P.J. Mediterranean trees vulnerability to climate change will not be minimized through hydraulic safety traits adjustments. In Proceedings of the EGU General Assembly Conference Abstracts, vEGU21: Gather Online, 19–30 April 2021; p. EGU21-3850.
3. Zindros, A.; Radoglou, K.; Milios, E.; Kitikidou, K. Tree Line Shift in the Olympus Mountain (Greece) and Climate Change. *Forests* **2020**, *11*, 985. [CrossRef]
4. Kramer, P.J. Drought stress and the origin of adaptations. In *Adaptation of Plants to Water and High Temperature Stress*; Turner, N.C., Kramer, P.J., Eds.; John Wiley & Sons: New York, NY, USA, 1980; pp. 7–21.
5. Chaves, M.M.; Pereira, J.S.; Maroco, J.; Rodrigues, M.L.; Ricardo, C.P.; Osorio, M.L.; Carvalho, I.; Faria, T.; Pinheiro, C. How plants cope with water stress in the field. Photosynthesis and growth. *Ann. Bot.* **2002**, *89*, 907–916. [CrossRef] [PubMed]
6. Margolis, H.A.; Ryan, M.G. A physiological basis for biosphere-atmosphere interactions in the boreal forest: An overview. *Tree Physiol.* **1997**, *17*, 491–499. [CrossRef] [PubMed]
7. Sanchez, M.; Sinclair, T.R.; Pradhan, D. Transpiration response to vapor pressure deficit and soil drying among quinoa genotypes (Chenopodium quinoa Willd.). *J. Crop Improv.* **2020**, *35*, 291–302. [CrossRef]
8. Pereira, J.S. Plant water deficits in Mediterranean ecosystems. In *Water Deficits: Plant Response from Cell to Community*; Smith, J.A.C., Griffiths, H., Eds.; Bios Scientific Publishers: Oxford, UK, 1993; pp. 237–251.
9. Kapsali, E.-I.; Karatassiou, M. Seasonal changes in leaf tissue rehydration of one annual and two perennial grass forage species induced by bioclimate. *Not. Bot. Horti Agrobot. Cluj-Napoca* **2015**, *43*, 479–487. [CrossRef]
10. Karatassiou, M.; Noitsakis, B. Changes of the photosynthetic behaviour in annual C_3 species at late successional stage under environmental drought conditions. *Photosynthetica* **2010**, *48*, 377–382. [CrossRef]
11. Choat, B.; Brodribb, T.J.; Brodersen, C.R.; Duursma, R.A.; Lopez, R.; Medlyn, B.E. Triggers of tree mortality under drought. *Nature* **2018**, *558*, 531–539. [CrossRef]
12. Fang, Y.L.; Leung, L.R.; Wolfe, B.T.; Detto, M.; Knox, R.G.; McDowell, N.G.; Grossiord, C.; Xu, C.G.; Christoffersen, B.O.; Gentine, P.; et al. Disentangling the effects of Vapor Pressure Deficit and Soil Water Availability on Canopy Conductance in a Seasonal Tropical Forest During the 2015 El Nino Drought. *J. Geophys. Res.-Atmos.* **2021**, *126*, e2021JD035004. [CrossRef]
13. Aranda, I.; Gil, L.; Pardos, J. Seasonal changes in apparent hydraulic conductance and their implications for water use of European beech (*Fagus sylvatica* L.) and sessile oak [Quercus petraea (Matt.) Liebl] in South Europe. *Plant Ecol.* **2005**, *179*, 155–167. [CrossRef]
14. Meinzer, F.C.; Clearwater, M.J.; Goldstein, G. Water transport in trees: Current perspectives, new insights and some controversies. *Environ. Exp. Bot.* **2001**, *45*, 239–262. [CrossRef]
15. Sack, L.; Cowan, P.D.; Jaikumar, N.; Holbrook, N.M. The 'hydrology' of leaves: Co-ordination of structure and function in temperate woody species. *Plant Cell Environ.* **2003**, *26*, 1343–1356. [CrossRef]
16. Lo Gullo, M.A.; Castro Noval, L.; Salleo, S.; Nardini, A. Hydraulic architecture of plants of Helianthus annuus L. cv. Margot: Evidence for plant segmentation in herbs. *J. Exp. Bot.* **2004**, *55*, 1549–1556. [CrossRef] [PubMed]
17. Nardini, A.; Salleo, S. Limitation of stomatal conductance by hydraulic traits: Sensing or preventing xylem cavitation? *Trees* **2000**, *15*, 14–24. [CrossRef]

18. Tyree, M.; Sobrado, M.; Stratton, L.; Becker, P. Diversity of hydraulic conductance in leaves of temperate and tropical species: Possible causes and consequences. *J. Trop. For. Sci.* **1999**, *11*, 47–60.
19. Brodribb, T.J.; Holbrook, N.M. Leaf physiology does not predict leaf habit; examples from tropical dry forest. *Trees* **2005**, *19*, 290–295. [CrossRef]
20. Sack, L.; Tyree, M.T. Leaf Hydraulics and Its Implications in Plant Structure and Function. In *Vascular Transport in Plants*; Holbrook, N.M., Zwieniecki, M.A., Eds.; Elsevier: Amsterdam, The Netherlands; Academic Press: Cambridge, MA, USA, 2005; pp. 93–114.
21. Pereira, J.S.; Pallardy, S. Water stress limitations to tree productivity. In *Biomass Production by Fast-Growing Trees*; Pereira, J.S., Landsberg, J.J., Eds.; Springer: Dordrecht, The Netherlands, 1989; pp. 37–56.
22. Jordan, W.R.; Dugas, W.A., Jr.; Shouse, P.J. Strategies for crop improvement for drought-prone regions. *Agric. Water Manage.* **1983**, *7*, 281–299. [CrossRef]
23. Daszkowska-Golec, A.; Szarejko, I. Open or close the gate–stomata action under the control of phytohormones in drought stress conditions. *Front. Plant Sci.* **2013**, *4*, 138. [CrossRef]
24. Choat, B.; Jansen, S.; Brodribb, T.J.; Cochard, H.; Delzon, S.; Bhaskar, R.; Bucci, S.J.; Feild, T.S.; Gleason, S.M.; Hacke, U.G.; et al. Global convergence in the vulnerability of forests to drought. *Nature* **2012**, *491*, 752–755. [CrossRef]
25. Baert, A.; De Schepper, V.; Steppe, K. Variable hydraulic resistances and their impact on plant drought response modelling. *Tree Physiol.* **2015**, *35*, 439–449. [CrossRef]
26. Martınez-Vilalta, J.; Piñol, J.; Beven, K. A hydraulic model to predict drought-induced mortality in woody plants: An application to climate change in the Mediterranean. *Ecol. Modell.* **2002**, *155*, 127–147. [CrossRef]
27. Grossiord, C.; Buckley, T.N.; Cernusak, L.A.; Novick, K.A.; Poulter, B.; Siegwolf, R.T.W.; Sperry, J.S.; McDowell, N.G. Plant responses to rising vapor pressure deficit. *New Phytol.* **2020**, *226*, 1550–1566. [CrossRef] [PubMed]
28. Breshears, D.D.; Adams, H.D.; Eamus, D.; McDowell, N.G.; Law, D.J.; Will, R.E.; Williams, A.P.; Zou, C.B. The critical amplifying role of increasing atmospheric moisture demand on tree mortality and associated regional die-off. *Front. Plant Sci.* **2013**, *4*, 266. [CrossRef] [PubMed]
29. Stovall, A.E.L.; Shugart, H.; Yang, X. Tree height explains mortality risk during an intense drought. *Nat. Commun.* **2019**, *10*, 4385. [CrossRef] [PubMed]
30. Oren, R.; Sperry, J.S.; Katul, G.G.; Pataki, D.E.; Ewers, B.E.; Phillips, N.; Schäfer, K.V.R. Survey and synthesis of intra- and interspecific variation in stomatal sensitivity to vapour pressure deficit. *Plant Cell Environ.* **1999**, *22*, 1515–1526. [CrossRef]
31. Franks, P.; Cowan, I.; Farquhar, G. The apparent feedforward response of stomata to air vapour pressure deficit: Information revealed by different experimental procedures with two rainforest trees. *Plant Cell Environ.* **1997**, *20*, 142–145. [CrossRef]
32. Carminati, A.; Javaux, M. Soil Rather than Xylem Vulnerability Controls Stomatal Response to Drought. *Trends Plant Sci.* **2020**, *25*, 868–880. [CrossRef]
33. Meinzer, F.; Hinckley, T.; Ceulemans, R. Apparent responses of stomata to transpiration and humidity in a hybrid poplar canopy. *Plant Cell Environ.* **1997**, *20*, 1301–1308. [CrossRef]
34. Monteith, J. A reinterpretation of stomatal responses to humidity. *Plant Cell Environ.* **1995**, *18*, 357–364. [CrossRef]
35. Addington, R.N.; Mitchell, R.J.; Oren, R.; Donovan, L.A. Stomatal sensitivity to vapor pressure deficit and its relationship to hydraulic conductance in Pinus palustris. *Tree Physiol.* **2004**, *24*, 561–569. [CrossRef]
36. Li, J.; Zhang, G.Z.; Li, X.; Wang, Y.; Wang, F.Z.; Li, X.M. Seasonal change in response of stomatal conductance to vapor pressure deficit and three phytohormones in three tree species. *Plant Signal Behav.* **2019**, *14*, 1682341. [CrossRef]
37. Körner, C.; Neumayer, M.; Menendez-Riedl, S.P.; Smeets-Scheel, A. Functional morphology of mountain plants. *Flora* **1989**, *182*, 353–383. [CrossRef]
38. McNaughton, K.; Jarvis, P. Effects of spatial scale on stomatal control of transpiration. *Agric. For. Meteorol.* **1991**, *54*, 279–302. [CrossRef]
39. Cunningham, S.C. Photosynthetic responses to vapour pressure deficit in temperate and tropical evergreen rainforest trees of Australia. *Oecologia* **2005**, *142*, 521–528. [CrossRef] [PubMed]
40. Gao, J.; Zhao, P.; Shen, W.; Niu, J.; Zhu, L.; Ni, G. Biophysical limits to responses of water flux to vapor pressure deficit in seven tree species with contrasting land use regimes. *Agric. For. Meteorol.* **2015**, *200*, 258–269. [CrossRef]
41. Aasamaa, K.; Sõber, A.; Rahi, M. Leaf anatomical characteristics associated with shoot hydraulic conductance, stomatal conductance and stomatal sensitivity to changes of leaf water status in temperate deciduous trees. *Funct. Plant Biol.* **2001**, *28*, 765–774. [CrossRef]
42. Raftoyannis, Y.; Radoglou, K. Physiological responses of beech and sessile oak in a natural mixed stand during a dry summer. *Ann. Bot.* **2002**, *89*, 723–730. [CrossRef]
43. Schütz, W.; Milberg, P.; Lamont, B.B. Germination requirements and seedling responses to water availability and soil type in four eucalypt species. *Acta Oecol.* **2002**, *23*, 23–30. [CrossRef]
44. Klein, T. The variability of stomatal sensitivity to leaf water potential across tree species indicates a continuum between isohydric and anisohydric behaviours. *Funct. Ecol.* **2014**, *28*, 1313–1320. [CrossRef]
45. Karatassiou, M.; Noitsakis, B.; Koukoura, Z. Drought adaptation ecophysiological mechanisms of two annual legumes on semi-arid Mediterranean grassland. *Sci. Res. Essays* **2009**, *4*, 493–500. [CrossRef]

46. Cochard, H.; Venisse, J.-S.; Barigah, T.S.; Brunel, N.; Herbette, S.; Guilliot, A.; Tyree, M.T.; Sakr, S. Putative role of aquaporins in variable hydraulic conductance of leaves in response to light. *Plant Physiol.* **2007**, *143*, 122–133. [CrossRef]
47. Guyot, G.; Scoffoni, C.; Sack, L. Combined impacts of irradiance and dehydration on leaf hydraulic conductance: Insights into vulnerability and stomatal control. *Plant Cell Environ.* **2012**, *35*, 857–871. [CrossRef] [PubMed]
48. Sellin, A.; Sack, L.; Ounapuu, E.; Karusion, A. Impact of light quality on leaf and shoot hydraulic properties: A case study in silver birch (Betula pendula). *Plant Cell Environ.* **2011**, *34*, 1079–1087. [CrossRef] [PubMed]
49. Zhang, Y.J.; Meinzer, F.C.; Qi, J.H.; Goldstein, G.; Cao, K.F. Midday stomatal conductance is more related to stem rather than leaf water status in subtropical deciduous and evergreen broadleaf trees. *Plant Cell Environ.* **2013**, *36*, 149–158. [CrossRef] [PubMed]
50. Grassi, G.; Ripullone, F.; Borghetti, M.; Raddi, S.; Magnani, F. Contribution of diffusional and non-diffusional limitations to midday depression of photosynthesis in *Arbutus unedo* L. *Trees* **2009**, *23*, 1149–1161. [CrossRef]
51. Brodribb, T.J.; Jordan, G.J. Internal coordination between hydraulics and stomatal control in leaves. *Plant Cell Environ.* **2008**, *31*, 1557–1564. [CrossRef] [PubMed]
52. Comstock, J.P. Hydraulic and chemical signalling in the control of stomatal conductance and transpiration. *J. Exp. Bot.* **2002**, *53*, 195–200. [CrossRef] [PubMed]
53. Aranda, I.; Cano, F.J.; Gasco, A.; Cochard, H.; Nardini, A.; Mancha, J.A.; Lopez, R.; Sanchez-Gomez, D. Variation in photosynthetic performance and hydraulic architecture across European beech (*Fagus sylvatica* L.) populations supports the case for local adaptation to water stress. *Tree Physiol.* **2015**, *35*, 34–46. [CrossRef]
54. Martínez-Vilalta, J.; Cochard, H.; Mencuccini, M.; Sterck, F.; Herrero, A.; Korhonen, J.; Llorens, P.; Nikinmaa, E.; Nole, A.; Poyatos, R. Hydraulic adjustment of Scots pine across Europe. *New Phytol.* **2009**, *184*, 353–364. [CrossRef]
55. Kolb, K.J.; Davis, S.D. Drought tolerance and xylem embolism in co-occurring species of coastal sage and chaparral. *Ecology* **1994**, *75*, 648–659. [CrossRef]
56. Iovi, K.; Kolovou, C.; Kyparissis, A. An ecophysiological approach of hydraulic performance for nine Mediterranean species. *Tree Physiol.* **2009**, *29*, 889–900. [CrossRef]
57. Nardini, A. Are sclerophylls and malacophylls hydraulically different? *Biol. Plant.* **2001**, *44*, 239–245. [CrossRef]
58. Zellweger, F.; De Frenne, P.; Lenoir, J.; Vangansbeke, P.; Verheyen, K.; Bernhardt-Römermann, M.; Baeten, L.; Hédl, R.; Berki, I.; Brunet, J. Forest microclimate dynamics drive plant responses to warming. *Science* **2020**, *368*, 772–775. [CrossRef] [PubMed]
59. McDaniel, M.D.; Wagner, R.J.; Rollinson, C.R.; Kimball, B.A.; Kaye, M.W.; Kaye, J.P. Microclimate and ecological threshold responses in a warming and wetting experiment following whole tree harvest. *Theor. Appl. Climatol.* **2014**, *116*, 287–299. [CrossRef]
60. Jones, H.G. *Plants and Microclimate: A Quantitative Approach to Environmental Plant Physiology*; Cambridge University Press: Cambridge, UK, 2013.
61. Dai, A. Recent climatology, variability, and trends in global surface humidity. *J. Clim.* **2006**, *19*, 3589–3606. [CrossRef]
62. Byrne, M.P.; O'Gorman, P.A. Trends in continental temperature and humidity directly linked to ocean warming. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, 4863–4868. [CrossRef]
63. Will, R.E.; Wilson, S.M.; Zou, C.B.; Hennessey, T.C. Increased vapor pressure deficit due to higher temperature leads to greater transpiration and faster mortality during drought for tree seedlings common to the forest-grassland ecotone. *New Phytol.* **2013**, *200*, 366–374. [CrossRef]
64. Rhizopoulou, S.; Mitrakos, K. Water relations of evergreen sclerophylls. I. Seasonal changes in the water relations of eleven species from the same environment. *Ann. Bot.* **1990**, *65*, 171–178. [CrossRef]
65. Sellin, A. Does pre-dawn water potential reflect conditions of equilibrium in plant and soil water status? *Acta Oecol.* **1999**, *20*, 51–59. [CrossRef]
66. Williams, L.E.; Trout, T.J. Relationships among vine-and soil-based measures of water status in a Thompson Seedless vineyard in response to high-frequency drip irrigation. *Am. J. Enol. Vitic.* **2005**, *56*, 357–366.
67. Cai, G.; Ahmed, M.A.; Dippold, M.A.; Zarebanadkouki, M.; Carminati, A. Linear relation between leaf xylem water potential and transpiration in pearl millet during soil drying. *Plant Soil* **2020**, *447*, 565–578. [CrossRef]
68. Knipfer, T.; Bambach, N.; Hernandez, M.I.; Bartlett, M.K.; Sinclair, G.; Duong, F.; Kluepfel, D.A.; McElrone, A.J. Predicting stomatal closure and turgor loss in woody plants using predawn and midday water potential. *Plant Physiol.* **2020**, *184*, 881–894. [CrossRef] [PubMed]
69. Brodribb, T.J.; Holbrook, N.M. Stomatal Closure during Leaf Dehydration, Correlation with Other Leaf Physiological Traits. *Plant Physiol.* **2003**, *132*, 2166–2173. [CrossRef] [PubMed]
70. Meinzer, F.C.; Johnson, D.M.; Lachenbruch, B.; McCulloh, K.A.; Woodruff, D.R. Xylem hydraulic safety margins in woody plants: Coordination of stomatal control of xylem tension with hydraulic capacitance. *Funct. Ecol.* **2009**, *23*, 922–930. [CrossRef]
71. Rhizopoulou, S.; Heberlein, K.; Kassianou, A. Field water relations of *Capparis spinosa* L. *J. Arid Environ.* **1997**, *36*, 237–248. [CrossRef]
72. Meletiou-Christou, M.-S.; Rhizopoulou, S. Constraints of photosynthetic performance and water status of four evergreen species co-occurring under field conditions. *Bot. Stud.* **2012**, *53*, 325–334.
73. Meletiou-Christou, M.-S.; Rhizopoulou, S. Leaf functional traits of four evergreen species growing in Mediterranean environmental conditions. *Acta Physiol. Plant.* **2017**, *39*, 34. [CrossRef]
74. Kramer, P.J.; Boyer, J.S. *Water Relations of Plants and Soils*; Academic Press: San Diego, CA, USA, 1995.

75. Rhizopoulou, S.; Kapolas, G. In situ study of deep roots of Capparis spinosa L. during the dry season: Evidence from a natural "rhizotron" in the ancient catacombs of Milos Island (Greece). *J. Arid Environ.* **2015**, *119*, 27–30. [CrossRef]
76. Kosugi, Y.; Matsuo, N. Seasonal fluctuations and temperature dependence of leaf gas exchange parameters of co-occurring evergreen and deciduous trees in a temperate broad-leaved forest. *Tree Physiol.* **2006**, *26*, 1173–1184. [CrossRef]
77. Auge, R.M.; Green, C.D.; Stodola, A.J.; Saxton, A.M.; Olinick, J.B.; Evans, R.M. Correlations of stomatal conductance with hydraulic and chemical factors in several deciduous tree species in a natural habitat. *New Phytol.* **2000**, *145*, 483–500. [CrossRef]
78. Medrano, H.; Flexas, J.; Galmes, J. Variability in water use efficiency at the leaf level among Mediterranean plants with different growth forms. *Plant Soil* **2009**, *317*, 17–29. [CrossRef]
79. Koukos, D.; Meletiou-Christou, M.-S.; Rhizopoulou, S. Leaf surface wettability and fatty acid composition of Arbutus unedo and Arbutus andrachne grown under ambient conditions in a natural macchia. *Acta Bot. Gall.* **2015**, *162*, 225–232. [CrossRef]
80. Christodoulakis, N.S.; Lampri, P.-N.; Fasseas, C. Structural and cytochemical investigation of the leaf of silverleaf nightshade (Solanum elaeagnifolium), a drought-resistant alien weed of the Greek flora. *Aust. J. Bot.* **2009**, *57*, 432–438. [CrossRef]
81. Río, S.D.; Álvarez Nogal, R.; Candelas, A.; González-Sierra, S.; Herrero, L.; Penas, A. Preliminary study on taxonomic review using histological sections of some Iberian species from the genus Quercus L. (Fagaceae). *Am. J. Plant Sci.* **2014**, *5*, 2773–2784. [CrossRef]
82. Duff, G.A.; Myers, B.A.; Williams, R.J.; Eamus, D.; OGrady, A.; Fordyce, I.R. Seasonal patterns in soil moisture, vapour pressure deficit, tree canopy cover and pre-dawn water potential in a northern Australian savanna. *Aust. J. Bot.* **1997**, *45*, 211–224. [CrossRef]
83. Prior, L.; Eamus, D.; Duff, G. Seasonal and diurnal patterns of carbon assimilation, stomatal conductance and leaf water potential in Eucalyptus tetrodonta saplings in a wet–dry savanna in northern Australia. *Aust. J. Bot.* **1997**, *45*, 241–258. [CrossRef]
84. Devi, M.J.; Reddy, V.R. Transpiration response of cotton to vapor pressure deficit and its relationship with stomatal traits. *Front. Plant Sci.* **2018**, *9*, 1572. [CrossRef]
85. Ben-Asher, J.; y Garcia, A.G.; Flitcroft, I.; Hoogenboom, G. Effect of atmospheric water vapor on photosynthesis, transpiration and canopy conductance: A case study in corn. *Plant Soil Environ.* **2013**, *59*, 549–555. [CrossRef]
86. Shibuya, T.; Kano, K.; Endo, R.; Kitaya, Y. Photosynthetic properties and response to drought in cucumber seedlings acclimatized to different vapor-pressure-deficit levels. *Hort. J.* **2017**, *86*, 334–339. [CrossRef]
87. Larcher, W. *Physiological Plant Ecology: Ecophysiology and Stress Physiology of Functional Groups*; Springer Science & Business Media: Berlin/Heidelberg, Germany, 2003.
88. Manzoni, S.; Vico, G.; Katul, G.; Palmroth, S.; Jackson, R.B.; Porporato, A. Hydraulic limits on maximum plant transpiration and the emergence of the safety–efficiency trade-off. *New Phytol.* **2013**, *198*, 169–178. [CrossRef]
89. Hopkins, W.G. *Introduction to Plant Physiology*, 4th ed.; John Wiley & Sons: Devon, UK, 2009.
90. Bucci, S.J.; Goldstein, G.; Meinzer, F.C.; Scholz, F.G.; Franco, A.C.; Bustamante, M. Functional convergence in hydraulic architecture and water relations of tropical savanna trees: From leaf to whole plant. *Tree Physiol.* **2004**, *24*, 891–899. [CrossRef]
91. Markesteijn, L.; Poorter, L.; Bongers, F.; Paz, H.; Sack, L. Hydraulics and life history of tropical dry forest tree species: Coordination of species' drought and shade tolerance. *New Phytol.* **2011**, *191*, 480–495. [CrossRef] [PubMed]
92. Choat, B.; Ball, M.C.; Luly, J.G.; Holtum, J.A. Hydraulic architecture of deciduous and evergreen dry rainforest tree species from north-eastern Australia. *Trees* **2005**, *19*, 305–311. [CrossRef]
93. Blumler, M.A. *Winter-Deciduous Versus Evergreen Habit in Mediterranean Regions: A Model*; Gen. Tech. Report PSW-126; USDA Forest Service: Berkeley, CA, USA, 1991; pp. 194–197.
94. Holbrook, N.M.; Whitebeck, J.; Mooney, H. Drought responses of neotropical dry forests. In *Seasonally Dry Tropical Forests*; Bullock, S.H., Mooney, H.A., Medina, E., Eds.; Cambridge University Press: Cambridge, UK, 1995; pp. 243–276.
95. Borchert, R. Soil and stem water storage determine phenology and distribution of tropical dry forest trees. *Ecology* **1994**, *75*, 1437–1449. [CrossRef]
96. Filippidis, E.; Mitsopoulos, I. Mapping forest fire risk zones based on historical fire data in Mount Olympus, Greece, using geographical information systems. *WIT Trans. Ecol. Environ.* **2004**, *77*, 13. [CrossRef]
97. Klesse, S.; Ziehmer, M.; Rousakis, G.; Trouet, V.; Frank, D. Synoptic drivers of 400 years of summer temperature and precipitation variability on Mt. Olympus, Greece. *Clim. Dyn.* **2015**, *45*, 807–824. [CrossRef]
98. Strid, A. The botanical exploration of Greece. *Plant Syst. Evol.* **2020**, *306*, 27. [CrossRef]
99. Donovan, L.; Grise, D.; West, J.; Pappert, R.; Alder, N.; Richards, J. Predawn disequilibrium between plant and soil water potentials in two cold-desert shrubs. *Oecologia* **1999**, *120*, 209–217. [CrossRef]
100. Kavanagh, K.; Bond, B.; Aitken, S.; Gartner, B.; Knowe, S. Shoot and root vulnerability to xylem cavitation in four populations of Douglas-fir seedlings. *Tree Physiol.* **1999**, *19*, 31–37. [CrossRef]

plants

MDPI

Article

Impact of Grazing on Diversity of Semi-Arid Rangelands in Crete Island in the Context of Climatic Change

Maria Karatassiou [1,*], Zoi M. Parissi [2], Sampson Panajiotidis [3] and Afroditi Stergiou [1]

[1] Laboratory of Rangeland Ecology, School of Forestry and Natural Environment, Aristotle University of Thessaloniki, P.O. Box 286, 54124 Thessaloniki, Greece; afroster@for.auth.gr
[2] Laboratory of Range Science, School of Forestry and Natural Environment, Aristotle University of Thessaloniki, P.O. Box 236, 54124 Thessaloniki, Greece; pz@for.auth.gr
[3] Laboratory of Forest Botany—Geobotany, School of Forestry and Natural Environment, Aristotle University of Thessaloniki, P.O. Box 270, 54124 Thessaloniki, Greece; pansamp@for.auth.gr
* Correspondence: karatass@for.auth.gr; Tel.: +30-2310992302

Abstract: The rangelands of Crete island (Greece) are typical Mediterranean habitats under high risk of degradation due to long-term grazing and harsh climatic conditions. We explored the effect of abiotic (climatic conditions, altitude) and biotic factors (long-term grazing by small ruminants) on the floristic composition and diversity of selected lowland (Pyrathi, Faistos) and highland (Vroulidia, Nida) rangelands. In each rangeland, the ground cover was measured, and the floristic composition was calculated in terms of five functional groups: grasses, legumes, forbs, phrygana, and shrubs. The aridity index, species turnover, species richness, Shannon entropy, and Gini–Simpson index (with the latter two converted to the effective number of species) were calculated. Our results reveal that highlands are characterized by the highest aridity index (wetter conditions). Lowland rangelands, compared to highland, exhibited a higher percentage contribution of grasses, legumes, and forbs, while species turnover decreased along the altitudinal gradient. The Shannon entropy index was correlated (a) positively with Gini–Simpson and mean annual temperature and (b) negatively with mean annual precipitation, aridity index, and altitude. Moreover, the Gini–Simpson index correlated positively with mean annual temperature and negatively with altitude. Our results could help to understand the effects of grazing on rangeland dynamics and sustainability in semi-arid regions in the context of climatic change.

Keywords: aridity index; effective number of species; Shannon entropy; richness; Gini–Simpson

Citation: Karatassiou, M.; Parissi, Z.M.; Panajiotidis, S.; Stergiou, A. Impact of Grazing on Diversity of Semi-Arid Rangelands in Crete Island in the Context of Climatic Change. *Plants* **2022**, *11*, 982. https://doi.org/10.3390/plants11070982

Academic Editor: Giuseppe Fenu

Received: 29 December 2021
Accepted: 1 April 2022
Published: 4 April 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Arid and semi-arid rangelands occupy approximately 40% of the Earth's land surface and influence the livelihood and well-being of one-fifth of the world's human population [1,2]. More than one billion people rely on rangelands for their living, primarily through extensive livestock production, and roughly two billion acquire animal protein, water, or other resources from these biomes [3,4]. Rangelands comprise many habitats and host economically important species offering support to approximately 50% of the world's livestock, providing forage production for both domestic and wildlife populations [5–7].

Despite their high importance, most of the non-marketed services of these rangelands and their economic value have often been neglected [8,9]. Moreover, they have faced increased risks resulting from overutilization and degradation [10–12]. The estimated extent of rangeland degradation varies extensively, from as little as 10–20% to as much as 70–80% [3]. Desertification is a cumulative threat that includes both climatic and land-use drivers that interact in space and time [13].

It is well demonstrated that rangelands are maintained by grazing. However, they can be severely affected by the high intensity of the latter, climate change, soil quality, nutrient depletion, fire, habitat fragmentation, as well as human activities [4,7,14]. In

most rangelands, precipitation [15] and grazing [16,17] are the most important factors determining species diversity and ecosystem function [18,19]. On the other hand, altitude, which greatly affects the abiotic environment by modifying climatic variables and the topography [20,21], is an indirect gradient that is correlated with resources and regulators of plant growth [22,23] and species composition [24].

Although it is known that the richness of vascular plant species decreases with an increase in altitude [25–27], the patterns of response are rather fickle [28]. Changes in plant species richness along altitudinal transects are of great importance in the study of global climate change [29,30]. The spatial change in species composition involves the study of beta diversity and species turnover. Because of the greater diversity of habitat conditions, mountains have higher levels of species turnover than lowland areas [31], and under a climate change scenario, mountains are considered significant for the maintenance of biodiversity [32–34]. The relationship between climatic conditions and species turnover is described by the relationship between climatic factors and regional species richness [35].

To assess the impact of grazing on vegetation, the effect of precipitation on species diversity should be thoroughly studied and considered [36]. Both low ground cover and plant diversity increase the vulnerability of rangelands to climate change [37,38]. Overgrazing, which is prescribed as a decrease in productivity [39] and loss of biodiversity [40,41], is considered one of the main causes of land degradation in arid and semi-arid regions worldwide [42]. Heavy grazing directly changes the floristic composition of plant communities selectively, changing the structure and composition of communities at the expense of palatable species [43,44], and may also indirectly modify the outcome of competitive interaction by changing light availability [45]. The impact of grazing intensity on plant diversity varies along the precipitation gradient [46,47].

There is a list of methods employed to study diversity, which is a multi-dimensional phenomenon [48]. The simplest measure of diversity is to calculate the number of species (richness) in an area, which, however, does not take into consideration species abundances and is sensitive to sample size. Other approaches consider species abundance (Shannon index) or give weight to dominant species (e.g., Gini–Simpson). The Shannon and Gini–Simpson measures of diversity are themselves mere indices and not "true" diversities [49–53]. The true diversity of an investigated community is simply the community of equally common species (effective number of species, ENS) required to give the same value of an index calculated for the community in question [52,54,55]. In recent years, the use of ENS has been established in ecological studies. After the conversion of classical indices (Shannon and Simpson) to ENS, diversity is always measured in the number of species, providing more interpretable and comparable assessments of diversity [54,56,57].

Thirty-five percent of the Greek land, and more specifically 37–50% of the land in Crete, is characterized as critically susceptible to desertification due to the combination of a warming climate with low precipitation and intensified human activities [58,59]. To the best of our knowledge, the effect of grazing and climatic conditions on grassland biodiversity has not yet been studied in Crete, a vulnerable Mediterranean region.

The current study aimed to investigate the effect of abiotic (climatic conditions, altitude) and biotic factors (long-term grazing) on the floristic composition and diversity of lowland and highland rangelands on the island of Crete, Greece, which are typical Mediterranean habitats at high risk of degradation. We aimed to answer the following questions:

(a) Do patterns of species diversity indices and composition differ among rangelands exposed to different grazing intensities?
(b) Do these differences vary among rangelands with different altitudes and climatic conditions?

2. Results and Discussion

The current study indicates that the existing high grazing pressure, in combination with climatic conditions, could result in rangeland degradation on Crete island.

Diverse climatic conditions prevail among the four studied rangelands (Figure 1). At Faistos and Pyrathi, the mean annual temperature was 19.17 ± 1.24 and 17.38 ± 1.18 °C, and the mean monthly precipitation was 45.77 ± 11.05 and 60.67 ± 15.1 mm, respectively. At Vroulidia and Nida, an inverse trend was observed as the mean annual temperature was 12.67 ± 1.98 °C with an average monthly precipitation of 107.05 ± 23.93 mm. Pyrathi had higher rates of precipitation and mean monthly temperature compared to Faistos (Figure 1b,c). The climatic data indicated a shorter drought period in Vroulidia and Nida, which implied that plant species faced a water deficit for a shorter period in these areas.

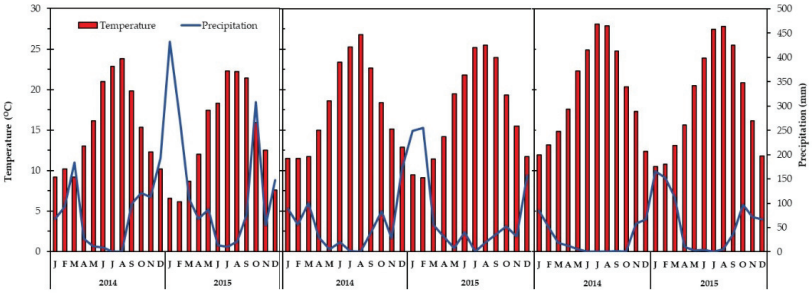


Figure 1. Monthly means of air temperature (°C) and precipitation (mm) at (**a**) Vroulidia—Nida, (**b**) Pyrathi, and (**c**) Faistos rangelands during the experimental period.

The aridity index (I_{dM}) classifies the type of climate in relation to water availability, and it is a crucial environmental factor affecting the growth of natural vegetation. In the present study, I_{dM} was negatively correlated with mean air temperature and positively with altitude and precipitation (Table 1). The Nida rangeland scored the highest aridity index, followed by Vroulidia, while Faistos had the lowest one (Figure 2). The higher values of I_{dM} in the highlands indicated higher humidity [60] and better climatic conditions for plant growth and development. Mallen-Cooper and coauthors [61] found similar results in eastern Australia, which support that aridity decreases when the height of precipitation and absorptivity of water increase. The higher I_{dM} correlated with the higher available water resources over time and, consequentlym lower vulnerability to desertification.

Table 1. Pearson correlation between richness (R), effective Shannon entropy (SE) and Gini–Simpson (GS), Martonne aridity index (I_{dM}), mean annual temperature (T), mean precipitation (P), and altitude for the four studied rangelands.

	R	SE	GS	I_{dM}	T	P	Altitude
R	1						
SE	−0.073	1					
GS	−0.280	0.861 **	1				
I_{dM}	0.059	−0.876 **	−0.678	1			
T	−0.295	0.894 **	0.795 *	−0.954 **	1		
P	−0.174	−0.766 *	−0.498	0.947 **	−0.809 *	1	
Altitude	0.218	−0.888 **	−0.763 *	0.980 **	−0.986 **	0.882 **	1

* Significant for $p < 0.05$, ** Significant for $p < 0.001$.

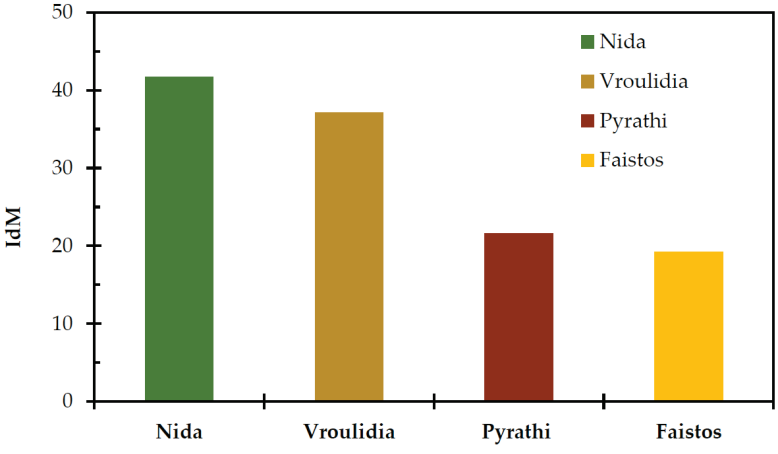


Figure 2. Martonne's aridity index (I_{dM}) for the studied rangelands for the period 1979–2013.

The aridity level interacts with plant traits related to stress resistance to determine the floristic composition and vegetation responses to domestic animal grazing [62–66]. The impact of grazing on species richness and composition under high-aridity conditions could be either high [67] or low [65].

Generalized Linear Model analysis showed significant differences ($p < 0.001$) in forage production in both fenced plots and grazed sites and for FUP (Table 2) among the studied rangelands. Additionally, there was a significant interaction between rangeland and year ($p < 0.001$), but only for forage production in grazed sites. The forage production in fenced plots ranged from 189.2 ± 24.2 to 116.5 ± 7.3 g m^{-2}, while in grazed from 128.9 ± 4.8 to 15.8 ± 1.4 g m^{-2}. The lowland rangelands had higher forage production in relation to the highland. This is in agreement with Bhandari and Zhang [68], who demonstrated that altitude is negatively related to aboveground biomass. Concerning the year, it was a significant predictor ($p < 0.001$) only for FUP. The FUP was 84.4–87.1% at Nida, 74.1–76.1% at Vroulidia, 74–75% at Pyrathi, and 15–17% at Faistos for 2014 and 2015, respectively (Table 2). The highest value of FUP was presented in Nida and the lowest in Faistos. In many cases, the high FUP is related to low vegetation percentage cover, a result of overgrazing [69].

Table 2. Forage production (g m^{-2}) in fenced plots and grazed sites and forage utilization percentage (FUP %) in the four studied rangelands in the study years. Values represent means ± SE (n = 9). Different letters in the same column indicated significant differences ($p < 0.05$).

	Forage Production (g m^{-2})				FUP (%)	
	Fenced Plots		Grazed Sites			
Rangeland (R)	**2014**	**2015**	**2014**	**2015**	**2014**	**2015**
Nida	122.2 ± 6.03 b	120.2 ± 5.5 bc	15.8 ± 1.4 d	18.8 ± 1.1 d	87.1 ± 0.9 a	84.4 ± 0.5 a
Vroulidia	126.7 ± 9.5 b	116.5 ± 7.3 c	29.9 ± 1.7 c	30.2 ± 2.2 c	76.1 ± 0.8 b	74.1 ± 1.0 b
Pyrathi	189.2 ± 24.2 a	173.6 ± 22.2 a	45.7 ± 6.5 b	42.2 ± 4.9 b	76 ± 0.7 b	75.1± 1.8 b
Faistos	134.4 ± 4.0 b	151.4 ± 5.3 b	111.5 ± 3.3 a	128.9 ± 4.8 a	17.00 ± 0.8 c	14.9 ± 0.8 c
R	<0.001 **		<0.001 **		<0.001 **	
Year	0.748 ns		0.084 ns		0.003 *	
R X Year	0.567 ns		0.017 *		0.761 ns	

* Significant for $p < 0.05$, ** Significant for $p < 0.001$, ns not significant.

It is known that the cover of vegetation is a health indicator of the rangelands. Our data analysis revealed significant differences ($p < 0.05$) in vegetation cover among the studied rangelands. The vegetation cover recorded in Nida and Vroulidia scored the lowest values, 52% to 66% and 64% to 70%, for 2014 and 2015, respectively, in comparison to the lowland sites of Pyrathi (78–90%) and Faistos (92–99%). The low vegetation cover and the high FUP at Nida and Vroulidia are likely the results of overgrazing, as the number of transhumant small ruminants, and, consequently, the grazing pressure is immoderate in the Psiloritis mountain [70]. Papanastasis and coauthors [71] point out that the Psiloritis mountain is overgrazed as the stocking rate is four times higher than the grazing capacity. Ojima and coauthors [72] found that overgrazing results in the loss of vegetation cover and increased erosion. The low vegetation cover provides low protection from soil erosion and a high risk for degradation [13] and reduced soil porosity [46,73]. This reduced vegetation cover and the lower plant diversity probably increase the susceptibility of rangelands to the effects of climate change as well [37,38,46].

Data analysis revealed that in all functional groups, there were no significant differences between years and no significant interaction between rangeland and year ($p \geq 0.05$). On the contrary, there was a significant interaction between rangeland and functional groups ($p < 0.001$) (Table 3, Figure 3). Overall, lowlands, compared to highlands, present a significantly ($p < 0.05$) higher percentage contribution of grasses, legumes, and forbs (Figure 3). On the other hand, shrubs had a significantly ($p < 0.05$) higher percentage in the highlands compared to the lowlands, while phrygana [74,75] had similar participation in both lowlands and highlands. Concerning the contribution of functional groups separately in highland and lowland, shrubs were significantly ($p < 0.05$) higher at Nida compared to Vroulidia, while the opposite trend was detected for forbs in highlands for both experimental years (Figure 3). As others point out as well, the Cretan landscape, especially in high elevations, is a mixture of woodland and open vegetation, where many woody species are found in various forms (from trees to small or dwarf shrubs) [76–78]. Many of these shrubby taxa are shaped by grazing and are adapted to this pressure, which includes prescribed fires. Moreover, woody plants are able to 'colonize' rocky places where soil can be scarce. Moreover, Papanastasis and coauthors [77] found that woody species on the Philoritis mountain cover 30% of the soil. Regarding the lowlands, there is significantly higher participation of grasses ($p < 0.05$) in the Faistos rangeland compared to Pyrathi (Figure 3). On the contrary, the participation of phrygana was higher at Pyrathi compared to Faistos.

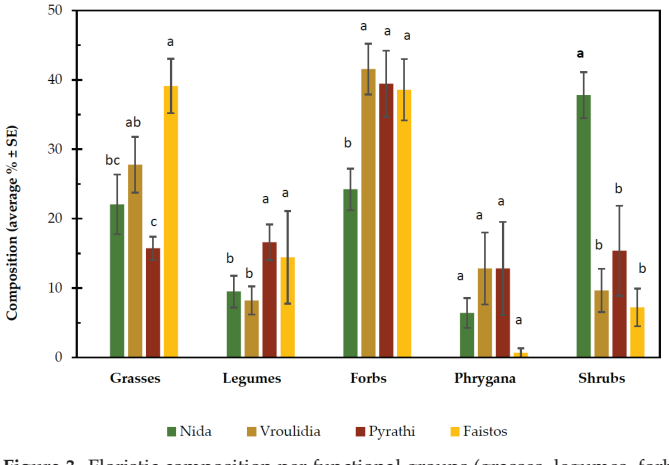


Figure 3. Floristic composition per functional groups (grasses, legumes, forbs, phrygana, shrubs) (%) at the four studied rangelands for the experimental period. Values represent means ± SE (n = 6). Different letters in columns indicate significant differences for the same parameter ($p < 0.001$).

Table 3. General linear model analysis for the effects of rangeland and year on participation of functional plants groups.

	Wald Chi-Square	df	*p*-Value
Year	0.033	1	$p \geq 0.05$ ns
Rangeland	0.045	3	$p \geq 0.05$ ns
Functional group	116.816	4	<0.001 *
Year * Rangeland	0.014	3	$p \geq 0.05$ ns
Rangeland * Functional group	80.120	12	<0.001 *

* Significant for $p < 0.001$, ns not significant.

On Crete island, as elsewhere in Greece, farmers traditionally improve grassland productivity [79] and quality through fire management of vegetation, mainly phrygana, which enables the modification of the floristic composition. The fires decrease the percentage of shrubs and phrygana and drive the ecosystem to a previous successional stage (secondary succession), where the percentage of grasses and legumes is higher, leading to higher herbage biomass production in terms of quantity and quality [80,81]. The floristic composition of the studied rangelands is strongly linked to habitat characteristics (abiotic factors: altitude, climatic conditions) and primary consumers (biotic factors) [82].

A drop in species turnover is observed between pairs of lowland to highland rangelands (Figure 4). Species turnover presented the highest value at an intermediate altitude from 355 to 1100 m a.s.l.; and decreased at higher altitudes, from 1100 to 1530 m a.s.l., due to the range of ecological adaptation and growth of plants at different altitudes. The same results were found by Mena and Vázquez-Domínguez [83] when the species turnover in mammals was studied, more specifically, small rodents, along an altitudinal gradient. Our results support the hypothesis that species turnover decreases with altitude only at the higher altitudinal zone. The lower species turnover in highlands could be attributed to the presence of sparse vegetation in mountainous areas generally [79], and probably, species turnover correlates with different rangeland management [84].

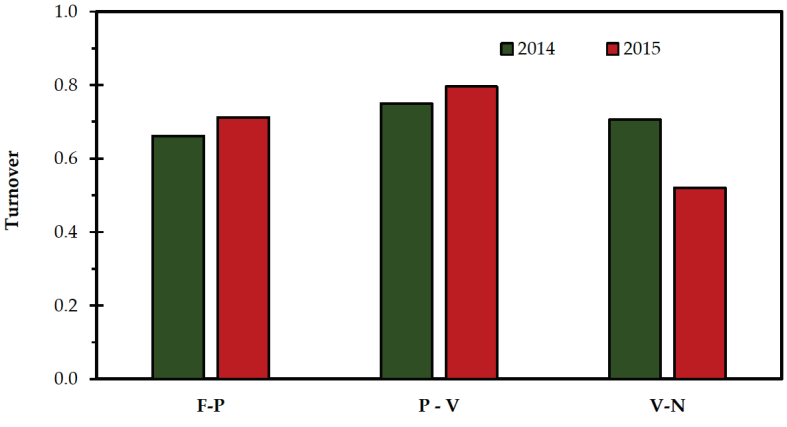


Figure 4. Species turnover in the studied rangelands Faistos—Pyrathi (F-P), Pyrathi—Vroulidia (P-V) and Vroulidia—Nida (V-N) for the experimental period.

Diversity in terms of abundance (ENS Shannon entropy) was lower than species richness, while diversity in terms of dominance (ENS Gini–Simpson index) was lower than the Shannon entropy for both years of the study (Figure 5). This result indicated that there is species dominance in all study areas. The greater the dominance in the community, the greater the differences among these three parameters [51,52]. In both years of the study,

the species richness, Shannon entropy, and Gini Simpson were higher in the lowlands of Faistos and Pyrathi compared to Vroulidia, while at Nida, the highest species richness was recorded. It is noteworthy that there are more plant species at Pyrathi than at Faistos, and the same trend for diversity (Figure 5). This was contrary to the theory that species richness and diversity decreased with an increase in altitude. The species richness may not be related to altitude, as it is demonstrated by Zawierucha and coauthors [85]. This unexpected result is probably due to the fire set by shepherds to improve forage production at Pyrathi last year and led to a change in the floristic composition. It has been proven that fire has important effects on diversity and plant community composition [86–90]. Shannon entropy could be used in situations where rare and abundant species or traits are expected to be equally important [91]. However, if dominant species or traits are expected to be more essential, then Gini–Simpson would be more relevant. Both indices were smaller than richness for all rangelands (Figure 5), as they were based more on abundant and dominant species, respectively [82]. Although the environmental conditions favored plant growth in mountainous areas, the grazing pressure significantly decreased species diversity. Nida had the highest species richness in relation to the other studied rangelands, but its species abundance (Shannon entropy) and dominance presented the lowest value. These results could be attributed to high FUP (overgrazing), lower vegetation cover, soil erosion, and unpalatable plant species encroachment that will be exacerbated by climate change [92].

Heavy grazing (high FUP) may result in high-level species replacement [93]. Plants at Pyrathi and Vroulidia are grown under different climatic conditions but under similar FUP, presented different species richness but similar diversity in terms of species abundance and dominance. At Faistos, under light grazing pressure, the rangeland presented similar high ratios of abundant and dominant species to total species recorded (richness). These results could be verified from the ratios of Shannon entropy/richness and Gini Simpson index/richness (Table 4). The highest ratio was recorded at Faistos and the lowest one at Nida. Faistos, with the longer semi-arid period under low FUP, presented a very diverse vegetation pattern, with three-quarters of all species showing the same abundance, while more than half were also dominant (Table 3). As grazing intensity escalates from Pyrathi to Nida, ratios of abundance diversity/richness and dominance diversity/richness decrease; this is more evident in terms of absolute ENS values of abundance and dominance diversity (Figure 5). Vroulidia shows higher ratios than Pyrathi but similar absolute ENS values for dominance diversity and lower for dominance diversity. These rangelands, without these disturbances (climate, grazing), would gradually decline due to the successional process to the next successional stages [94,95]. Animal grazing is a key factor in avoiding the successional processes of vegetation [82].

Table 4. Ratios of Shannon entropy/richness (HE/R) and Gini–Simpson (GS/R) index/richness for all of the studied rangelands for the experimental period.

	Lowland Rangelands				**Highland Rangelands**			
	Faistos		**Pyrathi**		**Vroulidia**		**Nida**	
	2014	**2015**	**2014**	**2015**	**2014**	**2015**	**2014**	**2015**
* HE/R	0.74	0.75	0.50	0.58	0.66	0.59	0.43	0.31
* GS/R	0.60	0.55	0.29	0.41	0.49	0.46	0.23	0.23

* Gini–Simpson and Shannon entropy are given as effective number of species (ENS).

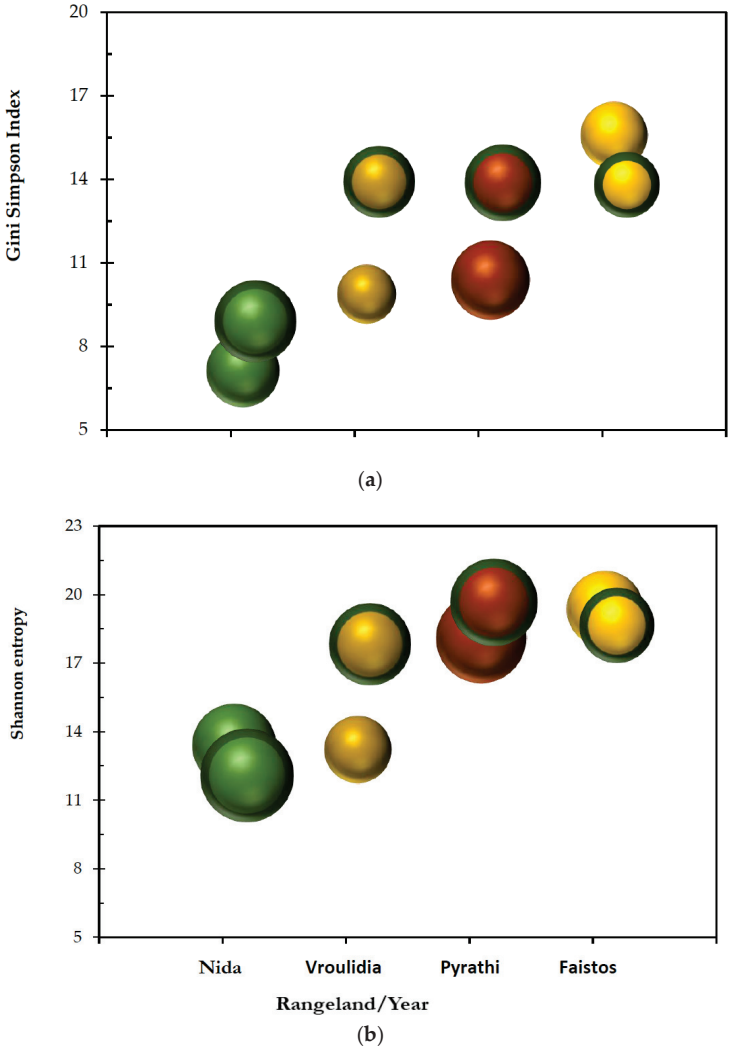


Figure 5. (**a**) Gini–Simpson vs. richness index and (**b**) Shannon entropy vs. richness index for all studied rangelands for the experimental period, 2014 and 2015 (symbols encircled). Cycle size is proportional to richness. Gini–Simpson and Shannon entropy are given as effective numbers of species (ENS).

According to the Pearson correlation coefficient, the Shannon entropy index was positively correlated with the Gini–Simpson and mean annual temperature and negatively with mean annual precipitation and altitude, while the Gini–Simpson index correlated negatively with altitude (Table 1). According to the results, the species diversity decreased with an increase in altitude and precipitation. This is in agreement and supports the theory that species richness and diversity decrease along the altitude gradient [27,96,97]. Altitude probably has the strongest effects on species richness, abundance, and ground cover [98]. Nevertheless, other studies found that overgrazing affects functional diversity more than climate, and species diversity declines with an increase in grazing intensity in areas with

different climatic conditions [41,46,99]. It is well known that the relationships between diversity indices do not always follow mathematically predicted patterns [100,101].

3. Materials and Methods

3.1. Study Area

The research was conducted during 2014–2015 on the island of Crete, the southernmost part of Greece. The selected experimental sites were two lowland rangelands of Heraklion prefecture: Faistos (F) (24°51′20″ E, 35°06′30″ N) 155 m a.s.l. and Pyrathi (P) (25°11′21″ E, 35°05′52″ N) 355 m a.s.l., and two in the highlands of Psiloritis mountain (Rethymnon prefecture): Vroulidia (V) (24°47′02″ E, 35°10′58″ N) 1100 m a.s.l. and Nida (N) (24°50′33″ E, 35°12′48″ N) 1530 m a.s.l. that have been subjected to grazing (Figure 6).

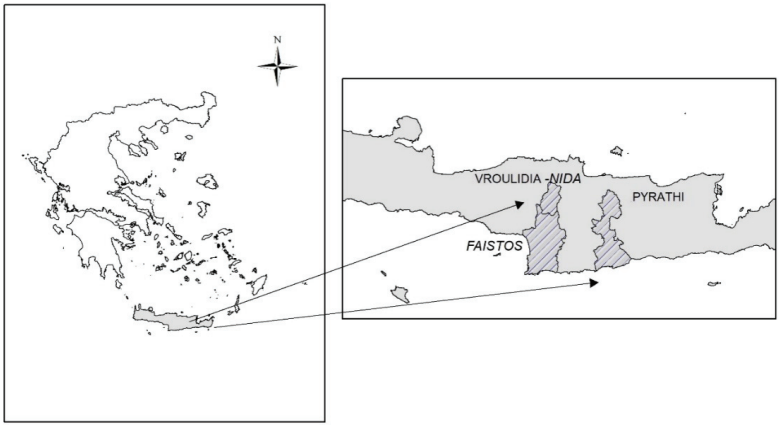


Figure 6. The experimental rangelands on the island of Crete, Greece.

The livestock farming system was introduced on the island about 8000 years ago, and animal husbandry has been used by humans to transform natural ecosystems to produce more grazing material and, therefore, more animal products for their own consumption and survival. Through these processes, the extensive forests of the island were turned into rangelands, while the abandoned fields due to grazing could not be reforested. Uncontrolled and random, both spatially and temporally, grazing is the rule on the island. In lowlands, e.g., Faistos, in recent years, a change in land-use has been observed with farming replacing pastoralism, so there is low grazing intensity in the area. On the other hand, the lowlands of Pyrathi are heavily grazed all year round by sheep and goat flocks. Concerningly, the highlands of Vroulidia are grazed all year by sheep and goats, while Nida, from April to October, by transhumant small ruminant flocks. The highlands are characterized by a long history of small ruminant overgrazing [71].

The climate of the lowland and highland rangelands is characterized as Csa and Csb, respectively, in the Köppen–Geiger system (www.en.climate-data.org, 12 December 2021). The daily climatic data (precipitation, average temperature) for the two lowland rangelands (P, F) (Figure 1b,c) were obtained from the nearest meteorological stations, while for the highlands (N, V) (Figure 1a) from the only one available meteorological station located between them, and are reported as mean monthly data for the period in which the study was conducted.

3.2. Field Data

The vegetation (ground) cover was measured at the end of the growing season according to the line and point method [102]. Three experimental transects (25 m each) [103,104] were established in each rangeland, as the habitats were homogeneous. After that, the floristic composition was calculated and presented in five functional plant groups: (1) grasses,

(2) legumes, (3) forbs, (4) phrygana, and (5) shrubs, according to their life form and by distinguishing legumes from forbs based on their nutritional value for small ruminants (Table S1). Moreover, two sampling quadrats of 0.35 x 0.35 m were established in every transection of each rangeland at 8 and 16 m in order to calculate: (a) species richness (equivalent to its own numbers) and (b) species diversity indices (Shannon entropy and Gini–Simpson), which were converted to the effective number of species (ENS). Shannon entropy was calculated following the formula in Equation (1) below

$$H = -\sum_{i=1}^{S} p_i ln p_i \quad (1)$$

and was converted to ENS by taking its exponential exp(H) (exponential of Shannon entropy index), where p_i is the population frequency of the ith species. The Gini–Simpson index (H_{GS}) was converted by the transformation

$$1/(1-H_{GS}),$$

which is the inverse of the index [51,52,57,105]. These measures easily pass interpretable counts and provide information at three different levels based on how rare and abundant taxa are weighted [53,105–107].

$$1/\left(\sum_{i=1}^{s} pi^2\right)$$

For every studied rangeland, the aridity index (de Martonne index, I_{dM}) was calculated following the formula in Equation (2) below [60]:

$$I_{dM} = P/(T + 10) \quad (2)$$

where P is the mean annual precipitation (mm), and T (°C) is the mean annual air temperature. The values of T and P for every rangeland were downloaded from Climatologies, at high resolution (30 × 30 s), for the Earth's land surface areas (CHELSA, http://chelsa-climate.org/, 2 February 2022), which is a global climate database covering the period 1979 to 2013.

The species turnover was calculated as the gain and loss of species between altitudes following the formula in Equation (3) below [108]:

$$\beta(H) = (g(H) + l(H))/(\alpha(H) + \alpha(H\text{-}1)) \quad (3)$$

where g(H) and l(H) are the number of species gained and lost, respectively, from altitude H-1 to altitude H, while α(H) and α(H-1) is the species richness at altitude H and H-1, respectively [108].

In order to estimate the forage utilization percentage (FUP) in the spring of 2012 in each of the four rangeland's three plots, 9 m^2 were fenced to be protected from grazing. The above-ground herbage production was collected by clipping three 0.5 × 0.5 m quadrats in each fenced plot (i.e., nine quadrats per fenced plot). In the same period into grazed rangelands (sites), the remaining above-ground biomass after grazing was collected by clipping in three similar quadrats in each transect (i.e., nine quadrats per rangeland), in May 2014–2015. Consequently, grazing intensity was expressed by FUP. The difference among herbage yields of fenced (UG) and grazed sites (G) was used to calculate FUP from the formula of Equation (4) below [109]:

$$FUP = [(UG\text{-}G)/UG] \times 100 \quad (4)$$

3.3. Statistical Analysis

The Generalized Linear Model (GLM), assuming a normal distribution, was used to assess whether the altitude of each rangeland, functional group, and year were significant predictors of ground cover and floristic composition. Before analysis, the data were converted to ln + 1 to meet assumptions of normality (tested with the Kolmogorov–Smirnov

test) and homogeneity of variances (Levene's test). Estimated marginal means for all the above factors were calculated with pairwise contrasts, and LSD adjustment was applied for the multiple comparisons ($\alpha = 0.05$). The data in the figures and tables depict values before the transformation. Pearson correlation was used to explore links among R, SE, GS, I_{dM}, T, P, and altitude. All statistical analyses were performed using the SPSS statistical package v. 27.0 (IBM Corp. in Armonk, NY). The Paleontological statistics software package for education and data analysis (Past) was used to calculate the diversity indices.

4. Conclusions

The Mediterranean basin includes a wide range of vegetation, climatic, and edaphic conditions that have been shaped by natural selection under the pressure of a distinct climate and human activities. Our results demonstrate the strong relationship between diversity and temperature and agree with the fact that vegetation diversification is strongly related to the climatic gradient and is more related to temperature than precipitation. Moreover, this research could help to understand how grazing intensity and climatic conditions interactively influence rangelands dynamics in semi-arid regions and monitor the livestock management and decision making in these areas.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/plants11070982/s1, Table S1: Plant species in floristic composition at the four studied rangelands (life form: Grass (G), Legume (L), Forb (F), Phrygana (PH), Shrub (S).

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

Funding: The authors gratefully acknowledge the financial support of the European Union through the Action "THALIS" of the Programme "Education and Life-long learning".

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

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 conflict of interest.

References

1. White, R.P.; Murray, S.; Rohweder, M. *Pilot Analyses of Global Ecosystems: Grassland Ecosystems*; Edeburn, M., Ed.; World Resources Institute: Washington, DC, USA, 2000.
2. Reynolds, J.F.; Smith, D.M.; Lambin, E.F.; Turner, B.L., II; Mortimore, M.; Batterbury, S.P.; Downing, T.E.; Dowlatabadi, H.; Fernandez, R.J.; Herrick, J.E.; et al. Global desertification: Building a science for dryland development. *Science* **2007**, *316*, 847–851. [CrossRef] [PubMed]
3. Reid, W.; Mooney, H.; Cropper, A.; Capistrano, D.; Carpenter, S.; Chopra, K.; Dasgupta, P.; Dietz, T.; Duraiappah, A.; Hassan, R.; et al. *Millenium Ecosystem Assessment Synthesis Report*; Island Press: Washington, DC, USA, 2005.
4. Neely, C.; Bunning, S.; Wilkes, A. *Review of Evidence on Drylands Pastoral Systems and Climate Change. Implications and Opportunities for Mitigation and Adaptation*; FAO: Rome Italy, 2009.
5. Allen-Diaz, B.; Chapin, F.S.; Díaz, S.; Howden, S.M.; Puigdefábregas, J.; Stafford Smith, M. Rangelands in a Changing Climate: Impacts, Adaptations, and Mitigation. In *Climate Change 1995: Impacts, Adaptations and Mitigation of Climate Change: Scientific-Technical Analyses*; Watson, R.T., Zinyowera, M.C., Moss, R.H., Dokken, D.J., Eds.; Cambridge University Press: Cambridge, UK, 1996; pp. 131–158.
6. Olff, H.; Ritchie, M.E.; Prins, H.H. Global environmental controls of diversity in large herbivores. *Nature* **2002**, *415*, 901–904. [CrossRef] [PubMed]
7. Alkemade, R.; Reid, R.S.; Van den Berg, M.; De Leeuw, J.; Jeuken, M. Assessing the impacts of livestock production on biodiversity in rangeland ecosystems. *PNAS* **2013**, *110*, 20900–20905. [CrossRef] [PubMed]
8. Hoffman, M. Experience with Grazing in Flemish Nature Reserves (Northern Belgium). Proceedings of Grazing as a Conservation Management Tool in Peatland, Goniadz, Poland, 22–26 April 2022; pp. 49–53.

9. Hayati, D.; Ranjbar, Z.; Karami, E. Measuring agricultural sustainability. In *Biodiversity, Biofuels, Agroforestry and Conservation Agriculture*; Lichtfause, E., Ed.; Springer: Dordrecht, The Netherlands, 2010; Volume 5.
10. Eteraf, H.; Telvari, A.A.R. Effects of animal grazing on some physical characteristics of loose soil in Maravetapeh Rangelands, Golestan, Iran. *Pajouhesh-Va-Sazandegi* **2005**, *17*, 8–13.
11. Rahmati, O.; Samani, A.N.; Mahmoodi, N.; Mahdavi, M. Assessment of the Contribution of N-Fertilizers to Nitrate Pollution of Groundwater in Western Iran (Case Study: Ghorveh–Dehgelan Aquifer). *Water Quall. Expos. Hea.* **2015**, *7*, 143–151. [CrossRef]
12. Maruşca, T.; Roman, A.; Taulescu, E.; Ursu, T.M.; Popa, R.D. Detecting trends in the quality and productivity of grasslands by analyzing the historical vegetation relevés: A case study from Southeastern Carpathians, Vlădeasa Mountains (Romania). *Not. Bot. Horti Agrobot. Cluj-Na.* **2021**, *49*. [CrossRef]
13. Peters, D.P.C.; Bestelmeyer, B.T.; Havstad, K.M.; Rango, A.; Archer, S.; Comrie, A.; Gimblett, R.; López-Hoffman, L.; Sala, O.E.; Vivoni, E.R. Desertification of rangelands. In *Climate Vulnerability: Understanding and Addressing Threats to Essential Resources*; Elsevier Inc.: Dublin, Ireland, 2013; pp. 239–258.
14. Karatassiou, M.; Koukoura, Z. Protection from grazing: A way to restore vegetation in semiarid grasslands in Northern Greece. *Options Mediterr. Serie A* **2009**, *85*, 99–104.
15. Adler, P.B.; Levine, J.M. Contrasting relationships between precipitation and species richness in space and time. *Oikos* **2007**, *116*, 221–232. [CrossRef]
16. Herrero-Jáuregui, C.; Oesterheld, M. Effects of grazing intensity on plant richness and diversity: A meta-analysis. *Oikos* **2018**, *127*, 757–766. [CrossRef]
17. Zhang, R.; Wang, Z.; Han, G.; Schellenberg, M.P.; Wu, Q.; Gu, C. Grazing induced changes in plant diversity is a critical factor controlling grassland productivity in the Desert Steppe, Northern China. *Agric. Ecosys. Environ.* **2018**, *265*, 73–83. [CrossRef]
18. Pinto, H.V.; Villa, P.M.; de Menezes, L.F.T.; Pereira, M.C.A. Effect of climate and altitude on plant community composition and richness in Brazilian inselbergs. *J. Mt. Sci* **2020**, *17*, 1931–1941. [CrossRef]
19. Koerner, S.E.; Burkepile, D.E.; Fynn, R.W.S.; Burns, C.E.; Eby, S.; Govender, N.; Hagenah, N.; Matchett, K.J.; Thompson, D.I.; Wilcox, K.R.; et al. Plant community response to loss of large herbivores differs between North American and South African savanna grasslands. *Ecology* **2014**, *95*, 808–816. [CrossRef] [PubMed]
20. Biggs, J.R.; VanLeeuwen, D.M.; Holechek, J.L.; Valdez, R. Multi-Scale Analyses of Habitat Use by Elk Following Wildfire. *Northwest Sci.* **2010**, *84*, 20–32. [CrossRef]
21. Holechek, J.L. An approach for setting the stocking rate. *Rangel. Archives* **1988**, *10*, 10–14.
22. Austin, M.P. Searching for a model for use in vegetation analysis. *Vegetatio* **1980**, *42*, 11–21. [CrossRef]
23. Austin, M.P.; Smith, T.M. A New Model for the Continuum Concept. *Vegetatio* **1989**, *83*, 35–47. [CrossRef]
24. Roukos, C.; Koutsoukis, C.; Akrida-Demertzi, K.; Karatassiou, M.; Demertzis, G.P.; Kandrelis, S. The effect of altitudinal zone on soil properties, species composition and forage production in a subalpine grassland in northwest Greece. *Appl. Eco. Environ. Res.* **2017**, *15*, 609–626. [CrossRef]
25. Begon, M.; Harper, J.L.; Townsend, C.R. *Ecology: Individuals, Populations and Communities*; Blackwell Scientific: Oxford, UK, 1996.
26. Odland, A.; Birks, H.J.B. The altitudinal gradient of vascular plant richness in Aurland, western Norway. *Ecography* **1999**, *22*, 548–566. [CrossRef]
27. Trigas, P.; Panitsa, M.; Tsiftsis, S. Elevational gradient of vascular plant species richness and endemism in Crete–the effect of post-isolation mountain uplift on a continental island system. *PLoS ONE* **2013**, *8*, e59425. [CrossRef]
28. Rahbek, C. The elevational gradient of species richness: A uniform pattern? *Ecography* **1995**, *18*, 200–205. [CrossRef]
29. Grabherr, G.; Gottfried, M.; Gruber, A.; Pauli, H. Patterns and current changes in alpine plant diversity. In *Arctic And Alpine Biodiversity: Patterns, Causes and Ecosystem Consequences*; Chapin, F.S., Körner, C., Eds.; Springer: Heidelberg/Berlin, Germany, 1995; Volume 113.
30. Sætersdal, M.; Birks, H.J.B.; Peglar, S. Predicting changes in Fennoscandian vascular-plant species richness as a result of future climatic change. *J. Biogeogr.* **1998**, *25*, 111–112. [CrossRef]
31. Kluge, J.; Bach, K.; Kessler, M. Elevational distribution and zonation of tropical pteridophyte assemblages in Costa Rica. *Basic Appl. Ecol.* **2008**, *9*, 35–43. [CrossRef]
32. Sandel, B.; Arge, L.; Dalsgaard, B.; Davies, R.; Gaston, K.; Sutherland, W.; Svenning, J.-C. The Influence of Late Quaternary Climate-Change Velocity on Species Endemism. *Science* **2011**, *334*, 660–664. [CrossRef] [PubMed]
33. Wang, L.; Schneider, H.; Zhang, X.C.; Xiang, Q.P. The rise of the Himalaya enforced the diversification of SE Asian ferns by altering the monsoon regimes. *BMC Plant Biol.* **2012**, *12*, 210. [CrossRef] [PubMed]
34. Hughes, C.; Atchison, G. The ubiquity of alpine plant radiations: From the Andes to the Hengduan Mountains. *New Phytol.* **2015**, *207*, 275–282. [CrossRef]
35. Olivares, I.; Kessler, M. Regional species richness determines local species turnover in ferns. *Front. Biogeogr.* **2020**, *12*, e46818. [CrossRef]
36. Yan, H.; Liang, C.; Li, Z.; Liu, Z.; Miao, B.; He, C.; Sheng, L. Impact of precipitation patterns on biomass and species richness of annuals in a dry steppe. *PLoS ONE* **2015**, *10*, e0125300. [CrossRef]
37. Belgacem, A.O.; Louhaichi, M. The vulnerability of native rangeland plant species to global climate change in the West Asia and North African regions. *Clim. Change* **2013**, *119*, 451–463. [CrossRef]

38. Hudson, L.N.; Newbold, T.; Contu, S.; Hill, S.L.L.; Lysenko, I.; De Palma, A.; Phillips, H.R.P.; Senior, R.A.; Bennett, D.J.; Booth, H.; et al. The PREDICTS database: A global database of how local terrestrial biodiversity responds to human impacts. *Ecol. Evol.* **2014**, *4*, 4701–4735. [CrossRef]
39. Adeel, Z.; Safriel, U.; Niemeijer, D.; White, R. *Ecosystems and Human Well-Being: Desertification Synthesis*; World Resources Institute (WRI): Washington, DC, USA, 2005.
40. Sala, O.E.; Chapin, F.S.; Armesto, J.J.; Berlow, E.; Bloomfield, J.; Dirzo, R.; Huber-Sanwald, E.; Huenneke, L.F.; Jackson, R.B.; Kinzig, A. Global biodiversity scenarios for the year 2100. *Science* **2000**, *287*, 1770–1774. [CrossRef]
41. Hanke, W.; Böhner, J.; Dreber, N.; Jürgens, N.; Schmiedel, U.; Wesuls, D.; Dengler, J. The impact of livestock grazing on plant diversity: An analysis across dryland ecosystems and scales in southern Africa. *Ecol. Appl.* **2014**, *24*, 1188–1203. [CrossRef] [PubMed]
42. Zhang, L.; Liu, J.; Wang, D.; Wang, H.; Wu, Y.; Lü, Z. Fencing for conservation?-The impacts of fencing on grasslands and the endangered Przewalski's gazelle on the Tibetan Plateau. *Sci. China Life Sci.* **2018**, *61*, 1593–1595. [CrossRef] [PubMed]
43. Metera, E.; Sakowski, T.; Sloniewski, K.; Romanowicz, B. Grazing as a tool to maintain biodiversity of grassland—A review. *Anim. Sci. Pap. Rep.* **2010**, *28*, 315–334.
44. Wan, H.W.; Bai, Y.F.; Hooper, D.U.; Schonbach, P.; Gierus, M.; Schiborra, A.; Taube, F. Selective grazing and seasonal precipitation play key roles in shaping plant community structure of semi-arid grasslands. *Landsc. Ecol.* **2015**, *30*, 1767–1782. [CrossRef]
45. Rook, A.J.; Dumont, B.; Isselstein, J.; Osoro, K.; WallisDeVries, M.F.; Parente, G.; Mills, J. Matching type of livestock to desired biodiversity outcomes in pastures—A review. *Biol. Conserv.* **2004**, *119*, 137–150. [CrossRef]
46. Rahmanian, S.; Hejda, M.; Ejtehadi, H.; Farzam, M.; Pysek, P.; Memariani, F. Effects of livestock grazing on plant species diversity vary along a climatic gradient in northeastern Iran. *Appl. Veg. Sci.* **2020**, *23*, 551–561. [CrossRef]
47. Schultz, N.L.; Morgan, J.W.; Lunt, I.D. Effects of grazing exclusion on plant species richness and phytomass accumulation vary across a regional productivity gradient. *J. Veg. Sci.* **2011**, *22*, 130–142. [CrossRef]
48. Purvis, A.; Hector, A. Getting the measure of biodiversity. *Nature* **2000**, *405*, 212–219. [CrossRef]
49. Tuomisto, H. Commentary: Do we have a consistent terminology for species diversity? Yes, if we choose to use it. *Oecologia* **2011**, *167*, 903–911. [CrossRef]
50. Tuomisto, H. A consistent terminology for quantifying species diversity? Yes, it does exist. *Oecologia* **2010**, *164*, 853–860. [CrossRef]
51. Jost, L. Partitioning diversity into independent alpha and beta components. *Ecology* **2007**, *88*, 2427–2439. [CrossRef] [PubMed]
52. Jost, L. Entropy and diversity. *Oikos* **2006**, *113*, 363–375. [CrossRef]
53. Jost, L. The relation between evenness and diversity. *Diversity* **2010**, *2*, 207–232. [CrossRef]
54. Cao, Y.; Hawkins, C.P. Weighting effective number of species measures by abundance weakens detection of diversity responses. *J. Appl. Ecol.* **2019**, *56*, 1200–1209. [CrossRef]
55. Beck, J.; Schwanghart, W. Comparing measures of species diversity from incomplete inventories: An update. *Methods Ecol. Evol.* **2010**, *1*, 38–44. [CrossRef]
56. Daly, A.J.; Baetens, J.M.; De Baets, B. Ecological Diversity: Measuring the Unmeasurable. *Mathematics* **2018**, *6*, 119. [CrossRef]
57. Jost, L.; DeVries, P.; Walla, T.; Greeney, H.; Chao, A.; Ricotta, C. Partitioning diversity for conservation analyses. *Divers. Distrib.* **2010**, *16*, 65–76. [CrossRef]
58. Tsakona, M.; Gekas, V. Desertification in Crete and the effect of global warming. In Proceedings of the 5th WSEAS, International Conference on Environment, Ecosystems and Development, Tenerife, Spain, 14–16 December 2007; pp. 211–216.
59. Morianou, G.G.; Kourgialas, N.N.; Psarras, G.; Koubouris, G.C. Mapping sensitivity to desertification in Crete (Greece), the risk for agricultural areas. *J. Water Clim. Change* **2018**, *9*, 691–702. [CrossRef]
60. Li, Z.; Liang, M.; Li, Z.; Mariotte, P.; Tong, X.; Zhang, J.; Dong, L.; Zheng, Y.; Ma, W.; Zhao, L.; et al. Plant functional groups mediate effects of climate and soil factors on species richness and community biomass in grasslands of Mongolian Plateau. *J. Plant Ecol.* **2021**, *14*, 679–691. [CrossRef]
61. Mallen-Cooper, M.; Eldridge, D.J.; Delgado-Baquerizo, M. Livestock grazing and aridity reduce the functional diversity of biocrusts. *Plant Soil* **2018**, *429*, 175–185. [CrossRef]
62. Agrawal, A.A. Overcompensation of plants in response to herbivory and the by-product benefits of mutualism. *Trends Plant Sci.* **2000**, *5*, 309–313. [CrossRef]
63. Louthan, A.M.; Doak, D.F.; Goheen, J.R.; Palmer, T.M.; Pringle, R.M. Climatic stress mediates the impacts of herbivory on plant population structure and components of individual fitness. *J. Ecol.* **2013**, *101*, 1074–1083. [CrossRef]
64. Milchunas, D.G.; Sala, O.E.; Lauenroth, W.K. A generalized model of the effects of grazing by large herbivores on grassland community structure. *Am. Nat.* **1988**, *132*, 87–106. [CrossRef]
65. Oñatibia, G.R.; Amengual, G.; Boyero, L.; Aguiar, M.R. Aridity exacerbates grazing-induced rangeland degradation: A population approach for dominant grasses. *J. Appl. Ecol* **2020**, *57*, 1999–2009. [CrossRef]
66. Stuth, J.W. Foraging behavior. In *Grazing Management: An Ecological Perspective*; Heitschmidt, R.K., Stuth, J.W., Eds.; Timber Press: Portland, OR, USA, 1991.
67. Eldridge, D.J.; Delgado-Baquerizo, M.; Travers, S.K.; Val, J.; Oliver, I.; Dorrough, J.W.; Soliveres, S. Livestock activity increases exotic plant richness, but wildlife increases native richness, with stronger effects under low productivity. *J. Appl. Ecol.* **2018**, *55*, 766–776. [CrossRef]

68. Bhandari, J.; Zhang, Y. Effect of altitude and soil properties on biomass and plant richness in the grasslands of Tibet, China, and Manang District, Nepal. *Ecosphere* **2019**, *10*, e02915. [CrossRef]
69. Abraham, E.; Karatassiou, M.; Parissi, Z.; Koukoura, Z.; Tsiouvaras, C. Long-term effects of grazing on composition in various habitats of a mountainous area in Central Greece. *Options Méditerr. Série A* **2009**, 73–78.
70. Tsiourlis, G.; Kasapidis, P.; Konstantinidis, P. The role of grazing on the maintain and degradation of Mediterranean Ecosystems in Central Crete, Greece. In Proceedings of the Forest Research: A Challenge for an Integrated European Approach, Thessaloniki, Greece, 27 August–1 September 2001; pp. 715–721.
71. Papanastasis, V.P.; Kyriakakis, S.; Kazakis, G. Plant diversity in relation to overgrazing and burning in mountain Mediterranean ecosystems. *J. Mediterr. Ecol.* **2002**, *3*, 53–64.
72. Ojima, D.S.; Aicher, R.; Archer, S.R.; Bailey, D.W.; Casby-Horton, S.M.; Cavallaro, N.; Reyes, J.J.; Tanaka, J.A.; Washington-Allen, R.A. A climate change indicator framework for rangelands and pastures of the USA. *Clim. Change* **2020**, *163*, 1733–1750. [CrossRef]
73. Lund, H.G. Accounting for the World's Rangelands. *Rangelands* **2007**, *29*, 3–10. [CrossRef]
74. Margaris, N.S.; Vokou, D. Structural and physiological features of woody plants in phryganic ecosystems related to adaptive mechanisms. *Ecol. Mediterr.* **1982**, *8*, 449–459. [CrossRef]
75. Thompson, J.D. *Plant Evolution in the Mediterranean: Insights for Conservation*; Oxford University Press: New York, NY, USA, 2020.
76. Atherden, M.; Hall, J. Human impact on vegetation in the White Mountains of Crete since AD 500. *Holocene* **1999**, *9*, 183–193. [CrossRef]
77. Papanastasis, V.P.; Kyriakakis, S.; Kazakis, G.; Abid, M.; Doulis, A. Plant cover as a tool for monitoring desertification in mountain Mediterranean rangelands. *Manag. Environ. Qual.* **2003**, *14*, 69–81. [CrossRef]
78. Vogiatzakis, I.; Griffiths, G.H.; Mannion, A.M. Environmental factors and vegetation composition, Lefka Ori massif, Crete, S. Aegean. *Glob. Ecol. Biogeogr.* **2003**, *12*, 131–146. [CrossRef]
79. Sklenár, P.; Ramsay, P.M. Diversity of zonal páramo plant communities in Ecuador. *Divers. Distrib.* **2001**, *7*, 113–124. [CrossRef]
80. Mekonnen, Z.A.; Riley, W.J.; Randerson, J.T.; Grant, R.F.; Rogers, B.M. Expansion of high-latitude deciduous forests driven by interactions between climate warming and fire. *Nat. Plants* **2019**, *5*, 952–958. [CrossRef]
81. Zupo, T.; Daibes, L.F.; Pausas, J.G.; Fidelis, A. Post-fire regeneration strategies in a frequently burned Cerrado community. *J. Veg. Sci.* **2021**, *32*, e12968. [CrossRef]
82. Gatti, R.C.; Amoroso, N.; Monaco, A. Estimating and comparing biodiversity with a single universal metric. *Ecol. Mod.* **2020**, *424*, 109020. [CrossRef]
83. Mena, J.L.; Vázquez-Domínguez, E. Species turnover on elevational gradients in small rodents. *Glob. Ecol. Biogeogr.* **2005**, *14*, 539–547. [CrossRef]
84. Xiang, M.; Wu, J.; Wu, J.; Guo, Y.; Lha, D.; Pan, Y.; Zhang, X. Heavy Grazing Altered the Biodiversity–Productivity Relationship of Alpine Grasslands in Lhasa River Valley, Tibet. *Front. Ecol. Evol.* **2021**, *9*, 698707. [CrossRef]
85. Zawierucha, K.; Smykla, J.; Michalczyk, Ł.; Gołdyn, B.; Kaczmarek, Ł. Distribution and diversity of Tardigrada along altitudinal gradients in the Hornsund, Spitsbergen (Arctic). *Polar Res.* **2015**, *34*, 24168. [CrossRef]
86. Lantz, T.C.; Gergel, S.E.; Henry, G.H.R. Response of green alder (Alnus viridis subsp. fruticosa) patch dynamics and plant community composition to fire and regional temperature in north-western Canada. *J. Biogeogr.* **2010**, *37*, 1597–1610. [CrossRef]
87. Coop, J.D.; Massatti, R.T.; Schoettle, A.W. Subalpine vegetation pattern three decades after stand-replacing fire: Effects of landscape context and topography on plant community composition, tree regeneration, and diversity. *J. Veg. Sci.* **2010**, *21*, 472–487. [CrossRef]
88. McKenzie, D.A.; Tinker, D.B. Fire-induced shifts in overstory tree species composition and associated understory plant composition in Glacier National Park, Montana. *Plant Ecol.* **2012**, *213*, 207–224. [CrossRef]
89. Staver, A.C.; Botha, J.; Hedin, L. Soils and fire jointly determine vegetation structure in an African savanna. *New Phytol.* **2017**, *216*, 1151–1160. [CrossRef] [PubMed]
90. Omidipour, R.; Tahmasebi, P.; Faal Faizabadi, M.; Faramarzi, M.; Ebrahimi, A. Does β diversity predict ecosystem productivity better than species diversity? *Ecol. Indic.* **2021**, *122*, 107212. [CrossRef]
91. Morris, E.K.; Caruso, T.; Buscot, F.; Fischer, M.; Hancock, C.; Maier, T.S.; Meiners, T.; Müller, C.; Obermaier, E.; Prati, D.; et al. Choosing and using diversity indices: Insights for ecological applications from the German Biodiversity Exploratories. *Ecol. Evol.* **2014**, *4*, 3514–3524. [CrossRef]
92. Gibson, D.J.; Newman, J.A. Grasslands and climate change: An overview. In *Grasslands and Climate Change*; Gibson, D.J., Newman, J.A., Eds.; Cambridge University Press: Cambridge, UK, 2019; pp. 3–18.
93. Sanaei, A.; Ali, A.; Chahouki, M.A.Z.; Jafari, M. Plant coverage is a potential ecological indicator for species diversity and aboveground biomass in semi-steppe rangelands. *Ecol. Indic.* **2018**, *93*, 256–266. [CrossRef]
94. WallisDeVries, M.F.; Poschlod, P.; Willems, J.H. Challenges for the conservation of calcareous grasslands in northwestern Europe: Integrating the requirements of flora and fauna. *Biol. Conserv.* **2002**, *104*, 265–273. [CrossRef]
95. Benthien, O.; Braun, M.; Riemann, J.C.; Stolter, C. Long-term effect of sheep and goat grazing on plant diversity in a semi-natural dry grassland habitat. *Heliyon* **2018**, *4*, e00556. [CrossRef]
96. Mohapatra, J.; Singh, C.P.; Hamid, M.; Khuroo, A.A.; Malik, A.H.; Pandya, H.A. Assessment of the alpine plant species biodiversity in the western Himalaya using Resourcesat-2 imagery and field survey. *J. Earth Syst. Sci.* **2019**, *128*, 1–16. [CrossRef]

97. Kazakis, G.; Ghosn, D.; Vogiatzakis, I.; Papanastasis, V. Vascular plant diversity and climate change in the alpine zone of the Lefka Ori, Crete. *Biodivers. Conserv.* **2007**, *16*, 1603–1615. [CrossRef]
98. Ibanez, T.; Hart, P.; Ainsworth, A.; Gross, J.; Monello, R. Factors associated with alien plant richness, cover and composition differ in tropical island forests. *Divers. Distrib.* **2019**, *25*, 1910–1923. [CrossRef]
99. Todd, S.W. Gradients in vegetation cover, structure and species richness of Nama-Karoo shrublands in relation to distance from livestock watering points. *J. Appl. Ecol.* **2006**, *43*, 293–304. [CrossRef]
100. Stirling, G.; Wilsey, B. Empirical Relationships between Species Richness, Evenness, and Proportional Diversity. *Am. Nat.* **2001**, *158*, 286–299. [CrossRef] [PubMed]
101. Nagendra, H. Opposite trends in response for the Shannon and Simpson indices of landscape diversity. *Appl. Geogr.* **2002**, *22*, 175–186. [CrossRef]
102. Cook, C.W.; Stubbendieck, J. *Range Research: Basic Problems and Techniques*; Society for Range Management: Denver, CO, USA, 1986.
103. Bonham, C.D. *Measurements for Terrestrial Vegetation*; John Wiley & Sons: Hoboken, NJ, USA, 2013.
104. Deléglise, C.; Loucougaray, G.; Alard, D. Effects of grazing exclusion on the spatial variability of subalpine plant communities: A multiscale approach. *Basic Appl. Ecol.* **2011**, *12*, 609–619. [CrossRef]
105. Hill, M.O. Diversity and evenness: A unifying notation and its consequences. *Ecology* **1973**, *54*, 427–432. [CrossRef]
106. Chao, A.N.; Chiu, C.H.; Jost, L. Unifying Species Diversity, Phylogenetic Diversity, Functional Diversity, and Related Similarity and Differentiation Measures Through Hill Numbers. *Annu. Rev. Ecol. Evol. Syst.* **2014**, *45*, 297–324. [CrossRef]
107. Chao, A.; Gotelli, N.J.; Hsieh, T.C.; Sander, E.L.; Ma, K.H.; Colwell, R.K.; Ellison, A.M. Rarefaction and extrapolation with Hill numbers: A framework for sampling and estimation in species diversity studies. *Ecol. Monogr.* **2014**, *84*, 45–67. [CrossRef]
108. Gurevitch, J.; Scheiner, S.M.; Fox, G.A. *The Ecology of Plants*, 3rd ed.; Sinauer Associates Sunderland, Oxfrord University Press: Cary, NC, USA, 2021.
109. Heady, H.F.; Child, R.D. *Rangeland Ecology and Management*; Routledge: New York, NY, USA, 2019.

plants

MDPI

Review

A Review and Evaluation of the Data Supporting Internal Use of *Helichrysum italicum*

Katja Kramberger [1,2], Saša Kenig [1], Zala Jenko Pražnikar [1], Nina Kočevar Glavač [3] and Darja Barlič-Maganja [1,*]

[1] Faculty of Health Sciences, University of Primorska, 6310 Izola, Slovenia; katja.kramberger@fvz.upr.si (K.K.); sasa.kenig@fvz.upr.si (S.K.); zala.praznikar@fvz.upr.si (Z.J.P.)
[2] Faculty of Medicine, University of Ljubljana, 1000 Ljubljana, Slovenia
[3] Faculty of Pharmacy, University of Ljubljana, 1000 Ljubljana, Slovenia; nina.kocevar.glavac@ffa.uni-lj.si
* Correspondence: darja.maganja@fvz.upr.si; Tel.: +386-5-662-6467

Abstract: *Helichrysum italicum* is a Mediterranean plant with various pharmacological activities. Despite extensive reports on the bioactivity of the plant, its clinically studied applications have not yet been reviewed. The aim of our study was to gather information on the internal use of *H. italicum* and its bioactive constituents to determine its efficacy and safety for human use. We reviewed research articles that have not been previously presented in this context and analyzed relevant clinical studies with *H. italicum*. Cochranelibrary.com revealed six eligible clinical trials with *H. italicum* that examined indications for pain management, cough, and mental exhaustion. Although the efficacy of *H. italicum* has been demonstrated both in in vitro tests and in humans, it is difficult to attribute results from clinical trials to *H. italicum* alone, as it has usually not been tested as the sole component. On the other hand, clinical trials provide positive information on the safety profile since no adverse effects have been reported. We conclude that *H. italicum* is safe to use internally, while new clinical studies with *H. italicum* as a single component are needed to prove its efficacy. Based on the recent trend in *H. italicum* research, further studies are to be expected.

Keywords: *Helichrysum italicum*; biological activity; internal use; clinical studies

Citation: Kramberger, K.; Kenig, S.; Jenko Pražnikar, Z.; Kočevar Glavač, N.; Barlič-Maganja, D. A Review and Evaluation of the Data Supporting Internal Use of *Helichrysum italicum*. *Plants* **2021**, *10*, 1738. https://doi.org/10.3390/plants10081738

Academic Editors: Sofia Rhizopoulou, Maria Karatassiou and Efi Levizou

Received: 23 July 2021
Accepted: 18 August 2021
Published: 23 August 2021

1. Introduction

Plants have a long history of use in medicine and have been used by all cultures or ethnic groups throughout history to improve human health [1]. They are considered to be the oldest form of medicine known to humankind but are, on the other hand, also an important source of modern medicines and govern synthetic drug development [1,2]. According to the World Health Organization (WHO), 70–95% of the world's population rely on traditional medicine for their primary health care [2]. This is especially true for the Mediterranean countries, where plants play a vital role in the diet habits, and sometimes there is no clear dividing line between food and medicinal plants, particularly in indigenous and local traditions [3].

The plants belonging to the genus *Helichrysum* (family Asteraceae) are known as everlasting flowers and are widely used in traditional medicine worldwide [4]. The plant species of this genus typically have inflorescences of a bright yellow color [5], which retain their form and color when dried, hence the name "everlasting" or "immortal" [4]. The stems are woody at the base and can reach 30–70 cm in height. The plant is well adapted to environments that lack water as it naturally grows on alkaline, dry, sandy and poor soil at the altitude from the sea level up to 2200 m [6]. The *Helichrysum* Miller genus includes more than a thousand of taxa, among them the most well-known and studied species are *Helichrysum italicum* (Roth) G. Don [7] (Figure 1), *Helichrysum stoechas* (L.) Moench [8], and *Helichrysum arenarium* (L.) Moench [9]. *H. italicum* and *H. stoechas* are distributed throughout the Mediterranean [10] but are especially characteristic to Adriatic region [11] and Iberian Peninsula [12], respectively, whereas *H. arenarium* is mostly found

in the Central Europe [9,10]. Despite the long tradition in treatment of various disorders for all three before-mentioned species, their traditional use for treating digestive problems (e.g., fullness and bloating) has been approved by the WHO and the European Medicines Agency (EMA) for the species *H. arenarium* alone [8]. Nevertheless, *H. italicum* has been recently investigated quite extensively, especially in the Balkan countries. The focus of this review article will be from herein after on *H. italicum* only. Among the *H. italicum* species, there are also several subspecies (abbreviated ssp.), which are difficult to distinguish due to a strong polymorphism in morphology [10]. The explanation of *H. italicum* classification is beyond the scope of this review article, but the name of subspecies is included, if available in the referenced article.

Figure 1. Cultivated *Helichrysum italicum* plant in flowering stage (Photo: Katja Kramberger).

Characteristic yellow fade-resistant inflorescences (seen in Figure 1) as well as vegetative aerial organs of *H. italicum* are a treasury of bioactive secondary metabolites that result from the plants' adaptation to this challenging environment. Apart from the volatile terpenes present in essential oils, absolutes and supercritical CO_2 extracts, *H. italicum* is also very rich in phenolic compounds, which are recognized as health promoting agents due to antioxidant properties they exert and their probable role in the prevention of various diseases associated with oxidative stress, such as cancer, cardiovascular and neurodegenerative diseases [13]. The health-beneficial potential of *H. italicum* has been reported in ethnopharmacological surveys and supported by numerous in vitro and in vivo experiments [7]. In the Greek-Roman system of medicine, *H. italicum* was used as an anti-inflammatory and anti-infective plant, and both uses are still well rooted in traditional medicine today [14]. Antunes Viegas et al. [7] emphasized that, in contrast to animal studies, there is a severe lack of clinical studies investigating the effects of the *H. italicum* extracts, which undermines the possibility of validating the traditional uses of this plant. One of the reasons why clinical and other comprehensive studies with herbal products are scarce probably lies in the difficulty of interpreting the results of such studies [2].

The aim of our study was to gather all information reported in the literature on internal applications of *H. italicum* or its bioactive constituents in humans to support the efficacy and safety of *H. italicum* preparations for human use. We reviewed studies that have not been outlined in this context before and analyzed relevant clinical trials with *H. italicum* as the main or one of the studied components.

2. Methodology

Research and review articles were searched via online databases including PubMed and Scopus until February 2021. Clinical trials on *H. italicum* and its constituents were searched through Cochrane Library (https://www.cochranelibrary.com/) and ClinicalTrials (https://clinicaltrials.gov/) (last accessed on 2 May 2021).

The reviewed literature on *H. italicum* use is presented in thematic sections; ethnopharmacological surveys and non-clinical research articles (Section 3), human clinical observations (Section 4), clinical trials (Section 5) and recently published articles (Section 6). The first section briefly reviews the use of *H. italicum* in traditional medicine, as well as the most prominent in vitro and in vivo scientific experiments. Section on human clinical observation summarizes the experiments in humans, which were not registered as clinical trials. Clinical trials section is divided in clinical trials with *H. italicum* (Section 5.1) and trials with isolated compounds commonly found in *H. italicum* (Section 5.2). Lastly, recently published articles on *H. italicum* are presented to provide insight into the current trend of *H. italicum* research.

3. Traditional Uses and Scientific Data

Ethnopharmacological surveys on *H. italicum*, summarized in the study by Antunes Viegas et al. [7], show that the most frequently reported traditional uses are related to respiratory, digestive and skin inflammatory conditions. Depending on the application, *H. italicum* preparations are administered via inhalation, ingestion or topically. Other therapeutic applications include wound healing and antimicrobial uses, as well as gall and bladder disorders and analgesic use. Common types of preparations are mostly infusions and decoctions, for both oral and external use, followed by vapors, juices and powders [7]. Several in vitro as well as in vivo studies have confirmed biological activity of compounds isolated from *H. italicum* or its fractionated extracts, whereas for some indications such as digestive non-inflammatory disorders, pain in the gastrointestinal tract, alopecia, helminthic infections, and sleeplessness, scientific validation is still missing [6]. Moreover, studies investigating crude extracts, especially aqueous extracts, which are the most commonly used in traditional medicine, are rather scarce.

A large variety of *H. italicum* extracts can be prepared, and the resulting products differ in their chemical composition, yet the main compound classes are terpenes and phenolics. A systematic review of *H. italicum* bioactive compounds with regard to extraction procedure used for their isolation was conducted by Maksimovic et al. [15] and for the most characteristic compounds this information is summarized in Table 1. Research on *H. italicum* regained interest in the 1990s with the studies by Facino et al. [16,17], Pietta et al. [18,19] and by Zapesochnaya et al. [20,21] on isolation and identification of bioactive substances of Italian *H. italicum*. Numerous studies were performed in the following decades, in which additional bioactive constituents were isolated and in vitro and in vivo tests performed to support their bioactivity [14,22–42]. Nostro et al. [22–25] investigated anti-cariogenic potential of *H. italicum* diethyl ether and ethanolic extracts, which is probably attributed to flavonoids. Studies by Sala et al. [26–29] dealt with a class of acetophenone compounds and flavonoids pinocembrin, gnaphallin and tiliroside, isolated from *H. italicum*, and tested their anti-inflammatory action in mice. Noteworthy are also the studies by Appendino et al. [14], Rosa et al. [33,34] and Bauer et al. [35], who discovered and investigated a main anti-inflammatory compound present in *H. italicum*, arzanol. Its anti-inflammatory effects have also been proved in vivo [35]. Arzanol's activity and its mechanism of action are summarized in review by Kothavade et al. [43]. The following studies are worth mentioning due to validating traditional uses at in vivo level. Rigano et al. [39] proved that ethanol extract elicited antispasmodic action in the isolated mouse ileum and inhibited transit preferentially in the inflamed gut. The suitability of the traditional use of *H. italicum* ssp. *italicum* flowers for intestinal diseases was thereby confirmed. De la Garza et al. [38] showed that methanol-water extract decreased blood glucose levels and reduced postprandial glucose levels, as well as improved hyperinsu-

linemia in a dietary model of insulin resistance in rats. Furthermore, Pereira et al. [42] investigated anti-diabetic activity of water-based preparations (infusions and decoctions) of *H. italicum* ssp. *picardii* via inhibitory activity towards α-glucosidase and found moderate effects. Although diabetes is not one of the conditions mentioned in traditional medicine of *H. italicum*, this study opened another possible application—treatment of metabolic syndrome.

In recent years, increased interest for *H. italicum* was also observed in many Southern European countries, predominantly due to *H. italicum* essential oil and its use in the perfume and cosmetic industry. This topic is reviewed by Ninčević et al. [10], who also focused on taxonomic classification and morphological characteristics arising from genetic diversity, in addition to bioactive compounds of *H. italicum* and their biological activity.

Table 1. The list of the most characteristic bioactive compounds found and investigated in *H. italicum*.

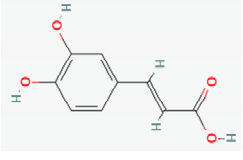
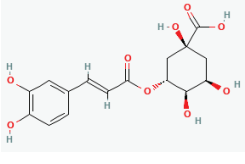
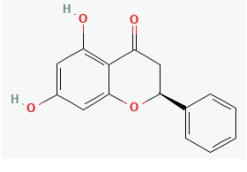
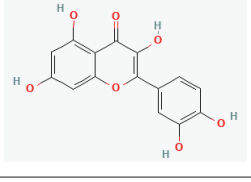
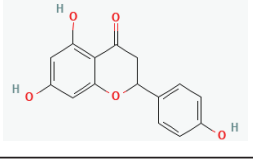
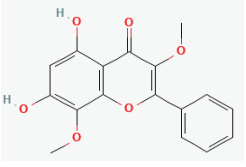
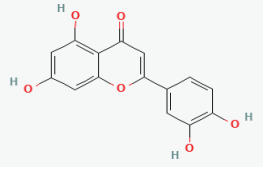
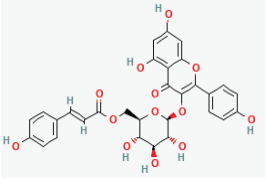
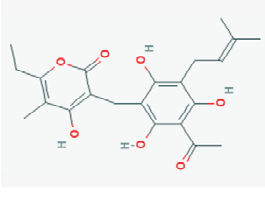
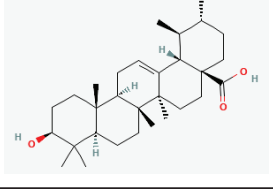
Compound Name	Chemical Structure [1]	Extraction Solvent [2]	Extraction Yield (from Starting Plant Material) [3]	Main Bioactivity [4]
Caffeic acid		Methanol	0.0067% (NS) [38] 0.00042% (flowers) [41] 0.0057–0.015% (aerial parts or flowers) [42]	Antioxidant, anti-inflammatory and anticancer activity [44]
Chlorogenic acid		Methanol	0.104% (NS) [38] 0.045% (flowers) [41] 0.52–0.77% (aerial parts or flowers) [42]	Antibacterial [36], antiviral, antioxidant, anti-inflammatory, anticardiovascular, hypoglycemic, and anticancer activity [45]
Pinocembrin		Acetone [32] Methanol [27]	NA (NS) [32] NA (aerial parts) [27]	Anti-inflammatory [29]
Quercetin		Methanol	0.015% (flowers) [41] 0.001–0.0015% (aerial parts or flowers) [42]	Antioxidant, anti-inflammatory, antimicrobial, cardioprotective, gastroprotective and anticancer activity [46]
Naringenin		Methanol	0.023% (NS) [38]	Antioxidant, antitumor, antiviral, antibacterial, anti-inflammatory, and cardioprotective activity [47]

Table 1. *Cont.*

Compound Name	Chemical Structure [1]	Extraction Solvent [2]	Extraction Yield (from Starting Plant Material) [3]	Main Bioactivity [4]
Gnaphaliin		Methanol	0.03% (aerial parts) [31]	Anti-inflammatory [29]
Luteolin		Ethanol	NA (flowers) [16]	Antiviral [24]
Tiliroside		Methanol	0.0063% (aerial parts) [31] 0.0015% (flowers) [41]	Anti-inflammatory and antioxidant [29]
Arzanol		Acetone	0.078% (aerial parts) [14] 0.064% (aerial parts and flowers) [33] 0.32% (aerial parts) [37]	Anti-inflammatory [14,35], antiviral [14], antioxidant [33] and antibacterial [37]
Ursolic acid		Acetone	0.40% (aerial parts) [37]	Anti-inflammatory, anticancer, antidiabetic, antioxidant and antibacterial effects [48]

[1] Images of 2D structures of compounds were obtained from PubChem database. [2] Only the primary solvent used for extraction is mentioned. Solvents used in subsequent fractionation process are not listed but can be found in the referenced articles. [3] Extraction yields were calculated from the masses obtained after isolation procedures in response to the amount of the starting plant material. In addition, information on the plant part used in the extraction is included, if specified. [4] Preferentially only activity related to *H. italicum* investigation is reported, when available. NA—not available, NS—not specified.

4. Human Clinical Observations

Despite several review articles on *H. italicum*, the following clinical observations are rarely mentioned. Systematic clinical studies on the anti-inflammatory properties of *H. italicum* were already carried out by Leonardo Santini, an Italian physician, in the 1940s. Despite the promising results, these investigations were largely overlooked at that time, but were later recognized as relevant for studies on anti-inflammatory activity of *H. italicum* [14]. The clinical experiments performed by Santini [49], Benigni [50], Vannini [51] and Campanini [52] are described in Italian literature and summarized in the

article by Appendino et al. [53]. These observations are presented in Table 2 along with additional studies by Facino et al. [16], Voinchet and Giraud-Robert [54], and a very recent one by Granger et al. [55]. These studies mainly cover treatments of respiratory and dermal conditions.

Table 2. Clinical observations in humans, listed in chronological order.

Author and Date [Reference]	Observation
Santini, 1930s [49]	*H. italicum* decoction administered to patients suffering from bronchitis and asthmatic cough led to improvement of their respiratory condition and unrelated conditions such as psoriasis and arthritis.
Santini, 1930–1950 [49]	Two decades of clinical observations led to the conclusion that clinical activity of a decoction and syrup from *H. italicum* is similar to that of cortisone. Aerosolized decoction of *H. italicum* showed positive results in the use for allergic rhinitis.
Santini, 1950s [49]	Two independent clinical studies on patient with psoriasis confirmed beneficial effects of *H. italicum* treatment.
Benigni, 1950s [50]	A series of clinical studies in various Italian centers substantially confirmed the findings of Santini, showing that "Fraction H" produced using an organic solvent, could, to varying degrees, replace corticosteroids in many of their uses and their adverse side effects were thus avoided.
Vannini, 1981 [51]	*H. italicum* decoction was found to be highly efficacious in treatment of tracheo-bronchitis in a small clinical study in children.
Facino, 1988 [16]	Flavonoid fraction was applied to humans 10 min before or after exposure to UVB radiation to evaluate their photoprotective and anti-erythematous activities, respectively. The onset of the erythematous response was completely prevented and a sun protection factor of approximately 5 was provided.
Campanini, 1995 [52]	Three weeks of treatment with 5% *H. italicum* decoction led to improvement of psoriasis in all participants, with relapses observed within two months post-treatment.
Voinchet and Giraud-Robert, 2007 [54]	Two drops of *H. italicum* essential oil, orally twice a day for 10 d, followed by its topical application for 2–3 months in post-operative scars of patients submitted to a plastic surgery of the thorax led to a reduction of local inflammation, edema, bruises, and hematomas.
Granger, 2020 [55]	Night cream containing melatonin, carnosine, and *H. italicum* extract reduced skin damage caused by environmental factors and its nightly use could improve clinical signs of aging with additional skin calming benefits. Hydration and trans-epidermal water loss values were improved within 1 h of use. Wrinkle counts were reduced by up to 18.9%, and brown and UV spot numbers by 5.5% and 13.2%, respectively. Lactic acid-induced stinging was significantly reduced within 7 d of use, with 86.7% of subjects reporting that their skin felt calmer.

5. Registered Clinical Trials

5.1. H. italicum Herb

To date, no review of clinical trials including *H. italicum* has been conducted. Searching the Cochrane Library for the term "Helichrysum" in Title Abstract Keyword (Word variations have been searched) resulted in seven hits (https://www.cochranelibrary.com/, accessed on 4 January 2021). An additional record was identified through PubMed database. After inspection, one duplicate record was identified, and another one was excluded based on an inappropriate *Helichrysum* species investigated. Other six records addressed *H. italicum* alone or in combination with other herbs. The indications studied were pain (chronic prostatitis, post-surgical pain), cough and mental exhaustion. Consequently, diverse dosage forms were used: granules, syrup, inhalation preparation and suppositories. All relevant records were included in the qualitative synthesis, although few were missing full-text articles. The above-described process of literature search and article selection is shown in Figure 2. No meta-analysis could be performed, as there were too few trials

published, and the presented trials possessed too much heterogeneity both in studied conditions and in the formulations tested.

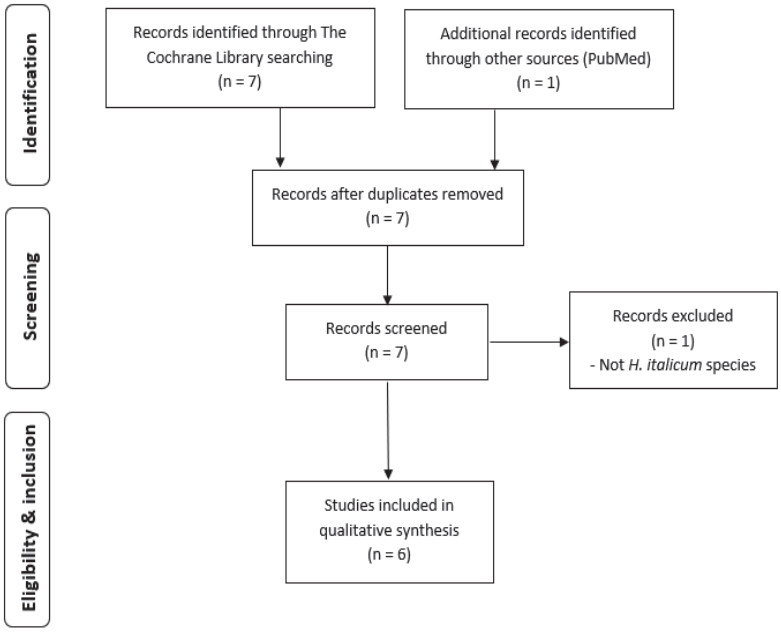


Figure 2. Flowchart of the trials' selection process. n—number of records/studies.

The reviewed clinical trials on *H. italicum* are summarized in Table A1 and described in more detail in the following paragraphs. The first trial, chronologically, was performed by Aboca S.p.A., an Italian company focused on innovative products based on natural substances. According to the EU clinical trials register record [56], two commercial products in the form of granules for oral suspension were administered as a pain treatment to adult patients with post-surgery pain. Freeze-dried extracts of *H. italicum* flowering tops and *Salvia officinalis* (sage) leaves were parallelly compared against placebo. Although trial status is "completed", the results are not available in the databases, and no articles have been published. We also tried contacting the company directly but were unsuccessful.

In the trial by Galeone et al. [57], *H. italicum* was incorporated in the medical device Proxelan® (Sala Bolognese, Italy) suppositories along with other plants: *Boswellia serrata*, *Centella asiatica* (Asiatic pennywort) and *Cucurbita pepo* (pumpkin) seeds. Altogether sixty subjects with bacterial and non-bacterial prostatitis were divided in two groups, one receiving antibiotic treatment and the other receiving antibiotics together with Proxelan® suppositories. Minor side effects were observed, but they did not cause trial interruption in any case. From a microbiological point of view, Proxelan® treatment was not better than antibiotics alone ($p = 0.46$). However, the combination of antibiotics and Proxelan® improved both symptoms associated to chronic prostatitis and urinary symptoms, which were two-fold decreased compared to control group after two months following the intervention ($p = 0.028$). The trial provides some relevant information on the safety since the rectal application can also have systemic effects. Conclusions regarding efficacy are not as straightforward, as *H. italicum* was not a single plant in that formulation.

Additional trial with the same product has been published more recently [58]. This time, authors aimed to investigate the effects of Proxelan® monotherapy on the pain symptoms of patients with a clinical diagnosis of chronic abacterial prostatitis or chronic pelvic pain syndrome. Proxelan® suppositories were prescribed to thirty male patients

for a month with a daily dosage of one suppository at bedtime. Subjective pain relief was obtained in all the patients ($p = 0.04$). Urinary symptoms, investigated by questionnaire, decreased significantly ($p = 0.04$), and the quality of life improved ($p = 0.04$). Further seminal investigations were performed on a subset of patients. In a one-month follow-up, leukocytospermia decreased substantially or disappeared, IL-6 decreased by 11.55%, while IL-8 values did not show significant variation. The sperm motility increased by 17.3% and spermatozoa concentration remained unchanged. The medical device showed efficiency in pain reduction, as well as in improvement of semen quality by addressing the inflammatory component of this condition. This trial thus confirmed that Proxelan® monotherapy can be successfully used without antibiotics combination treatment to obtain comparable clinical outcomes in patients with chronic prostatitis or chronic pelvic pain syndrome symptoms. However, the results obtained should be investigated on a larger cohort of patients in randomized controlled trials.

Another trial was published by Varney and Buckle [59], who investigated the effect of *H. italicum* essential oil on mental exhaustion and moderate burnout. Patients were given a personal inhaler with mixture of essential oils (peppermint, basil, and everlasting) or placebo (rose water), which they administered themselves three times in each nostril every hour of the working day (approx. seven times per day) for the duration of five days. According to the authors, this mixture contained two stimulant essential oils to address the fatigue, and one balancing essential oil to address the anxiety. In aromatherapy, *Mentha* x *piperita* (peppermint) essential oil is used to increase alertness and mental clarity, and *Ocimum basilicum* ct *linalol* (basil) essential oil to reduce mental fatigue and achieve antidepressant properties. *H. italicum* essential oil is known for its calming and soothing properties. The participants self-assessed their feelings via questionnaire three times per day in the intervention week, as well as one week before and after. Both groups reported reduction in perception of mental exhaustion or moderate burnout, whereas for the aromatherapy group, reduction was two times greater. Although the results were encouraging, they may not be generalizable due to the small population tested and due to some reported inconsistency in the administration.

The trial led by Cohen et al. [60] was by far the most extensive and multicentered. It included 150 children over four pediatric clinics in Israel. The purpose was to determine if there is comparable efficacy between mucolytic substance carbocysteine and a protective cough syrup (Grintuss®, Sansepolcro, Italy) based on natural ingredients on children's cough due to upper respiratory tract infections, such as the common cold. Mucolytic agents have been shown to be helpful, but serious side effects have been reported, and the use has been prohibited for children under two years of age. Therefore, safer alternatives for cough management, which function via other mechanisms such as irritated pharynx mucosa protection, were explored. Grintuss® syrup contained a combination of specific substances such as resins, polysaccharides, saponins, flavonoids and sugars derived from *Grindelia robusta* (gumweed), *Plantago lanceolata* (ribwort plantain), *H. italicum*, and honey. The protective effect of the syrup on the mucosa of the upper respiratory tract was exerted by a local mechanical barrier (limiting cough stimuli with a non-pharmacological approach, but with an indirect anti-inflammatory action), as well as by radical scavenging action. A survey was conducted among parents on four consecutive days, where treatment was Grintuss® or Mucolit® (Kiryat Malachi, Israel), with single-blinded randomization, 3 times per day for 3 days. Both syrups were well tolerated, and the cough was alleviated. There was a significantly better result throughout for Grintuss® ($p < 0.05$) after one day for all the main outcome measures (cough frequency, cough severity, bothersome nature of cough, and sleep quality for both a child and a parent). The trend for improvement over the four days was steeper for Grintuss® ($p < 0.05$) for all cough parameters. Although both syrups were effective and safe treatments for children over two years of age, Grintuss® appeared to produce faster (first night) and more effective response (over four days of treatment) as to clinical cough symptoms. This trial reveals important information on the safety of *H. italicum* even for young children.

In the trial by Canciani et al. [61], Grintuss® syrup was compared to a placebo syrup in young children suffering from persisting cough. Both syrups were taken in four doses per day for eight days. None of the patients discontinued the trial for adverse events, or other safety reasons. The authors, however, state that Grintuss® should not be used in case of known hypersensitivity to the components of the medical device, but no other contraindications have been registered. It is worth mentioning that at the time of the research, Grintuss® syrup has been on the market for more than ten years, registered as a medical device (class IIa), during which the post-marketing surveillance system, in compliance with Directive 93/42/EC, has not registered incident or side effects related to the medical device, which further supports the safety of this device.

A similar trial by Calapai et al. [62] investigated the effect of KalobaTuss® (Egna–Neumarkt, Italy) syrup in children with persisting cough. This product contained *H. stoechas* as a component, therefore the record was not evaluated further. However, as the *H. stoechas* is closely related to *H. italicum* [7], these findings might also be relevant.

5.2. Individual Bioactive Substances

Several factors, such as the growing conditions, drying, storage and extraction procedure, can greatly affect quality and the composition of an herbal preparation and hence its therapeutic outcome [63]. In modern phytotherapy and traditional medicine, mostly extracts with complex chemical composition are used, rather than isolated substances, favoring occurrence of synergistic effects and polyvalent activity. It needs to be stressed out, that also less abundant compounds can be potent. Consequently, identifying the active constituent in many herbal extracts has often proved to be difficult [64]. Several bioactive compounds were previously isolated from *H. italicum* and their activity investigated (already discussed in Section 3). Pereira et al. [42] investigated the composition of infusions and decoctions of *H. italicum* ssp. *picardii* and established that the main compounds were chlorogenic and quinic acids, dicaffeoylquinic acid isomers and flavonoid gnaphaliin A. According to Karača et al. [65], the most abundant phenolic compounds present in the water extract prepared from commercially available *H. italicum* flowers, were caffeic acid, chlorogenic acid, and its derivatives. Kramberger et al. [66] found that caffeoylquinic acids and pyrones were the most prevalent compounds in *H. italicum* ssp. *italicum* water extracts. Composition of the essential oils, on the other hand, is completely different. In essential oil volatile terpenes predominate to the contrary of polar and semi-polar phenols, which are common in water-based preparations and organic solvent extracts [15]. Some terpenes can also be extracted with non-polar organic solvents or supercritical fluids, but this extraction procedure deviates from the traditional methods of preparations. In the following sub-section, clinical trials on the most relevant individual substances that are confirmed to be present in *H. italicum* water and hydroalcoholic extracts are briefly described.

5.2.1. Phenolic Acids

In contrast to clinical trials evaluating *H. italicum* extracts of a whole plant, trials on isolated substances are more numerous. One of the most studied isolated substances found in *H. italicum* is chlorogenic acid, an ester of caffeic and quinic acid. It is a widely distributed natural compound with many important activities. *In vitro* and in vivo studies have found that the main pharmacological effects of chlorogenic acid are antioxidant, anti-inflammatory, antibacterial, antiviral, hypoglycemic, lipid lowering, anti-cardiovascular, antimutagenic, anticancer and immunomodulatory [45].

In the Cochrane Library, there are 160 Trials matching "chlorogenic acid" in Title Abstract Keyword (Word variations have been searched) (https://www.cochranelibrary.com/, accessed on 4 January 2021), and among them, 71 have been published in the recent four years [67]. The trials mostly investigated effects on cardiovascular system, weight loss, chronic inflammatory diseases, cognition, and lung cancer as well as bioavailability. Chlorogenic acid has been investigated alone or as the main component of some dietary supplements (i.e., green coffee extract). As this ubiquitous phenolic acid is present in largest

quantities in coffee [68], which is a widely consumed beverage, action in the *H. italicum* should not pose health concerns.

Similarly, caffeic acid—a very common phenolic acid with antioxidant, anti-inflammatory and anticarcinogenic activity [44], is also a well investigated compound (68 registered trials, accessed on 17.3.2021) [69]. Trials include effects on esophageal cancer, non-alcoholic fatty liver disease, photoprotection, immune thrombocytopenia, and bioavailability studies.

5.2.2. Flavonoids

Pinocembrin is a well investigated flavonoid of *H. italicum* with demonstrated anti-inflammatory action in vivo [29]. To date, there are only two registered clinical trials on pinocembrin, and both have investigated its neuroprotective effect. Pinocembrin was injected into patients with ischemic stroke [70], and in another trial by Cao et al. [71], pharmacokinetics and safety of pinocembrin injection was investigated. When administered intravenously to healthy adults, pinocembrin was well tolerated up to 120 mg/d. Furthermore, no major safety concerns were identified that would preclude further clinical development of pinocembrin injection.

Quercetin is a versatile antioxidant known to possess protective abilities against tissue injury induced by various drug toxicities [46]. It is present in over twenty plants, in *H. italicum* mostly in the form of various glycosides. There are over 497 registered trials in Cochrane Library (https://www.cochranelibrary.com/, accessed on 17 March 2021) investigating versatile interventions [72]. These include effects on the vascular system (cerebral blood flow, vascular function, blood pressure, thalassemia), inflammation (sarcoidosis, asthma, chronic obstructive pulmonary disease), sex hormone disorders (prostate cancer, prostatitis, polycystic ovary syndrome and estrogen deficiency), metabolic disorders (dyslipidemia, glucose absorption, non-alcoholic fatty liver disease), performance (neuromuscular function, endurance, recovery) and other (immune response, stroke, myocardial infarction, hyperuricemia and oral mucositis).

Naringenin is a commonly found flavonoid in citrus fruits but is also found in its glycoside forms in *H. italicum*. Several biological activities have been ascribed to this phytochemical, above all cardioprotective action is the best investigated clinically [47]. Its effects on liver markers and blood pressure or on metabolic rate, insulin sensitivity and blood glucose have been studied in patients with non-alcoholic fatty liver disease or diabetes, respectively [73]. In the trial by Rebello et al. [74] on safety and pharmacokinetics of naringenin consumption, naringenin proved to be safe in healthy adults (up to 900 mg), and serum concentrations were proportional to the dose administered.

Clinical trials have been performed with luteolin as well. Effects on obesity and cardiometabolic risk factors in metabolic syndrome and on memory and behavior in children with autism were investigated [75]. In addition, its effect on exercise performance was also investigated.

5.2.2. Other Compounds

A triterpene ursolic acid, is one of the non-polar compounds of *H. italicum*, that has been isolated from acetone [37] and methanol extracts [26]. Although it possesses anti-inflammatory, anticancer, antidiabetic, antioxidant and antibacterial effects, its bioavailability and solubility limit its clinical application [48]. Its activity has been investigated in patients with metabolic syndrome and on muscle function. Furthermore, ursolic acid was also injected to patients with solid tumors, where it was shown that ursolic acid liposome does not accumulate in the body. The administration was tolerable, had manageable toxicity, and could potentially improve patient remission rates [76].

Lastly, we want to mention also arzanol, which is probably the most characteristic compound for *H. italicum*. Although extensively investigated in vitro and in vivo, to date, no clinical trial has been registered.

6. Recent Advances in *H. italicum* Studies

Concurrently with the clinical studies, other publications from the past three years were also evaluated. While the majority of the published articles is still focused on *H. italicum* essential oil, quite some of the recent research has been devoted to minimizing waste from the production process and herewith to follow emerging sustainability and upcycling approaches. Essential oil is produced preferentially from the flowerheads, while the rest of the plant, which contains significant amounts of secondary metabolites and could be used for extract production, is left in situ [53]. Dzamic et al. [77] investigated wastewater extracts of *H. italicum* produced after distillation. The highest phenolic concentration was measured in deodorized aqueous extract. The deodorized aqueous extract also possessed the highest antioxidant activity, followed by deodorized methanol extract, while essential oil had the lowest radical scavenging activity. Addis et al. [78] investigated wastewater extracts of aromatic plants, among them also *H. italicum*. They determined that water decoction not only retains antioxidant activity, but is also effective in wound healing, as it promotes tissue re-establishment after environmental stress exposure. Environmental topics continue to arise as Pilić and Martinović [79] investigated effect of *H. italicum* macerate on the corrosion of copper in simulated acid rain solution and Eksi et al. [80] assessed *H. italicum* as a green roof substrate.

Recently, detailed chemical composition and antioxidant activity of hydroalcoholic and water extracts has been evaluated and compared by Kramberger et al. [66]. In addition, further functional studies with water extracts (infusions) have been performed on cell models and gene expression of oxidative-stress related genes has been carried out [81]. In this comparative study, two morphologically distinct *H. italicum* subspecies were compared with a recognized medicinal plant of *H. arenarium*, on a genetic, chemical, and functional level. Both *H. italicum* subspecies exhibited superior antioxidant activity in vitro as well as cytoprotective activity. As it has been emphasized before [7], genetic or morphological description of the plant material used is often lacking in studies, probably due to the difficult characterization of *H. italicum*, arising from great diversity of the species and disunited classification keys available. In the recently published study by Baruca Arbeiter et al. [82], a set of new microsatellites as DNA markers was developed, which will serve for selection of most promising genotypes for propagation and their implementation in agricultural production.

7. Critical Perspective on Safety and Efficacy

H. italicum toxicity has been investigated in some in vitro studies, but in general, this information is scarce. Pereira et al. [42] investigated cytotoxicity of *H. italicum* ssp. *picardii* tisanes towards different mammalian cell lines: hepatocarcinoma (HepG2), microglia (N9) and bone marrow stromal (S17) cell line. The extracts in the tested concentration of 100 µg/mL and after 72 h of exposure had low toxicity, with cell viability values similar or higher than those obtained for green (*Camellia sinensis*) and red bush (*Aspalathus linearis*) teas, which suggest that these aqueous extracts can be regarded as non-toxic beverages. Kramberger et al. [81] evaluated cell viability on lymphoma cell line (U937), adenocarcinoma cell line (Caco-2) and primary colon fibroblasts (CCD112CoN) after exposure to *H. italicum* infusions. Concerning U937 cells infusion was not toxic up to 5% *v/v* concentration, whereas for Caco-2 it was toxic at 1% *v/v*. Interestingly, higher concentration (2% *v/v*) was toxic for CCD112CoN cells, than for cancerous cell line Caco-2. Genotoxic activity of *H. italicum* has been evaluated by Nostro et al. [24], where diethyl ether extract showed no DNA-damaging activity at concentrations up to 2000 µg/disc. Some potential cytochrome P450 enzyme interactions have been discussed by Antunes Viegas et al. [7], more specifically for flavonoid tiliroside. It should be emphasized that the concentrations that are achievable via oral route in vivo may not be sufficient to cause medical important interactions due to low bioavailability. Such interactions are even less likely to occur when administering traditional preparations, where effect of one minor compound usually does not prevail. From the safety perspective, essential oils are potentially more problematic,

as they are more concentrated mixtures. In this case, allergic reactions in hypersensitive individuals can occur [83].

From the current review of the clinical trials, no adverse events that could be attributed to the tested herbal products, were reported. Very importantly, the review also included trials on a large number of young children. Furthermore, *H. italicum* has a long traditional use and several products containing *H. italicum* are already on the market. However, the products are mostly not registered as therapeutics, but rather as dietary supplements or cosmetics. These products include oral supplements developed to favor venous circulation or cough treatment, while cosmetic products, claim the calming and antimicrobial properties of *H. italicum* essential oil incorporated in their formulas [7]. Appendino [84] strongly believes that the former focus on *H. italicum* essential oil and its application in cosmetics, should be turned to development of novel ingredients for medicinal and health-food products. The use of herbal products in general is very complex; apart from medicines, food supplements and cosmetics, botanicals could be marketed also as food (for example spices or herbal teas) or as medical devices, when the plant product can demonstrate a sole mechanical and non-pharmacological action, such as protection of mucous membranes or skin cooling/warming effect [85]. As these products are not proposed as treatments of diseases, demonstration of their clinical profile is not legally required [86]. Nevertheless, consumers usually choose the ones that better respond to their health needs, often ignoring the fact that diverse marketing categories imply profound differences in terms of manufacturing processes, chemical composition, quality controls, and studies of efficacy [85]. Based on these data all together, it can be concluded that *H. italicum* in the orally acceptable formulations is safe to use but would need further evaluation in the case of consideration as an herbal medicine with well-established use.

Efficacy of *H. italicum* has been established several times both in in vitro tests and in humans; first in human clinical observations and more recently also in clinical trials. Although, there are proper clinical trials that demonstrated its tested efficacy, findings are difficult to attribute to *H. italicum* alone, as it was usually not the only plant component tested. From the only one monotherapy trial performed by the company Aboca S.p.A., the findings were unfortunately not available. On the other hand, there are numerous studies and trials conducted with individual compounds present in *H. italicum*, which can contribute to identification of bioactive substances and elucidation of their mechanism of action. However, the findings can be misleading, as the effect of single isolated compound can be more potent due to higher concentrations achieved than in a plant, or even less manifested due to cumulative effects that occur in a compound mixture. For that reason, such findings cannot be directly extrapolated to whole plant extracts.

8. Conclusions

With this review, *H. italicum* has been evaluated in terms of efficacy and safety for internal use. The clinical trials provide rather more insight into the safety profile than into the efficacy, due to lack of trials performed with *H. italicum* alone. The efficacy of *H. italicum*, however, is evident from reports on traditional use, human observational studies, or in vitro research. From the data gathered, particularly the trials in young children, we conclude that the ingestion of *H. italicum* does not pose a risk to human health. Although *H. italicum* is a plant with documented immense potential in several aspects of health, it still lacks regulatory recognition. *H. italicum* could be considered for evaluation by regulatory bodies such as the Committee on Herbal Medicinal Products under the EMA based solely on its traditional use. Although ethnopharmacological reports are available, for that purpose, traditional use of *H. italicum* in European territory would have to be evaluated more thoroughly. On the other hand, to meet the well-established medicinal use criteria, novel clinical studies with *H. italicum* as a single component are needed. Based on the recent trend in *H. italicum* research, it is evident that the current interest in *H. italicum*, especially at Balkan Peninsula, is considerable and expands far beyond cosmetic

applications. As various publications continue to emerge, further clinical trials can also be expected in the future.

Author Contributions: Conceptualization, K.K., S.K., D.B.-M. and N.K.G.; methodology, K.K.; validation, S.K.; investigation, K.K.; resources, K.K.; data curation, K.K.; writing—original draft preparation, K.K.; writing—review and editing, S.K., N.K.G., Z.J.P. and D.B.-M.; visualization, K.K.; supervision, S.K. and D.B.-M.; project administration, Z.J.P. and D.B.-M.; funding acquisition, Z.J.P. and D.B.-M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financially supported by the Slovenian Research Agency (research program P1-0386 and grant number 1000-18-1988 for junior researcher Katja Kramberger).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data is presented within the article.

Conflicts of Interest: The authors declare no conflict 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.

Appendix A

Table A1. Characteristics of included clinical trials, listed in chronological order.

Author, Year, Country	Study Type	Condition	Spec. "Italicum" Specie, Plant Parts, Compos.	Formulation, Dosage	No. of Participants, Sex	Age (y)	Duration	Main Outcome/ Results	Safety, Adverse Events
NA (Aboca S.p.A.), 2005, Italy [56]	Randomized, controlled, parallel	Post-surgery pain	Yes, flowering tops, single component	Granules for oral suspension, 2.5 g	45, m/f	18–65, >65	NA	NA	NA
Galeone, 2012, Italy [57]	Randomized, controlled	Bacterial and non-bacterial prostatitis	No, NA, comb. with other herbs	Suppository (2 g)	60, m	20–50	28 d, follow-up after 60 and 120 d	Improvement of urinary symptoms, no difference in microbiological results	Minor side effects
Varney, 2013, USA [59]	Pilot, randomized, controlled, double-blind	Mental exhaustion, moderate burnout	Yes, essential oil, mixture with two other EOs	Plastic inhaler, 2 drops of *H. italicum* EO per inhaler	14, m/f	25–45, 45–65, >65	3 weeks	Reduction in perceived level of mental fatigue/burnout	Not reported
Cohen, 2013, Israel [60]	Randomized, controlled, single-blind, parallel, multi-center	Cough associated with upper respiratory tract infections	Yes, NA, comb. with other herbs and honey	Syrup, 20 mL in three doses per day	150, m/f	2–5	4 d	Reduced cough frequency, severity, better sleep quality	Safe, well tolerated
Canciani, 2014, Italy [61]	Randomized, double-blind, multicenter, placebo-controlled	Cough associated with upper respiratory tract infections, persisting > 7 d	Yes, NA, comb. with other herbs and honey	Syrup, 4 doses per day (5 mL each)	102, m/f	3–6	8 d	The significant decrease of cough, especially evident at night	Adverse events unrelated to the treatment
Di Vico, 2019, Italy [58]	Pilot, randomized, controlled	Chronic prostatitis, chronic pelvic pain syndrome	No, NA, comb. with other herbs	Suppository (2 g), 1 supp./day	30, m	23–49	1 month	Subjective pain relief, decrease in urinary symptoms	None reported

NA—information not available, EO—essential oil, m—male, f—female, no.—number, comb.—combination, supp.—suppository, spec.—specified, compos.—composition of a product when *H. italicum* is not the single component, y—years.

References

1. Faqi, A.S.; Yan, J.S. Chapter 30—Nonclinical safety assessment of botanical products. In *A Comprehensive Guide to Toxicology in Nonclinical Drug Development*, 2nd ed.; Faqi, A.S., Ed.; Academic Press: Boston, MA, USA, 2017; pp. 813–823, ISBN 978–0-12-803620-4.
2. Carmona, F.; Soares Pereira, A.M. Herbal medicines: Old and new concepts, truths and misunderstandings. *Rev. Bras. Farmacogn.* **2013**, *23*, 379–385. [CrossRef]
3. Rivera, D.; Obon, C.; Inocencio, C.; Heinrich, M.; Verde, A.; Fajardo, J.; Llorach, R. The ethnobotanical study of local mediterranean food plants as medicinal resources in southern Spain. *J. Physiol. Pharmacol.* **2005**, *56*, 97–114.
4. Akaberi, M.; Sahebkar, A.; Azizi, N.; Emami, S.A. Everlasting flowers: Phytochemistry and pharmacology of the genus *Helichrysum*. *Ind. Crop. Prod.* **2019**, *138*, 111471. [CrossRef]
5. Perrini, R.; Alba, V.; Ruta, C.; Morone-Fortunato, I.; Blanco, A.; Montemurro, C. An evaluation of a new approach to the regeneration of *Helichrysum italicum* (Roth) G. Don, and the molecular characterization of the variation among sets of differently derived regenerants. *Cell. Mol. Biol. Lett.* **2009**, *14*, 377–394. [CrossRef]

6. Galbany-Casals, M.; Blanco-Moreno, J.M.; Garcia-Jacas, N.; Breitwieser, I.; Smissen, R.D. Genetic variation in mediterranean *Helichrysum italicum* (asteraceae; gnaphalieae): Do disjunct populations of subsp. microphyllum have a common origin? *Plant Biol.* **2011**, *13*, 678–687. [CrossRef]
7. Antunes Viegas, D.; Palmeira-de-Oliveira, A.; Salgueiro, L.; Martinez-de-Oliveira, J.; Palmeira-de-Oliveira, R. *Helichrysum italicum*: From traditional use to scientific data. *J. Ethnopharmacol.* **2014**, *151*, 54–65. [CrossRef]
8. Les, F.; Venditti, A.; Cásedas, G.; Frezza, C.; Guiso, M.; Sciubba, F.; Serafini, M.; Bianco, A.; Valero, M.S.; López, V. Everlasting Flower (*Helichrysum* stoechas moench) as a potential source of bioactive molecules with antiproliferative, antioxidant, antidiabetic and neuroprotective properties. *Ind. Crop. Prod.* **2017**, *108*, 295–302. [CrossRef]
9. Pljevljakušić, D.; Bigović, D.; Janković, T.; Jelačić, S.; Šavikin, K. Sandy everlasting (*Helichrysum* arenarium (l.) moench): Botanical, chemical and biological properties. *Front. Plant. Sci.* **2018**, *9*, 1123. [CrossRef]
10. Ninčević, T.; Grdiša, M.; Šatović, Z.; Jug-Dujaković, M. *Helichrysum italicum* (Roth) G. Don: Taxonomy, biological activity, biochemical and genetic diversity. *Ind. Crop. Prod.* **2019**, *138*, 111487. [CrossRef]
11. Blažević, N.; Petričić, J.; Stanić, G.; Maleš, Ž. Variations in yields and composition of immortelle (*Helichrysum italicum*, Roth Guss.) essential oil from different locations and vegetation periods along adriatic coast. *Acta Pharm.* **1995**, *45*, 517–522.
12. Benítez, G.; González-Tejero, M.R.; Molero-Mesa, J. Pharmaceutical ethnobotany in the western part of granada province (southern Spain): Ethnopharmacological synthesis. *J. Ethnopharmacol.* **2010**, *129*, 87–105. [CrossRef] [PubMed]
13. Manach, C.; Scalbert, A.; Morand, C.; Rémésy, C.; Jiménez, L. Polyphenols: Food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79*, 727–747. [CrossRef] [PubMed]
14. Appendino, G.; Ottino, M.; Marquez, N.; Bianchi, F.; Giana, A.; Ballero, M.; Sterner, O.; Fiebich, B.L.; Munoz, E. Arzanol, an anti-inflammatory and anti-HIV-1 Phloroglucinol alpha-pyrone from *Helichrysum italicum* Ssp. microphyllum. *J. Nat. Prod.* **2007**, *70*, 608–612. [CrossRef]
15. Maksimovic, S.; Tadic, V.; Skala, D.; Zizovic, I. Separation of phytochemicals from *Helichrysum italicum*: An analysis of different isolation techniques and biological activity of prepared extracts. *Phytochemistry* **2017**, *138*, 9–28. [CrossRef]
16. Facino, R.M.; Carini, M.; Mariani, M.; Cipriani, C. Anti erythematous and photoprotective activities in guinea pigs and in man of topically applied flavonoids from *Helichrysum italicum* G. Don. *Acta Ther.* **1988**, *14*, 323–346.
17. Facino, R.M.; Carini, M.; Franzoi, L.; Pirola, O.; Bosisio, E. Phytochemical characterization and radical scavenger activity of flavonoids from *Helichrysum italicum* G. Don (compositae). *Pharmacol. Res.* **1990**, *22*, 709–721. [CrossRef]
18. Pietta, P.; Mauri, P.; Gardana, C.; Facino, R.M.; Carini, M. High-performance liquid chromatographic determination of flavonoid glucosides from *Helichrysum italicum*. *J. Chromatogr. A* **1991**, *537*, 449–452. [CrossRef]
19. Pietta, P.; Mauri, P.; Facino, R.M.; Carini, M. Analysis of flavonoids by MECC with ultraviolet diode array detection. *J. Pharm. Biomed. Anal.* **1992**, *10*, 1041–1045. [CrossRef]
20. Zapesochnaya, G.G.; Dzyadevich, T.V.; Karasartov, B.S. Phenolic compounds of *Helichrysum italicum*. *Chem. Nat. Compd.* **1990**, *26*, 342–343. [CrossRef]
21. Zapesochnaya, G.G.; Kurkin, V.A.; Kudryavtseva, T.V.; Karasartov, B.S.; Cholponbaev, K.S.; Tyukavkina, N.A.; Ruchkin, V.E. Dicaffeolyquinic acids from *Helichrysum italicum* and achillea cartilaginea. *Chem. Nat. Compd.* **1992**, *28*, 40–44. [CrossRef]
22. Nostro, A.; Bisignano, G.; Angela Cannatelli, M.; Crisafi, G.; Paola Germanò, M.; Alonzo, V. Effects of *Helichrysum italicum* extract on growth and enzymatic activity of staphylococcus aureus. *Int. J. Antimicrob. Agents* **2001**, *17*, 517–520. [CrossRef]
23. Nostro, A.; Cannatelli, M.A.; Musolino, A.D.; Procopio, F.; Alonzo, V. *Helichrysum italicum* extract interferes with the production of enterotoxins by staphylococcus aureus. *Lett. Appl. Microbiol.* **2002**, *35*, 181–184. [CrossRef]
24. Nostro, A.; Cannatelli, M.A.; Marino, A.; Picerno, I.; Pizzimenti, F.C.; Scoglio, M.E.; Spataro, P. Evaluation of antiherpesvirus-1 and genotoxic activities of *Helichrysum italicum* extract. *New Microbiol.* **2003**, *26*, 125–128. [PubMed]
25. Nostro, A.; Cannatelli, M.A.; Crisafi, G.; Musolino, A.D.; Procopio, F.; Alonzo, V. Modifications of hydrophobicity, In Vitro adherence and cellular aggregation of streptococcus mutans by *Helichrysum italicum* extract. *Lett. Appl. Microbiol.* **2004**, *38*, 423–427. [CrossRef] [PubMed]
26. Sala, A.; Recio, M.C.; Giner, R.M.; Máñez, S.; Ríos, J.L. New acetophenone glucosides isolated from extracts of *Helichrysum italicum* with antiinflammatory activity. *J. Nat. Prod.* **2001**, *64*, 1360–1362. [CrossRef]
27. Sala, A.; Recio, M.; Giner, R.M.; Máñez, S.; Tournier, H.; Schinella, G.; Ríos, J.-L. Anti-inflammatory and antioxidant properties of *Helichrysum italicum*. *J. Pharm. Pharmacol.* **2002**, *54*, 365–371. [CrossRef] [PubMed]
28. Sala, A.; Recio, M.C.; Schinella, G.R.; Máñez, S.; Giner, R.M.; Ríos, J.-L. A new dual inhibitor of arachidonate metabolism isolated from *Helichrysum italicum*. *Eur. J. Pharmacol.* **2003**, *460*, 219–226. [CrossRef]
29. Sala, A.; Recio, M.C.; Schinella, G.R.; Máñez, S.; Giner, R.M.; Cerdá-Nicolás, M.; Ríos, J.-L. Assessment of the anti-inflammatory activity and free radical scavenger activity of tiliroside. *Eur. J. Pharmacol.* **2003**, *461*, 53–61. [CrossRef]
30. Schinella, G.R.; Tournier, H.A.; Prieto, J.M.; de Buschiazzo, P.M.; Ríos, J.L. Antioxidant activity of anti-inflammatory plant extracts. *Life Sci.* **2002**, *70*, 1023–1033. [CrossRef]
31. Schinella, G.R.; Tournier, H.A.; Máñez, S.; de Buschiazzo, P.M.; del Carmen Recio, M.; Ríos, J.L. Tiliroside and gnaphaliin inhibit human low density lipoprotein oxidation. *Fitoterapia* **2007**, *78*, 1–6. [CrossRef] [PubMed]
32. Wollenweber, E.; Christ, M.; Dunstan, R.H.; Roitman, J.N.; Stevens, J.F. Exudate Flavonoids in some gnaphalieae and inuleae (asteraceae). *Z. Nat. C* **2005**, *60*, 671–678. [CrossRef] [PubMed]

33. Rosa, A.; Deiana, M.; Atzeri, A.; Corona, G.; Incani, A.; Melis, M.P.; Appendino, G.; Dessì, M.A. Evaluation of the antioxidant and cytotoxic activity of arzanol, a prenylated α-pyrone–phloroglucinol etherodimer from *Helichrysum italicum* subsp.microphyllum. *Chem. Biol. Interact.* **2007**, *165*, 117–126. [CrossRef]
34. Rosa, A.; Pollastro, F.; Atzeri, A.; Appendino, G.; Melis, M.P.; Deiana, M.; Incani, A.; Loru, D.; Dessì, M.A. Protective role of arzanol against lipid peroxidation in biological systems. *Chem. Phys. Lipids* **2011**, *164*, 24–32. [CrossRef] [PubMed]
35. Bauer, J.; Koeberle, A.; Dehm, F.; Pollastro, F.; Appendino, G.; Northoff, H.; Rossi, A.; Sautebin, L.; Werz, O. Arzanol, a prenylated heterodimeric phloroglucinyl pyrone, inhibits eicosanoid biosynthesis and exhibits anti-inflammatory efficacy In Vivo. *Biochem. Pharmacol.* **2011**, *81*, 259–268. [CrossRef] [PubMed]
36. D'Abrosca, B.; Buommino, E.; D'Angelo, G.; Coretti, L.; Scognamiglio, M.; Severino, V.; Pacifico, S.; Donnarumma, G.; Fiorentino, A. Spectroscopic identification and anti-biofilm properties of polar metabolites from the medicinal plant *Helichrysum italicum* against pseudomonas aeruginosa. *Bioorg. Med. Chem.* **2013**, *21*, 7038–7046. [CrossRef]
37. Taglialatela-Scafati, O.; Pollastro, F.; Chianese, G.; Minassi, A.; Gibbons, S.; Arunotayanun, W.; Mabebie, B.; Ballero, M.; Appendino, G. Antimicrobial phenolics and unusual glycerides from *Helichrysum italicum* subsp. microphyllum. *J. Nat. Prod.* **2013**, *76*, 346–353. [CrossRef]
38. de la Garza, A.L.; Etxeberria, U.; Lostao, M.P.; San Román, B.; Barrenetxe, J.; Martínez, J.A.; Milagro, F.I. *Helichrysum* and grapefruit extracts inhibit carbohydrate digestion and absorption, improving postprandial glucose levels and hyperinsulinemia in rats. *J. Agric. Food Chem.* **2013**, *61*, 12012–12019. [CrossRef] [PubMed]
39. Rigano, D.; Formisano, C.; Senatore, F.; Piacente, S.; Pagano, E.; Capasso, R.; Borrelli, F.; Izzo, A.A. Intestinal antispasmodic effects of *Helichrysum italicum* (Roth) Don ssp. italicum and chemical identification of the active ingredients. *J Ethnopharmacol.* **2013**, *150*, 901–906. [CrossRef] [PubMed]
40. Rigano, D.; Formisano, C.; Pagano, E.; Senatore, F.; Piacente, S.; Masullo, M.; Capasso, R.; Izzo, A.A.; Borrelli, F. A new acetophenone derivative from flowers of *Helichrysum italicum* (Roth) Don ssp. italicum. *Fitoterapia* **2014**, *99*, 198–203. [CrossRef] [PubMed]
41. Mari, A.; Napolitano, A.; Masullo, M.; Pizza, C.; Piacente, S. Identification and quantitative determination of the polar constituents in *Helichrysum italicum* flowers and derived food supplements. *J Pharm. Biomed. Anal.* **2014**, *96*, 249–255. [CrossRef]
42. Pereira, C.G.; Barreira, L.; Bijttebier, S.; Pieters, L.; Neves, V.; Rodrigues, M.; Rivas, R.; Varela, J.; Custodio, L. Chemical profiling of infusions and decoctions of *Helichrysum italicum* Subsp. picardii by UHPLC-PDA-MS and In Vitro biological activities comparatively with green tea (Camellia sinensis) and rooibos tisane (Aspalathus linearis). *J. Pharm. Biomed. Anal.* **2017**, *145*, 593–603. [CrossRef]
43. Kothavade, P.S.; Nagmoti, D.M.; Bulani, V.D.; Juvekar, A.R. Arzanol, a potent MPGES-1 inhibitor: Novel anti-inflammatory agent. *Sci. World J.* **2013**, *2013*, 1–9. [CrossRef]
44. Espíndola, K.M.M.; Ferreira, R.G.; Narvaez, L.E.M.; Silva Rosario, A.C.R.; da Silva, A.H.M.; Silva, A.G.B.; Vieira, A.P.O.; Monteiro, M.C. Chemical and pharmacological aspects of caffeic acid and its activity in hepatocarcinoma. *Front. Oncol.* **2019**, *9*, 541. [CrossRef]
45. Miao, M.; Xiang, L. Chapter Three—Pharmacological action and potential targets of chlorogenic acid. In *Advances in Pharmacology*; Du, G., Ed.; Pharmacological Advances in Natural Product Drug Discovery; Academic Press: Boston, MA, USA, 2020; Volume 87, pp. 71–88.
46. Anand David, A.V.; Arulmoli, R.; Parasuraman, S. Overviews of biological importance of quercetin: A bioactive flavonoid. *Pharmacogn. Rev.* **2016**, *10*, 84–89. [CrossRef]
47. Salehi, B.; Fokou, P.V.T.; Sharifi-Rad, M.; Zucca, P.; Pezzani, R.; Martins, N.; Sharifi-Rad, J. The therapeutic potential of naringenin: A review of clinical trials. *Pharmaceuticals* **2019**, *12*, 11. [CrossRef] [PubMed]
48. Mlala, S.; Oyedeji, A.O.; Gondwe, M.; Oyedeji, O.O. Ursolic acid and its derivatives as bioactive agents. *Molecules* **2019**, *24*, 2751. [CrossRef] [PubMed]
49. Santini, L. Rassegna clinico-statistica sulle proprietà terapeutiche dell élicrisio. *Minerva Med.* **1952**, *43*, 714–719. [PubMed]
50. Benigni, R.; Capra, C.; Cattorini, P. *Piante Medicinali: Chimica, Farmacologia e Terapia*; Inverni & Della Beffa: Milano, Italy, 1962.
51. Vannini, C. *Atti II Seminario Internazionale Piante Medicinali e Medicina Tradizionale*; Maremagnum: Città di Castello, Italy, 1981.
52. Campanini, E. *Helichrysum* angustifolium: Esperienze cliniche sulla psoriasi. *Acta Phytother.* **1995**, *1*, 8–10.
53. Appendino, G.; Taglialatela-Scafati, O.; Minassi, A.; Pollastro, F.; Ballero, M.; Maxia, A.; Sanna, C. *Helichrysum italicum*: The sleeping giant of mediterranean herbal medicine. *Herb. J. Am. Bot. Counc.* **2015**, *105*, 34–45.
54. Voinchet, V.; Giraud-Robert, A.-M. Utilisation de l'huile essentielle d'hélichryse italienne et de l'huile végétale de rose musquée après intervention de chirurgie plastique réparatrice et esthétique. *Phytothérapie* **2007**, *5*, 67–72. [CrossRef]
55. Granger, C.; Brown, A.; Aladren, S.; Narda, M. Night cream containing melatonin, carnosine and *Helichrysum italicum* extract helps reduce skin reactivity and signs of photodamage: Ex Vivo and clinical studies. *Dermatol. Ther.* **2020**, *10*, 1315–1329. [CrossRef] [PubMed]
56. Randomised Parallel Study against Placebo for the Determination of Efficacy of Liophilised Salvia Officinalis and Helichrysum italicum in Pain Treatment from Post-Surgery Pain in 45 Male or Female Patients. Available online: https://www.clinicaltrialsregister.eu/ctr-search/trial/2005-000958-71/IT/ (accessed on 30 December 2020).
57. Galeone, G.; Spadavecchia, R.; Balducci, M.T.; Pagliarulo, V. The role of proxelan in the treatment of chronic prostatitis. Results of a randomized trial. *Minerva Urol. Nefrol.* **2012**, *64*, 135–141. [PubMed]

58. Di Vico, T.; Durante, J.; Polito, C.; Tognarelli, A.; Canale, D.; Caglieresi, C.; Morelli, G.; Bartoletti, R. Pumpkin seeds, centella asiatica, boswellia, *Helichrysum*, acetate vitamin e, melaleuca alternifolia and hyaluronic acid phytocomplex monotherapy effects in patients with chronic pelvic pain syndrome. *Minerva Urol. Nefrol.* **2020**, *72*, 236–242. [CrossRef]
59. Varney, E.; Buckle, J. Effect of inhaled essential oils on mental exhaustion and moderate burnout: A small pilot study. *J. Altern. Complement. Med.* **2013**, *19*, 69–71. [CrossRef] [PubMed]
60. Cohen, H.A.; Blau, H.; Gur, S.; Moshe, H.; Ran, B. Randomized, single-blinded study to evaluate the efficacy of grintuss and mucolit pediatric syrups for cough due to upper respiratory tract infection. *Pediatric Pulmonol.* **2015**, *50*, S53–S83. [CrossRef]
61. Canciani, M.; Murgia, V.; Caimmi, D.; Anapurapu, S.; Licari, A.; Marseglia, G.L. Efficacy of Grintuss® pediatric syrup in treating cough in children: A randomized, multicenter, double blind, placebo-controlled clinical trial. *Ital. J. Pediatr.* **2014**, *40*, 56. [CrossRef] [PubMed]
62. Calapai, G. Randomized, Controlled, Double Blind Clinical Study on the Assessment of Efficacy, Safety and Palatability of "KalobaTuss®Children", a Syrup for the Treatment of Cough in Pediatric Age (3–6 Years). Available online: https://clinicaltrials.gov/ct2/show/NCT04073251 (accessed on 30 December 2020).
63. Sendker, J.; Sheridan, H. Composition and quality control of herbal medicines. In *Toxicology of Herbal Products*; Pelkonen, O., Duez, P., Vuorela, P.M., Vuorela, H., Eds.; Springer International Publishing: Cham, Switzerland, 2017; pp. 29–65, ISBN 978-3-319-43806-1.
64. 2-Principles of herbal pharmacology. In *Principles and Practice of Phytotherapy*, 2nd ed.; Bone, K.; Mills, S. (Eds.) Churchill Livingstone: Saint Louis, MO, USA, 2013; pp. 17–82, ISBN 978-0-443-06992-5.
65. Karača, S.; Trifković, K.; Bušić, A.; Đorđević, V.; Belščak-Cvitanović, A.; Cebin, A.V.; Bugarski, B.; Komes, D. The functional potential of immortelle (*Helichrysum italicum*) based edible films reinforced with proteins and hydrogel particles. *LWT* **2019**, *99*, 387–395. [CrossRef]
66. Kramberger, K.; Barlič-Maganja, D.; Bandelj, D.; Baruca Arbeiter, A.; Peeters, K.; Miklavčič Višnjevec, A.; Jenko Pražnikar, Z. HPLC-DAD-ESI-QTOF-MS determination of bioactive compounds and antioxidant activity comparison of the hydroalcoholic and water extracts from two *Helichrysum italicum* species. *Metabolites* **2020**, *10*, 403. [CrossRef] [PubMed]
67. Records in Cochrane Library on Chlorogenic Acid. Available online: https://www.cochranelibrary.com/advanced-search?q=chlorogenic%20acid&t=1 (accessed on 2 May 2021).
68. Naveed, M.; Hejazi, V.; Abbas, M.; Kamboh, A.A.; Khan, G.J.; Shumzaid, M.; Ahmad, F.; Babazadeh, D.; FangFang, X.; Modarresi-Ghazani, F.; et al. Chlorogenic acid (CGA): A pharmacological review and call for further research. *Biomed. Pharmacother.* **2018**, *97*, 67–74. [CrossRef] [PubMed]
69. Records in Cochrane Library on Caffeic Acid. Available online: https://www.cochranelibrary.com/advanced-search?q=caffeic%20acid&t=1 (accessed on 2 May 2021).
70. Phase II Study of Pinocembrin Injection to Treat Ischemic Stroke—Randomized, Double-Blind, Placebo-Controlled, Multicenter Study. Available online: https://clinicaltrials.gov/ct2/show/NCT02059785 (accessed on 29 April 2021).
71. Cao, G.; Ying, P.; Yan, B.; Xue, W.; Li, K.; Shi, A.; Sun, T.; Yan, J.; Hu, X. Pharmacokinetics, safety, and tolerability of single and multiple-doses of pinocembrin injection administered intravenously in healthy subjects. *J. Ethnopharmacol.* **2015**, *168*, 31–36. [CrossRef] [PubMed]
72. Records in Cochrane Library on Quercetin. Available online: https://www.cochranelibrary.com/advanced-search (accessed on 2 May 2021).
73. Records in Cochrane Library on Naringenin. Available online: https://www.cochranelibrary.com/advanced-search?q=naringenin&t=1 (accessed on 2 May 2021).
74. Rebello, C.J.; Beyl, R.A.; Lertora, J.J.L.; Greenway, F.L.; Ravussin, E.; Ribnicky, D.M.; Poulev, A.; Kennedy, B.J.; Castro, H.F.; Campagna, S.R.; et al. Safety and pharmacokinetics of naringenin: A randomized, controlled, single-ascending-dose clinical trial. *Diabetes Obes. Metab.* **2020**, *22*, 91–98. [CrossRef] [PubMed]
75. Records in Cochrane Library on Luteolin. Available online: https://www.cochranelibrary.com/advanced-search?q=luteolin&t=1 (accessed on 2 May 2021).
76. Qian, Z.; Wang, X.; Song, Z.; Zhang, H.; Zhou, S.; Zhao, J.; Wang, H. A phase I trial to evaluate the multiple-dose safety and antitumor activity of ursolic acid liposomes in subjects with advanced solid tumors. *BioMed Res. Int.* **2015**, *2015*, e809714. [CrossRef]
77. Dzamic, A.M.; Mileski, K.S.; Ciric, A.D.; Ristic, M.S.; Sokovic, M.D.; Marin, P.D. Essential oil composition, antioxidant and antimicrobial properties of essential oil and deodorized extracts of *Helichrysum italicum* (Roth) G. Don. *J. Essent. Oil Bear. Plants* **2019**, *22*, 493–503. [CrossRef]
78. Addis, R.; Cruciani, S.; Santaniello, S.; Bellu, E.; Sarais, G.; Ventura, C.; Maioli, M.; Pintore, G. Fibroblast proliferation and migration in wound healing by phytochemicals: Evidence for a novel synergic outcome. *Int. J. Med. Sci.* **2020**, *17*, 1030–1042. [CrossRef] [PubMed]
79. Pilić, Z.; Martinović, I. Effect of *Helichrysum italicum* on the corrosion of copper in simulated acid rain solution. *Chem. Biochem. Eng. Q.* **2019**, *33*, 449–457. [CrossRef]
80. Eksi, M.; Sevgi, O.; Akburak, S.; Yurtseven, H.; Esin, İ. Assessment of recycled or locally available materials as green roof substrates. *Ecol. Eng.* **2020**, *156*, 105966. [CrossRef]

81. Kramberger, K.; Pražnikar, Z.J.; Baruca Arbeiter, A.; Petelin, A.; Bandelj, D.; Kenig, S. A comparative study of the antioxidative effects of *Helichrysum italicum* and *Helichrysum* arenarium infusions. *Antioxidants* **2021**, *10*, 380. [CrossRef] [PubMed]
82. Baruca Arbeiter, A.; Hladnik, M.; Jakše, J.; Bandelj, D. First set of microsatellite markers for immortelle (*Helichrysum italicum* (Roth) G. Don): A step towards the selection of the most promising genotypes for cultivation. *Ind. Crop. Prod.* **2021**, *162*, 113298. [CrossRef]
83. Foti, C.; Guida, S.; Antelmi, A.; Romita, P.; Corazza, M. Allergic contact dermatitis caused by *Helichrysum italicum* contained in an emollient cream. *Contact Dermat.* **2013**, *69*, 62–63. [CrossRef]
84. Appendino, G. *Helichrysum italicum*: Back to medicine from the tinsel of luxury. *Planta Med.* **2015**, *81*, PL_09. [CrossRef]
85. Biagi, M.; Pecorari, R.; Appendino, G.; Miraldi, E.; Magnano, A.R.; Governa, P.; Cettolin, G.; Giachetti, D. Herbal products in Italy: The thin line between phytotherapy, nutrition and parapharmaceuticals; a normative overview of the fastest growing market in Europe. *Pharmaceuticals* **2016**, *9*, 65. [CrossRef] [PubMed]
86. Thakkar, S.; Anklam, E.; Xu, A.; Ulberth, F.; Li, J.; Li, B.; Hugas, M.; Sarma, N.; Crerar, S.; Swift, S.; et al. Regulatory landscape of dietary supplements and herbal medicines from a global perspective. *Regul. Toxicol. Pharmacol.* **2020**, *114*, 104647. [CrossRef] [PubMed]

plants

MDPI

Article

Climatic Drivers of the Complex Phenology of the Mediterranean Semi-Deciduous Shrub *Phlomis fruticosa* Based on Satellite-Derived EVI

Aris Kyparissis * and Efi Levizou

Department of Agriculture Crop Production and Rural Environment, University of Thessaly, Fytokou Str., 384 46 Volos, Greece; elevizou@uth.gr
* Correspondence: akypar@uth.gr

Abstract: A 21-year Enhanced Vegetation Index (EVI) time-series produced from MODIS satellite images was used to study the complex phenological cycle of the drought semi-deciduous shrub *Phlomis fruticosa* and additionally to identify and compare phenological events between two Mediterranean sites with different microclimates. In the more xeric Araxos site, spring leaf fall starts earlier, autumn revival occurs later, and the dry period is longer, compared with the more favorable Louros site. Accordingly, the control of climatic factors on phenological events was examined and found that the Araxos site is mostly influenced by rain related events while Louros site by both rain and temperature. Spring phenological events showed significant shifts at a rate of 1–4.9 days per year in Araxos, which were positively related to trends for decreasing spring precipitation and increasing summer temperature. Furthermore, the climatic control on the inter-annual EVI fluctuation was examined through multiple linear regression and machine learning approaches. For both sites, temperature during the previous 2–3 months and rain days of the previous 3 months were identified as the main drivers of the EVI profile. Our results emphasize the importance of focusing on a single species and small-spatial-scale information in connecting vegetation responses to the climate crisis.

Keywords: remote sensing; MODIS; enhanced vegetation index; temperature; precipitation; rain days; inter-annual variability; time-series; machine learning; climate change

Citation: Kyparissis, A.; Levizou, E. Climatic Drivers of the Complex Phenology of the Mediterranean Semi-Deciduous Shrub *Phlomis fruticosa* Based on Satellite-Derived EVI. *Plants* **2022**, *11*, 584. https://doi.org/10.3390/plants11050584

Academic Editor: Stefan Zerbe

Received: 30 December 2021
Accepted: 20 February 2022
Published: 22 February 2022

1. Introduction

Vegetation response to climate variability is becoming increasingly important, especially under the frame of the ongoing global climate change [1–3]. Our understanding of vegetation function, its interactions with the climate, the key controlling mechanisms, and its vulnerability to climate change are far from complete. Evidently, understanding climatic influences on processes and interactions enables the prediction of changes under different climatic scenarios [4,5].

The most consistent results of climate change experiments are the species-specific responses. Many experiments that have been conducted worldwide, including manipulations of the CO_2 and UV-B environment, temperature rise, and N deposition, have manifested that plant species differ in their sensitivity to damage and their morphological, biochemical, and physiological responses to altered environmental factors [6,7]. The exact position held by a certain species in the sensitivity-tolerance continuum, as well as its specific responses, could cascade upwards to alter community and ecosystem composition and structure through changes in the competitive balance between species [8,9]. Additionally, the proposed conceptual frameworks for analysis of the species and ecosystem response to changing climate underline the importance of thresholds for interpreting experimental results and predicting effects [10]. Rapid, nonlinear changes in some plant processes or responses can be triggered by even small differences in environmental conditions if threshold values are exceeded. This evidence stresses the importance of studies focusing

on direct and indirect effects of environmental change on plant species and not only on large formations and ecosystem-level spatial scales.

Plant phenology is considered an important factor that mediates vegetation and climate relationships, through affecting a diverse set of processes [2]. Phenology is not merely the succession of recurrent biological events in the plant's lifecycle, but it also greatly relates to plant activity, since different phenophases affect plant function and resource allocation patterns [11]. The phenology–climate feedbacks are bi-directional. At one end is the climate impact on the timing and duration of phenological events [3,12]. At the other end is the influence of phenological events and, moreover, transitions on vegetation feedbacks to the microclimate, i.e., humidity, temperature, wind speed, as well as soil moisture and topsoil temperature [2]. Scaling up at the community and ecosystem level, phenology influences processes and mechanisms such as water, CO_2, and energy fluxes which feedback to large-scale vegetation–atmosphere interactions. The well-established sensitivity of phenology to year-to-year variability in climate could also serve as an indicator of the long-term biological impacts of climate change on terrestrial ecosystems [13,14].

Remotely sensed data proved to be a valuable tool in coupling climate and vegetation distribution/performance at large spatial and temporal scales. As a result, the objective of many studies was the assessment of the effects of certain environmental factors on remote-sensing-derived vegetation parameters [15–17]. A common feature in most of the above-mentioned research efforts is the large spatial scale used, i.e., regional, continental or global, exploiting satellite-derived simultaneous estimates of ecosystem function over wide areas. Indeed, remote sensing of vegetation offers a promising and urgently needed assessment of ecosystem function at a spatial scale that is comparable with the extent of human-caused environmental change. However, information on specific species performance, which is possibly incorporated in remote sensed data, is rather lost in the inevitably vague picture given by large spatial-scale studies [18]. Ecophysiological field surveys could address this issue, but because of laborious and time-consuming measurements, they have the disadvantage of temporal and spatial limitations. Alternatively, satellite imagery in the context of studying a particular species' behavior, i.e., at small spatial scale, may render an accurate picture of species responses to natural climate variability, as well as climate change [19,20].

The focus on species and use of satellite data to study species-level responses, from a phenological and especially an eco-physiological point of view, to climate forcing has an essential prerequisite: strong correspondence with ground-measured plant processes or features [21,22]. Indeed, established relationships between ground-determined characteristics and their satellite-derived surrogates in terms of vegetation indices allow for an explicit physiological meaning to the latter. This in turn permits understanding, monitoring, and explaining species behavior, as well as identifying broad patterns in space and time, including a species' relationship with environmental determinants. Collectively, established correlations enable exploiting the advantages of remote sensed data, i.e., large spatial and temporal scales, with direct reference to species phenological/physiological characteristics. The above-mentioned benefits justify the intensive research efforts of the last two decades devoted in establishing such correlations [22–25].

The Enhanced Vegetation Index (EVI) has been shown to be well correlated with LAI, biomass, canopy cover, and the fraction of absorbed photosynthetically active radiation [26,27], and is therefore useful for monitoring seasonal, inter-annual, and long-term variation of the vegetation structure and function [28]. EVI has been used instead of the Normalized Difference Vegetation Index (NDVI) because it reduces sensitivity to soil and atmospheric effects and remains sensitive to variation in canopy density where NDVI becomes saturated [29–31]. Given these characteristics, modelers have begun using EVI data to predict net primary production in ecosystem modelling applications [24,29].

Under the above-described framework, a 21-year EVI time-series is used in the present study to assess climatic effects on the growth cycle of the drought semi-deciduous Mediterranean shrub *Phlomis fruticosa*. *P. fruticosa* was chosen for the following reasons: (a) it is

a typical drought shrub of Mediterranean ecosystems and, moreover is considered a key species of the garrigue vegetation which dominates the most xeric parts of the Mediterranean basin [32]; (b) we consider this species an ecological indicator of overgrazed ecosystems in which it forms large, continuous, and undisturbed stands, exclusively covered by this particular species, because it is not eatable by major farm animals (sheep, goats); (c) it has a multi-phase intra-annual growth cycle, with distinct phases being possible targets to climatic effects, which makes *P. fruticosa* a good model plant for satellite-derived phenology studies; (d) its growth cycle has been extensively studied through eco-physiological field measurements [33,34]; thus, there are established relationships between growth/eco-physiological parameters and satellite indices [22,25]; (e) the plasticity and adaptability of *P. fruticosa*, although established by field eco-physiological measurements have not been validated in large space and time scales, as those provided by satellite imagery.

For the purposes of the present study, two distant *P. fruticosa* ecosystems, with differences in climatic characteristics were chosen. The aims were (a) to depict the complex phenological cycle of this species through satellite-derived EVI and extract metrics that analyze the phenological events and transitions, (b) to determine the climatic drivers that influence the phenophases and their possible differences between the two sites, and (c) to identify trends for change, which could further be used as diagnostic and prognostic tool for climate crisis effects.

2. Materials and Methods

2.1. Study Sites

Two ecosystems dominated by *P. fruticosa* with different climatic characteristics were chosen in order to study possible differences in climate control and plant responses (Figure 1): (a) Araxos area, the southern one (NW Peloponessos, Greece, 38.18° N, 21.37° E), characterized by a prolonged summer stress period where high temperature co-exists with a severe water shortage and (b) Louros area, the northern one (Epirus, Greece, 39.17° N, 20.85° E), with more favorable temperature and water availability conditions for plant growth.

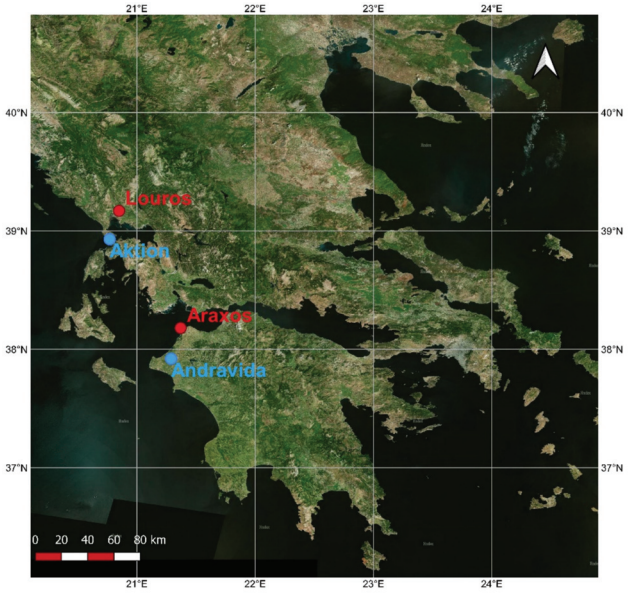


Figure 1. Map of Greece with the two study sites indicated with red dots and the locations of the meteorological stations with blue dots.

2.2. Meteorological Data

Meteorological data (average daily temperature and daily precipitation) of the 21-year study period (2000–2020) for Araxos site were recorded by a meteorological station situated in Andravida, about 29 km from the study area, while for Louros site, in Aktion airport, about 28 km from the study site. Data were downloaded from U.S. National Oceanic and Atmospheric Administration (NOAA) National Climatic Data Center (NCDC, www.ncdc.noaa.gov, accessed on 1 March 2021). In Figure 2, the annual profile of the average total monthly precipitation and the average monthly temperature for the 21-year study period is presented for both study sites. Increased rain amounts in Louros site throughout the year and a slightly lower temperature during the stressful summer period are evident (average temperature of June to August 25.93 ± 0.62 °C for Araxos and 25.25 ± 0.63 °C for Louros). For Araxos, the average annual temperature is 17.86 ± 0.35 °C and total annual precipitation 759.5 ± 151.8 mm, while for Louros, the temperature and precipitation were 17.64 ± 0.42 °C and 919.3 ± 217.2 mm, respectively.

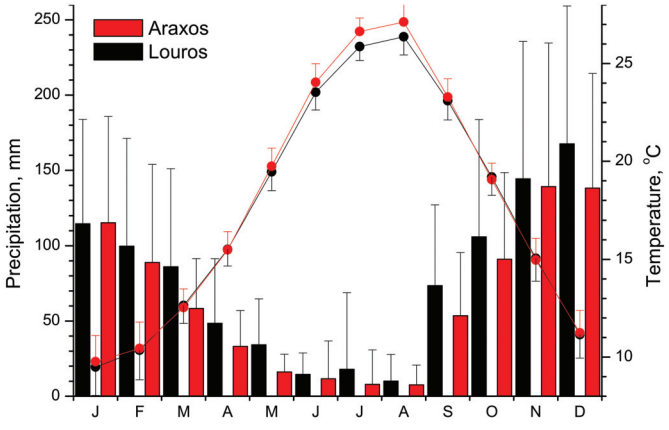


Figure 2. Annual profile of the average total monthly precipitation (bars) and the average monthly temperature (points) for the 21-year study period (2000–2020) for Araxos and Louros study sites.

2.3. Species Description

Phlomis fruticosa is a dimorphic, semi-deciduous shrub of the eastern Mediterranean areas. It bears two kinds of leaves—winter and summer ones—with different biochemical and structural characteristics [30]. Winter leaves and summer leaves of the previous growing season are massively shed during mid to late spring, resulting in a decrease in Leaf Area Index (LAI). Hereinafter, we refer to this event as spring drop. During the summer dry period, plants bear summer leaves developed during spring, which are smaller than winter leaves and have lower chlorophyll content. After the onset of the autumn rainy period, summer leaves absorb water rapidly (within days) and increase their area, while they alter their biochemical characteristics, including chlorophyll content increase. Additionally, new winter leaves with high chlorophyll content appear during November. Hereinafter, we refer to this event as autumn revival. The transformation of summer leaves and the appearance of winter leaves during autumn result in an increase in LAI, which remains almost stable during winter, until next spring. Even though this phenological/physiological cycle is repeated every year, the extent and/or the exact date for each particular phenological event seem to depend on the microenvironmental conditions [31]. The main physiological advantage of the semi-deciduous habit is the decrease in the transpiring leaf area during the summer dry months, resulting in more efficient water economy.

2.4. Satellite Data

The Enhanced Vegetation Index (EVI) was used in the present study. For the calculation of EVI, data from the Moderate Resolution Imaging Spectroradiometer (MODIS) onboard the Terra satellite (part of the NASA Earth Observing System) were used. MODIS scans the entire Earth surface every 1–2 days, acquiring data in 36 spectral bands. Out of the 36 spectral bands, 7 bands are designed for the study of vegetation and land surfaces: blue (459–479 nm), green (545–565 nm), red (620–670 nm), near infrared (NIR1: 841–875 nm, NIR2: 1230–1250 nm), and shortwave infrared (SWIR1: 1628–1652 nm, SWIR2: 2105–2155 nm). Several products with differences in spectral, spatial, and temporal resolution, as well as in correction levels are freely provided by the MODIS Land Science Team to users. In the present study, the geometrically and atmospherically corrected Surface Reflectance 8-Day L3 Global 500 m product (MOD09A1), available to the public from the US Geological Survey EROS Data Center (USGS EROS Center, http://eros.usgs.gov/, accessed on 1 March 2021), was used. EVI was calculated according to the equation:

$$EVI = 2.5\frac{R_{nir} - R_{red}}{R_{nir} + 6R_{red} - 7.5R_{blue} + 1} \quad (1)$$

where R_{nir} is reflectance between 841 and 875 nm, R_{red} between 620 and 670 nm and R_{blue} between 459 and 479 nm [35].

The MOD09A1 datasets (2000–2020), which have a 500 m spatial resolution and 8-day temporal resolution, were downloaded from the USGS EDC website using the geographical coordinates of each study site and 21 years EVI time-series were produced for 4 and 3 pixels for Araxos and Louros sites, respectively. These pixels were selected for each site after land observations and GPS recording, as being homogenous and dominated exclusively by *P. fruticosa*. The time-series of each pixel were corrected for erroneous values during cloudy dates or other reasons using the BISE (Best Index Slope Extraction) algorithm [32]. Accordingly, for each date the average of the corresponding pixels was calculated for each site and used for the construction of time-series over the 21-year study period. The time-series of the two sites were further smoothed using an adjusted Fast Fourier Transform [36]. Additionally, the average annual EVI profile for each site was calculated by averaging EVI values for the same 8-day period between years (Figure 4).

2.5. Phenology Metrics

The phenological cycle of *P. fruticosa*—as described above—may be depicted by the seasonal EVI fluctuation shown in Figure 2. High and rather stable values of EVI during winter (December to March), corresponding to high LAI and chlorophyll content values, are followed by the spring drop period (April to June). The steep reduction in EVI during that period corresponds to the massive loss of winter leaves and summer leaves of the previous year. During summer dry period (July to September), plants bear a small number (low LAI) of low chlorophyll content summer leaves (low and stable EVI values). The subsequent autumn revival period, coinciding with the onset of the autumn rainy period (October–November), is characterized by an abrupt rise of EVI, as a result of the "resurrection" of summer leaves (rapid increase in leaf area due to water absorption, accompanied by chlorophyll content increase) followed by the burst of new winter leaves at the end of autumn. This pattern is repeated every year in both study sites, but remarkable differences in parameters of spring drop, dry period and autumn revival may occur among sites and/or years.

In order to quantify the phenological events described above (spring drop, dry period, and autumn revival), the parameters presented in Table 1 were calculated for each site and year. For the calculation of spring drop and autumn revival related parameters, the 1st derivative of the EVI curve was used. Day of Year for spring drop onset (SDO) and autumn revival onset (ARO) are determined when the 1st derivative departs from near zero values (Figure 3) and spring drop end (SDE) and autumn revival end (ARE) when the 1st derivative returns to near-zero values, during the spring and autumn EVI steep

change period. The differences between SDE-SDO and ARE-ARO result in the spring drop duration (SDD) and autumn revival duration (ARD), respectively.

Table 1. Phenological events derived from the EVI curves, their abbreviations, and characteristics.

Phenological event		Abbreviation	Characteristics
Spring drop	onset	SDO	Day of Year
	end	SDE	Day of Year
	duration	SDD	Number of days. SDD = SDE – SDO
Autumn revival	onset	ARO	Day of Year
	end	ARE	Day of Year
	duration	ARD	Number of days. ARD = ARE – ARO
Dry period	onset	DPO	Day of Year
	end	DPE	Day of Year
	duration	DPD	Number of days. DPD = DPE – DPO
Annual maximum EVI Date of Max EVI		Max EVI	 Day of Year
Annual minimum EVI Date of Min EVI		Min EVI	 Day of Year

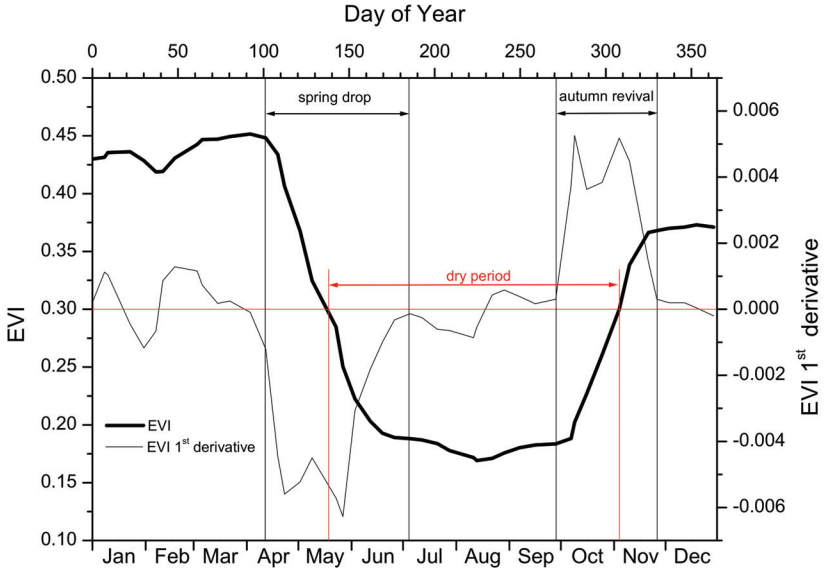


Figure 3. Typical profile of the annual fluctuation of EVI (thick line) and its 1st derivative (thin line). Red horizontal line corresponds to EVI = 0.3 and EVI 1st derivative = 0. Black vertical lines indicate the onset and end of the spring drop and the autumn revival period, when EVI 1st derivative departs or returns to zero values for onset and end correspondingly. Red vertical lines indicate the onset and end of the dry period, when EVI is lower than 0.3.

Concerning dry period parameterization, onset (DPO) and end (DPE) of the dry period were calculated according to the threshold method [37,38]. The EVI value of 0.3 was defined as the threshold for DPO and DPE and was chosen because it represents the midpoint between absolute maximum and absolute minimum (all 21 years) EVI values. Thus, DPO and DPE were quantified as the Day of Year at which EVI reaches or leaves 0.3, respectively, and the dry period duration as their difference (Figure 3).

Additionally, in an attempt to exploit all the information contained in the shape of EVI curve, annual maximum and minimum values and the date of their occurrence were also determined for each site and year (Table 1). Finally, mean monthly EVI was calculated and used as a surrogate of ecosystem dynamics in the assessment of climate control on growth features. All the above-mentioned extracted parameters were used as independent phenology metrics in the statistical analyses (see below) for the identification of the most influential climatic factor.

2.6. Statistical Analysis

The relationships between the above described phenological events and climatic parameters were assessed using Pearson correlations and stepwise multiple linear regressions. The examined climatic parameters concern total monthly precipitation (P), monthly sum of rainy days (RD) and mean monthly temperature (T), of different time intervals-concurrent and lagged-in relation to the corresponding phenological event. More specifically, phenological events were examined against the following combinations:

1. Average temperature, total precipitation, and total rain days of one to six consecutive months before each phenological event, e.g., for a phenological event occurring in October, total precipitation of October and September (two months combination).
2. Average temperature, total precipitation, and total rain days of one to five consecutive months with one to five months lag before the event, e.g., for a phenological event occurring in October, total precipitation of August and July (two months combination with two months lag time).

The first step was to perform a Pearson correlation analysis for each phenological event and the above-mentioned climatic parameters. Accordingly, the independent variables that exhibited the maximum correlations in each case were employed in multiple linear regression analyses with stepwise selection. Collinearity of predictor variables was automatically detected by the statistical software and subsequently dealt by omitting variables and re-running the regression analysis.

The influence of climatic parameters on inter-annual EVI variation was examined following two different regression analysis methods, i.e., multiple linear regression and random forest machine learning. As in the case of phenology and climate control, all combinations of the climatic parameters (precipitation, rain days, temperature) of various time intervals and time lags of consecutive months up to six months before the event (EVI of a particular month) were considered. Initially, a monthly step EVI time-series was produced for each site, by averaging the analytical time-series data for each month. For the first approach, the most significant climatic parameters were determined through single linear regressions between monthly EVI and climatic parameters. Accordingly, combinations of the most important parameters were examined through stepwise multiple linear regression. Analyses showed that a high regression coefficient was achieved with two climatic parameters, i.e., adding additional parameters did not significantly enhance the efficiency of the regression (data not shown). On the second approach, all climatic parameters were used in a random forest machine learning procedure. During this procedure, data were randomly split into a training set (64% of data), a validation set (16% of data) for performance optimization and a test set (20% of data) for assessment of performance efficiency. However, for an overall comparison of machine learning with multiple linear regression all data were used in Figure 8. The efficiency of the two approaches was assessed through the coefficient of determination (R^2) and Root Mean Square Error (RMSE) of the predicted EVI against actual EVI values.

All statistical analyses were performed with JASP v.0.16 software (JASP Team 2021 Computer Software), which includes a machine learning module.

3. Results

3.1. Phenology

In Figure 4, the annual EVI profile for the two study sites is presented as average ± SD from the 21-year study period data. In Araxos, spring drop as well as dry period (EVI < 0.3) starts earlier and dry period ends later in autumn, accompanied by a retarded autumn revival. Additionally, annual maximum and minimum EVI values appear higher for the Louros site compared with Araxos, possibly as a result of better physiological performance and/or higher shrub density under the more favorable conditions of Louros.

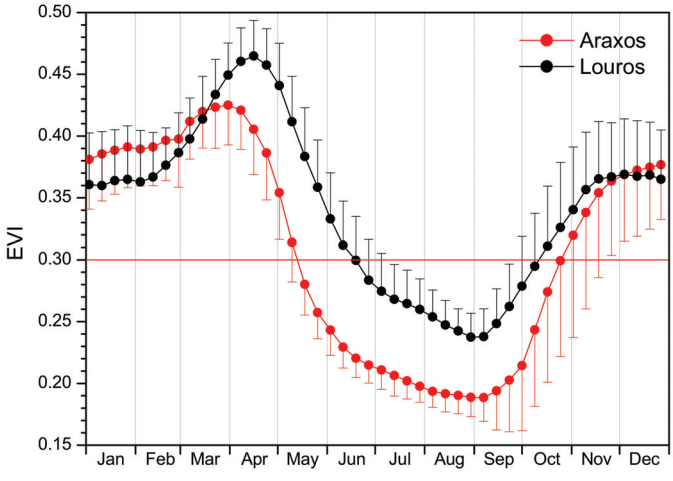


Figure 4. Annual EVI profile for the two study sites as average ± SD from the 21-year study period data. The red line marks EVI = 0.3, the value which was defined as the threshold for dry period onset and end.

These general differences among sites are usually followed every year throughout the 21-year study period (Figure 5). Additionally, it is clear from Figure 5 that the annual profiles for both sites show strong differences from year to year. These interannual variations may be rather large, especially during summer periods, as seen by comparing the very dry summer of 2001 with the wet summer of 2016 (Figure 5).

In an attempt to reveal the detailed differences between sites, the phenological events described in Table 1, were determined for all years and sites and their average values are presented in Table 2, whereas the most important among them, i.e., events concerning spring drop, dry period, and autumn revival, are depicted in Figure 6.

As shown in Table 2, onset and end of spring drop occur 15 and 27 days earlier in Araxos compared with Louros, respectively, with both events showing statistically significant differences. Additionally, spring drop in Araxos occurs more rapidly, as indicated by the smallest duration, compared with Louros. Dry period starts 40 days earlier and finishes 11 days later in Araxos compared with Louros, resulting in a significantly longer dry period duration by 51 days. Concerning autumn revival, onset and end appear 13 and 5 days later, respectively, in Araxos, even though only onset is significant. However, as in the case of spring drop, autumn revival occurs more rapidly in Araxos (shorter duration), compared with Louros. Maximum and minimum EVI values are both significantly higher in the Louros site, where more favorable climatic conditions prevail. Accordingly, the date of maximum EVI appears 22 days earlier in Araxos, whereas no statistical difference is evident for the date of minimum EVI.

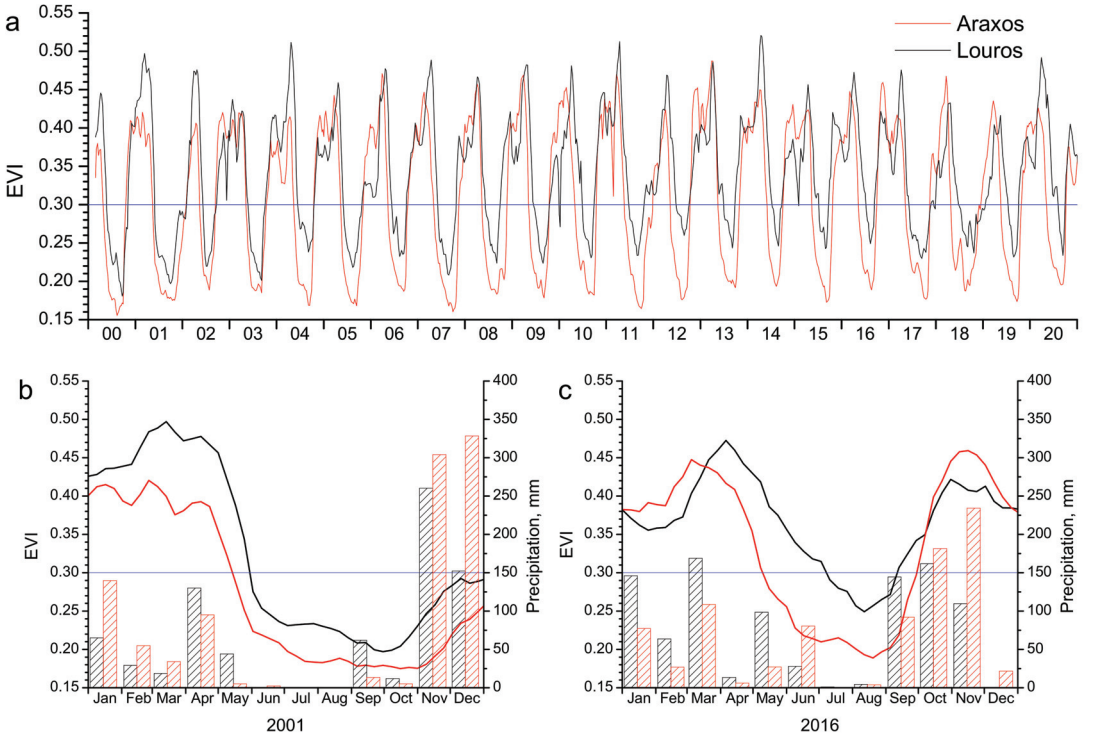


Figure 5. Interannual EVI fluctuation for the 21-year study period for the two study sites (**a**); EVI fluctuation and the corresponding precipitation profile during a dry (2001, **b**) and a wet (2016, **c**) year in the two study sites. The blue line marks the threshold for dry period onset and end (EVI = 0.3).

Table 2. Average data (±SD) for the phenological events described in Table 1 for the two study sites, their difference (Araxos–Louros), and the significance of their difference (P, paired *t*-test). DOY, Day of Year; ND, Number of Days.

Phenological Event		Araxos	Louros	Difference	P
Spring Drop	Onset (DOY)	95 ± 14	110 ± 11	−15	<0.001
	End (DOY)	212 ± 33	239 ± 17	−27	0.004
	Duration (ND)	117 ± 38	129 ± 23	−11	0.242
Dry Period	Onset (DOY)	131 ± 8	171 ± 19	−40	<0.001
	End (DOY)	304 ± 33	292 ± 37	11	0.033
	Duration (ND)	172 ± 34	121 ± 47	51	<0.001
Autumn Revival	Onset (DOY)	268 ± 23	255 ± 22	13	0.007
	End (DOY)	345 ± 40	340 ± 42	5	0.486
	Duration (ND)	77 ± 33	85 ± 32	−8	0.238
Max EVI		0.439 ± 0.026	0.473 ± 0.028	−0.034	<0.001
Date of Max EVI	(DOY)	81 ± 17	103 ± 10	−22	<0.001
Min EVI		0.179 ± 0.011	0.226 ± 0.021	−0.048	<0.001
Date of Min EVI	(DOY)	253 ± 23	245 ± 20	8	0.171

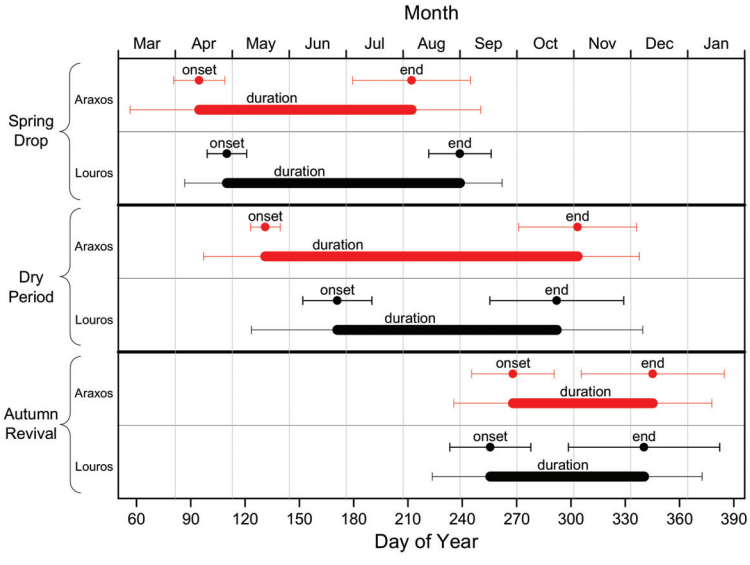


Figure 6. Graphical depiction of onset, duration, and end of the main phenological events (Spring Drop, Dry Period and Autumn Revival) for the two study sites. For all events, onset and end correspond to Day of the Year, whereas duration corresponds to number of days (data from Table 2).

3.2. Phenology and Climatic Control

In order to account for climatic controls of phenology, average monthly temperature, total monthly precipitation and total monthly rain days of time windows relevant to each event and transition were examined. More specifically, phenological events were examined against climatic parameters of various time intervals and time lags up to six months before the event (see Section 2.6).

Spring drop onset for Araxos is influenced by precipitation of April (Figure 7), with more rain delaying SDO. Accordingly, Louros is similarly influenced by the rain days of April and March, whereas the temperature during January and February also plays a role; low temperature delays SDO, probably through sustaining higher soil water capacity.

In Araxos, where precipitation during summer months is minimal and with low interannual variability (July and August rainfall of 15 $\pm$ 33 mm), the main influential factor on SDE is temperature of July and August with high temperature delaying the SDE and resulting in lower minimum EVI values compared with Louros (Table 2). On the contrary, in the wetter and more variable Louros site (July and August rainfall of 29 $\pm$ 61 mm), SDE is mainly influenced by summer precipitation: the more it rains the earlier SDE appears and at higher minimum EVI value compared with Araxos.

Dry period onset for Araxos, is affected by the precipitation of March and April (higher precipitation delays onset) and the temperature of April and May (higher temperature advances onset). For Louros, DPO is mildly affected by the February temperature, with higher temperature delaying the event. DPE for Araxos is strongly affected by summer-early autumn rain, since both precipitation and rain days of July to October period influence the event, causing a delay at drier years. Similar effects of precipitation are evident for Louros, but for this site, the temperature in July also plays a minor role; a higher temperature results in earlier DPE and shorter duration.

Figure 7. Climatic control on phenological events and transitions of *P. fruticosa* in the two sites as derived by single or multiple linear regressions. The phenological metrics are: Spring drop onset (SDO); spring drop end (SDE); spring drop duration (SDD); Dry period onset (DPO); dry period end (DPE); dry period duration (DPD); Autumn revival onset (ARO); autumn revival end (ARE), autumn revival duration (ARD); MaxEVI the maximum value of EVI; MaxDate, the date it is achieved; MinEVI the minimum value of EVI; MinDate, the date it is achieved. For each phenological event, the partial regression coefficient(s) of the most significant climatic variable(s) (precipitation (Pr), number of rain days (RD) and temperature (T)) of single or multiple months (top line) is presented in the corresponding colored horizontal lines, according to the chromatic scale appearing in the right. The regression coefficient (R^2) of the model which includes the factors influencing each event and the corresponding level of significance (p) is presented in the right column of each site.

Autumn revival onset is also strongly affected by rain (precipitation of July to October and rain days of August and September), with earlier onset during wetter years. A similar effect of rain is apparent for Louros, but only through precipitation (June to October). For both sites, ARE is affected by autumn rain days (July to October for Araxos and September to October for Louros). Nevertheless, a rather unreasonable effect of June rain days is evident in Araxos, where more rain days during June delay the ARE. However, this peculiar effect is also recorded for ARD for both sites (rain days of June for Araxos and July for Louros).

The value of maximum EVI seems to be positively affected by spring rain days for both sites (of March for Araxos and March–April for Louros). Winter precipitation affects the date in which the maximum EVI is achieved for both sites. More specifically, more rainfall during January to March for Araxos and January for Louros causes a delayed appearance of maximum EVI. Minimum EVI occurs during late August or early September in both sites. The rain days of August is the determinant of minimum EVI in Araxos, whereas precipitation over a longer period, February to June, positively affects the minimum EVI of

Louros. Additionally, Louros seems to be affected by temperature of July, but in a rather unexpected way, since higher temperature results in higher EVI. Finally, for both sites the date that the minimum EVI appears is affected by the spring–summer precipitation (May–June for Araxos and March to August for Louros), with more rain transferring the date earlier.

Collectively, the 13 phenological events analyzed above are influenced mostly by rain related parameters for the Araxos site; more specifically 10 events by precipitation and/or rain days, 2 events by temperature and 1 event by both rain and temperature. On the contrary, both rain and temperature play crucial roles in Louros phenology, since 6 events are influenced by precipitation and/or rain days, 6 events by both rain and temperature, and 1 event by temperature.

3.3. Phenology and Climate Change

All phenological events examined above could be potentially related to the ongoing climate change. Our dataset of 21 years is long enough to permit the analysis of the trends of phenological events' interannual fluctuation in the context of climate change. As shown in Figure 8, spring-drop-related events show significant trends for the Araxos site, but not for Louros, whereas no significant trends appear for the rest of the phenological events (data not shown). The trends appearing for Araxos seem to be explained by similar trends in the main climatic factors that these events are related to (Figure 8). SDO tends to commence earlier in the season by 1 day per year, whereas April precipitation—the main influential climatic parameter (Figure 7)—tends to decrease by 1.7 mm per year. Accordingly, SDE experiences a delay by 3.8 days per year and spring drop duration is elongated by 4.9 days per year. Both events are influenced by July–August temperature (Figure 7), which shows a similar trend, increasing by 0.06 °C per year during the study period (Figure 8).

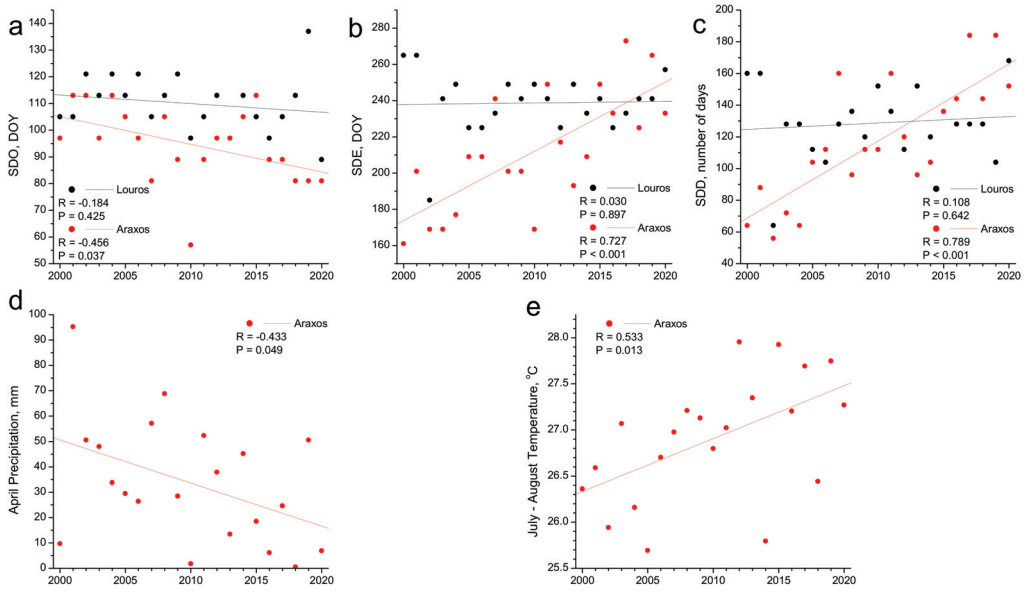


Figure 8. Interannual fluctuation of the spring drop related phenological events (dots) and their trends (lines) for the two study sites during the study period: (**a**) Spring drop onset (SDO,), (**b**) spring drop end (SDE), (**c**) spring drop duration (SDD. Interannual fluctuation of the main climatic parameters influencing the phenological events of the Araxos site (dots) and their trends (lines) during the study period: (**d**) April precipitation, (**e**) July–August temperature.

3.4. EVI and Climatic Control

Since all phenological parameters are extracted from the EVI time-series, the influence of climatic parameters on EVI per se was examined as a final integrating step. To that purpose, as in the case of climate control on phenological events, all combinations of the climatic parameters (precipitation, rain days, temperature) of various time intervals and time lags of consecutive months up to six months before the event (EVI of a particular month) were considered through two regression analysis methods, i.e., multiple linear regression and random forest machine learning.

As shown on Figure 9, EVI may be predicted by similar parameters for both sites through multiple linear regression analysis, i.e., temperature of the previous two months for Araxos and three months for Louros and rain days of the precious three months for both sites. However, the machine learning approach—in which all climatic parameters are included—results in much stronger models compared with the multiple linear regression approach, as judged by R^2, RMSE and the regression line which is closer to the 1:1 line. It is worth to note, that the parameters determined by the multiple linear regression approach are among the most important ones determined by the machine learning approach, but the inclusion of additional parameters significantly enhances model efficiency.

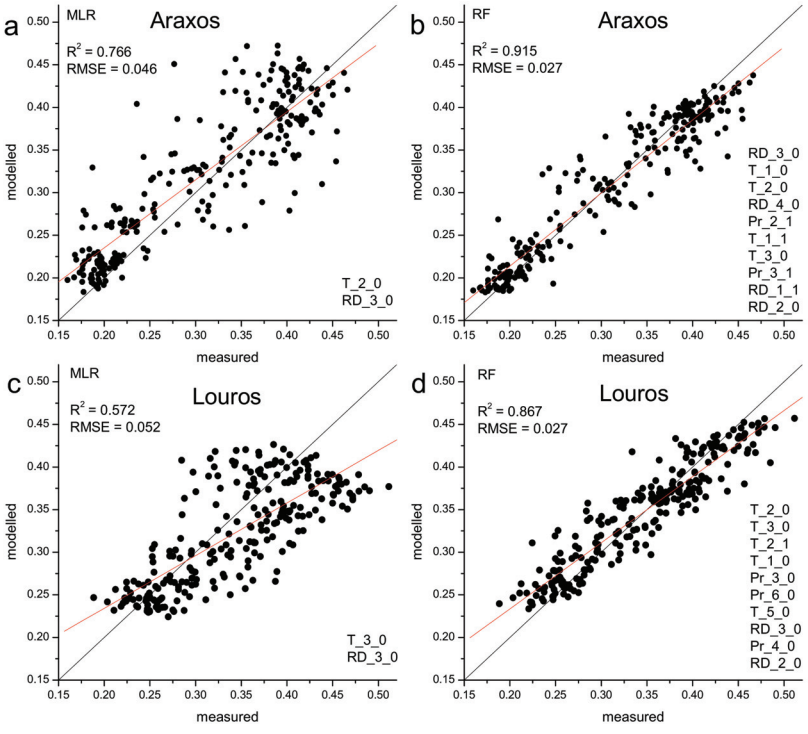


Figure 9. Regressions between measured and modelled EVI through multiple linear regression (MLR, **a**,**c**) and random forest machine learning (RF, **b**,**d**) for Araxos (**a**,**b**) and Louros (**c**,**d**). In multiple linear regressions (**a**,**c**) the two climatic parameters participating in the models are shown in the right lower corner of each graph. In machine learning models (**b**,**d**) the ten most important parameters are shown. In both cases parameter importance decreases from top to bottom. Black lines correspond to the 1:1 lines and red ones to the regression lines. Climatic parameters are described by an acronym for parameter description (T for temperature, Pr for precipitation, and RD for rain days) followed by a number showing the number of the corresponding months and a number corresponding to the lag time, for example T_2_1 refer to temperature of two months before one month.

4. Discussion

In this study, the phenological differences between two sites dominated by the semi-deciduous shrub *Phlomis fruticosa*, were examined using MODIS EVI time-series. *P. fruticosa* has been extensively studied from an ecophysiological point of view with both field measurements [33,34] and in combination with satellite data [22,25]. The most important characteristic of its growth pattern is the massive leaf shedding during spring, as an adaptation to the adverse conditions of the hot and dry Mediterranean summer, accompanied by autumn revival after the onset of the autumn rains.

4.1. EVI Tntra- and Inter-Annual Fluctuation and Phenology Metrics

The first target of the present study was to monitor seasonal and inter-annual fluctuation of EVI and, subsequently, to identify key phenological events, in order to analyze the temporal dynamics of *P. fruticosa* community in two distinct sites.

The two sites examined in this study are located near to the shoreline of western Greece but have a latitude difference of about 1°. Accordingly, during the 21-year study period, the southern site (Araxos) appeared more xeric compared with the northern site (Louros, Figure 2).

As shown in Figures 3–5, satellite data can capture and effectively describe the complex phenology of the semi-deciduous shrub. The inter-annual variability of EVI values is considerable and this is well depicted in two extreme years, the dry 2001 and wet 2016, which are presented in Figure 5 in relation to precipitation. Both sites exhibit an analogous profile concerning the prolonged drought phase (denoted by EVI < 0.3) at the dry year, which is considerably shortened during the wet year. In the relevant remote sensing literature, several studies have reported a detailed single-species phenology monitoring, emphasizing the importance of spatially explicit analyses and the study of phenological trends in small-scale level [15,39,40]. Especially in the fragmented and highly heterogeneous Mediterranean vegetation, this approach connects small- to large-scale information on community or ecosystem function, which would be otherwise lost if only regional level is considered [41].

The timing of certain phenological events that describe the annual cycle of *P. fruticosa* differs in various degrees between the two sites. In the southern Araxos, the earlier spring drop onset, the later autumn revival onset, the prolonged dry period and the earlier appearance of maximum EVI are important in shaping the annual picture and statistically significant. It is well documented that the above-described phenological transitions relate to greening-up or senescence processes which control the community function, state, and productivity [22,25]. Thus, the phenological transitions are influenced and in turn may influence the microclimate, generating gradients of humidity and temperature and affecting topsoil characteristics. Playing such a crucial role in microclimate modification the phenological patterns may have long-term feedback on larger-scale vegetation–climate interactions [2].

4.2. Climatic Control on Phenological Events

In semi-arid Mediterranean ecosystems, the high inter-annual variability of EVI suggests that the "memory effects" of previous year's climate are minimized, as proposed by Catorci et al. [42]. Therefore, our analysis of the relationship between phenological events and climatic drivers was focused on up to 6 months preceding each certain transition. The spring and dry-period-related events were influenced by both precipitation and temperature of a short preceding period. The spring drop of winter leaves indicates the end of winter growth and the "preparation" of *P. fruticosa* to face summer adverse period. Spring precipitation was the major driver of SDO, whereas its duration was influenced by summer precipitation in Louros and summer temperature in the drier Araxos site. Spring phenology has been proved to be sensitive to climatic control in Mediterranean-type ecosystems, possibly due to the high variability of climatic parameters during spring [4,43]. Working with Mediterranean grasslands Catorci et al. [42] reported that rainfall in March and

drought stress in April and May were the main drivers of satellite-derived spring biomass production. Climatic parameters linked to moisture control are predominant in shaping vegetation response in Mediterranean, semi-arid and arid ecosystems [44]. Precipitation totals over the preceding three months have been found to correlate with the start of growth season in various Mediterranean regions [4]. Piedallu et al. [45] highlighted the positive correlation of spring temperature with vegetation greenness in an elevation gradient in South France, while stating that rainfall played a minor role in the overall region response, except for the most arid microsites.

The onset of the dry period for *P. fruticosa* was determined as the time-point at which the EVI reaches the 0.3 threshold, denoting a massive leaf loss thus a significant decrease in LAI. Increased spring precipitation retards DPO, but increased spring temperature advances it in Araxos, whereas the temperature of February is the only important parameter in Louros. The duration of dry period and the DPE is influenced mainly by precipitation in both study sites. The seasonal timing of rainfall events is important in determining their effects on phenology [43]. Especially in semi-arid systems and drylands, the precipitation during water-deficit periods is significantly more important in driving phenology than the rain during favorable moisture conditions. Broich et al. [31] suggested that the stronger correlation patterns with single-month compared with multi-month aggregated drivers indicate that rainfall at a specific time-point determines most phenological events. Corroborating this conclusion, our findings suggest that 7 out of 13 phenology metrics were influenced by single or double-month precipitation related parameters in the drier site of Araxos.

The autumn revival of *P. fruticosa* is characterized by the resurrection of summer leaves and the massive production of the winter leaves, thus it is related to an abrupt increase in EVI values. The ARO is primarily driven by the precipitation of the previous 5 months in Louros, whereas in Araxos an equal contribution of the previous 4 months precipitation and the rain days of previous 3 months is recorded. This result is in accordance with Cabello et al. [43], who reported that the earlier arrival of the first rains after the summer adverse period significantly account for the acceleration of growth period onset in Mediterranean drylands. To the same direction was the effect of precipitation on vegetation greening in semi-arid sites of Tunisia [17]. On the contrary, Horion et al. [46] stated that temperature of the last month was the major climatic constraint for growth from the start of vegetative growth to flowering in two studied sites in the Mediterranean basin.

The study of possible climate change effects on phenology was significantly advanced by the use of satellite remote sensing as an efficient tool for continuous vegetation monitoring in large temporal scales [47,48]. The present 21-year study covers an adequate period to evaluate trends in timing of phenological events. A significant trend for earlier spring drop onset in Araxos site was evident following the similar downward trend of April precipitation, which was found to be the most influential climatic factor at this particular event and site. On the contrary, the SDE showed a delay rate of 3.8 days year^{-1}, in response to July-August temperature increase. Both SDO advancement and SDE delay resulted in a significant trend for prolonged SDD, at a rate of 4.9 days year^{-1}. Spring events in Louros and the other phenophases of *P. fruticosa* showed weak or no trends for change. The advancement of spring phenology is one of the consistent observations across Northern Europe [18], North America [30], and China [5] during the two recent decades. In Mediterranean-type ecosystems, the spring phenology trends show scattered spatial pattern according to a comprehensive study of Ivits et al. [4]; over the southern Mediterranean region, an earlier start-of-season was observed, whereas over parts of the northern Mediterranean basin a growing season shift towards later dates was evident. Concerning the climate forcing of spring phenology trends, March rainfall was reported as the main driver of NDVI variability [42], whereas in accordance with our results Cabelo et al. [43] also reported a trend for reduced spring precipitation which accounted for spring phenology variations. Temperature rising has also well documented consequences in vegetation phenology, especially in semi-arid ecosystems [49–51]. An interesting outcome of the

analyses presented here is that the contribution of both temperature and precipitation is higher in shaping the Louros phenological profile, whereas the more xeric Araxos depend more on rainfall. This is in accordance with the relevant literature, where a positive link of precipitation and satellite-derived phenology has been generally observed, but a stronger relationship has been reported for xeric compared with colder and wetter areas [45,52].

Temperature and rain days proved to be the main climatic drivers of EVI profile for both Araxos and Louros sites, according to multiple linear regressions and machine learning approach (Figure 9). Specifically, the temperature of the previous two (Araxos) or three (Louros) months and the number of rain days for the preceding three months account for the overall variation of EVI in the 21-year period. The same climatic factors with variable time windows are present in the set of the 10 most influential parameters derived from the machine learning approach, which is increasingly adopted in studies involving time-series. It is interesting to examine these results in the context of climate change. Mediterranean ecosystems are vulnerable due to intense anthropogenic pressure, natural disturbances (i.e., drought and fires), and a highly fluctuating climate, with main characteristic the erratic precipitation patterns [4]. The scenarios of climate change impact on the existing precipitation and temperature regimes include a large reduction in annual precipitation and an increase in inter-annual variability. The latter is expected to result in more heavy rainfall concentrated in fewer rain days, thus prolonged and frequent drought events. Thus, it is crucial to include the parameter of rain days in the studies of climatic forcing on phenology and ecosystem productivity.

The findings of the present work revealed differences in both *P. fruticosa* phenology and its climatic drivers between two sites being only 1° apart with small differences in climate. We may expect even more significant differences between regions with completely different climatic profile, though the direction and magnitude of response cannot be predicted. The single-species sites examined in this work facilitated EVI signal analysis and drawing conclusions but simultaneously may be considered a study's limitation concerning the applicability of the methodology in more diverse and species-rich ecosystems. Future studies may examine distant Mediterranean sites where significantly different climatic conditions prevail. Moreover, incorporating other key Mediterranean species will be crucial for understanding the dynamics of Mediterranean species phenology in regard to climate. Since *P. fruticosa* is a key species in garrigue formations in Greece (also called phrygana), the findings of the present study may give valuable baseline information for future studies on more complex garrigue ecosystems phenology and the involved climatic drivers.

5. Conclusions

The complex phenological cycle of the drought semi-deciduous *P. fruticosa* was clearly depicted in the satellite-derived EVI seasonal fluctuations in both studied sites, the southern Araxos and the northern Louros. The phenology metrics were differentiated between the two sites. The contribution of both temperature and precipitation is higher in shaping the Louros phenological profile, whereas the more xeric Araxos depends more on rainfall. In Araxos, a trend for SDO advancement and more prolonged SDD was recorded during the last 21 years, closely related to certain precipitation and temperature trends. The results of the present study revealed the importance of analyzing the seasonal timing of the phenological events in the lifecycle of a typical species of the Mediterranean ecosystem and of identifying the climatic drivers of their profiles changes. This approach, which focuses on a single species and explicit small-spatial-scale information, will be crucial in connecting small- and large-scale vegetation responses to climate crisis.

Author Contributions: Conceptualization, A.K. and E.L.; investigation, A.K. and E.L.; writing—original draft preparation, A.K. and E.L.; writing—review and editing, A.K. and E.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data is available from the authors upon request.

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

References

1. Zhou, Y.; Zhang, L.; Fensholt, R.; Wang, K.; Vitkovskaya, I.; Tian, F. Climate Contributions to Vegetation Variations in Central Asian Drylands: Pre- and Post-USSR Collapse. *Remote Sens.* **2015**, *7*, 2449–2470. [CrossRef]
2. Richardson, A.D.; Keenan, T.F.; Migliavacca, M.; Ryu, Y.; Sonnentag, O.; Toomey, M. Climate Change, Phenology, and Phenological Control of Vegetation Feedbacks to the Climate System. *Agric. For. Meteorol.* **2013**, *169*, 156–173. [CrossRef]
3. Gordo, O.; Sanz, J.J. Long-Term Temporal Changes of Plant Phenology in the Western Mediterranean. *Glob. Change Biol.* **2009**, *15*, 1930–1948. [CrossRef]
4. Ivits, E.; Cherlet, M.; Tóth, G.; Sommer, S.; Mehl, W.; Vogt, J.; Micale, F. Combining Satellite Derived Phenology with Climate Data for Climate Change Impact Assessment. *Glob. Planet. Change* **2012**, *88–89*, 85–97. [CrossRef]
5. Chen, F.; Liu, Z.; Zhong, H.; Wang, S. Exploring the Applicability and Scaling Effects of Satellite-Observed Spring and Autumn Phenology in Complex Terrain Regions Using Four Different Spatial Resolution Products. *Remote Sens.* **2021**, *13*, 4582. [CrossRef]
6. Levizou, E.; Manetas, Y. Combined Effects of Enhanced UV-B Radiation and Additional Nutrients on Growth of Two Mediterranean Plant Species. In *Responses of Plants to UV-B Radiation*; Advances in Vegetation Science; Rozema, J., Manetas, Y., Björn, L.-O., Eds.; Springer: Dordrecht, The Netherlands, 2001; pp. 179–186. ISBN 978-94-017-2892-8.
7. Caldwell, M.M.; Bornman, J.F.; Ballaré, C.L.; Flint, S.D.; Kulandaivelu, G. Terrestrial Ecosystems, Increased Solar Ultraviolet Radiation, and Interactions with Other Climate Change Factors. *Photochem. Photobiol. Sci.* **2007**, *6*, 252–266. [CrossRef]
8. Körner, C.; Paulsen, J. A World-wide Study of High Altitude Treeline Temperatures. Available online: https://onlinelibrary.wiley.com/doi/full/10.1111/j.1365-2699.2003.01043.x (accessed on 23 December 2021).
9. Dahlin, K.M.; Asner, G.P.; Field, C.B. Environmental and Community Controls on Plant Canopy Chemistry in a Mediterranean-Type Ecosystem. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 6895–6900. [CrossRef]
10. Burkett, V.R.; Wilcox, D.A.; Stottlemyer, R.; Barrow, W.; Fagre, D.; Baron, J.; Price, J.; Nielsen, J.L.; Allen, C.D.; Peterson, D.L.; et al. Nonlinear Dynamics in Ecosystem Response to Climatic Change: Case Studies and Policy Implications. *Ecol. Complex.* **2005**, *2*, 357–394. [CrossRef]
11. Cao, R.; Chen, J.; Shen, M.; Tang, Y. An Improved Logistic Method for Detecting Spring Vegetation Phenology in Grasslands from MODIS EVI Time-Series Data. *Agric. For. Meteorol.* **2015**, *200*, 9–20. [CrossRef]
12. Cerlini, P.B.; Saraceni, M.; Orlandi, F.; Silvestri, L.; Fornaciari, M. Phenological Response to Temperature Variability and Orography in Central Italy. *Int. J. Biometeorol.* **2022**, *66*, 71–86. [CrossRef]
13. Liu, Y.; Hill, M.J.; Zhang, X.; Wang, Z.; Richardson, A.D.; Hufkens, K.; Filippa, G.; Baldocchi, D.D.; Ma, S.; Verfaillie, J.; et al. Using Data from Landsat, MODIS, VIIRS and PhenoCams to Monitor the Phenology of California Oak/Grass Savanna and Open Grassland across Spatial Scales. *Agric. For. Meteorol.* **2017**, *237–238*, 311–325. [CrossRef]
14. Sangüesa-Barreda, G.; Di Filippo, A.; Piovesan, G.; Rozas, V.; Di Fiore, L.; García-Hidalgo, M.; García-Cervigón, A.I.; Muñoz-Garachana, D.; Baliva, M.; Olano, J.M. Warmer Springs Have Increased the Frequency and Extension of Late-Frost Defoliations in Southern European Beech Forests. *Sci. Total Environ.* **2021**, *775*, 145860. [CrossRef] [PubMed]
15. Busetto, L.; Colombo, R.; Migliavacca, M.; Cremonese, E.; Meroni, M.; Galvagno, M.; Rossini, M.; Siniscalco, C.; Morra Di Cella, U.; Pari, E. Remote Sensing of Larch Phenological Cycle and Analysis of Relationships with Climate in the Alpine Region. *Glob. Change Biol.* **2010**, *16*, 2504–2517. [CrossRef]
16. Carpintero, E.; Andreu, A.; Gómez-Giráldez, P.J.; Blázquez, Á.; González-Dugo, M.P. Remote-Sensing-Based Water Balance for Monitoring of Evapotranspiration and Water Stress of a Mediterranean Oak–Grass Savanna. *Water* **2020**, *12*, 1418. [CrossRef]
17. Touhami, I.; Moutahir, H.; Assoul, D.; Bergaoui, K.; Aouinti, H.; Bellot, J.; Andreu, J.M. Multi-Year Monitoring Land Surface Phenology in Relation to Climatic Variables Using MODIS-NDVI Time-Series in Mediterranean Forest, Northeast Tunisia. *Acta Oecologica* **2022**, *114*, 103804. [CrossRef]
18. Liu, L.; Cao, R.; Shen, M.; Chen, J.; Wang, J.; Zhang, X. How Does Scale Effect Influence Spring Vegetation Phenology Estimated from Satellite-Derived Vegetation Indexes? *Remote Sens.* **2019**, *11*, 2137. [CrossRef]
19. Isaacson, S.; Ephrath, J.E.; Rachmilevitch, S.; Maman, S.; Ginat, H.; Blumberg, D.G. Long and Short Term Population Dynamics of Acacia Trees via Remote Sensing and Spatial Analysis: Case Study in the Southern Negev Desert. *Remote Sens. Environ.* **2017**, *198*, 95–104. [CrossRef]
20. Paz-Kagan, T.; Silver, M.; Panov, N.; Karnieli, A. Multispectral Approach for Identifying Invasive Plant Species Based on Flowering Phenology Characteristics. *Remote Sens.* **2019**, *11*, 953. [CrossRef]
21. Liang, L.; Schwartz, M.D.; Fei, S. Validating Satellite Phenology through Intensive Ground Observation and Landscape Scaling in a Mixed Seasonal Forest. *Remote Sens. Environ.* **2011**, *115*, 143–157. [CrossRef]
22. Stagakis, S.; Markos, N.; Sykioti, O.; Kyparissis, A. Monitoring Canopy Biophysical and Biochemical Parameters in Ecosystem Scale Using Satellite Hyperspectral Imagery: An Application on a Phlomis Fruticosa Mediterranean Ecosystem Using Multiangular CHRIS/PROBA Observations. *Remote Sens. Environ.* **2010**, *114*, 977–994. [CrossRef]

23. Pan, Y.; Li, L.; Zhang, J.; Liang, S.; Zhu, X.; Sulla-Menashe, D. Winter Wheat Area Estimation from MODIS-EVI Time Series Data Using the Crop Proportion Phenology Index. *Remote Sens. Environ.* **2012**, *119*, 232–242. [CrossRef]
24. Sjöström, M.; Ardö, J.; Eklundh, L.; El-Tahir, B.A.; El-Khidir, H.A.M.; Hellström, M.; Pilesjö, P.; Seaquist, J. Evaluation of Satellite Based Indices for Gross Primary Production Estimates in a Sparse Savanna in the Sudan. *Biogeosciences* **2009**, *6*, 129–138. [CrossRef]
25. Stagakis, S.; Markos, N.; Sykioti, O.; Kyparissis, A. Tracking Seasonal Changes of Leaf and Canopy Light Use Efficiency in a Phlomis Fruticosa Mediterranean Ecosystem Using Field Measurements and Multi-Angular Satellite Hyperspectral Imagery. *ISPRS J. Photogramm. Remote Sens.* **2014**, *97*, 138–151. [CrossRef]
26. Hinojo-Hinojo, C.; Goulden, M.L. Plant Traits Help Explain the Tight Relationship between Vegetation Indices and Gross Primary Production. *Remote Sens.* **2020**, *12*, 1405. [CrossRef]
27. Restrepo-Coupe, N.; Huete, A.; Davies, K.; Cleverly, J.; Beringer, J.; Eamus, D.; van Gorsel, E.; Hutley, L.B.; Meyer, W.S. MODIS Vegetation Products as Proxies of Photosynthetic Potential along a Gradient of Meteorologically and Biologically Driven Ecosystem Productivity. *Biogeosciences* **2016**, *13*, 5587–5608. [CrossRef]
28. Huete, A.; Didan, K.; Miura, T.; Rodriguez, E.P.; Gao, X.; Ferreira, L.G. Overview of the Radiometric and Biophysical Performance of the MODIS Vegetation Indices. *Remote Sens. Environ.* **2002**, *83*, 195–213. [CrossRef]
29. Cavalaris, C.; Megoudi, S.; Maxouri, M.; Anatolitis, K.; Sifakis, M.; Levizou, E.; Kyparissis, A. Modeling of Durum Wheat Yield Based on Sentinel-2 Imagery. *Agronomy* **2021**, *11*, 1486. [CrossRef]
30. Peng, D.; Wu, C.; Li, C.; Zhang, X.; Liu, Z.; Ye, H.; Luo, S.; Liu, X.; Hu, Y.; Fang, B. Spring Green-up Phenology Products Derived from MODIS NDVI and EVI: Intercomparison, Interpretation and Validation Using National Phenology Network and AmeriFlux Observations. *Ecol. Indic.* **2017**, *77*, 323–336. [CrossRef]
31. Broich, M.; Huete, A.; Tulbure, M.G.; Ma, X.; Xin, Q.; Paget, M.; Restrepo-Coupe, N.; Davies, K.; Devadas, R.; Held, A. Land Surface Phenological Response to Decadal Climate Variability across Australia Using Satellite Remote Sensing. *Biogeosciences* **2014**, *11*, 5181–5198. [CrossRef]
32. Fanelli, G.; Attorre, F.; Del Giudice, M.; Gjeta, E.; De Sanctis, M. Phlomis Fruticosa Scrublands in the Central Mediterranean Region: Syntaxonomy and Ecology. *Phytocoenologia* **2015**, *45*, 49–68. [CrossRef]
33. Kyparissis, A.; Manetas, Y. Seasonal Leaf Dimorphism in a Semi-Deciduous Mediterranean Shrub: Ecophysiological Comparisons between Winter and Summer Leaves. *Acta Oecol.* **1993**, *14*, 23–32.
34. Kyparissis, A.; Grammatikopoulos, G.; Manetas, Y. Leaf Demography and Photosynthesis as Affected by the Environment in the Drought Semi-Deciduous Mediterranean Shrub *Phlomis fruticosa* L. *Acta Oecol.* **1997**, *18*, 543–555. [CrossRef]
35. Huete, A.R.; Liu, H.Q.; Batchily, K.; van Leeuwen, W. A Comparison of Vegetation Indices over a Global Set of TM Images for EOS-MODIS. *Remote Sens. Environ.* **1997**, *59*, 440–451. [CrossRef]
36. Roerink, G.J.; Su, Z.; Menenti, M. S-SEBI: A Simple Remote Sensing Algorithm to Estimate the Surface Energy Balance. *Phys. Chem. Earth Part B Hydrol. Oceans Atmos.* **2000**, *25*, 147–157. [CrossRef]
37. White, M.A.; Thornton, P.E.; Running, S.W. A Continental Phenology Model for Monitoring Vegetation Responses to Interannual Climatic Variability. *Glob. Biogeochem. Cycles* **1997**, *11*, 217–234. [CrossRef]
38. Schwartz, M.D.; Reed, B.C.; White, M.A. Assessing Satellite-Derived Start-of-Season Measures in the Conterminous USA. *Int. J. Climatol.* **2002**, *22*, 1793–1805. [CrossRef]
39. Gao, F.; Anderson, M.; Daughtry, C.; Karnieli, A.; Hively, D.; Kustas, W. A Within-Season Approach for Detecting Early Growth Stages in Corn and Soybean Using High Temporal and Spatial Resolution Imagery. *Remote Sens. Environ.* **2020**, *242*, 111752. [CrossRef]
40. Le Maire, G.; Marsden, C.; Nouvellon, Y.; Stape, J.-L.; Ponzoni, F.J. Calibration of a Species-Specific Spectral Vegetation Index for Leaf Area Index (LAI) Monitoring: Example with MODIS Reflectance Time-Series on Eucalyptus Plantations. *Remote Sens.* **2012**, *4*, 3766–3780. [CrossRef]
41. Féret, J.-B.; Corbane, C.; Alleaume, S. Detecting the Phenology and Discriminating Mediterranean Natural Habitats With Multispectral Sensors—An Analysis Based on Multiseasonal Field Spectra. *IEEE J. Sel. Top. Appl. Earth Obs. Remote Sens.* **2015**, *8*, 2294–2305. [CrossRef]
42. Catorci, A.; Lulli, R.; Malatesta, L.; Tavoloni, M.; Tardella, F.M. How the Interplay between Management and Interannual Climatic Variability Influences the NDVI Variation in a Sub-Mediterranean Pastoral System: Insight into Sustainable Grassland Use under Climate Change. *Agric. Ecosyst. Environ.* **2021**, *314*, 107372. [CrossRef]
43. Cabello, J.; Alcaraz-Segura, D.; Ferrero, R.; Castro, A.J.; Liras, E. The Role of Vegetation and Lithology in the Spatial and Inter-Annual Response of EVI to Climate in Drylands of Southeastern Spain. *J. Arid Environ.* **2012**, *79*, 76–83. [CrossRef]
44. Stöckli, R.; Rutishauser, T.; Dragoni, D.; O'Keefe, J.; Thornton, P.E.; Jolly, M.; Lu, L.; Denning, A.S. Remote Sensing Data Assimilation for a Prognostic Phenology Model. *J. Geophys. Res. Biogeosci.* **2008**, *113*. [CrossRef]
45. Piedallu, C.; Chéret, V.; Denux, J.P.; Perez, V.; Azcona, J.S.; Seynave, I.; Gégout, J.C. Soil and Climate Differently Impact NDVI Patterns According to the Season and the Stand Type. *Sci. Total Environ.* **2019**, *651*, 2874–2885. [CrossRef] [PubMed]
46. Horion, S.; Cornet, Y.; Erpicum, M.; Tychon, B. Studying Interactions between Climate Variability and Vegetation Dynamic Using a Phenology Based Approach. *Int. J. Appl. Earth Obs. Geoinf.* **2013**, *20*, 20–32. [CrossRef]
47. Sousa, D.; Davis, F.W. Scalable Mapping and Monitoring of Mediterranean-Climate Oak Landscapes with Temporal Mixture Models. *Remote Sens. Environ.* **2020**, *247*, 111937. [CrossRef]

48. Stanimirova, R.; Cai, Z.; Melaas, E.K.; Gray, J.M.; Eklundh, L.; Jönsson, P.; Friedl, M.A. An Empirical Assessment of the MODIS Land Cover Dynamics and TIMESAT Land Surface Phenology Algorithms. *Remote Sens.* **2019**, *11*, 2201. [CrossRef]
49. Jin, H.; Jönsson, A.M.; Olsson, C.; Lindström, J.; Jönsson, P.; Eklundh, L. New Satellite-Based Estimates Show Significant Trends in Spring Phenology and Complex Sensitivities to Temperature and Precipitation at Northern European Latitudes. *Int. J. Biometeorol.* **2019**, *63*, 763–775. [CrossRef]
50. Workie, T.G.; Debella, H.J. Climate Change and Its Effects on Vegetation Phenology across Ecoregions of Ethiopia. *Glob. Ecol. Conserv.* **2018**, *13*, e00366. [CrossRef]
51. Liu, Y.; Li, Y.; Li, S.; Motesharrei, S. Spatial and Temporal Patterns of Global NDVI Trends: Correlations with Climate and Human Factors. *Remote Sens.* **2015**, *7*, 13233–13250. [CrossRef]
52. Vicente-Serrano, S.M.; Gouveia, C.; Camarero, J.J.; Beguería, S.; Trigo, R.; López-Moreno, J.I.; Azorín-Molina, C.; Pasho, E.; Lorenzo-Lacruz, J.; Revuelto, J.; et al. Response of Vegetation to Drought Time-Scales across Global Land Biomes. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 52–57. [CrossRef]

plants

MDPI

Article

The Influence of the Partitioning of Sugars, Starch, and Free Proline in Various Organs of *Cyclamen graecum* on the Biology of the Species and Its Resistance to Abiotic Stressors

John Pouris [1], Efi Levizou [2], Maria Karatassiou [3], Maria-Sonia Meletiou-Christou [1] and Sophia Rhizopoulou [1,*]

[1] Section of Botany, Department of Biology, National and Kapodistrian University of Athens, Panepistimiopolis Athens, 15784 Athens, Greece; jopouris@biol.uoa.gr (J.P.); mmeleti@biol.uoa.gr (M.-S.M.-C.)
[2] Department of Agriculture Crop Production and Rural Environment, University of Thessaly, Fytokou Str., 38446 Volos, Greece; elevizou@uth.gr
[3] Laboratory of Rangeland Ecology (PO 286), School of Forestry and Natural Environnent, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece; karatass@for.auth.gr
* Correspondence: srhizop@biol.uoa.gr; Tel.: +30-210-727-4513

Abstract: The geophyte *Cyclamen graecum* is native to the eastern Mediterranean. Its beautiful flowers with upswept pink petals appear during early autumn, after the summer drought period and before leaf expansion in late autumn. The floral and leaf development alternates with their cessation in early winter and late spring, respectively. Ecophysiological parameters and processes underlining the life-cycle of *C. graecum* have not previously been published. Seasonal fluctuations of sugars, starch, and free proline have been investigated in tubers, leaves, pedicels, and petals, as well as petal and leaf water status. At the whole plant level, the seasonal co-existence of leaves and flowers is marked by an elevated soluble sugar content, which was gradually reduced as the above-ground plant parts shed. The sugar content of petals and pedicels was lower than that of leaves and tubers. Leaf starch content increased from late autumn to spring and was comparable to that of tubers. The starch content in petals and pedicels was substantially lower than that of tubers and leaves. In tubers, monthly proline accumulation was sustained at relatively constant values. Although the partitioning of proline in various organs did not show a considerable seasonal variation, resulting in an unchanged profile of the trends between tubers, leaves, and flowers, the seasonal differences in proline accumulation were remarkable at the whole plant level. The pronounced petal proline content during the flowering period seems to be associated with the maintenance of floral turgor. Leaf proline content increased with the advance of the growth season. The values of leaf relative water content were sustained fairly constant before the senescence stage, but lower than the typical values of turgid and transpiring leaves. Relationships of the studied parameters with rainfall indicate the responsiveness of *C. graecum* to water availability in its habitat in the Mediterranean ecosystem.

Keywords: *Cyclamen graecum*; geophyte; Mediterranean; phenology; seasonality

Citation: Pouris, J.; Levizou, E.; Karatassiou, M.; Meletiou-Christou, M.-S.; Rhizopoulou, S. The Influence of the Partitioning of Sugars, Starch, and Free Proline in Various Organs of *Cyclamen graecum* on the Biology of the Species and Its Resistance to Abiotic Stressors. *Plants* **2022**, *11*, 1254. https://doi.org/10.3390/plants11091254

Academic Editors: Jess K. Zimmerman and Matthew Paul

Received: 29 March 2022
Accepted: 3 May 2022
Published: 5 May 2022

1. Introduction

The geophytes exhibit a life-cycle associated with temporal separation of the vegetative phase from the flowering phase and possess perennial tubers, which support their annual growth [1–3]. *Cyclamen* L. (Primulaceae) is commercially important and a very popular horticultural genus native to the area around the Mediterranean Basin [4–6]. Wild cyclamens are perennial geophytes of woods and rocky areas. *Cyclamen* was mentioned as kyklaminos (κυκλάμινος) in Theophrastus' writings (4th century BC) [2,7]. The Cyclamen Society [8] recognizes 20 *Cyclamen* species. The cultivated cyclamens are usually hybrids of the spring flowering species *Cyclamen persicum*.

Cyclamen graecum Link grows in the wild; it is a perennial, tuberous geophyte, naturally distributed in bushy, stony, and sunlit ground in southern parts of the mainland of Greece and Aegean islands, as well as in coastal areas of Turkey and Cyprus, sometimes being confined into crevices in rocks. Environmental conditions, especially temperature, control annual development and florogenesis in geophytes [9]. *C. graecum* is an autumn flowering geophyte. Floral pedicels and leaf petioles arise from the upper part and/or the crown of the over summering, large, perennial tubers that have nodes marked by small buds [10,11]. It has been published that *Cyclamen* species with more than 30 chromosomes, e.g., *C. persicum* (2n = 48) and *C. graecum* (2n = 84), can develop very large tubers [12,13]. Actually, cyclamens do not produce sister tubers, but their tubers enlarge with age [14,15]. In *C. graecum*, thick anchor roots are developed between fibrous roots, from the center of the base of the tuber [8], but their role is not yet fully clarified; it is expected that they penetrate deep into the texture of stony substrate [4,5], which is a habitat of distinct seasonal aridity and moistness. The dark green, heart-shaped leaves of *C. graecum* appear in November. The leaves are slightly angular with variegated green spots on the adaxial surface; parts of the adaxial leaf surface are light green and other parts are dark green, while the abaxial leaf surface is violet-mauve.

Partitioning of total sugars between plant parts is directly linked to developmental flux of carbon molecules, osmolytes, and energy [16]. Moreover, soluble sugars and proline play pivotal roles in plants' stress responses [16–18]. In geophytes, the mobilization of total sugars and starch from below-ground organs generates crucial metabolites to support vegetative and reproductive growth, and synthesize essential compounds. Starch as a storage compound is easily hydrolyzed to soluble sugars that can be transported to growing plant organs. Subsequently, after leaf shedding, total sugars and starch accumulate in below-ground plant parts to sustain metabolism and serve as transient energy storage during the oncoming above-ground vegetative stage [16]. It may be worth noting that sowbread (*panis porcinus*), a common name for the genus *Cyclamen*, is a reference to tubers supposedly being a favorable food for pigs [19,20].

Proline has proven to be a multi-functional tool in plant metabolism during the last two decades. Beyond its well-established roles as an osmoticum and generally a protectant against abiotic stresses [21], its involvement in various developmental processes has also been recognized [18,22]. Its contribution to stress adaptation extends from the protection of photosynthetic apparatus and the involved enzymes to the stabilization of redox balance in the chloroplast, along with reactive oxygen species detoxification. Proline's involvement in re-adjustment of growth once the stress is relieved relates to its catabolism to support new growth with energy [22]. Under normal non-stress conditions, proline acts as a metabolic signal impacting plant growth and development via regulating metabolite pools and the expression of several genes [18]. Moreover, the widespread phenomenon of proline accumulation in reproductive organs has been attributed to its role as a developmental regulator and flowering signal [18,23]. Interestingly, proline-rich floral nectar has been found in ornamental tobacco flowers and connected with the attraction and reward of visiting pollinators with an energy source to sustain insect flight [24].

The objective of this study was to identify and compare the partitioning of soluble sugars, starch, and proline among above- and below-ground plant parts of Cyclamen graecum, which are exposed to fluctuating environmental conditions, as well as the water status of petals and leaves, which according to the best of our knowledge has not hitherto been published, in order to evaluate ecophysiological traits of tissues exposed to ambient conditions.

2. Results

2.1. Phenological Stages

Within the context of seasonality, the life-cycle of *C. graecum* is characterized by two phenological stages in the course of a year: the active phase (from flower emergence in September to leaf senescence in April) and the dormant phase spanning the prolonged drought period (from May to August) in the eastern Mediterranean, when the aboveground

plant parts are not visible. Concerning the active phenological stage, the flowers splay open in September (Figures 1 and 2) before leaf emergence in November (Figure 2). The seasonal floral and leaf development of *C. graecum* in early and late autumn (Figure 2) respectively alternates with cessation of flowers and leaves in winter and before summer, respectively. There is a marked variation in the length of the period of flowering and leafing that affects the capacity of this species for resource acquisition.

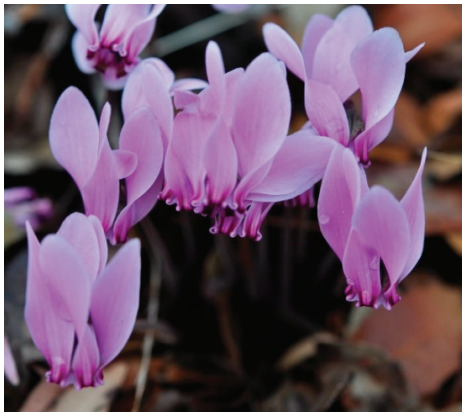


Figure 1. The flowers of *C. graecum* that attract the eye consist of five upswept petals.

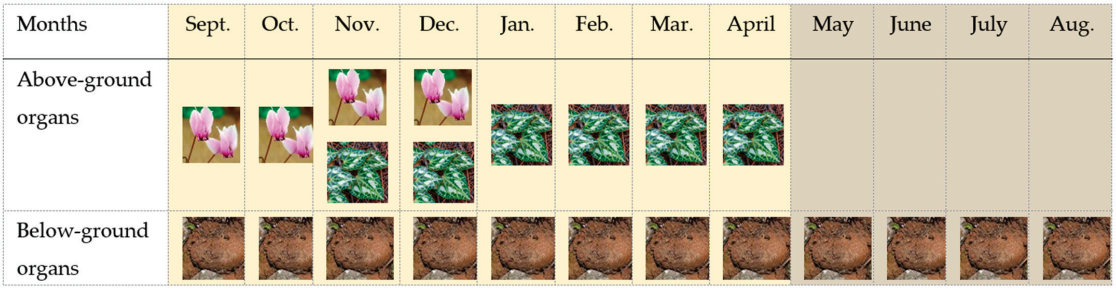


Figure 2. Iconographic presentation of the annual cycle and the phenological stages of *C. graecum* (using illustrations of flowers ( ), leaves ( ) and tubers ( )), i.e., the active phase indicated by the ivory area that includes flower initiation and longevity (September–December), and leaf emergence, development, longevity, and senescence (November–April), and the dormant phase indicated by the gray area that includes only the subterranean tubers, because above-ground growth is not visible.

2.2. Sugars

Tubers accumulated large quantities of soluble sugars throughout the year (Figure 3). A gradual reduction of stored sugars in the tubers was detected in October, during the flowering stage, and continued during November and December, when flowers and leaves co-existed. Sugar content of petals and pedicels was lower than that of leaves and tubers, reaching maxima in October. Strong monthly fluctuation of soluble sugars in leaves was detected from November to April, with the maximum values being achieved by mature leaves of December. The considerable sugar content, which was estimated in tubers, declined during the coexistence of floral and leaf stage in November and December. At the whole plant level, the co-existence of leaves and flowers marked the highest sugar content, which was gradually reduced as the above-ground plant parts shed, eventually entering the dormant phase. Monthly values of petal sugar content were positively related with those of tubers ($y = 0.523x - 3.882$, $R^2 = 0.889$, $p < 0.05$) and pedicels ($y = 1.227x - 30.461$,

R^2 = 0.609, $p < 0.05$), while leaf sugar content was negatively related with that of tubers (y = −0.494x + 337.030, R^2 = 0.591). In addition, the seasonal rainfall was negatively correlated with the sugar content of petals (y = −0.213x + 87.849, R^2 = 0.619, $p < 0.05$) and positively correlated with the sugar content of leaves (y = 0.098x + 17.322, R^2 = 0.709, $p < 0.05$).

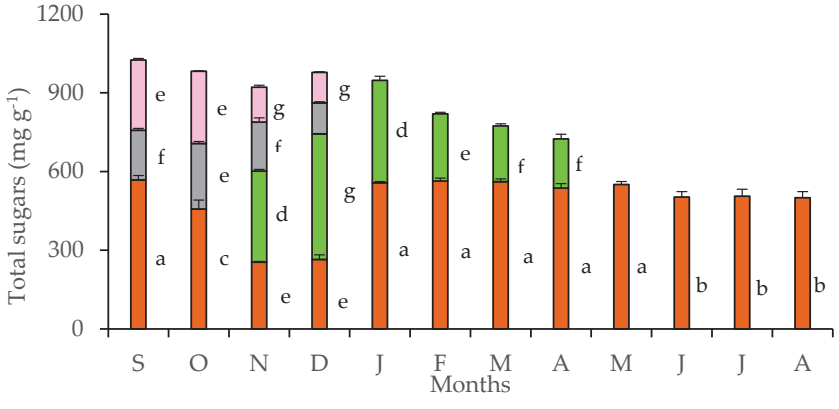


Figure 3. Sugar content in tubers (brown bars), leaves (green bars), pedicels (gray bars), and petals (purple bars) of *Cyclamen graecum*, from September (S) to August (A). Each column denotes means of five replicates ± standard error. Standard Errors smaller than the line thickness of the columns are not shown. Significant differences ($p < 0.05$) of mean values are marked using lowercase letters.

2.3. Starch

The highest starch content in tubers of *C. graecum* was observed in April (256 mg g^{-1}) when the climatic conditions were favorable for photosynthesis in the Mediterranean ecosystems, and before the cessation of foliar growth of *C. graecum*. Thereafter, from May to October, starch content declined (Figure 4). The lowest values of starch content in tubers (80 mg g^{-1}) were measured from June to October; actually, this period coincides the three-month summer drought (June to August), the cessation of foliar growth, and the early stage of flower formation in September, before the development of new expanding leaves in November (Figure 2). Intermediate values of starch content were detected in tubers from November to January during the development of new leaves. The values of starch content in leaves increased from November to April and were comparable to those of the tubers (Figure 4); in fact, seasonal variation of leaf starch content was positively related with that of tubers (y = 1.027x + 13.422, R^2 = 0.784, $p < 0.05$). The starch content in petals and pedicels was substantially lower than that of tubers and leaves during the flowering stage (from September to December) (Figure 2). The relatively elevated values of starch content in petals in September and October decreased in November and December (Figure 4), coinciding with elevated starch content in leaves (approximately from 100 to 140 mg g^{-1} d.w.). Negative relationships were detected between petal starch content with the corresponding tuber starch content (y = −0.632x + 125.930, R^2 = 0.892, $p < 0.05$) and the precipitation in the study site (y = −1.146x + 117.010, R^2 = 0.839, $p < 0.05$).

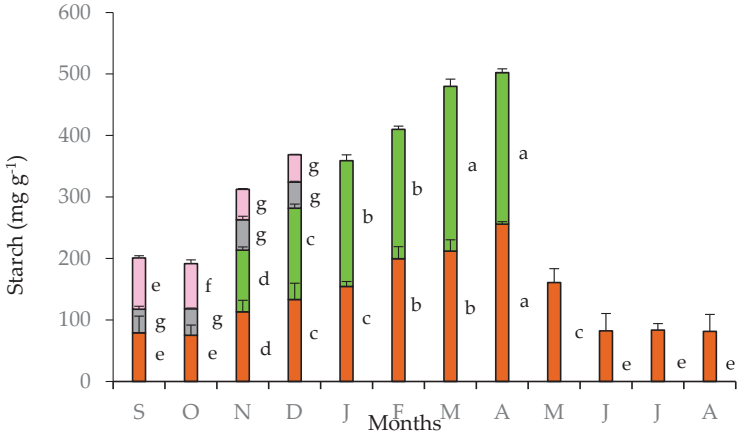


Figure 4. Starch content in tubers (brown bars), leaves (green bars), pedicels (gray bars), and petals (purple bars) of *Cyclamen graecum*, from September (S) to August (A). Each column denotes means of five replicates ± standard error; Standard Errors smaller than the line thickness of the columns are not shown. Significant differences ($p < 0.05$) of mean values are marked using lowercase letters.

2.4. Proline

The partitioning of free proline in tubers, leaves, pedicels, and petals are presented in Figure 5. The tubers sustained an almost stable free proline content throughout the year, with a slight but statistically significant increase during the cold months (November to March). The pronounced free proline accumulation in petals was evident during the flowering stage showing a substantial enhancement during October and November, the period of full blossom. The pedicels contained a quantity of proline at a comparative level with that of the tubers. The proline content found in leaves during the first two months of their appearance is comparable with that of tubers, but it significantly increased during the next four months of the leaf-stage for this species. Accordingly, the partitioning of proline in the various plant parts did not show a considerable seasonal variation resulting in an unchanged profile of the trends between tubers, leaves, and flowers. Nevertheless, seasonal differences in proline accumulation were obvious at the whole plant level, with the contribution of the proline-rich petals being remarkable.

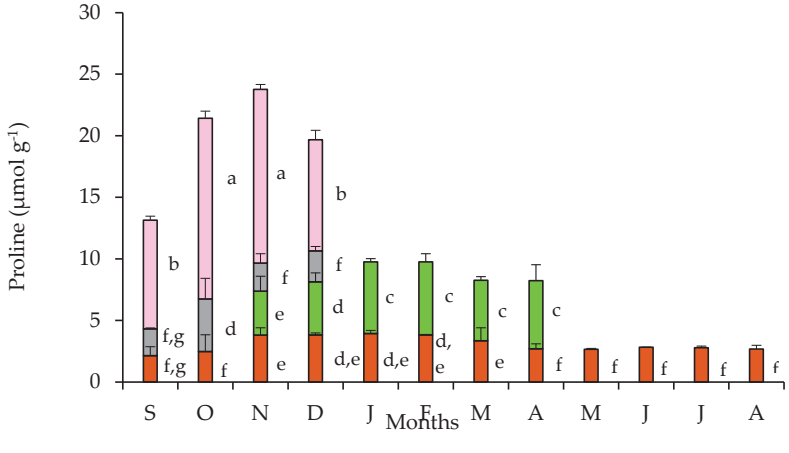


Figure 5. Proline content in tubers (brown bars), leaves (green bars), pedicels (gray bars), and petals (purple bars) of *Cyclamen graecum*, from September (S) to August (A). Each column denotes means of

five replicates ± standard error; Standard Errors smaller than the line thickness of the columns are not shown. Significant differences ($p < 0.05$) of mean values are marked using lowercase letters.

2.5. Water Status of above-Ground Plant Parts

Low values of petal water potential (Ψ) were detected in September (Table 1), after the summer drought period. In October Ψ, osmotic potential (Ψ_s) and turgor (Ψ_p) values were increased (less negative) (Table 1), and the same holds true in November. In December, the elevated values of Ψ and Ψ_s coincided with the lowest values of Ψ_p (Table 1), most probably due to cease of flowering by late December. Regarding Ψ, Ψ_s, and Ψ_p values of petals, significant differences ($p < 0.05$) were found among sampling dates (Table 1).

Table 1. Seasonal water potential (Ψ), osmotic potential (Ψ_s), and turgor (Ψ_p) of petals. The values are means of five replicates ± SE.

Months	Ψ (MPa)	Ψ_s (MPa)	Ψ_p (MPa)
September	-1.08 ± 0.02^{d}	-1.18 ± 0.05^{d}	$0.10 \pm 0.03^{b,c}$
October	-0.74 ± 0.04^{c}	-0.97 ± 0.02^{c}	0.23 ± 0.03^{a}
November	-0.56 ± 0.05^{b}	-0.72 ± 0.04^{b}	0.16 ± 0.04^{b}
December	-0.40 ± 0.02^{a}	-0.46 ± 0.03^{a}	0.06 ± 0.02^{c}

Significant differences ($p < 0.05$) of mean values are marked using lowercase superscript letters that are given separately on each column variable.

In petals, significant positive correlations were detected between Ψ and rainfall ($y = 0.0123x - 1.2499$, $R^2 = 0.954$, $p < 0.05$) and Ψ_s ($y = 0.0122x - 1.3865$, $R^2 = 0.811$, $p < 0.05$); also, negative relations were detected between soluble sugars and Ψ ($y = -0.0029x - 0.1193$, $R^2 = 0.730$, $p < 0.05$), and Ψ_s ($y = -0.0034x - 0.6634$, $R^2 = 0.843$, $p < 0.05$), as well as between starch and Ψ ($y = -0.0149x + 0.2407$, $R^2 = 0.908$, $p < 0.05$) and Ψ_s ($y = -0.0164x + 0.1905$, $R^2 = 0.936$, $p < 0.05$), while a positive relationship between Ψ_p and proline content was found ($y = 0.022x - 0.1168$, $R^2 = 0.822$, $p < 0.05$), as well as Ψ_p and soluble sugars ($y = 0.0007x + 0.00146$, $R^2 = 0.785$, $p < 0.05$).

The relative water content (RWC) of leaves was sustained fairly constant until March and declined in April (Table 2). Additionally, a positive relationship was detected between RWC and rainfall ($y = 0.2768x + 63.088$, $R^2 = 0.835$, $p < 0.05$).

Table 2. Relative water content (RWC) of leaves. The values are means of five replicates ± SE.

Months	RWC
November	79.34 ± 0.06^{a}
December	78.83 ± 0.10^{a}
January	77.33 ± 0.14^{a}
February	77.15 ± 0.08^{a}
March	77.23 ± 0.05^{a}
April	68.38 ± 0.12^{b}

Significant differences ($p < 0.05$) of mean values are marked using lowercase superscript letters.

3. Discussion

The seasonal accumulation of starch and soluble sugars in the tubers of *C. graecum* confirms to their role in storing photoassimilates and providing a supply of energy to drive new growth [17,25]. The distribution between soluble carbohydrates and starch differed between leaves and tubers. The partitioning of starch to tubers was reasonably similar to that of leaves and a positive linear relationship was detected between tubers and leaves ($y = 1.027x + 13.422$, $R^2 = 0.784$, $p < 0.05$); starch partitioning may be linked to source-sink relationships; the photosynthetically active leaves (the source) provide assimilated carbon (available for transport) to a storage organ (sink), which will utilize it to support metabolic requirements. Furthermore, the elevated values of starch from February to April in both

tubers and leaves coincide with the leaf photosynthetic efficiency, and the mild winter temperatures in the Mediterranean area that favor photosynthetic rates. It seems likely that in *C. graecum*, starch may represent a commitment of resources that are acquired by the above-ground tissues simultaneously with the growth of those drawn from stored reserves. This finding is in contrast to data published elsewhere for the summer flowering geophyte *Pancratium maritimum*, where starch was mainly stored in underground bulbs [26].

At the phenological stage of flowering, when the ambient temperatures begin to fall, the concentration of soluble sugars in the tubers rapidly decreased to support the metabolically demanding reproductive growth with carbon source and energy [27,28]; this is also indicated by the positive linear relationship between sugar content between tubers and petals ($y = 0.523x - 3.882$, $R^2 = 0.881$, $p < 0.05$). Floral growth during the early autumn takes precedence over allocation. A further decrease of soluble sugars in tubers at the period of leaf emergence indicates a translocation of stored sugars to sustain leaf development and floral exhibition, when winter lies ahead, via a transition from sink to source. The leaf sugar content during winter and spring denotes an active photosynthetic machinery for this species grown under ambient, environmental conditions, coinciding with the values of leaf RWC before wilting; leaf RWC may also be considered as a measure of the relative cellular volume, affecting interactions among macromolecules. Usually, levels of RWC below 70% imply a water potential at the order of −1.5 MPa or less, and this would cause changes in the metabolism with ceasing of photosynthesis [29], concomitantly with leaf senescence in April. Leaf sugar content may also be associated with the argument that leaves of cyclamen species are a static export pool of sucrose, and the sugar transport is probably linked to a time lag in the export of newly fixed carbon from leaves and low velocity of phloem transport [30]. Concerning the sugar partitioning, some geophytes seem to follow a pattern of relatively higher sugar concentration in the subterranean organs compared to leaves and in some cases, with flowers [31]. In petals, the reduced osmotic potential was significantly related with increased soluble sugar content ($y = -0.0034x - 0.1663$, $R^2 = 0.834$, $p < 0.05$), presumably contributing to turgor maintenance, expansion and water status of these tissues [32–34]. Actually, anthesis appears to be due to a pulsed increase in the concentration of soluble sugars [35–38]. A relationship linked to transfer of sugars between leaves and petals, which might be interesting, was not evaluated, because in *C. graecum*, flower and leaf development are concomitantly exhibited only during a two-month period, i.e., November and December.

Proline content in tubers of *C. graecum* showed a small but statistically significant increase from November to March, compared to the rest of the year. This accumulation may be driven by the low temperatures of the corresponding months and may be considered a stress-related response. An analogous profile was followed by its leaves, resulting in unchanged partitioning of proline between leaves and tubers, when leaves are coming through during the life-cycle of C. graecum; nevertheless, the greatest variation was found between petal and leaf concentrations. It has been published that *C. graecum* is a cold-tolerant species [37,39]. The increased proline biosynthesis and accumulation may partly account for a cold hardiness feature in both tubers and leaves, due to its protectiveness regarding stress and radical scavenging role [40]. Concerning the latter, proline has been related to scavenging of hydroxyl radicals (OH) and possibly other ROS [41], while indirectly modifies the plant's antioxidant response through increasing the capacity of the involved enzymes, especially ascorbate peroxidase [22]. Additionally, proline accumulation patterns may have implications on nitrogen storage and partitioning, especially under stress conditions [22,27,42]. Proline pool has been reported to expand during transition phase, i.e., from vegetative to reproductive growth [43–46].

Ecophysiological traits of plant organs that are seasonally either renewed or shed may be a suitable criterion of plant's adaptation to environmental conditions. *C. graecum* survives the hot summer in a state of dormancy. *C. graecum* blooms in autumn, before leaf emergence. Thereafter, leaves grow and accumulate metabolic reserves throughout the wet and cool season, until the dormancy period, which begins in late spring. In geophytes, long

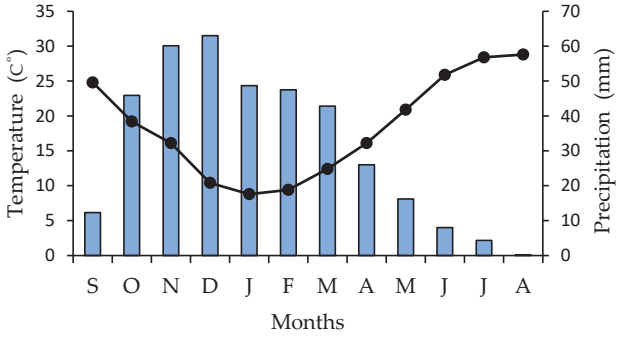
days can initiate the transition to bud dormancy [47,48]. The summer dormancy protects plants from negative effects of water shortage and elevated temperatures on vegetative and reproductive organs, and forces their active development in a more favorable season. *C. graecum* is released from dormancy when the ambient temperatures decrease; hence, shifts from the vegetative to reproductive stage, and floral initiation and differentiation occur in the mature tubers [8,49].

Free proline accumulation in petals was remarkable and significantly increased compared to the other plant organs. Flowers exhibited a 2.5- to 5-fold enhanced proline content in comparison to tubers and 2- to 3-fold compared to leaves. The transportation of proline into the reproductive organs, even under non-stress conditions, has been repeatedly reported [49]. Corroborating our results, a 60-fold higher proline concentration was detected in tomato flowers than in all the vegetative tissues [50]. Enhanced proline content was also documented in petals of *P. maritimum* and was attributed to a requirement for osmotic adjustment, because this geophyte is exposed to dry and saline ambient environmental conditions [26,46]. Multiple explanations of proline accumulation in flowers have been published. For example, the increased proline content has been connected to the high yield of ATP resulting from its oxidation, thus considering proline a molecule well-suited to sustain high energy-demanding processes in reproductive tissues [44]. Low values of petal osmotic potential coincided with enhanced proline accumulation. Additionally, the protective role of proline, which was positively correlated with the turgor of petals of *C. graecum*, has been highlighted during floral developmental processes that include dehydration, as spontaneously occurring during pollen formation or embryogenesis [51,52]. Furthermore, proline may provide a convenient source of energy and nitrogen during immediate post-stress metabolism [46,49,53].

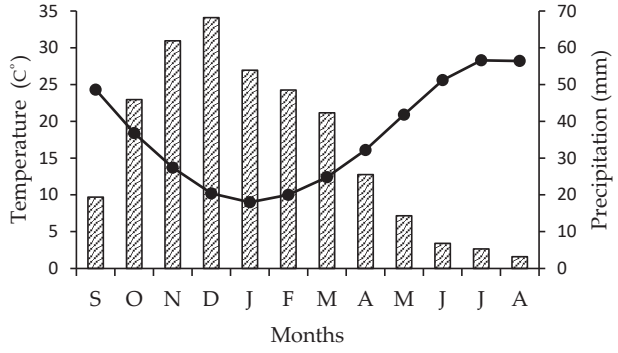
4. Material and Methods

4.1. Research Site and Plant Phenology

The study was conducted in naturally occurring patches of *Cyclamen graecum* Link distributed in the campus of the National and Kapodistrian University of Athens in Greece (latitude: 37.9664, longitude: 23.756971, altitude 260 m), at the foothills of Hymettus Mountain, where travertine limestone appears along discontinuities of strongly fractured gray dolomite limestone; also, father soil characteristics, texture, and composition have been previously published [54,55]. Concerning perennial geophytes, most bulbs and tubers must reach a critical size before floral induction can occur [56]. In addition, large bulbs and tubers generally produce vigorous above-ground organs and/or many flowers. Therefore, the active and the dormant phenological stage (Figure 2) were monitored, via detailed field observations in distinct niches of *C. graecum* [57], on a monthly basis for two consecutive years (2017 and 2018). The selected at random plants were growing under natural conditions; tubers, leaves, and flowers of *C. graecum* were sampled from a single stand of *C. graecum* surrounded by uniform Mediterranean phryganic vegetation [54], at monthly intervals during the course of a year, i.e., from September of 2018 to August of the next year (2019). The first flowers of *C. graecum* [58] appear in September, while the heart-shaped leaves are coming through in November, arising from the crown of the tubers [59]. Values of mean monthly precipitation and temperature, obtained from a meteorological enclosure, provided by the National Observatory of weather conditions in Greece, are presented in Figure 6a (annual data during the study period) and Figure 6b (multiannual data).



(**a**)



(**b**)

Figure 6. (**a**) Ombrothermic diagram (Precipitation scale = 2 × Temperature scale) for the study site; the order to months is from September (S) of 2018 to August (A) of 2019. Mean monthly precipitation is indicated by blue bars and mean monthly temperature by closed circles and the black line; (**b**) A multiannual ombrothermic diagram (Precipitation scale = 2 × Temperature scale) for the study site from September of 1955 to August of 2010; the order to months is from September (S) to August (A). Mean monthly precipitation is indicated by shaded bars, and mean monthly temperature by closed circles and the black line.

4.2. Determination of Total Soluble Sugar and Starch

Soluble sugars were extracted from dry, finely powdered samples (leaves, tubers, pedicels, petals) that were placed in 10 mL 80% ethanol (v/v), in a shaker, and the extracts were filtered using Whatman # 2 filter paper. Soluble sugar concentration was investigated colorimetrically, according to a modified phenol-sulphuric acid method [60,61], at 490 nm, using a spectrophotometer (Novaspec III$^+$ Spectrophotometer, Biochrom, Cambridge, UK). The determination of starch was made in the residue after the extraction of sugars, using the anthrone method [26,62]. D-glucose (Serva, Heidelberg, Germany) aqueous solutions were used for the standard curve. The values are expressed as mg g^{-1} d.w.

4.3. Determination of Proline

Free proline content was determined colorimetrically on 4 mL samples of the condensed fluid extracted from the plant material [63,64]. The extraction procedure from plant samples (finely powdered dried tubers, leaves, pedicels, and petals) and colorimetric determination were carried out as we have analytically published [26]. Dried, powdered samples were homogenized with aqueous sulphosalicylic acid (20 mL, 3% v/v), and the

homogenate filtered through Whatman # 2 filter paper; 2 mL of the filtrate reacted with acid-ninhydrin solution (2 mL) and glacial acetic acid (2 mL) in test tubes, which were placed in a water bath at 100 °C for 1 h, and the reaction terminated in an ice bath. After cooling, the reaction mixture was extracted with 4 mL toluene and homogenized in a vortex. The chromophore containing the toluene was aspirated from the aqueous phase and the absorbance was read at 520 nm; immediately after, the terminated reaction in glass tubes placed in an ice bath, using toluene as a blank sample and the spectrophotometer mentioned in paragraph 5.2. The proline concentration was estimated using a standard curve of relevant L-proline solutions (Serva, Heidelberg, Germany) and calculated on a dry weight basis.

4.4. Determination of Water Status

Petal water potential (Ψ) was measured psychometrically on 6 mm diameter fresh discs (five replicates) from fully expanded petals, which were placed in five C-52 psychrometric chambers (Wescor Inc., Logan, UT, USA) attached to a dew point psychrometer (HR-33T, Wescor Inc.) using the psychrometer switchbox (PS-10, Wescor); the time required for equilibration between the water vapor pressure of leaf sample and that of the psychrometer chamber was 2 h. The osmotic potential (Ψ_s) was measured using the same leaf discs after freezing and thawing [65,66]. Turgor pressure (Ψ_p) was calculated as the algebraic difference between Ψ and Ψ_s. The relative water content (RWC) of fully expanded leaves was determined according to the disc method [29], using the equation RWC (%) = [(FW − DW)/(TW − DW)] × 100, where: FW is the sample fresh weight, TW is the sample turgid weight, and DW is the sample dry weight.

4.5. Statistical Analysis

The results are presented as mean ± Standard Error (SE). In order to determine differences in the studied parameters of plant parts of *C. graecum*, a two-way analysis of variance (ANOVA) was performed on the studied parameters at $p < 0.05$ and the Duncan's multiple range test was applied for comparing the means. All statistical tests were performed using the SPSS statistical v. 23.0 (SPSS Inc., Chicago, IL, USA). Regression analysis was used to determine relationships among results obtained by plant tissues of *C. graecum* and precipitation.

5. Conclusions

The life form of *C. graecum* is characterized by two phenological stages in the course of a year, the active phase (from flower emergence in September to leaf senescence in April), and the dormant phase spanning the prolonged drought period, when above-ground plant parts are not exposed to the severity of summer in the eastern Mediterranean. Partitioning patterns of soluble sugars, starch, and free proline in above- and below-ground parts of *C. graecum* contribute to the maintenance of its annual rhythm and phenophases in fluctuating environmental conditions. The remarkable concentration of proline in petals, in comparison to other plant parts during autumn, seems to be associated with the maintenance of their turgor; without turgor, the exposed petals to ambient environmental conditions of the sharply reflexed corolla of *C. graecum* could not be standing so firm and erect. The leaf relative water content was found lower than the typical values of turgid and transpiring leaves; this may indicate that leaves of *C. graecum* subjected to ambient conditions are not susceptible to low temperatures. However, further work will be required to fully test this hypothesis.

Author Contributions: Conceptualization: J.P. and S.R.; methodology: J.P., M.-S.M.-C. and S.R.; investigation: J.P.; resources: S.R.; writing—original draft preparation. J.P., E.L., M.K., M.-S.M.-C. and S.R.; writing—review and editing: E.L., M.K., M.-S.M.-C. and S.R.; visualization: J.P., M.-S.M.-C. and S.R.; supervision: S.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received funding from the National and Kapodistrian University of Athens (Greece).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data are available from the authors upon request.

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

References

1. Dafni, A.; Cohen, D.; Noy-Mier, I. Life-cycle variation in geophytes. *Ann. Mo. Bot. Gard.* **1981**, *68*, 652–660. [CrossRef]
2. Negbi, M. Theophrastus on geophytes. *Bot. J. Linn. Soc.* **1989**, *100*, 15–43. [CrossRef]
3. Akita, Y.; Ishizaka, H.; Nakayama, M.; Shimada, A.; Kitamura, S.; Hase, Y.; Narumi, I.; Tanaka, A. Comparative analysis of floral pigmentation between wild-type and white-flowered varieties of *Cyclamen graecum*. *J. Hortic. Sci. Biotechnol.* **2010**, *85*, 437–443. [CrossRef]
4. Grey-Wilson, C. *The Genus Cyclamen*; Batsford, 2015. Available online: https://www.perlego.com/book/2425483/cyclamen-pdf (accessed on 12 March 2022).
5. Mazouz, W.; Djeddi, S. A biological overview on the genus *Cyclamen*. *Eur. J. Sci. Res.* **2013**, *110*, 7–22. Available online: http://www.europeanjournalofscientificresearch.com (accessed on 10 March 2022).
6. Panitsa, M.; Trigas, P.; Kontakos, D.; Valli, A.T.; Iatrou, G. Natural and cultural heritage interaction: Aspects of plant diversity in three East Peloponnesian castles (Greece) and conservation evaluation. *Plant Biosyst.* **2021**, 1–9. [CrossRef]
7. Scarborough, J. Theophrastus on herbals and herbal remedies. *J. Hist. Biol.* **1978**, *11*, 353–385. Available online: https://www.jstor.org/stable/4330714 (accessed on 20 February 2022). [CrossRef]
8. The Cyclamen Society. Available online: https://www.cyclamen.org/plants/species/ (accessed on 7 April 2022).
9. Le Nard, M.; De Hertogh, A.A. Bulb growth and development and flowering. In *The Physiology of Flower Bulbs*; De Hertogh, A.A., Le Nard, M., Eds.; Elsevier: Amsterdam, The Netherlands, 1993; pp. 29–44.
10. Dole, J.M. Research approaches for determining cold requirements for forcing and flowering of geophytes. *Hort. Sci.* **2003**, *38*, 341–346. [CrossRef]
11. Adkins, J.A.; Miller, W.B. Storage Organs. In *Plant Propagation Concepts and Laboratory Exercises*; Beyl, C.A., Trigiano, R.N., Eds.; CRC Press: London, UK, 2008; pp. 303–309.
12. Clennett, J.C.B. An analysis and revision of *Cyclamen* L. with emphasis on subgenus *Gyrophoebe* O. Schwarz. *Bot. J. Linn. Soc.* **2002**, *138*, 473–481. [CrossRef]
13. Liveri, E.; Phitos, D.; Kamari, G. Karyosystematic study of some plant taxa from Greece. *Fl. Medit.* **2021**, *31*, 346–354. [CrossRef]
14. Le Nard, M.; De Hertogh, A.A. General chapter on spring flowering bulbs. In *The Physiology of Flower Bulbs*; De Hertogh, A.A., Le Nard, M., Eds.; Elsevier: Amsterdam, The Netherlands, 1993; pp. 705–739.
15. Curuk, P.; Sogut, Z.; Izgu, T.; Sevindik, B.; Tagipur, E.M.; da Silva, J.A.T.; Serce, S.; Solmaz, I.; Kacar, Y.A.; Mendi, N.Y.Y.Y. Morphological characterization of *Cyclamen* sp. grown naturally in Turkey: Part II. *Acta Sci. Pol. Hortorum Cultus* **2016**, *15*, 205–224. [CrossRef]
16. Chen, L.-Q.; Cheung, L.S.; Feng, L.; Tanner, W.; Frommer, W.B. Transport of sugars. *Annu. Rev. Biochem.* **2015**, *84*, 865–894. [CrossRef] [PubMed]
17. Ruan, Y.-L. Sucrose metabolism: Gateway to diverse carbon use and sugar signaling. *Annu. Rev. Plant Biol.* **2014**, *65*, 33–67. [CrossRef] [PubMed]
18. Szabados, L.; Savouré, A. Proline: A multifunctional amino acid. *Trends Plant Sci.* **2010**, *15*, 89–97. [CrossRef] [PubMed]
19. Harris, S. The Persian Cyclamen. In *The Temple of Flora Commentary*; The Folio Society: London, UK, 2008; p. 67.
20. Sherwood, S.; Rix, M. *Treasures of Botanical Art*; Kew Publishing: Kew, Australia, 2008; pp. 20–21.
21. Ru, Q.; Wang, X.; Liu, T.; Zheng, H. Physiological and comparative proteomic analyses in response to nitrogen application in an Amaryllidaceae plant, *Lycoris aurea*. *Acta Physiol. Plant.* **2013**, *35*, 271–282. [CrossRef]
22. Kishor, K.P.B.; Sreenivasulu, N. Is proline accumulation *per se* correlated with stress tolerance or is proline homeostasis a more critical issue? *Plant Cell Environ.* **2014**, *37*, 300–311. [CrossRef]
23. Mattioli, R.; Costantino, P.; Trovato, M. Proline accumulation in plants-Not only stress. *Plant Signal. Behav.* **2009**, *4*, 1016–1018. [CrossRef]
24. Carter, C.; Shafir, S.; Yehonatan, L.; Palmer, R.G.; Thornburg, R. A novel role for proline in plant floral nectars. *Naturwissenschaften* **2006**, *93*, 72–79. [CrossRef]
25. Kamenetsky, R. Patterns of dormancy and florogenesis in herbaceous perennial plants: Environmental and internal regulation. *Crop Sci.* **2009**, *49*, 2400–2404. [CrossRef]
26. Pouris, J.; Meletiou-Christou, M.S.; Chimona, C.; Rhizopoulou, S. Seasonal functional partitioning of carbohydrates and proline among plant parts of the sand daffodil. *Agronomy* **2020**, *10*, 539. [CrossRef]
27. Orthen, B. Sprouting of the fructan-and starch-storing geophyte *Lachenalia minima*: Effects on carbohydrate and water content within the bulbs. *Physiol. Plant* **2001**, *113*, 308–314. [CrossRef]
28. Lundgren, M.R.; Des Marais, D.L. Life history variation as a model for understanding trade-offs in plant- environment interactions. *Curr. Biol.* **2020**, *30*, R180–R189. [CrossRef] [PubMed]

29. González, L.; González-Vilar, M. Determination of relative water content. In *Handbook of Plant Ecophysiology Techniques*; Springer: Dordrecht, The Netherlands, 2011; pp. 207–212.
30. Rothe, K.; Porzel, A.; Neumann, S.; Grimm, E. Characteristics of the phloem path: Analysis and distribution of carbohydrates in the petiole of *Cyclamen*. *J. Exp. Bot.* **1999**, *50*, 1807–1816. [CrossRef]
31. Li, W.; Huang, D.; Wang, B.; Hou, X.; Zhang, R.; Yan, M.; Liao, W. Changes of starch and sucrose content and related gene expression during the growth and development of Lanzhou lily bulb. *PLoS ONE* **2022**, *17*, e0262506. [CrossRef] [PubMed]
32. Tarpley, L.; Sassenrath, G.F. Carbohydrate profiles during cotton floral bud (square) development. *J. Agron. Crop Sci.* **2006**, *192*, 363–372. [CrossRef]
33. Beauzamy, L.; Nakayama, N.; Boudaoud, A. Flowers under pressure: Ins and outs of turgor regulation in development. *Ann. Bot.* **2014**, *114*, 1517–1533. [CrossRef]
34. Rhizopoulou, S.; Pantazi, H. Constraints on floral water status of successively blossoming Mediterranean plants under natural conditions. *Acta Bot. Gall.* **2015**, *162*, 97–102. [CrossRef]
35. van Doorn, W.G. Is petal senescence due to sugar starvation? *Plant Physiol.* **2004**, *134*, 35–42. [CrossRef]
36. van Doorn, W.G.; Kamdee, C. Flower opening and closure: An update. *J. Exp. Bot.* **2014**, *65*, 5749–5757. [CrossRef]
37. Khodorova, N.V.; Boitel-Conti, M. The role of temperature in the growth and flowering of geophytes. *Plants* **2013**, *2*, 699–711. [CrossRef]
38. Borghi, M.; Perez de Souza, L.; Yoshida, T.; Fernie, A.R. Flowers and climate change: A metabolic perspective. *New Phytol.* **2019**, *224*, 1425–1441. [CrossRef]
39. Ishizaka, H. Interspecific hybrids of *Cyclamen persicum* and *C. graecum*. *Euphytica* **1996**, *91*, 109–117. [CrossRef]
40. Hajihashemi, S.; Brestic, M.; Landi, M.; Skalicky, M. Resistance of *Fritillaria imperialis* to freezing stress through gene expression, osmotic adjustment and antioxidants. *Sci. Rep.* **2020**, *10*, 10427. [CrossRef] [PubMed]
41. Signorelli, S.; Arellano, J.-B.; Melo, T.-B.; Borsani, O.; Monza, J. Proline does not quench singlet oxygen: Evidence to reconsider its protective role in plants. *Plant Physiol. Biochem.* **2013**, *64*, 80–83. [CrossRef] [PubMed]
42. Ncube, B.; Finnie, J.F.; Van Staden, J. Carbon–nitrogen ratio and in vitro assimilate partitioning patterns in *Cyrtanthus guthrieae* L. *Plant Physiol. Biochem.* **2014**, *74*, 246–254. [CrossRef] [PubMed]
43. Tegeder, M.; Masclaux-Daubresse, C. Source and sink mechanisms of nitrogen transport and use. *New Phytol.* **2018**, *217*, 35–53. [CrossRef] [PubMed]
44. Chiang, H.H.; Dandekar, A.M. Regulation of proline accumulation in *Arabidopsis* during development and in response to dessication. *Plant Cell Environ.* **1995**, *18*, 1280–1290. [CrossRef]
45. Wainwright, H.; Harwood, A.C. In vitro organogenesis and plant regeneration of *Cyclamen persicum* Mill. using seedling tissue. *J. Hortic. Sci.* **1985**, *60*, 397–403. [CrossRef]
46. Carfagna, S.; Salbitani, G.; Innangi, M.; Menale, B.; De Castro, O.; Di Martino, C.; Crawford, T.W. Simultaneous biochemical and physiological responses of the roots and leaves of *Pancratium maritimum* (Amaryllidaceae) to mild salt stress. *Plants* **2021**, *10*, 345. [CrossRef]
47. Larcher, W. *Physiological Plant Ecology: Ecophysiology and Stress Physiology of Functional Groups*; Springer: Berlin, Germany, 2003; p. 233.
48. Proietti, S.; Scariot, V.; De Pascale, S.; Paradiso, R. Flowering mechanisms and environmental stimuli for flower transition: Bases for production scheduling in greenhouse floriculture. *Plants* **2022**, *11*, 432. [CrossRef]
49. Ghosh, U.K.; Islam, M.N.; Siddiqui, M.N.; Cao, X.; Khan, M.A.R. Proline, a multifaceted signaling molecule in plant responses to abiotic stress: Understanding the physiological mechanisms. *Plant Biol.* **2022**, *24*, 227–239. [CrossRef]
50. Schwacke, R.; Grallath, S.; Breitkreuz, K.E.; Stransky, E.; Stransky, H.; Frommer, W.B.; Rentsch, D. LeProT1, a transporter for proline, glycine betaine, and gamma-amino butyric acid in tomato pollen. *Plant Cell* **1999**, *11*, 377–392. [CrossRef] [PubMed]
51. Funck, D.; Winter, G.; Baumgarten, L.; Forlani, G. Requirement of proline synthesis during *Arabidopsis* reproductive development. *BMC Plant Biol.* **2012**, *12*, 191. [CrossRef] [PubMed]
52. Ishizaka, H. Cytogenetic studies in *Cyclamen persicum*, *C. graecum* (Primulaceae) and their hybrids. *Plant Syst. Evol.* **2003**, *239*, 1–14. [CrossRef]
53. Stewart, C.R. The effect of wilting on proline metabolism in excised bean leaves in the dark. *Plant Physiol.* **1973**, *51*, 508–511. [CrossRef]
54. Margaris, N.S. Structure and dynamics in a phryganic (East Mediterranean) ecosystem. *J. Biogeogr.* **1976**, *3*, 249–259. [CrossRef]
55. Kampouroglou, E.; Economou-Eliopoulos, M. Assessment of the environmental impact by As and heavy metals in lacustrine travertine limestone and soil in Attica, Greece: Mapping of potentially contaminated sites. *Catena* **2016**, *139*, 137–166. [CrossRef]
56. Khalafalla, M.M.; Menesy, F.; Magouz, M.R.; Hamed, E.B. Growth and flowering of endemic wild Libyan geophyte *Cyclamen rohlfsianum* Ascher, with a high ornamental value. *Appl. Ecol. Environ. Res.* **2020**, *218*, 4583–4594. [CrossRef]
57. Yesson, C.; Culham, A. A phyloclimatic study of *Cyclamen*. *BMC Evol. Biol.* **2006**, *6*, 72. [CrossRef]
58. Leven, S. Cyclamen graecum. 2004. Available online: https://www.srgc.org.uk/monthfeature/nov2004/content.html (accessed on 5 March 2022).
59. Debussche, M.; Garnier, E.; Thompson, J.D. Exploring the causes of variation in phenology and morphology in Mediterranean geophytes: A genus-wide study of *Cyclamen*. *Bot. J. Linn. Soc.* **2004**, *145*, 469–484. [CrossRef]

60. Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350–356. [CrossRef]
61. Buysse, J.; Merckx, R. An improved colorimetric method to quantify sugar content of plant tissue. *J. Exp. Bot.* **1993**, *4*, 1627–1629. [CrossRef]
62. Meletiou-Christou, M.S.; Rhizopoulou, S. Leaf functional traits of four evergreen species growing in Mediterranean environmental conditions. *Acta Physiol. Plant.* **2017**, *39*, 34. [CrossRef]
63. Bates, L.S.; Waldren, R.P.; Teare, I.D. Rapid determination of free proline for water studies. *Plant Soil* **1973**, *39*, 205–207. [CrossRef]
64. Ain-Lhout, F.; Zunzunegui, M.; Barradas, M.D.; Tirado, R.; Clavijo, A.; Novo, F.G. Comparison of proline accumulation in two Mediterranean shrubs subjected to natural and experimental water deficit. *Plant Soil* **2001**, *230*, 175–183. [CrossRef]
65. Richter, H. A diagram for the description of water relations in plant cells and organs. *J. Exp. Bot.* **1978**, *29*, 1197–1203. [CrossRef]
66. Rhizopoulou, S.; Meletiou-Christou, M.S.; Diamantoglou, S. Water relations for sun and shade leaves of four Mediterranean evergreen sclerophylls. *J. Exp. Bot.* **1991**, *42*, 627–635. [CrossRef]

plants

MDPI

Brief Report

Effect of Temperature on the Germination of Five Coastal Provenances of *Nothofagus glauca* (Phil.) Krasser, the Most Representative Species of the Mediterranean Forests of South America

Rómulo E. Santelices-Moya [1], Marta González Ortega [2], Manuel Acevedo Tapia [2], Eduardo Cartes Rodríguez [2] and Antonio M. Cabrera-Ariza [3,*]

[1] Centro de Desarrollo del Secano Interior, Facultad de Ciencias Agrarias y Forestales, Universidad Católica del Maule, Talca 3466706, Chile; rsanteli@ucm.cl
[2] Centro Tecnólogico de la Planta Forestal, Instituto Forestal Sede Biobío, Camino a Coronel Km 7.5, San Pedro de la Paz 4130000, Chile; mgonzale@infor.cl (M.G.O.); macevedo@infor.cl (M.A.T.); ecartes@infor.cl (E.C.R.)
[3] Centro de Investigación y Estudios Avanzados del Maule, Vicerrectoría de Investigación y Postgrado, Universidad Católica del Maule, Talca 3466706, Chile
* Correspondence: acabrera@ucm.cl

Citation: Santelices-Moya, R.E.; González Ortega, M.; Acevedo Tapia, M.; Cartes Rodríguez, E.; Cabrera-Ariza, A.M. Effect of Temperature on the Germination of Five Coastal Provenances of *Nothofagus glauca* (Phil.) Krasser, the Most Representative Species of the Mediterranean Forests of South America. *Plants* **2022**, *11*, 297. https://doi.org/10.3390/plants11030297

Academic Editors: Sofia Rhizopoulou, Maria Karatassiou and Efi Levizou

Received: 12 January 2022
Accepted: 21 January 2022
Published: 24 January 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Abstract: Temperature is one of the most important abiotic factors affecting seed germination, and it is strongly influenced by local site conditions. Seeds of *Nothofagus glauca*, an endemic and vulnerable species of the Mediterranean region of Chile and the most representative of the Mediterranean forests of South America, were collected. In this study, we evaluated the effect of temperature on different germinative attributes of five *N. glauca* provenances representative of their natural distribution. The seeds were treated at a constant temperature (i.e., 18 °C, 22 °C, 26 °C, or 30 °C) in the absence of light for 40 days. The results show that in all the provenances, the germination ratio and energy increase linearly with temperature until reaching an optimum temperature (i.e., 22 °C), above which they decrease severely. At 22 °C, the response of average germination speed and germination vigor was significantly higher than with the other temperatures (performance of germination start day was not clear). The base temperature was around 18 °C and the maximum, above 30 °C, which may be close to thermo-inhibition. Given the threat of climate change, it is necessary to increase research in terms of the possible adaptation of this species to increased temperatures and prolonged periods of drought

Keywords: hualo; Mediterranean plants; seeds

1. Introduction

Nothofagus glauca (Phil.) Krasser (common name, hualo or roble maulino) is an endemic species of Central Chile that belongs to the Nothofagaceae family and is the most representative of the Mediterranean forests of its genus in South America. It is a deciduous, monoecious tree that can reach up to 30 m in height and 2 m in diameter [1], although at present it is difficult to find individuals that are more than 40 cm in diameter. The species is listed as vulnerable, and its populations are currently severely fragmented and trending toward decreasing [2].

The *N. glauca* forests have a discontinuous distribution in a latitudinal range of about 400 km, from 33°58′ S, 71°05′ W to 37°27′ S, 71°58′ W, although they are mostly concentrated in the Maule Region [3]. They are a transitional system between xerophytic formations and the southernmost temperate forests. This type of deciduous forest has adapted to the prolonged dry periods of summer and plays very important roles in the conservation of water and organic soil and in the biogeochemical carbon cycle as well as offering a great variety of ecological niches and habitats to the flora, fauna, and associated microbiota [4]. The

natural range of this species has been considered a hotspot of biodiversity for conservation and is characterized by a great diversity of endemic species, although this has decreased to critical levels in terms of dominance and variability mainly because of anthropogenic factors [5]. The highest population density in Chile is concentrated in *N. glauca* distribution area, with the consequent pressure on natural resources, including the *N. glauca* forests.

The anthropogenic pressure has strongly shaped the landscape in the natural distribution area of *N. glauca* in recent years, affecting its spatial distribution, among other variables. Forty-five years ago, Urzúa [6] reported that there were 900,000 ha of these forests, whereas today, there are only 157,000 ha [3]. Another threat looming over these and other forests in the region is global climate change. Indeed, an increase in both temperatures and prolonged periods of drought has been recorded, factors that predispose the vegetation to being more prone to damage caused by biotic and abiotic agents. As an example, it can be noted that in the summer of 2017, 184,000 ha of the forest were consumed in a single fire, which affected an important part of this forest system [7]. In addition, considering that its regeneration by natural seeding is currently almost nonexistent, it is necessary to study its propagation by seeds to provide the necessary background to produce plants intended for afforestation and/or restoration of its populations. It is also important to consider the temperatures that limit the germination of seeds, especially if climate change is a factor that is affecting ecosystems.

Temperature is a crucial factor in the germination process of seeds [8]. It plays an important role in determining the periodicity of seed germination and the distribution of species (among other factors, it affects enzymatic activity) [9]. The germination rate generally increases linearly with temperatures up to an optimum temperature; subsequently, germination decreases severely with higher temperatures. Moreover, in the seed germination process, there are three levels of temperature: minimum, optimal, and maximum. The minimum, or base, temperature is the lowest temperature at which the seeds can germinate. The optimum temperature is the temperature at which the seeds reach their highest germination rate, and the maximum, or ceiling, temperature is the temperature above which the seeds cannot germinate [10]. The germination rate will increase between the minimum and optimum temperatures, whereas temperatures ranging from optimum to maximum will lead to a decrease in this attribute [11]. If the temperature is changing because of global climate change, it is evident that some species will have to adapt to these new environmental conditions, among other aspects, in the germination process.

N. glauca is a species whose seed production cycles are becoming longer, with years without seed production (unpublished data) and has seeds that present endogenous dormancy. There are mechanisms to break this condition, for example, through variable stratification periods at 4 °C (i.e., between 4 and 6 weeks) or by soaking them in gibberellic acid in concentrations from 0.1 to 0.8 g L^{-1} [12], reaching a germination ratio even above 95% using only viable seeds. However, the germination percentage may be different depending on the origin of the seeds [13]. Until today, no report has been made on the effect of temperature on the germination of any population of *N. glauca*. Therefore, this study aimed to analyze the effect of temperature in the germination process of seeds from five coastal provenances of *N. glauca*.

2. Results

The results show that temperature has a significant effect on the germination of *N. glauca* seeds from different provenances (Table 1, Figures 1 and 2). In all the provenances, the highest germination percentage was obtained at 22 °C, although very different values were recorded in the germination ratio, from 34.0 ± 0.6% in the Los Ruiles provenance to 78 ± 0.3% in the Las Cañas provenance, observing the same tendency in the germinative energy. In general, in the northernmost provenances, a higher germination ratio was observed. These results show that *N. glauca* has a significant germination potential at 22 °C. In addition, the highest slope in the germination curves was observed at this temperature (Figure 2), which is an indicator that with endogenous application of gibberellic

acid at 22 °C, latency is better overcome; it could be considered, then, that the optimum germination temperature for most provenances of *N. glauca* is around 22 °C (in the Las Cañas provenance, there were no significant differences between 22 °C and 26 °C).

Table 1. Effect of temperature on different germination parameters of *Nothofagus glauca* (mean ± SE). Different letters indicate significant differences by Tukey's multiple comparison test ($p < 0.05$).

Provenance	Temperature (°C)	Germination Ratio (%)	Germination Energy (%)	Germination Start Day	Average Germination Speed (Seed/Day)	Germination Vigor
Licantén	18	6.7 ± 0.67 d	6.8 ± 0.33 d	25.0 ± 0.0 c	0.1 ± 0.22 c	0.0 ± 0.0 d
	22	52.0 ± 0.01 a	45.0 ± 2.26 a	11.0 ± 0.94 a	1.5 ± 0.07 a	2.4 ± 0.11 a
	26	32.0 ± 0.01 b	31.4 ± 1.44 b	16.0 ± 0.94 b	0.7 ± 0.03 b	0.9 ± 0.04 b
	30	20.7 ± 0.67 c	14.6 ± 0.71 c	13.0 ± 0.47 ab	0.6 ± 0.03 b	0.4 ± 0.02 c
Las Cañas	18	10.7 ± 0.54 c	6.2 ± 0.11 c	24.3 ± 0.27 b	0.2 ± 0.01 d	0.1 ± 0.0 d
	22	78.7 ± 0.54 a	77.1 ± 3.42 a	12.0 ± 0.94 a	2.4 ± 0.11 a	9.1 ± 0.41 a
	26	65.3 ± 0.54 a	57.5 ± 2.97 a	10.7 ± 0.72 a	1.7 ± 0.08 b	4.0 ± 0.2 b
	30	22.7 ± 0.54 b	14.0 ± 0.65 b	12.0 ± 0.47 a	0.7 ± 0.04 c	0.6 ± 0.03 c
Los Ruiles	18	0.7 ± 0.54 d	0.7 ± 0.54 d	0.0 ± 0.0 c	0.0 ± 0.0 d	0.0 ± 0.0 d
	22	34.0 ± 0.0 a	31.2 ± 0.0 a	16.0 ± 0.94 ab	0.8 ± 0.04 a	1.3 ± 0.05 a
	26	23.3 ± 0.54 b	19.3 ± 0.9 b	21.0 ± 0.94 b	0.5 ± 0.02 b	0.5 ± 0.03 b
	30	8.7 ± 0.54 c	6.7 ± 0.49 c	14.0 ± 0.47 a	0.3 ± 0.01 c	0.2 ± 0.02 c
Curanipe	18	8.0 ± 0.94 d	8.0 ± 0.42 b	24.3 ± 1.19 b	0.1 ± 0.01 d	0.0 ± 0.0 d
	22	40.7 ± 0.54 a	34.6 ± 1.62 a	12.0 ± 0.47 a	1.3 ± 0.06 a	2.3 ± 0.11 a
	26	34.0 ± 0.0 b	34.0 ± 0.0 a	13.0 ± 0.47 a	0.8 ± 0.03 b	1.3 ± 0.06 b
	30	13.3 ± 0.54 c	8.1 ± 0.38 b	14.0 ± 0.47 a	0.4 ± 0.02 c	0.2 ± 0.01 c
Quirihue	18	11.3 ± 0.54 c	10.8 ± 0.47 c	24.0 ± 0.94 b	0.2 ± 0.02 c	0.3 ± 0.01 c
	22	42.0 ± 0.0 a	35.4 ± 1.72 a	10.0 ± 0.47 a	1.3 ± 0.08 a	5.2 ± 0.25 a
	26	26.0 ± 0.0 b	23.3 ± 1.1 b	13.0 ± 0.47 a	0.6 ± 0.03 b	1.7 ± 0.08 b
	30	4.0 ± 0.0 d	3.8 ± 0.14 d	12.0 ± 0.47 a	0.1 ± 0.01 d	0.1 ± 0.01 d

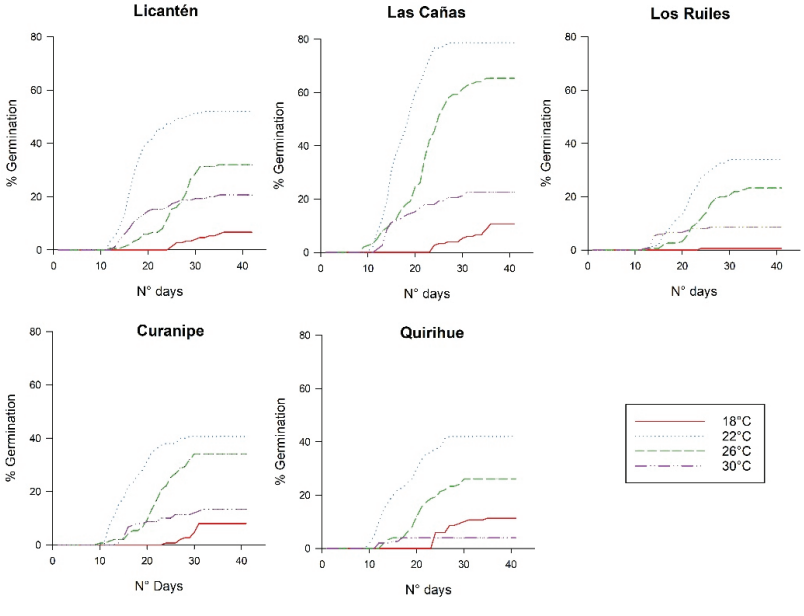


Figure 1. Cumulative germination percentage during 40 days for five *Nothofagus glauca* provenances treated at different temperatures in the absence of light.

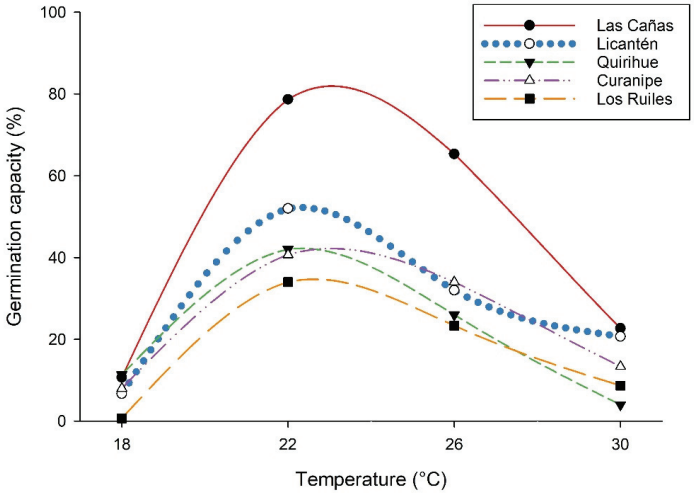


Figure 2. Final germination percentage of five provenances of *Nothofagus glauca* seeds treated at different temperatures in the absence of light.

In most of the provenances, once the seeds had begun to germinate, higher temperatures stimulated germination to an optimum temperature (22 °C), after which at 26 °C and 30 °C, the germination rate decreased. A clear pattern was also observed on the germination start day because of temperature, although seeds treated at 18 °C generally took longer to germinate. In general, average germination speed and vigor were higher at 22 °C, to later decrease with increasing temperatures; at 18 °C the lowest values were recorded in these two variables, although in some provenances, no significant differences were observed regarding those seeds treated at the highest temperature (30 °C).

3. Discussion

Seed germination is a process in which endogenous and exogenous factors intervene. On the one hand, two growth regulators that play a fundamental role in this process are gibberellic acid, a promoter of germination, and abscisic acid (ABA), which is an inhibitor. On the other hand, in addition to humidity and oxygen availability, temperature is the environmental factor with the greatest effect on the germination process of seeds, directly affecting their metabolism and germination speed [8]. With super-optimal temperatures, seed germination is delayed because a high level of ABA is maintained in the embryo and endosperm and the synthesis of gibberellic acid is suppressed [14,15]. The results of our research show a significant drop in germination at 30 °C, and it is likely that above this temperature, the seeds of *N. glauca* approach the thermo-inhibition process and prevent gibberellin biosynthesis.

The cardinal temperatures for germination are related to the environmental range of adaptation of a particular species and serve to match the germination time with the favorable conditions for the growth and subsequent development of the seedlings [10]. In our research in all the provenances studied, it was observed that from 18 °C, as the temperature increased, so did the germination until reaching a maximum at 22 °C, and then decreased. Thus, the optimum germination temperature for *N. glauca* would be at 22 °C, whereas the minimum, or basal, temperature would be around 18 °C, and the maximum, or ceiling, temperature would be above 30 °C. While the optimum temperature germination rate may vary between seed lots of the same species as a result of different genetic and environmental conditions [9], in the five provenances of *N. glauca*, this was not the case; the optimum temperature was clearly 22 °C, although for the Las Cañas provenance, it would be between 22 °C and 26 °C. It is striking that in the Los Ruiles provenance (area

protected by the state), germination at 18 °C was practically null and the basal temperature for that provenance would likely be above that level. In the case of the southernmost provenance (Quirihue), at 30 °C, germination fell to levels below those reached at 18 °C; in this case, it would likely be closer to thermo-inhibition at that temperature. According to these results, it is probable that there is a genetic effect that conditions the germination process of the seeds.

Temperature not only plays a fundamental role in capacity and energy germination, but it also affects the germination start day, average germination speed, and germination vigor (Table 2). Once the seed has been rehydrated after imbibition, under suitable temperature conditions, physiological processes are triggered that allow the germination process to develop [8]. Although germination occurred at 18 °C, with variable rates according to the geographic origin of the seeds, the process is significantly slower at that temperature, taking at least 24 days to begin germination; instead, with the optimum temperature (i.e., 22 °C), it begins in some cases at 12 days. This same trend is also observed in both the average germination speed and vigor, meaning that the germination process of *N. glauca* is strongly conditioned by temperature. The germination ratio at the optimal temperature (i.e., 22 °C), is in the range found by other authors for the same species [12,13].

Table 2. Geographic and climatic data for provenances sampled.

Provenance	Latitude	Longitude	Elevation (m a.s.l.)	M.A.T [1] (°C)	M.A.R [2] (mm Year^{-1})
Licantén	34°55′49″ S	72° 5′11″ W	403	12.5	829
Las Cañas	35°27′27″ S	72°28′11″ W	165	12.9	831
Los Ruiles	35°50′2″ S	72°30′36″ W	202	12.4	851
Curanipe	35°51′24″ S	72°37′13″ W	129	13.1	817
Quirihue	36°2′18″ S	72°36′35″ W	342	12.0	898

[1] M.A.T.: mean annual temperature; [2] M.A.R.: mean annual rainfall.

In the northernmost provenances, a higher percentage of germination was recorded, although no relationship was observed with the weight and morphometric characteristics of the seeds. In other species of the genus *Nothofagus*, it has been observed that the larger and heavier seeds have a greater germination ratio than those that are smaller and lighter, observing a clinal variation, although in a broader latitudinal distribution than that of this study [16]. This pattern was not observed in our study.

All species have a temperature range in which the germination process occurs. It has been described that for *N. glauca*, this range is likely between 10 °C and 30 °C [17]. On the one hand, in our research, we observed that the minimum temperature would be around 18 °C, especially for the Los Ruiles provenance, which is why it would be advisable to investigate the behavior of different provenances of this species under 18 °C. On the other hand, at 30 °C, germination strongly decreases, and above that level would be the maximum temperature and occurrence of thermo-inhibition. Consequently, temperatures under 18 °C and above 30 °C in the germination process of *N. glauca* should be evaluated.

Temperature is one of the main environmental factors that regulate seed physiology across plant taxa [10]. Because of climate change (i.e., increased temperatures and prolonged periods of drought), plants in general are being subjected to greater water stress, and therefore, their physiological processes are being affected. In the Mediterranean region of Chile, where *N. glauca* is distributed, there has been a significant increase in extremely hot events affecting the average temperature, and a deficit in rainfall [18]. *N. glauca* is widely known for its strong tendency for alternate bearing, which severely affects the fruit yield from year to year, and it has been observed that the cycles in seed production are becoming longer, with years without seed production (unpublished data). Given the threat of climate change on the reproductive cycle of the species and considering the effect that temperature has on the germination of *N. glauca* seeds, it is urgent and necessary to study in greater depth the adaptation capacity that this species would have to these new

environmental conditions. Santelices, Espinoza, Magni, Cabrera, Donoso, and Peña [13] reported that the intra-provenance variability of *N. glauca* is systematically greater than that of inter-provenance, indicating a high potential capacity of the species to adapt to climate change. However, these authors affirm that there are differences in germination between Andean and coastal origins (in our research, we evaluated only costal provenances); this reaffirms the need to deepen research on the potential adaptation of *N. glauca* to climate change and the capacity of the species to regenerate and self-perpetuate.

4. Material and Methods

4.1. Seed Collection and Preparation

In March 2017, mature seeds were collected from different provenances of *N. glauca* in the Maule Region of Chile (Table 3, Figure 3), except those from Licantén, which were collected in March 2015. Seeds were transported to the laboratory, where they were manually separated from the rest of the plant material and the damaged seeds were discarded; then, they were weighed, dried, and stored in the dark in glass containers in an environment at 4 °C until they were used (i.e., January 2020). The standards of the International Seed Testing Association (ISTA) were followed to characterize the seeds [19]. One hundred seeds were weighed separately for eight repetitions to determine the weight of the seeds, which was expressed as the average weight of 1000 seeds. Then, the average weight of 1000 seeds and its equivalence in number of seeds per kilogram were calculated. In addition, the dimerous seeds were measured for length and width, and the trimerous seeds for length, width, and thickness (Table 2). To break the dormancy, the seeds were soaked in a 200 mg L^{-1} gibberellic acid solution (Giberplus® Tabletas, Anasac Chile S.A., Santiago, Chile) for 24 h before starting the germination tests [12].

The mean annual precipitation and temperature were obtained from WorldClim (version 2) at a spatial resolution of 30 s (~1 km^2) by interpolation of the records of meteorological stations from 1970 to 2000 [20].

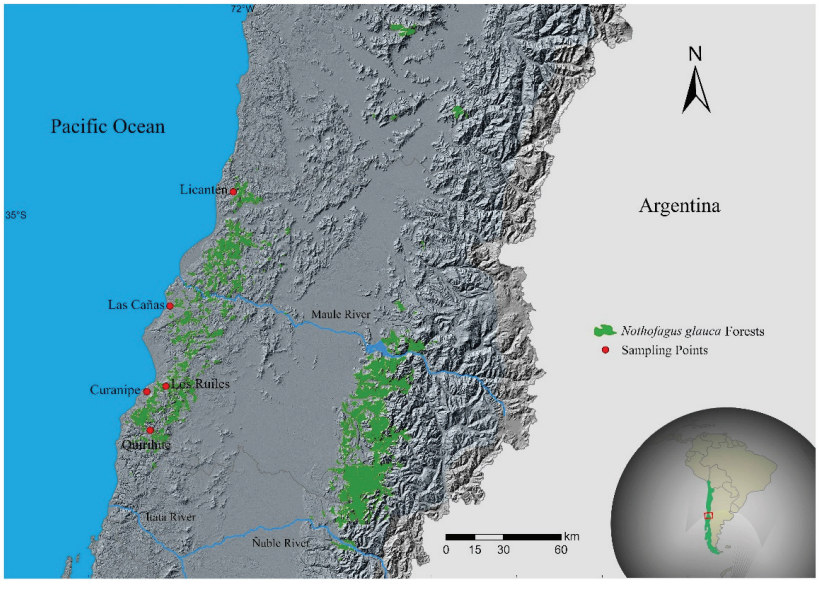


Figure 3. Seed provenances of *Nothofagus glauca*.

Table 3. Weight and morphometric characterization of the seeds from five provenances of *Nothofagus glauca* (mean ± Standard Error).

Provenance	Weight of 1000 Seeds (g)	Number of Seeds per Kilogram	Dimerous Seeds		Trimerous Seeds		Thickness (mm)
			Length (mm)	Width (mm)	Lenght (mm)	Width (mm)	
Licantén	468.3 ± 0.3	2136 ± 10.4	17.1 ± 1.8	14.2 ± 1.8	17.2 ± 0.1	10.4 ± 0.1	9.7 ± 0.1
Las Cañas	537.5 ± 0.4	1861 ± 14.0	19.5 ± 0.1	12.1 ± 0.2	19.0 ± 0.1	10.8 ± 0.1	9.9 ± 0.1
Los Ruiles	683.7 ± 0.5	1463 ± 10.3	19.7 ± 1.6	13.2 ± 1.8	19.6 ± 0.2	11.3 ± 0.2	11.2 ± 0.1
Curanipe	395.5 ± 0.3	2530 ± 9.9	19.5 ± 0.3	11.3 ± 0.2	17.5 ± 0.3	10.3 ± 0.1	8.2 ± 0.2
Quirihue	379.0 ± 0.4	2641 ± 14.9	19.3 ± 0.1	12.1 ± 0.1	18.5 ± 0.2	9.7 ± 0.1	9.1 ± 0.1

4.2. Germination Experiments

The study was carried out in a laboratory at the Universidad Católica del Maule, Talca, Chile (35°26′10″ S, 71°37′13″ W, 131 m a.s.l.), during January and February 2020. The seeds were soaked in a Gibberellic Acid (GA_3) solution at 200 mg L^{-1} for 24 h using distilled water, and those that floated were excluded as they were considered unviable. To determine the effect of temperature on the germination of *N. glauca* seeds from different provenances, four different temperatures were tested: 18 °C, 22 °C, 26 °C, and 30 °C. The cultivation was carried out in germinating chambers in the absence of light, maintaining fixed temperatures according to each treatment and using filter paper as the substrate. To not interfere with the treatments, the ambient temperature of the laboratory was constantly maintained below 16 °C. Irrigation was manual, and care was taken that the seeds were always wet.

The germination process was monitored daily until germination ceased over a period of 40 days. Seeds were considered to have germinated when the emerging radicles were over 2 mm long. The following germination parameters, adapted from [21,22], were calculated:

$$GR = \left[\frac{Sg}{Ss}\right] \times 100 \quad (1)$$

Germination ratio (GR) (1): represents the final percentage of seeds that germinate (Sg) in relation to the total number of seeds sown (Ss):

Germination energy (GE): accumulated percentage of germination on the day when the maximum value occurs (maximum value is maximum ratio from cultivated germination percentage on day X divided by X).

Germination start day (GSD): the time elapsed from the sowing of the seeds to the germination of 5% of the sown seeds.

Average germination speed (AGS) [2]: corresponds to the average number of germinated seeds per day, calculated by the expression:

$$AGS = \sum_1^k \frac{ni}{ti} \quad (2)$$

where *ni* corresponds to the number of seeds germinated in the *i*th data collection, *ti* is the time (in days) of the *i*th data collection, and *k* is the time (in days) of the germination test duration.

Germination vigor (GV): reflects in a single value the changes in the germination peak, the total germination, and the germination speed, calculated as the product between the maximum value and the average germination speed.

4.3. Trial Design and Statistical Analysis

There were two factors tested in the trial: temperature (four levels) and provenance (five levels). The factorial combination of 20 treatments (4 × 5) was replicated three times in a split-plot design. Fixed effects were randomly assigned within subplots (considering the homogeneity of the temperature, whole plots were not randomized). There were 50 viable

seeds per factorial combination, giving a total of 150 seeds per treatment. Temperature was applied to the whole plots and provenance, to the subplots. The treatments imposed follow:

- Temperature: 18 °C, 22 °C, 26 °C, and 30 °C
- Provenance: Licantén, Las Cañas, Los Ruiles, Curanipe, and Quirihue

Analyses of variance (ANOVAs) and comparisons of the means were conducted using the general linear model (GLM) procedure from the statistical software SPSS for Windows (SPSS, Chicago, IL, USA). Mean values with significant differences were compared using the Tukey test at the 5% significance level.

5. Conclusions

Based on the results of this research, it can be concluded that temperature is an abiotic factor that significantly affects the germination of *N. glauca*. In the absence of light conditions, the optimum germination temperature for all the provenances studied was 22 °C. By maintaining the temperature at 18 °C, germination was induced, although at a low percentage. At 30 °C, the germination percentage was also low, and above this level is the maximum germination temperature and risk of thermo-inhibition.

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

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

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

References

1. Rodríguez, R.; Quezada, M. Fagaceae. In *Flora de Chile 2*; Marticorena, C., Rodríguez, R., Eds.; Universidad de Concepción: Concepción, Chile, 2003; pp. 64–76.
2. Barstow, M.; Rivers, M.C.; Baldwin, H. Nothofagus Glauca. *IUCN Red List. Threat. Species* **2017**, eT32034A2809142. [CrossRef]
3. Santelices-Moya, R.; Vergara, R.; Cabrera-Ariza, A.; Espinoza-Meza, S.; Silva-Flores, P. Variación intra-específica en *Nothofagus glauca* una especie endémica de los bosques mediterráneos de Chile. *Bosque* **2020**, *41*, 221–231. [CrossRef]
4. Arroyo, M.T.K.; Riveros, M.; Peñaloza, A.; Cavieres, L.; Faggi, A.M. Phytogeographic relationships and regional richness patterns of the cool temperate rainforest flora of southern South America. In *High-Latitude Rainforests and Associated Ecosystems of the West Coasts of the Americas: Climate, Hydrology, Ecology and Conservation*; Lawford, R.G., Alaback, P.B., Fuentes, E., Eds.; Springer: New York, NY, USA, 1996; pp. 134–172.
5. Myers, N.; Mittermeier, R.A.; Mittermeier, C.G.; da Fonseca, G.A.B.; Kent, J. Biodiversity hotspots for conservation priorities. *Nature* **2000**, *403*, 853–858. [CrossRef] [PubMed]
6. Urzúa, A. Cambio de Estructura en el Bosque de *Nothofagus glauca* (Phil.) Krasser. Ph.D. Thesis, Universidad de Chile, Santiago, Chile, 1975; Tesis Ingeniería Forestal.
7. Valencia, D.; Saavedra, J.; Brull, J.; Santelices, R. Severidad del daño causado por los incendios forestales en los bosques remanentes de *Nothofagus alessandrii* Espinosa en la región del Maule de Chile. *Gayana Botánica* **2018**, *75*, 531–534. [CrossRef]
8. Baskin, C.C.; Baskin, J.M. *Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination*; Academic Press: San Diego, CA, USA, 2014; p. 1600.
9. Belmehdi, O.; El Harsal, A.; Benmoussi, M.; Laghmouchi, Y.; Senhaji, N.S.; Abrini, J. Effect of light, temperature, salt stress and pH on seed germination of medicinal plant *Origanum elongatum* (Bonnet) Emb. & Maire. *Biocatal. Agric. Biotechnol.* **2018**, *16*, 126–131. [CrossRef]
10. Bewley, J.D.; Bradford, K.J.; Hilhorst, H.W.M.; Nonogaki, H. *Seeds: Physiology of Development, Germination and Dormancy*, 3rd ed.; Springer: New York, NY, USA, 2013; p. 392.

11. Bradford, K.J. Applications of hydrothermal time to quantifying and modeling seed germination and dormancy. *Weed Sci.* **2002**, *50*, 248–260. [CrossRef]
12. Cabello, A.; Espinoza, N.; Espinoza, S.; Cabrera, A.; Santelices, R. Effect of pre-germinative treatments on *Nothofagus glauca* seed germination and seedling growth. *N. Z. J. For. Sci.* **2019**, *49*, 1–9. [CrossRef]
13. Moya, R.S.; Meza, S.E.; Díaz, C.M.; Ariza, A.C.; Calderón, S.D.; Peña-Rojas, K. Variability in seed germination and seedling growth at the intra- and inter-provenance levels of *Nothofagus glauca* (*Lophozonia glauca*), an endemic species of Central Chile. *N. Z. J. For. Sci.* **2017**, *47*, 10. [CrossRef]
14. Toh, S.; Imamura, A.; Watanabe, A.; Nakabayashi, K.; Okamoto, M.; Jikumaru, Y.; Hanada, A.; Aso, Y.; Ishiyama, K.; Tamura, N.; et al. High Temperature-Induced Abscisic Acid Biosynthesis and Its Role in the Inhibition of Gibberellin Action in Arabidopsis Seeds. *Plant Physiol.* **2008**, *146*, 1368–1385. [CrossRef] [PubMed]
15. Izydorczyk, C.; Nguyen, T.N.; Jo, S.; Son, S.; Tuan, P.A.; Ayele, B.T. Spatiotemporal modulation of abscisic acid and gibberellin metabolism and signalling mediates the effects of suboptimal and supraoptimal temperatures on seed germination in wheat (*Triticum aestivum* L.). *Plant Cell Environ.* **2018**, *41*, 1022–1037. [CrossRef] [PubMed]
16. Donoso, C. Variación Natural en Especies de Nothofagus en Chile. *Bosque* **1987**, *8*, 85–97. [CrossRef]
17. Buamscha, M.G.; Contardi, L.T.; Dumroese, R.K.; Enricci, J.A.; Escobar, R.; Gonda, H.E.; Jacobs, D.F.; Landis, T.D.; Luna, T.; Mexal, J.G.; et al. *Producción de Plantas en Viveros Forestales*; Contardi, L.T., Gonda, H.E., Tolone, G., Salimbeni, J., Eds.; Consejo Federal de Inversiones; Universidad Nacional de la Patagonia San Juan Bosco; Centro de Investigación y Extensión Forestal Andino: Patagonia, Argentina, 2012; p. 220.
18. DMC. *Reporte Anual de la Evolución del Clima en Chile*; Dirección Meteorológica de Chile: Santiago, Chile, 2020; p. 44.
19. ISTA. *International Rules for Seed Testing*; ISTA (International Seed Testing Association): Zurich, Switzerland, 2006.
20. Fick, S.E.; Hijmans, R.J. WorldClim 2: New 1-km spatial resolution climate surfaces for global land areas. *Int. J. Climatol.* **2017**, *37*, 4302–4315. [CrossRef]
21. Kamran, M.; Wang, D.; Xie, K.; Lu, Y.; Shi, C.; El Sabagh, A.; Gu, W.; Xu, P. Pre-sowing seed treatment with kinetin and calcium mitigates salt induced inhibition of seed germination and seedling growth of choysum (*Brassica rapa* var. *parachinensis*). *Ecotoxicol. Environ. Saf.* **2021**, *227*, 112921. [CrossRef] [PubMed]
22. Czabator, F.J. Germination value: An index combining speed and completeness of pine seed germination. *For. Sci.* **1962**, *8*, 386–396.

MDPI
St. Alban-Anlage 66
4052 Basel
Switzerland
Tel. +41 61 683 77 34
Fax +41 61 302 89 18
www.mdpi.com

Plants Editorial Office
E-mail: plants@mdpi.com
www.mdpi.com/journal/plants

MDPI
St. Alban-Anlage 66
4052 Basel
Switzerland

Tel: +41 61 683 77 34

www.mdpi.com

ISBN 978-3-0365-4862-3