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Abiotic Stresses, Biostimulant and Plant Activity

Series II

Edited by
Daniele Del Buono, Primo Proietti and Luca Regni

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Guest Editors

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Editorial

Abiotic Stresses, Biostimulants and Plant Activity—Series II

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1. Introduction

Agricultural practices often mainly focus on maximizing productivity. For this reason, cultivation systems often have significant environmental and ecological impacts, as well as pose risks to the safety of the final products. The intensive use of soils, the consumption of freshwater, and the utilization of fertilizers and synthetic compounds, such as herbicides and pesticides, have severe repercussions throughout the global ecosystem [1]. All of these factors negatively impact the status of primary resources and contribute to greenhouse gas emissions and waste production [1]. In addition, agriculture follows a linear production model that can lead to an unsustainable use of natural resources [1].

We should also consider that agriculture is impacted by climate change, facing abiotic stresses like salinity, drought, and extreme temperatures [1]. These stressors significantly threaten plant growth, crop development, and overall agricultural output. These stresses are expected to increase in frequency and severity as climate change progresses. As a result, there is a serious risk of a significant decrease in crop yields, which is worrying, given the need to feed the growing global population.

We urgently need innovative strategies and smart solutions to address a major challenge for our farming systems: reducing the impact of climate change on agriculture while increasing its resilience and productivity.

In this context, the use of biostimulants is becoming increasingly attractive as they become more effective. These include various organic materials and microorganisms designed to improve plant performance in both normal and stressful conditions. Biostimulants are obtained from a range of natural sources, including protein hydrolysates, mainly of vegetal origin, plant and algal extracts, humic substances, some organic compounds, and bioactive inorganic elements. Biostimulants enhance plant growth, stress tolerance, and their water and nutrient use efficiency. In addition to these effects on crops, biostimulants can also induce benefits in soils, improving quality and fertility [2]. By optimizing crop growth conditions, even in challenging environments, and enabling plants to counteract the effects of abiotic and biotic stresses, biostimulants have the potential to enhance agricultural productivity.

In this context, the aim of this Special Issue of *Agriculture*, "Abiotic Stresses, Biostimulants, and Plant Activity—Series II", was to advance knowledge on the effect of biostimulants but also other materials and techniques (i.e., nanomaterials, priming, etc.) on promoting plants' growth, yield, and product quality, as well as in abiotic stress conditions. Therefore, this Special Issue considered scientific contributions regarding the stimulatory and protective effects of different biostimulants on crops, their mechanisms of action, and their qualitative, economic, or environmental benefits.

2. Special Issue Overview

In pursuing sustainable agriculture, researchers are exploring innovative strategies to mitigate the detrimental effects of abiotic stresses on agriculture and enhance crop productivity. Salt stress, one of the most impactful abiotic stressors, poses significant challenges

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to crop cultivation by impairing plant growth and compromising crop yield and quality. To provide increasingly valuable tools for containing such adversity, researchers have increasingly turned to biostimulants as a promising avenue for safeguarding crops against abiotic stresses and promoting sustainable agricultural practices. In line with the above, the study by Regni et al. [3] underscores the potential of plant biostimulants in mitigating salt stress effects on crop plants. Their investigation focused on assessing the effects of an aqueous extract from *Lemna minor* L. (duckweed) in alleviating the harmful effects of salt stress in olive plants (cv. Arbequina) grown in hydroponic systems. The application of duckweed extract resulted in a notable recovery in olive plant functionality and mitigated the detrimental effects of salt stress. Such a result highlights the biostimulant's ability to enhance physiological and biometric traits, including improved photosynthetic activity and stomatal conductance. In addressing the soil salinity issue, the effects of foliar-applied biostimulants on Chinese silver grass plants under salt stress conditions were investigated [4]. The author of this research demonstrated the efficacy of biostimulants on enhancing physiological properties and alleviating the adverse effects of salinity stress, thereby contributing to sustainable farming practices. The critical mechanisms underlying mung bean tolerance to salt stress facilitated by silicon application were elucidated in another study [5]. In this frame, the role of silicon in enhancing antioxidant capacity and proteomes has been revealed, thereby mitigating the adverse effects of salinity stress on mung bean plants [5].

Similarly, the use of three different commercial organo-mineral fertilizers with biostimulating action on young almond trees in semiarid climates was explored [6]. Despite adverse weather conditions in certain years, biostimulant treatments exhibited enhanced vegetative and reproductive performance, emphasizing the potential of biofertilizers to improve soil fertility and crop productivity.

Aquaponics, an integrated agri-aquaculture system, offers a unique approach to improving crop quality and bioactive compound content in medicinal plants. In this context, the modification of bioactive compound concentrations in Cuphea spp. irrigated with aquaponic waters was explored, highlighting the potential of aquaponics in promoting the biostimulation of medicinal plants [7].

Understanding the impact of weather variables on crop yields is crucial for sustainable farming practices. The influence of weather events on winter wheat yields, emphasizing the significance of extreme weather events, such as heat waves and dry periods, in affecting crop productivity, was examined [8]. The study's main finding was that in the observation period, years with reduced yield, compared with a multiannual trend, were frequently well explained by extreme weather events.

Furthermore, the detrimental environmental impacts of pesticide use in agriculture necessitate the exploration of alternative biostimulants. To this end, the biostimulant effect of mannosylerythritol lipids (MELs) on lettuce germination and growth was evaluated, highlighting their potential as eco-friendly alternatives to chemical pesticides [9].

In addition, the use of beneficial microorganisms, such as plant growth-promoting rhizobacteria (PGPR) and mycorrhizal fungi, holds promise for improving crop productivity and resilience. The effects of PGPR-based products on snap bean yield and quality were evaluated, highlighting their potential to regenerate soils and enhance crop productivity in organic farming systems [10].

From a circular economy perspective, an aqueous extract was obtained from a non-food and invasive species (duckweed) rich in bioactive compounds and used to biostimulate young tomato plants [11]. The results showed that the extract improved the activity and functionality of photosystems I and II, the linear flow of electrons, and the electrochemical gradient across the thylakoid membrane. In particular, the photosystems of the treated plants showed a greater ability to use light for biochemical and biosynthetic purposes, reducing the amount of radiation dissipated as heat, which is potentially toxic to chloroplasts and capable of triggering oxidative stress. These benefits justified the increases in aerial biomass production and root phenotyping, which, again, showed benefits promoted by the extract. The extract also induced pigment content and some metabolic clusters of interest.

Finally, a review synthesizes the existing literature to highlight the positive aspects of intercropping in nut production, as well as the challenges and limitations faced in different regions regarding agricultural production [12]. Indeed, it should not be underestimated that both the global population growth and intensive agriculture have had detrimental effects on the environment. Consequently, there is a growing interest in sustainable alternatives to promote better use of natural resources and create a balance between agriculture and the environment. In this context, intercropping aims to optimize land use economically while enhancing biodiversity through plant–microorganism interactions, thereby increasing crop productivity.

3. Conclusions

The Special Issue of *Agriculture*, "Abiotic Stresses, Biostimulants, and Plant Activity—Series II" highlights that the integration of biostimulant uses and sustainable practices in agriculture offers promising solutions for mitigating abiotic stresses, enhancing crop productivity, and promoting environmental sustainability. Research on this topic and the adoption of these innovative approaches are essential for building resilient and sustainable food systems that meet the challenges of global agriculture. The academic editors of this Special Issue hope that the collected articles will substantially enhance our understanding and spur additional exploration in this pivotal domain, essential for the future of agriculture, particularly in light of ongoing climate change, which is predicted to intensify the impacts of abiotic stresses on crops.

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Review

Intercropping Systems: An Opportunity for Environment Conservation within Nut Production

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Abstract: Global population growth and intensive agriculture have both contributed negatively to the environment. As a result, there is increasing interest in the use of sustainable alternatives is increasing to promote better use of natural resources and create an equilibrium between agriculture and the environment. Intercropping, the simultaneous cultivation of multiple crops, aims to optimize land use economically while enhancing biodiversity through plant–microorganism interactions, thereby boosting crop productivity. This practice has particularly benefited nut production by combining the nutrient-sequestering capacity of trees with continuous annual crop production, improving soil nutrient and water utilization. Intercropping systems not only enhance nut yield and quality but also offer economic advantages to farmers. This review synthesized the existing literature with the aim of highlighting not only the positive aspects that intercropping brings to the production of nuts, but also the challenges and limitations faced in different regions when it comes to agricultural production.

Keywords: diversified crop cultivation; nuts; sustainability; biodiversity; polyculture; intercropping

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1. Introduction

In recent decades, consumers have become aware of the importance of functional foods in their diet plan due to the increase in chronic diseases that have been their main concern [1]. For this reason, nuts have become the most popular snack, with a global market estimated at $295.8 billion in the year 2022 and forecast to grow by 5.7% through 2030, reaching $459.1 billion over the next 8 years [2]. This growth is due to their nutraceutical properties, as well as their unique flavor and, above all, their health-promoting bioactive compounds [3].

Nuts such as almonds, Brazil nuts, hazelnuts, macadamias, pine nuts, pecans, pistachios, and walnuts, are rich in minerals (e.g., calcium, magnesium, and potassium), high-quality protein, fiber, vitamins (e.g., folic acid, niacin, vitamin E and B6) and unsaturated fat specifically, monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) [4,5]. These dry fruits are considered to be the main sources of procyanidins, one of the most abundant polyphenols in plants, which has been found to be beneficial for human health and used to prevent cancers, diabetes, and cardiovascular diseases [6]. It was reported by Balakrishna et al. [7] that, the consumption of 28 g/day of nuts reduced the risk of cardiovascular disease by 21%, cancer deaths by 11% and all-cause mortality by 22% compared to those who did not eat the nuts. However, the nutritional value of the nut

varies significantly according to the type of nut, the genotype, and the differences between cultivars [3]. Sokolow et al. [8], also pointed out that the nutrient content of food can change depending on the ecological and climatic conditions, such as temperature, rainfall, and solar radiation during growth [3]. To guarantee sustainable food production systems adapting to extreme climatic conditions, intercropping has become an excellent option for effective land use, by implementing agricultural practices that increase productivity [9].

Intercropping is considered an old agricultural practice technology that involves the planting of diversified crops with low input, improving the quality of the agroecosystem, thus focusing on food production in healthy environmental conditions [10,11]. Reportedly, the first agricultural settlements in South Asia (2500–2000 BCE) began using a double cropping system, with summer (wheat, barley, etc.) and winter (rice, etc.) crops grown in the same year. This period was marked by great agricultural development, stemming from the need to take advantage of the seasonal floods of the Ganges and its emerging rice plains [12].

Also known as mixed cropping or polyculture [11], intercropping aims for the efficient use of resources generated by the interrelationship between morphologically different crops, so that the result is an improvement in soil fertility through an increase in organic matter, as well as in crop yields [13,14]. In addition to these benefits, others can be generated from this agroecological approach such as, improving the management of diseases and pests and, meanwhile, increasing the proportion of pollinators and natural enemies due to the floral intercropping (Figure 1) [15,16]. Compared to monocultures, intercropping tends to increase yield stability, reduce inputs of agrochemicals through association with leguminous plants capable of fixing atmospheric nitrogen and, consequently, environmental costs through the reduction of water and soil pollution and greenhouse gas emissions [17].

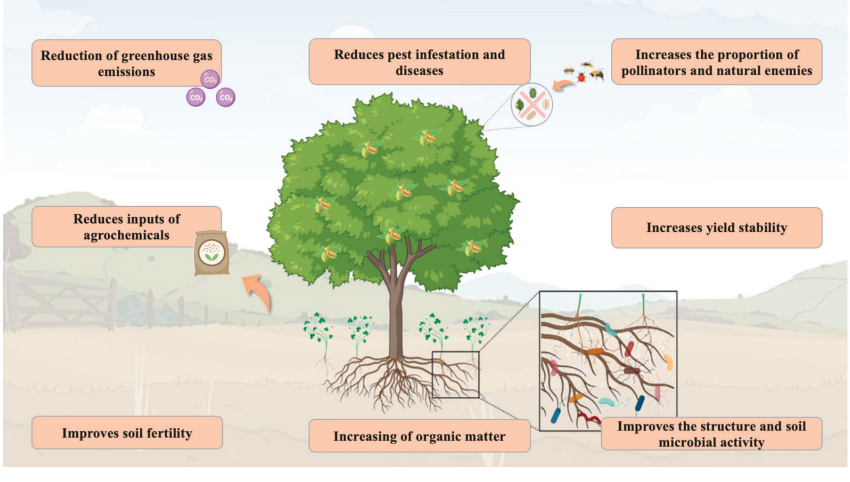


Figure 1. Benefits generated by the interspecific relationship between crops in the intercropping system.

Among these strategies, the tree system has proven to be a tool to support agricultural production, playing a substantial role in reducing the atmospheric concentration of CO_2 through the carbon sequestration power of trees, besides continuing to produce annual crops, and reducing soil erosion [18,19]. In addition, trees play another fundamental role in intercropping systems by modifying the microclimate, reducing radiation on the plant, thus forming a barrier against the force of the wind and temperature oscillations [20]. Agroforestry, which consist of growing woody vegetation (trees or shrubs) with plant and/or animal production systems, has become a practice used by farmers around the

world due to the constant availability of food, fruit, timber, fodder, and fuelwood, which has created sustainability in their livelihoods [21,22].

Regarding the categories of agroforestry systems, intercropped fruit trees [23] are used in the nuts production system, with the exception of peanuts, which is botanically classified as a legume. Nutritionists and consumers have included them in the nuts group because of their similar nutritional composition [24]. Research confirms the benefits generated by the intercropping of nuts and other crops, where the levels of soil nutrients such as nitrogen, phosphorus, potassium and organic matter, were significantly higher in walnut–tea intercropped forests than in monocrops [24].

However, many factors affect the balance of this system, where competition between species for resources, such as water, light and nutrients, can become crucial for the overall productivity of agroforestry intercrops [20,25]. Therefore, the main challenge for agriculture is to adopt tillage strategies that guarantee the long-term stability of agroecosystems, with an emphasis on preserving and improving soil health [26]. There are still several limitations that need to be analyzed in order to improve the deficiencies of this system, with the aim of allowing global food production to grow in a more sustainable way.

2. Features and Advantageous of Intercropping System

The intercropping system consists of a mixture of crops of different species grown in the same field, with the aim of achieving more sustainable and profitable crop cultivation. Intercropping generates several benefits for the agricultural ecosystem, such as the increased use of natural resources like water and nutrients, the greater conservation of resources and the promotion of soil biodiversity [27]. According to Maitra et al. [11], in order to maximize benefits from this system, farmers must consider suitable intercropping and management practices, planting geometry, and the operation of intercropping and plant protection.

2.1. Improvement in the Soil Quality

The intercropping system generates a positive impact on soil quality, including increased and maintained soil organic matter, nitrogen fixation when the crop is associated with a legume in the system, increased phosphorus availability and reduced erosion by providing more soil cover [28]. Researchers consider the intercropping system a suitable option because it manages to produce more on a smaller area of land compared to monoculture [29,30]. In addition to land use, intercropping can increase organic matter [31], and enzyme activity [32] and, improve structure [33] and soil microbial activity [34]. Roohi et al. [33] also highlighted that the practice of intercropping, combined with the use of fertilizers containing organic additives, has the potential to improve the structural integrity of the soil, promote the sequestration of soil organic carbon and ensure optimum crop production.

Sharma and Banik [35] revealed that the intercropping system of baby corn tested with four several plant species such as chickpea, pea, groundnut, and lentil provided advantages in increasing the economic yield due to the rise in the number of cobs/pods per plant. Regarding soil, the intercrop improved soil fertility in terms of nitrogen, phosphorus and potassium availability, organic carbon, cation exchange capacity, soil enzymes, microbial respiration, and microbial biomass carbon. In addition to the above benefits, intercropping also helps to reduce soil erosion, where excessive runoff can result in infertile soils with unproductive characteristics for farming systems.

Roots play an important role in this function, as deeper roots act in the deeper layers to retain soil moisture and nutrients. On the other hand, roots keep the soil on the surface, reducing erosion [36]. Diversity among crops and the healthy competition generated tend to increase the extent of root networks, leading to more efficient use of natural soil resources due to greater absorption [37,38].

Nyawade et al. [39] highlighted that intercropping with legumes reduces the loss of nitrogen from the soil, increases productivity and increases the efficiency of nutrient use. It

also improves drought resistance in shallow soil plants by encouraging deeper root growth, leading to higher growth rates and nutrient levels in leaves [40]. Crop diversification stabilizes yields under variable climatic conditions, leveraging beneficial interspecific relationships, optimizing resource use spatially, temporally or chemically [41]. This approach is essential, especially considering that multi-species agroforestry systems show remarkable potential for increasing agricultural productivity, thanks to the sequestration of organic carbon by trees [42].

This carbon sequestration occurs in the above-ground biomass, including stems, branches and foliage, and in the underground biomass, such as roots, in addition to the soil itself [43]. In the same way, these systems are effective at recovering nutrients from below the rooting zone of crops, promoting a more efficient and sustainable nutrient cycle [42]. Another function performed by agroforestry systems is the contribution to the mitigation of climate change, in which the sequestration of C should slow or even reverse the increase in atmospheric concentration of CO_2 [42]. Agroforestry systems not only influence the chemical and physical properties of the soil, but also the microbial population, making the soil more productive and the plant growing indirectly [44].

2.2. Biodiversity Conservation

In intercropping, the benefits generated in the agroecosystem also come from the increased biodiversity of microorganisms, which results in a greater concentration of nutrients for the soil, increasing its fertility [36]. In the intercropping system, biodiversity does not only apply to the soil, as crop mixtures tend to increase the population of different arthropods, insects and birds [11].

The research of Cai et al. [45] revealed that the highest abundance of arthropod predators was found in intercropping systems of Chinese cabbage with lettuce (141.67 predators/plot), presenting lower values when intercropped with green cabbage (97.67 predators/plot). This study aimed to elucidate the importance of intercropping in the conservation of natural enemies and in the ecological management of pests, making the system more sustainable for the environment.

Regarding the benefit of the plant interactions with microorganisms, this relationship can be divided into three categories, (i) microorganisms in association with plants that are responsible for providing nutrients, (ii) the groups of microorganisms that stimulate plant growth indirectly, by growth prevention or by the activity of pathogens, and finally, (iii) microorganisms responsible for direct plant growth, due to the production of phytohormones [46].

Microorganisms and enzymes act as regulators of soil health, as they are essential components that catalyze various biochemical processes, such as the decomposition of organic matter and the renewal of nutrients [47]. Soil enzymes such as urease, catalase and invertase catalyze the decomposition reactions of microorganisms, contributing to nutrient cycling and being important for plant maintenance [48,49]. Therefore, soil enzyme activity is strongly related to soil microbial functions, influencing the ability of soils to perform critical environmental functions, such as participation in biogeochemical cycles, where enzymes like β-glycosidases are related to an indicator of C cycling in soils, while urease, phosphatase and sulfatase are enzymes responsible for the generation of available N, P and S [49].

In agroforestry systems, microbial abundance is higher due to the influence of the trees, organic matter deposition and root exudates, thus creating a favorable environment for the increase of beneficial soil organisms such as nematodes, collembola, mites, diplopods, earthworms, fungi, and various insects are involved in carbon transformation and nutrient cycling [44].

2.3. Yield Stability

Production instability is one of the main negative points suffered by the monoculture system, due to its lower resistance to environmental disturbances and extreme climatic

conditions, such as frosts, droughts, and floods [36]. Climate change projects instability in the field, and significantly reduces yields in monocrops. The mixture of species in an intercrop may be the way to adapt crops that face these changes by providing the means to protect the plant against abiotic or biotic stresses suffered by them [50].

The stability of intercropping can be associated with an increase in biodiversity attributed to the relationship between crops, unlike monocultures. Intercropping offers greater production security, especially in high-risk areas, such as those subject to climate change, generating financial stability for farmers [36]. In farming systems with less use of pesticides or synthetic fertilizers, yield stability becomes more important, being higher compared to organic farming. However, profitability can suffer due to the high cost of inputs, in terms of seed costs and mechanization [50,51].

Madembo et al. [52] demonstrated that intercropping maize with jack bean and cowpea enhances yield stability compared to monoculture. This stability is attributed to nitrogen fixation by the intercropping system, weed suppression, and soil cover provided by cowpea, resulting in consistent yields across multiple growing seasons. Similarly, studies have shown that intercropping maize with legumes, particularly soybean, improves yield stability and nitrogen use efficiency, with maize–soybean rotations being notably stable [53]. Intercropping sugarcane with soybean has also proven effective in stabilizing soybean yields while reducing nitrogen fertilizer inputs by 40%, thereby lowering CO_2 and N_2O emissions associated with synthetic fertilizers [54]. Further analysis by Raseduzzaman & Jensen [55] underscored that intercropping legume with cereals significantly enhances yield stability compared to monocropping, contributing to higher and more reliable crop yields. These findings highlight that increasing diversity through cereal–legume intercrops promote stability and enhances global food security efforts.

2.4. Valorization of Bioactive Compounds

In order to produce healthier and more nutritious food, intercropping has become a more sustainable option, along with the use of N-fixing legumes, bio-fertilizers and biological control methods. Researchers have shown that intensive nitrogen fertilization has a negative effect on plant growth and biomass production, consequently interfering with the concentration of bioactive compounds [56]. To resolve this problem, Mohammadzadeh et al. [57] implemented a sustainable strategy using the intercropping of legumes with medicinal and aromatic plants. The results showed that intercropping improved essential oil content and quality by increasing compounds such as carvacrol, gamma-terpinene, p-cymene and carvacrol methyl ether. In addition, productivity was increased compared to monoculture cultivation.

Over a 5-year period, Rodríguez et al. [26] carried out experiments in an organic production system in a dryland almond orchard located in the Mediterranean region, assessing the impact of no-till and legume cover crops. An improvement in soil physical characteristics was observed with legume cover crops, including bulk density, water holding capacity and aggregate stability. Furthermore, there were improvements in chemical properties, such as an increase in soil organic carbon, nitrogen, potassium and micronutrient content, along with an increase in soil microbial activity. In summary, the implementation of mulch resulted in an increase in the antioxidant activity and total polyphenol content of the almonds, which contributed to improving their nutritional value.

In previous research, the intercropping of chicory (*Cichorium intybus* L.) with legumes resulted in a higher production of condensed tannins compared to monocrops, being 51.4 mg/g of TC in mixtures of Antler chicory and red clover (*Trifolium pratense* L.) and 30.9 mg/g of TC for Antler chicory monocrop. In addition, the intercropping system of chicory and red clover showed higher dry mass yield and forage of better nutritional quality compared to the solo chicory crop. Most of the condensed tannins produced were in the unbound form, suggesting that most of the forages evaluated would provide benefits for ruminant nutrition and health [58,59].

Wu et al. [60], reported in their study that intercropping green tea with Chinese chestnut obtained 100 differential positively regulated metabolites, including amino acids, organic acids, lipids, carbohydrates and flavonoids, in tea leaves from the intercropping system when compared to monocropping. Many of these compounds were responsible for the flavor and bioactivity, providing improvements in the quality of green tea, as well as benefits for human health.

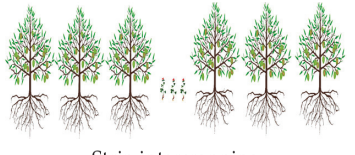
3. Types of Intercropping Systems

By growing genetically different plants, it is necessary to consider the factors that differ in each crop, such as crop maturity, planting time, irrigation, planting density, sunlight, and nutritional requirements [11,61]. There are also the abiotic factors (heat, cold, drought, salinity, among others) to which the crop is exposed during its growth, and these stress conditions lead the plant to adapt and create resistance mechanisms [62]. Each form of crop intercropping presents unique methods of planting, maintenance and harvesting to avoid competition between crop species. Therefore, there are various types of intercropping systems to suit planting conditions, such as mixed, row, relay and strip cropping systems [28].

3.1. Mixed Intercropping

This intercropping practice consists of sowing two or more plant species on a plot of land, co-existing with each other without any defined proportion between rows [11,28]. As it is a system that consists of a greater number of crops in an area, it can bring benefits to the crop depending on the type of species, where it will provide resistance to abiotic and biotic stresses, as well as an increase in biodiversity, protecting the primary crop from the wind, frost, drought and other severe weather conditions [61,63]. According to Pan & Qin [64], mixed cropping was shown to be effective in pest control due to the increase in natural enemies. Specifically, three predators—ladybirds, lacewings, and hoverflies—increased compared to monocultures, resulting in a decrease in herbivores (aphids, leafhoppers, and whiteflies) in soybean cultivation. One of the disadvantages of this system is the problem of selecting the correct herbicide (Table 1) in the case of the combination of cereals and legumes, which generates a variable yield at the end of the harvest and, consequently, is the limiting factor in the use of mixed intercropping in organic farming [65,66]. There is also the difficulty of developing appropriate management practices and sowing ratios, due to the mixture of roots, leaves, and microbiome, which can generate greater interspecific interactions between crops and, with this, undesirable competition [28].

Table 1. Types of intercropping considering their use, advantages and disadvantages in agricultural production.

Schematic	Uses	Advantages	Disadvantages
 Mixed intercropping	• Temporary pasture and annual forages as part of a rotation; • Perennial pasture and hayfields; • Extending the grazing season; • Grain and pulse production; • Crop-livestock integrated systems.	• Reduces the risk of crop failure due to environmental stress; • Pest infestation of crops is greatly reduced; • Increases soil fertility and yields due to crop mix.	• Applying fertilizers and pesticides to individual crops is very difficult; • Harvesting and threshing of crops separately are not possible; • Crops compete fiercely for resources like water, sunlight, and space.
 Row intercropping	• Used in the production of cereals and pulses; • Staple crops; • Forage species; • Sugar cane; • Small-scale horticultural production.	• Lower seed expenses; • Better controlling of stubble; • Less work on the soil; • Easier control of weeds between the rows.	• Lower rate of crop competition with weeds; • Decreased yield in some situations; • Higher rate of evaporation from the soil surface; • Less water efficiency.
 Relay intercropping	• Used in maize and soybean production; • Annual self-seeding legumes; • Rice—cauliflower—onion-summer.	• Reduced need for soil tillage; • Better use of resources in farm management, such as labour, time and equipment; • Some diseases and insects appear to spread more slowly when crops are intercropped; • Better erosion control as a result of increased ground cover; • Reduced leaching of mineral N from the agricultural environment.	• Inadaptable to extremely heavy, poorly drained or dry clay soils; • An increase in pests and nematodes is highly possible; • Some crops may be affected by early autumn frosts; • There may be potential expenditure on additional machinery, which requires rapid field operations.
 Strip intercropping	• Grain and pulse production; • Annual and perennial forage crops.	• Allows the use of optimal agricultural techniques and makes it possible to harvest separately for each species; • The soil is filtered into the runoff through the strip with the nearby crop.	• Limits the efficient use of machinery, so it is not suitable for highly mechanized systems; • One crop may host a plant disease and pest that is harmful to the other crop.

3.2. Row Intercropping

Row cropping is the cultivation of crops planted in a single or double row, allowing for interspecific interactions such as root-mixing, shading and competition for water and nutrients (Table 1) [11,28]. Positive points worth highlighting in this system are that intercropping in rows has the potential to alter the light environment to improve overall interception by crops [67], help minimize soil erosion [68], decrease surface runoff and reduce soil nutrient loss [69].

Experiments carried out by Perdoná and Soratto [70] evaluated the growth and productivity of a macadamia plantation, as well as the profitability and investment return period during 7 years of cultivation. The treatments consisted of two types of cultivation: macadamia in monoculture and macadamia–coffee in intercropping, with irrigation methods varying between dry and drip. Organized in a 2 × 2 row arrangement, both the consortium with arabica coffee and drip irrigation resulted in the greater vertical growth of the macadamias, reaching 5.41 m for both systems, compared to 3.76 m in irrigated macadamia monocropping.

A study conducted by Lu et al. [71] examined the effects of different configurations of row proportions and strip widths in intercropping systems. Five treatments were tested in a field study: maize soil (SM), peanut soil (SP), four rows of maize interspersed with eight rows of peanuts (M4P8), four rows of maize interspersed with four rows of peanuts (M4P4) and four rows of maize interspersed with two rows of peanuts (M4P2). The results showed that the M4P8 configuration presented the highest yield and land use efficiency, offering substantial yield benefits with the increase in the proportion of peanut rows. Compared to the other intercropping systems, the M4P8 treatment showed a significant increase of 40.99% compared to the M4P4 treatment and 79.01% compared to the M4P2 treatment.

3.3. Relay Intercropping

Relay intercropping is the cultivation of two or more crops at the same time during part of the growing period of each, being planted and harvested at different times (Table 1). In this system, the crop is rotated for periods, that is, while the first crop completes its life cycle, close to being harvested, the second crop is sown [11,28]. According to Glaze-Corcoran et al. [28], competitive inhibition can be reduced with the better coordination of the life cycles of different crops through relay intercropping. Other features, such as the extended period of individual growth and the recovery period between the two cultures, are part of the process of this system.

Amossé et al. [72] reported that the success of the application depends on the choices between cereals and legumes, in function of the competitiveness generated between them. According to Raza et al. [73], the productivity benefits of relay intercropping systems are many times greater than other types of intercropping because crops do not have to compete for nutrients, light or water due to rotation. In an experiment formulated by Fan et al. [74], they showed that the maize–soybean relay strip planting system had a significant increase in relation to the uptake of nutrients such as nitrogen, phosphorus, and potassium, which may lead to greater crop development. Chen et al. [75] also reported that dry matter is a determining factor for nitrogen concentration in the crop, differing between species due to growth state and photosynthetic variations.

3.4. Strip Intercropping

Strip cropping can be defined as planting crops in parallel strips, where the strip width interferes with production yield (Table 1) [28,76]. According to the research conducted by Oort et al. [76], the benefits of intercropping decreased as strip width increased. This study also pointed out that wheat and corn intercropping obtained better results with widths of less than 1 m, which may be a limiting factor in relation to the use of machines with larger widths. In this system, crops can be harvested at the same time if cultivars of the same species reach the same maturity or, in the case of grain and legume crops, are harvested separately [28].

The studies conducted by Wang et al. [77] revealed that crop yields are affected by the variation in the proportion of border rows, influenced by the width of the strip, which varies from 1 to 4 m. Thus, strip width plays a crucial role in regulating plant interactions and relative yields in strip intercropping. Although wide strips in intercropping facilitate mechanization, increasing the width of the strip reduces the benefits of border lines at the field scale.

4. Intercropped Species

Some requirements can be adopted to optimize the process of crop productivity through intercropping systems. For this purpose, it is necessary to increase the set of crop combinations already tested to know how they behave through the interspecific relationship, as well as to cultivate regional cultivars with complementary genotypes and to test plantings in different regions, since the dose of inputs is adjusted according to each crop [78,79]. Other practices can be adopted to obtain a more successful production: (i) select species that have the same water requirements; (ii) select plants that do not compete for sunlight; (iii) avoid grouping crops of the same family to mitigate pest invasion; (iv) sow herbs to obtain a repellent effect and attractive species to attract pollinators [61]. This is why it is so important to choose the species to be intercropped with the main production crop to achieve the efficient use of resources, high and stable yields, and sustainable agriculture.

4.1. Legumes

In the intercropping system, legumes are valued for providing an important service to the field by reducing interspecific competition between crops by improving the exploitation of soil resource yields, reflected in increased productivity, and making the process more environmentally sustainable [9,80]. Intercropping with legumes has several positive effects such as biological nitrogen fixation in the soil, improving biodiversity, positively affecting the composition of rhizospheres, and thus increasing the availability of nutrients for plants [11,61]. Legumes are the only ones capable of obtaining free atmospheric nitrogen through symbiotic association with Rhizobia, which are nitrogen-fixing bacteria found in the root nodules of these plants. In addition to being available to the plant, this nitrogen also enriches the soil when the organic matter decomposes [81].

In the rhizosphere, microorganisms supply their host plants with essential assimilable nutrients, stimulate plant development by means of plant growth promoting bacteria (PGPB) and induce the production of antibiotics [82]. The intercropping of legumes with other crops such as maize, wheat, soybean, cowpea and fava bean results in improved nitrogen balance, P availability, root exudates, and increased microbial biomass and crop yield under stress conditions [9].

For the cereal–legume consortium to be successful in productivity, there are several conditions to be followed: (i) the periods of peak nutrient demand should not overlap (ii) there should be minimum competition for light between crops; (iii) there should be a complementarity between crops for the use of growth resources in time and space; (iv) there should be a difference in crop maturity of at least 30 days to reduce com- petition [83]. Research by Yu et al. [84] revealed that, through a meta-analysis of published studies, that the yield of cereals and legumes in a consortium is affected by seeding densities, seeding seasons and nitrogen fertilizers, which tends to reduce the yield of legumes in intercropping. Therefore, to increase food production, proper cultivation practices as well as the use of legumes at their maximum genetic potential and inoculation with compatible rhizobia are important [85].

Rodríguez et al. [26] evaluated the effect of legumes on almond production, analyzing various physical, chemical and biological aspects relevant to soil health and their implications for yield and the physical and chemical quality of the almonds. Three types of legumes were used as cover crops: fava bean (*Vicia faba* L.), vetch (*Vicia sativa* L.) and ervil (*Vicia ervilia* L.), to assess the influence of different soil management strategies. In terms of water capacity, the combination of *Vicia sativa* and *Vicia ervilia* (VS-VE) increased by more

than 21%, while the available water capacity of the soil increased by 23% at a depth of 10 to 25 cm. In terms of soil organic carbon (SOC) sequestration, the systems intercropped with Vicia sativa and VS-VE were statistically superior when compared to the system with fava beans. Long-term studies such as this one are essential to demonstrate how the use of mulch on crops such as dryland almonds can improve soil health and influence the nutritional composition of the almonds. This type of research is essential to promote sustainable agricultural practices and guarantee the quality of the food produced.

4.2. Oilseeds

The intercropping of pulses and oilseeds seems to change the traditional agronomic scenario, where cereals and legumes, are the more common crops to be used in the environment. In studies conducted by Shah et al. [86], oilseed crops such as soybean, sesame, sunflower and Brassica were shown to suppress weeds through the production of different compounds in the air and rhizosphere by the release of isothiocyanates which are potent inhibitors of weed germination.

In other field trials, intercropping chickpeas with oilseed species of flax and canola resulted in yield maintenance or even yield increases in other cases compared to single crops. A significant reduction in fertilizer and fungicide inputs was also evaluated. In addition to production, intercropping an oilseed and a legume also offers other profit options for farmers, providing a high-quality hay or pasture option in years of low grain productivity [87].

The intercropping of legume/oilseed rape proved to be advantageous compared to monocropping in relation to maize biomass production and P uptake. Moreover, P uptake by intercropped maize averaged 0.58–0.92, significantly higher than biomass production (0.51–0.78), proving to be a resource that tends to exploit the biological potential of plants [88]. Other studies conducted on the intercropping of oilseed rape and legumes showed it to be advantageous with respect to yield and number of grains, being three times higher in the intercropping of oilseed rape (*Brassica napus* L.) with the faba bean (*Vicia faba* L.) compared to oilseed rape alone. Moreover, in this interspecific relationship, the above- ground biomass and N accumulation of weeds were reduced by 35% and 11%, respectively [89].

Regarding the structure of the microbial community, the intercropping of rape with white lupins (*Lupinus albus* L.) was able to enrich the rhizosphere with phosphorus solubilizing bacteria, such as *Streptomyces*, *Actinomadura* and *Bacillus*, and phosphorus solubilizing fungi, such as *Chaetomium, Aspergillus* and *Penicillium*. These phosphorus solubilizing microorganisms performed an important function in improving the uptake of this macronutrient in the soil. In addition to organic acids, 23 other metabolites from root exudates were significantly positively correlated with this microbial community established from the oilseed rape/white lupin intercropping system [90].

In addition to the increase in biodiversity, the oilseed intercropping system also contributes to the biological control of pests and the increase in the diversity of arthropod predators. Studies by Alarcón-Segura et al. [91] demonstrated a 50% reduction in wheat aphid densities and a 20% reduction in pollen beetle larvae in wheat and oilseed rape intercropping areas. An increase in carabid beetles was observed in canola strips and spiders in wheat strips in the intercropped area. In this context, the biological control implemented by the intercropping strips had a synergistic relationship among the system, causing a balance and, consequently, a decrease in the use of pesticides [91].

4.3. Aromatic Plants

Also known as herbs and spices, aromatic plants began to be used thousands of years ago in the Middle East due to their preservative and medicinal properties, in addition to enhancing the aroma and flavour of foods [92]. They are plants rich in bioactive compounds, mainly polyphenolic, which promote antimicrobial, antioxidant, antiparasitic, antiprotozoal, antifungal, and anti-inflammatory activities [93,94].

Aromatic plants are characterised as perennials, flat growing, shade tolerant and adapted to dry and hot climatic conditions. Due to the increasing demand for products derived from aromatic plants, they become suitable for combining short-term returns with environmental benefits [95]. In response to abiotic and biotic stresses, the crop synthesizes considerable amounts of secondary metabolites and harvested plant materials, whether crude or processed, which are used in various applications in the food, cosmetic and pharmaceutical sectors [96,97].

Research has proven that intercropping with aromatic plants significantly increased soil organic matter and water content and decreased pH values. The exudates from the roots of aromatic plants, such as saccharides, lipids, organic acids, aromatic compounds, and amine, had the power to shape microbial diversity and promote enzymatic activities in the soil. In addition, it also regulated the nutrients C and N during the decomposition of soil organic matter [31].

Intercropping aromatic and medicinal plants (AMP) with nut trees in integrated management systems has been shown to have significant potential for increasing yields, and controlling pests/pathogens and weeds, as well as improving soil health and the quality of commercial crops [98]. The practice of diversifying into woody crops, such as almonds, offers a short-term annual balance of carbon (C) in the soil in semi-arid rainfed regions. This practice can be a sustainable strategy to reduce greenhouse gas (GHG) emissions in the soil and improve carbon sequestration and storage [99–101].

In studies carried out by Almagro et al. [101], the short-term effects on rainfed almond orchards (*Prunus dulcis* Mill.) grown under semi-arid Mediterranean conditions were evaluated, in intercropping with an aromatic plant such as thyme (*Thymus hyemalis* Lange). The results showed that diversification with winter thyme improved the aggregate stability of the soil and the availability of water for the plants. It also increased the organic carbon content of the topsoil from 3.7 gkg^{-1} in the first year to 4.6 gkg^{-1} in the third year of production. This highlights the importance of choosing species that provide carbon and plant cover all year round, such as winter thyme, to improve water regulation and soil formation.

4.4. Vegetables

Vegetables in intercropping systems improve nitrogen utilization and uptake, and complementary root growth and therefore can increase productivity by mixing complementary species in terms of resource usage. The research showed that beans grown in a monoculture system had a higher risk of nitrate leaching because their roots have shallow growth, reducing N uptake in the deep soil layers. On the other hand, the crop associated with the vegetable increased the intensity of roots in the bean rows, playing an important role in the use of soil resources [102].

Other intercropping systems were proven advantageous with respect to economic returns. Yield loss decreased with an increasing proportion of legumes in potato intercropping systems (in patterns of 1:2.4), which generated higher economic gain from intercropping compared to monocropping, indicating a benefit ratio: cost of 4.98 versus 4.55 for pure potato stand. The productivity of the intercropped systems was higher due to the harvest being carried out at different times. This is due to the fact that dolichos (*Lablab purpureus* L.) were still in the flowering phase, which allowed them to take advantage of the moisture present in the soil and the nutrients resulting from the mineralization of harvested potato waste, resulting in an excellent yield [103].

Studies conducted by Hu et al. (2020) [104], demonstrated that the association of cauliflower with grasses brought the effect of soil salinity control and nitrate reduction by the vegetable, associated with the capacity of absorption and accumulation of salts and nitrates of the grass species. The species that obtained the most significant results of soil salinity control was *Paspalum vaginatum*, which reduced 37.8% of nitrate content, increased 50.7% of vitamin C and increased 21.1% of soluble protein in cauliflower curd.

Research has shown that plants also perform other functions in intercropping systems, such as reducing competition for light and for production factors, which is favourable for plant development [105]. Besides nitrogen, other nutrient elements may increase with intercropping, such as P, K, Ca, Mg, Mn and Zn, according to the intercropped crop species, compounds that are important for plant growth and development [106].

Grass species have been used to increase productivity and make yields more sustainable. Grass/wolfberry intercropping systems have increased the nutrient content and enzymatic activity of rhizosphere soils. This change was reflected in a 21% increase in carotenoids (0.41 ± 0.05 g/kg), a 56% increase in flavonoids (2.32 ± 0.48 g/kg) and, a significant 127% increase in fruit ascorbic acid (0.50 ± 0.05 g/kg) compared to wolfberry monoculture. The association of grasses with other plants aims to maximize their productivity and the beneficial effects they produce on the soil, rendering this interaction favourable to the environment [107].

5. Successful Intercropping Systems

In terms of nut production, intercropping plays a crucial role in sustainable environmental conservation, providing significant benefits (Table 2) [20,108–110]. The integration of nut trees with other crops in agroforestry systems not only improves productivity, but also contributes to the preservation and improvement of the agricultural ecosystem. Furthermore, this practice provides additional sources of income and increases food security [111]. These agroforestry systems provide a variety of agricultural products that can be harvested throughout the year, diversifying farmers' sources of income and making them less dependent on a single crop. This not only helps to mitigate the risks associated with price fluctuations and adverse weather conditions, but also promotes the economic resilience of rural communities [112].

The research conducted by Abbasi Surki et al. [113] showed the effects of the almond–cereal agroforestry system on wheat and barley grain crops, in which the highest yields were 2985 and 2180 kg ha^{-1}, respectively. These results were obtained by arranging the trees in rows over the crops at a distance of 2.5 m between the systems, and the grain yields of wheat (35%) and barley (39%) were higher than their respective monocultures. Following the research of Abbasi Surki et al. [114], higher carbon contents (56 t ha^{-1}) were detected in the agroforestry plots 0.5 m from the almond tree rows, doubling those of the wheat and clover monoculture. During the 8 years of research, it was observed that both the moisture retained in the field and the soil organic carbon content were higher for crops close to almond trees, especially for barley, where they were 28% and 1.82%, respectively.

Abourayya et al. [115] evaluated not only the chemical properties and fertility of the soil, but also the growth and nutritional status of almond trees (*Prunus amygdalus* B.) intercropped with snap bean (*Phaseolus vulgaris* L.), with a planting space of 5×5 m cultivated under a drip irrigation system. The results showed that intercropping had a significant effect on the vegetative growth characteristics of the almond trees, recording greater stem length and diameter, number of branches, number of leaves, leaf area, and fresh mass and dry mass of leaves, compared to almond trees grown alone. As far as the soil is concerned, incorporating snap bean into the soil improved the levels of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) exchangeable in both growing seasons.

Table 2. Successful intercropping systems on nut production.

Nuts	Intercropped Crops	Positive Effects	Refs.
Almond (*Prunus dulcis* Mill.)	Legume cover (*Vicia faba* L., *Vicia sativa* L. and *Vicia ervilia* L.)	• Improved the physical, chemical (soil organic carbon content, N, K and micronutrients) and biological (increased microbial activity) properties of the soil. • Improved the nutritional value of the almonds by increasing antioxidant activity and total polyphenol content.	[26]
Almond (*Prunus amygdalus* B.)	Snap bean (*Phaseolus vulgaris* L.)	• The intercropping treatment had a significant impact on the nutrient composition of leaves, particularly in terms of nitrogen, phosphorus and potassium percentages. Additionally, it also resulted in higher total chlorophyll content when compared to a single tree system.	[115]
Almond (*Prunus dulcis* Mill.)	Caper (*Capparis spinosa* L.) and thyme (*Thymus hyemalis* L.)	• Both crops significantly reduced CO_2 emissions from the soil; • Thyme cultivation significantly increased the moisture content and organic carbon of the soil compared to monoculture, due to its perennial nature.	[99]
Walnut (*Juglans* spp.)	Tea (*Camellia sinensis* L.)	• Improved the soil's nutritional conditions by increasing soil nitrogen, phosphorus, potassium and organic matter; • Increased bacterial and fungal diversity, including Proteobacteria, Bacteroidetes, Firmicutes, Chlamydiae, Rozellomycota and Zoopagomycota in higher proportions.	[116]
Areca nut (*Areca catechu* L.)	Pandan (*Pandanus amaryllifolius* Roxb.)	• Areca nut and pandan intercropping cultivation had a positive impact on soil microbial diversity and dynamic balance.	[117]
Peanut (*Arachis hypogaea* L.)	Millet (*Setaria itálica* L.)	• The net income of the millet/peanut intercrop was the highest, reaching 2479 USD ha^{-1}, representing an increase of 13.5% over the millet and 8.6% over the groundnut monocrop.	[107,118]
Macadamia (*Macadamia integriolia* Maiden & Betche)	Coffee (*Coffee arabica* L.)	• Macadamia nut production in an intercropping system irrigated with coffee was 251% higher than the rainfed macadamia monocropping. • Intercropping with arabica coffee contributed positively to macadamia growth, regardless of the use of drip irrigation. • Intercropping increased the number of fruits by 32% and the production of nuts in shell per tree by 30% compared to monoculture, increasing macadamia production.	[70]

Table 2. *Cont.*

Nuts	Intercropped Crops	Positive Effects	Refs.
Cashew (*Anacardium occidentale* L.)	Mango ginger (*Curcuma amada* Roxb), elephant foot yam (*Amorphophallus paeoniifolius* (Dennst.)), turmeric (*Curcuma longa* L.), east Indian arrowroot (*Curcuma angustifólia* Roxb), taro (*Colocasia esculenta* (L.))	• The cashew and turmeric intercropping system recorded significantly higher cashew equivalent yield (3521.58 kg ha^{-1}) being attributed to the higher yield of turmeric. • The land equivalence ratio and production efficiency of the cashew + taro intercropping system were higher compared to the other systems, being 1.67 and 54.80 kg/ha/day, respectively.	[119]
Peanut (*Arachis hypogaea* L.)	Sugarcane (*Saccharum officinarum* L.)	• The rhizosphere soil of the intercropped peanut had a higher pH and nutrient content (P, K and N) than the soil of the monocultured peanut. • The sugarcane/peanut intercrop significantly increased the activities of acid phosphatase and urease in the rhizosphere soil, which are essential for the nitrogen cycle and the hydrolysis of phosphorus compounds in the soil.	[120]
Peanut (*Arachis hypogaea* L.)	Maize (*Zea mays* L.)	• Land use efficiency was significantly increased in the three treatments with two, four, and eight rows of peanut intercropped, when compared to monocultures of peanut and maize.	[71]
Peanut (Arachis hypogaea Linn.)	Maize (*Zea mays* L.)	• The yield of intercropped peanuts was directly proportional to the number of strips, with an average of 98.4 gm^{-2} for M8P8. In comparison, this result was significantly higher than in M4P4, with an increase of 30%, and M2P2, with an increase of 99%.	[77]

Integrating perennial cultivation with other crops in agroforestry systems can be an effective strategy for reducing CO_2 emissions from the soil, without harming production, while increasing the overall productivity of the agroecosystem [99]. For this reason, Sánchez-Navarro et al. [99] implemented a diversified almond intercropping system with *Capparis spinosa* L. and the aromatic species *Thymus hyemalis* Lange. The orchard was kept dry, but the thyme and capers were irrigated on four occasions to ensure proper establishment. The results showed that thyme significantly increased soil moisture when compared to monoculture, by 11.1% and 10.2% respectively. Thyme also showed significant improvements in soil carbon sequestration, with its values increasing from 3.85 g/kg in 2019 to 4.62 g/kg in 2021.

Another study involving intercrops with nuts confirmed that the integration of different crops significantly increased bacterial and fungal diversity compared to monoculture forests. Bai et al. [116] examined changes in soil physicochemical properties, enzyme activity and microbial community composition when walnut (*Juglans* spp.) was intercropped with tea plants (*Camellia sinensis* L.) in a forest system, comparing the results with a monoculture walnut and tea system. Bacterial and fungal diversity increased significantly as a result of intercropping, compared to tea and walnut monocultures, with the most abundant being *Proteobacteria, Bacteroidetes, Firmicutes, Chlamydiae, Rozellomycota* and *Zoopagomycota*. As a result, through intercropping, there was an increase in the presence of beneficial organisms responsible for nutrient cycling, protection against disease and improving abiotic stress.

The research conducted by Zhong et al. [117] also investigated soil microbial communities, as well as their effects on environmental factors, by establishing a field experiment in randomized blocks of pandan (*Pandanus amaryllifolius* Roxb) intercropped with areca nut (*Areca catechu* L.). The results showed that intercropping significantly improved soil bacterial indices, reducing organic carbon and total phosphorus content. Soil microbial communities such as *Firmicutes, Methylomirabilota, Proteobacteria, Actinobacteria, Chloroflexi, Verrucomicrobia* and *Ascomycota* responded significantly to changes in planting methods. These results suggest that intercropping with pandanus positively influences soil microbial homeostasis in areca nut plantations in the long term.

Perdoná and Soratto [70] conducted additional investigations to examine the growth, productivity and economic viability of the macadamia crop when grown in association with coffee, under two different irrigation systems: rainfed and drip irrigation. These results showed that intercropping resulted in higher macadamia production than monoculture, with a yield of 536 kg ha^{-1} compared to 197 kg ha^{-1} for the intercropped and monoculture systems, respectively.

Through the results obtained by Perdoná and Soratto [121], it was shown that intercropping with macadamia trees increased the productivity of Arabica coffee beans by 10% compared with monoculture under drought conditions. When combining drip irrigation and intercropping, the crop was more profitable, being 276% more than the monoculture irrigated coffee after the first five harvests. Ramteke et al. [119], presented data favourable to the system in which cashew in combination with taro (*Colocasia esculenta*) recorded the highest yield of 210.61 q/ha, compared to 4.18 q/ha from the cashew cultivation alone. In addition, a production efficiency of 54.80 kg/ha/day was obtained.

Regarding pest control, the intercropping of cashew and banana resulted in a significant decrease in the attack of the chestnut moth (*Anacampsis phytomiella* Busck), with 5.8% of the nuts punctured compared with the other treatments. It is assumed that this reduction in the number of pests is due to the degree of shading that the banana trees provide, which prevents the formation of a more favourable environment for the moth population [122].

Studies by Žalac et al. [20] showed that intercropping was more productive (28.986 € ha^{-1}) than the separate production of field crops and walnut trees for all tree density scenarios in the first 6 years, with a difference in net margin of 1.435 € ha^{-1}. Other research has proven the benefits of intercropping in walnut production, where results revealed that the composition and structure of the soil bacterial community changed significantly with the

intercropping of walnut/hairy vetch (*Vicia villosa* Roth.) compared to clear tillage. Soil microorganisms from this interspecific relationship were also found to have a potential for nitrogen cycling and carbohydrate metabolism, and may be related to the functions of *Burkholderia*, *Rhodopseudomonas*, *Pseudomonas*, *Agrobacterium*, *Paraburkholderia* and *Flavobacterium* [123].

Furthermore, a walnut intercropping system was shown to increase soil bacterial diversity, with *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Acidobacteria* being the dominant soil bacterial phyla. Soil pH and soil density were significantly correlated with bacterial diversity [124]. According to studies by Wu et al. [60], the intercropping of tea (*Camellia sinensis*) with Chinese chestnut influenced the amino acid metabolism of the crop, positively modifying the taste of the tea. The quality of the tea may be associated with the levels of allantoic acid, sugars, sugar alcohols and oleic acid, which were higher and the flavonoids less bitter in the intercropping system when compared to the monocropping system. Previous research confirmed the positive effect of intercropping chestnut (*Castanea mollissima* Blume) on tea (*Camellia sinensis* L.) production, where this crop integration has shown promise for tea quality and quantity. It had an effect on reducing the content of amino acids and catechins, while increasing theanine and caffeine, making the tea more refreshing and tastier [32].

Although taxonomically classified as a legume of the fabaceae family, peanuts (*Arachis hypogaea* L.) are also considered within the oilseed group due to their chemical composition [125]. Studies by Tang et al. [120] showed that pH, total phosphorus, total potassium, available nitrogen, available phosphorus, and available potassium were higher in the soil of a sugarcane–groundnut intercropping system compared to peanut monocropping system. It is also worth noting that the intercropping of sugarcane and groundnut also significantly increased the activities of acid phosphatase and urease in the rhizosphere soil, having a positive effect on improving the soil nutrition of groundnut.

The intercropping of sugarcane and peanut makes the most of the land resources by increasing nutrients in the soil through the rise of beneficial microorganisms, renewal of organic matter and cycling of compounds such as nitrogen and phosphorus. In addition to bringing economic benefits to farmers, it contributes to the development of efficient and sustainable production [120].

Another investigation into peanut production in intercropping was carried out by Wang et al. [77]. They tested four different combinations of intercropped crops, all with equal proportions of maize and peanuts, but varying the number of rows per strip: M2P2 (two rows of maize to two of peanuts), M4P4, M6P6 and M8P8, as well as maize soil (SM) and peanut soil (SP). The results indicated that the most effective combination for peanut yield was M8P8, achieving 98.4 gm^{-2}. This highlights the influence of strip width on yield results. Evidence such as this promotes an improvement in the layout of rows during intercropping, further highlighting the viability of the strip system for sustainable intensification.

6. Challenges and Limitations for the Establishment of Service Crops

Despite its many benefits, intercropping also faces challenges and limitations in productivity between crops [15]. Often, the complexity and cost of management means that some farmers do not use this technique in the field [126]. The biggest challenges of this system are the lack of information on yield and crop performance in a mixture, the more complicated crop management and harvesting, and the economic risk associated with new combinations [127]. In some cases, multiple cropping systems can result in undesirable outcomes when crop selection is carried out erroneously as well as by geographical region [128].

Special bioactive chemicals (allelochemicals) released by plants can damage the growth and productivity of the other crop when they interact with each other, becoming a negative turnout of this relationship [11]. A case in point is that of the black walnut tree (*Juglans nigra* L.), the ideal tree species for intercropping due to its rapid growth and, production of high-

quality wood, as well as its ability to produce nuts. This walnut tree generates a detrimental effect on other plants, negatively affecting the growth of other species due to juglone, a chemical compound produced by this tree, which has an allelopathic effect on different crop species [129]. Research by Žalac et al. [130] showed that there was a 30% reduction in maize yield due to juglone excretion, which significantly reduced plant density. Jose & Holzmuelle [129] also pointed out in their research that some management techniques can reduce the allelopathic effects of juglone, including the use of polyethylene root barriers, the opening of trenches or discs and the planting and management of companion species during the initial establishment phase of black walnut.

A major disadvantage in intercropping is the difficulty in practical management of essential agronomic operations, particularly where farm mechanization is adopted or when the component crops grown in intercropping have dissimilar requirements for fertilizers, water, and plant protection requirements [11]. The use of machinery is very important in regular agronomic operations such as seeding, weeding or harvesting, and intercropping negatively interferes in the individual management of crops. Another setback is the need for more labour per unit area, which potentially leads to a decrease in yield if not effectively handled. This is found to be relevant in the case of maize–soybean intercropping, where mechanization proceedings lead to the damage of soybean, as it is harvested secondly and ends up being indirectly harmed due to the machinery use [131].

In agrosystems, the age of the tree also influences competition between species, because as the tree ages, interspecific competition increases, affecting the yield and physiology of the plant compared to the same plant grown in monoculture [132]. In relation to pest control, the intercropping system may face some risks in the potential of the intercrop, where some flowers may be toxic to predators [15]. In one instance, the pollen of *Lilium martagone* L. and *Hippeastrum* sp., which that caused 100% mortality of the predatory mite *Amblyseius swirskii*, was used as a biological control agent against several pests in greenhouses [133]. There is also the fact that the cover crop acts as a 'green bridge', tending to behave as a reservoir of pests and pathogens that can transfer to the following cash crop, ruining the main crop [134].

Solar radiation tends to be a limiting factor for agroforestry crops in many regions and can generate a reduction in yield by increasing shade, which can be solved by the regular pruning of trees to increase the light transmission rate [25,135]. Other agronomic measures can be taken to reduce the competition generated by the cropping system, such as adjustments between the rows of trees and crops, the selection of the most suitable crop variety, and root barriers, additional irrigation and fertilization should also be applied [25]. However, growing two or more crops together requires the careful planning of field operations and may require special interventions to reduce competition between the intercropped species, thus maintaining a balance [11].

7. Future Perspectives

Research has advanced in order to improve the crop proportions as well as the relationships of resource availability, capture and partitioning, thus leading to the better yield performance of the intercropping systems [136]. Other studies have also focused on expanding crop diversity and, signal-controlled interactions between intercrop species, analyzing below- and aboveground diversity relationships and developing functional, structural and empirical models for crop optimization [137]. Crop growth models based on mathematical processes have already been used in order to combine plant characteristics and environmental conditions in a systemic approach. Thus, they simulate the functioning of plants considering their individual properties and growing conditions, allowing for the evaluation of yields and genetic improvements in an accurate and efficient way, without relying on extensive field trials [138].

In this sense, research has been advancing to bring new resources to make intercropping systems more effective. In future studies, it will be necessary to focus on investigating the effects of intercropping on the composition and function of the microbial community,

focusing especially on the relationship between extracellular enzymes present in the soil and the genes responsible for encoding them. Long-term research is essential to deepen the understanding of the effects of subsurface processes on soil fertility, ecosystem stability and the contributions of sustainable agroecosystems to climate change adaptation and mitigation [139]. In addition, future dissemination efforts aimed at increasing the adoption of consortia systems can benefit from a more robust integration of farmers' perspectives in generating and providing information [140].

Regarding the quality of food from intercropping systems, there is still a lot of area for research, as the information available is limited. However, studies indicate that sustainably produced foods generally have lower amounts of nitrate residues, nitrites, pesticides, heavy metals and other pollutants harmful to human health. Furthermore, nutritional quality tends to be improved through this agricultural production system, resulting in greater antioxidant activity, vitamin content, total sugars and improved protein quality. Crop rotation proves to be more profitable and beneficial to the environment, in addition to being equally or even more nutritious. However, due to its lower yields and different costs, it is still a smaller alternative compared to conventional agriculture [141]. Therefore, the biggest challenge of intercropping systems in nuts is to select compatible crops to minimize competitive inhibition, allowing for easy field management and consequently increasing the profit compared to monocultures [28].

8. Conclusions

In the face of challenges in agriculture, such as the need to increase food production in relation to a growing population, steps are being taken to provide sustainable and profitable crop production through intercropping systems. Coupled with climate change, intercropping can be an option to increase crop production in nuts, especially as this interaction between crops can increase nitrogen and nutrient availability in the soil, provide yield stability and decrease nutrient leaching and infestation by pathogens and weeds. As a potentially labor-intensive technique, it still faces challenges and limitations in production, because the success of this system depends on the interactions between the component species, available management practices and environmental conditions. Therefore, further research is needed to find the ideal growing conditions for each nut species, as well as to find suitable crops that can generate high quality fruits from this interspecific interaction.

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Insight into the Biostimulant Effect of an Aqueous Duckweed Extract on Tomato Plants

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Abstract: Agricultural systems must improve their sustainability and productivity to meet the growing global demand for food. A cost-effective and sustainable way is the development of biostimulants from plants rich in bioactive compounds. This study aimed to test an aqueous extract from *Lemna minor* L. (duckweed) on tomato plants at different concentrations (LE—0.1, 0.5 and 1.0%—*weight/volume*, *w/v*). Photosystem I and II activity, linear electron flow (LEF), electrochemical gradient across the thylakoid membrane (ECSt), shoot biomass production, root phenotyping, pigment and metabolite content were studied. LE improved many of these traits, with LE 0.5% being the most effective dosage. Compared to the untreated samples, LE significantly stimulated photosystems to use light energy while reducing the amount lost as heat (PhiNPQ and NPQt) or potentially toxic to chloroplasts (PhiNO). These results were supported by the improved shoot biomass production (number of leaves and fresh and dry weight) and root traits (number of tips, surface, volume and fresh and dry weight) found for LE-treated samples compared to untreated controls. Finally, the study highlighted that LE increased pigment and flavonoid contents. In conclusion, the research indicates that this species can be an effective and eco-friendly tool to stimulate beneficial responses in tomato.

Keywords: plant extract; horticultural crop; *Lycopersicon esculentum*; photosynthesis; biomass production; pigment content; antioxidants

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1. Introduction

One of the biggest challenges facing agriculture in the coming years is the growing demand for food, as the world population could reach 9.7 billion by 2050 [1]. In addition, human activities are compromising the quality of natural resources by reducing, for instance, the area dedicated to crop cultivation, and the situation is further exacerbated by climate change [2]. In this context, it should also be considered that agricultural systems based on the extensive use of synthetic chemicals administered to crops to increase yield result in environmental pollution and the degradation of primary resources such as soil and freshwater [3]. Therefore, in a circular economy logic, there is a cogent need for innovative and sustainable biobased solutions to mitigate the impact of cropping systems. Indeed, this vision aims to exploit more efficiently biological resources, even those derived from agroindustrial waste, for application in agriculture, with the scope of increasing crop productivity and quality, reducing pressure on the environment and safeguarding the health of ecosystems [4].

For these reasons, eco-friendly solutions should be searched for in unexplored natural resources and applied in agriculture to improve crop performance. As is well known, biostimulants are natural substances that can stimulate seed germination, affect plant nutrition, improve water uptake and use, influence plant growth and biomass production

and improve primary and secondary metabolism [3]. Moreover, these materials can make crops more tolerant to environmental biotic and abiotic stressors [3]. Based on their origin, biostimulants have been classified as microbial and non-microbial [5,6]. Non-microbial biostimulants can be obtained from plant extracts (plants and algae), protein hydrolysates (both of plant and animal origin), fulvic and humic substances and inorganic (salts) and organic compounds (chitosan) [7].

Currently, there is growing attention being paid to finding new plant extracts rich in bioactive molecules to be exploited as biostimulants and used in agriculture. In particular, plant extracts can show noticeable contents of bioactive compounds such as phenols, amino acids, small peptides, micro- and macro-elements and numerous other components that can stimulate crop metabolism and biomass production and improve the end product's quality [8]. These beneficial effects can be due to the ability of biostimulants to prompt some crucial physiological, morphological and biochemical processes, such as photosynthesis and metabolism [8]. Furthermore, specific molecules with signaling or hormonal activity have been identified in plant extracts, and they are responsible for increased plant biomass production, interactions with proteins to regulate genes and amino acid and metabolite synthesis [9,10]. For instance, it is well known that applying protein hydrolysates to plants can determine many plant-stimulatory effects, as they contain peptides and amino acids that can act as signal molecules [9,11].

Among the species that can be used to obtain plant extracts with bioactive properties and promote benefits in crops, duckweed is attracting increasing interest. This species is a small free-floating aquatic plant belonging to the Lemnaceae family that naturally occurs worldwide in wetland ecosystems, such as lagoons, swamps and ponds, as well as in irrigation ditches. Duckweed is considered invasive due to its fast growth rate and high capacity to adapt to different climatic conditions (temperatures in the range of 5–35 °C) and unfavorable aquatic environments (pH levels between 3.5 and 10.5) [11]. In addition, duckweed can tolerate and survive high concentrations of toxic compounds, and this resistance makes it a suitable species for phytoremediation and ecotoxicity studies [12,13]. Indeed, it was successfully used for phytoremediation purposes, such as wastewater treatment, as it can remove and bioaccumulate pollutants, ranging from organic compounds to metal trace elements [12,13].

Moreover, recent studies demonstrated that duckweed produces a plethora of secondary metabolites with bioactive properties, especially glucosinolates and phenols [11]. Indeed, metabolomics studies conducted by the authors of this research have revealed that this aquatic plant has a broad spectrum of bioactive substances [11,14–17]. Among them, compounds including phenols, glucosinolates, flavonoids and substances with antioxidant properties and protective action should be mentioned because they correlate with biostimulant properties [11,14–16]. For instance, glucosinolates, extensively studied for other species such as Brassicaceae, can exert protective action in plants against physical damage, such as wounds and those caused by pest attacks and even abiotic stressors (high temperatures, salinity and UV) [11]. In addition, it has been proposed that these compounds may act as signaling molecules capable of activating plant defense systems [18]. Phenols and their exogenous applications have shown a wide range of benefits in treated plants, due to their involvement in regulating and stimulating various physiological processes. These include growth regulation, photosynthesis, pigment synthesis induction and oxidative stress mitigation [19]. These bioactives can play a crucial role in the adaptation to challenging environmental conditions, and it has been demonstrated that their application to crops can help plants cope with abiotic stresses [20].

Despite the interesting traits of duckweed and its richness in bioactive compounds, few studies have explored its biostimulatory potential on crops, except for some recent research on olive and maize crops [11,14–16]. For horticultural species, on the other hand, there are no traces in the literature that address the biostimulant effect of duckweed on these crops. To fill this gap, the present work aimed to evaluate the effects of an aqueous duckweed extract (LE) on a horticultural species. In particular, LE was applied by foliar

spraying on tomato, which was chosen because this crop is one of the most important and widespread worldwide. The photosynthetic machinery and specific aspects, such as the photosystem functionality, aerial and root biomass development, pigment content and some major classes of antioxidants, were then investigated in LE-treated tomato plants. All this was undertaken to ascertain any beneficial effects of the extract on the horticultural species in question.

2. Materials and Methods

2.1. Preparation of the Aqueous Extract of Duckweed

All chemicals used in this research were purchased from Merck Life Science S.r.l. (Milan, Italy) and used as received without further purification.

Duckweed was grown in polyethylene trays (35 × 28 × 14 cm) according to a previously published procedure, renewing the culture medium every two weeks [21]. Briefly, the nutrient solution (pH 6.5) contained 3.46 mmol L^{-1} KNO_3, 1.25 mmol L^{-1}, $Ca(NO_3)_2{\cdot}4H_20$, 0.66 mmol L^{-1} KH_2PO_4, 0.071 mmol L^{-1} K_2HPO_4, 0.41 mmol L^{-1} $MgSO_4{\cdot}7H_2O$, 0.28 mmol L^{-1} K_2SO_4, 1.94 μmol L^{-1}, H_3BO_3, 0.63 μmol L^{-1} $ZnSO{\cdot}7H_2O$, 0.18 μmol L^{-1} $Na_2MoO_4{\cdot}2H_2O$, 1 μmol L^{-1} $MnSO_4{\cdot}H_2O$, 21.80 μmol L^{-1} Fe-EDTA and 1 μmol L^{-1} $CuSO_4$. Trays were maintained in a growth chamber at 24 ± 2 °C, 80 μmol m^{-2} s^{-1} of light intensity, and a photoperiod of 8 h light and 16 h dark.

Three different concentrations of LE were chosen for the experiments on tomato plants: LE 0.1, 0.5 and 1.0% (dry weight/water volume—wt/v). To this scope, 20 g of fresh plant material was thoroughly rinsed with water and dried at 40 °C for 72 h. Then, 1 g of dry biomass was mixed with 100 mL of deionized water (pH value = 7.00), extracted using a mortar with a pestle for 5 min in the presence of small amounts of quartz sand, and left in an orbital shaker overnight (100 rpm) at 23 °C, to complete the extraction. Finally, the suspension was filtered using filter paper and brought to the final volume of 100 mL with deionized water. This allowed us to obtain the most concentrated LE extract (LE 1.0%). This extract was appropriately diluted with deionized water to obtain the other two solutions, designated as LE 0.5 and 0.1%. The three concentrations were selected as previous studies showed they were capable of prompting biostimulatory effects in crops [11,14,15]. Differently, LE can be phytotoxic at higher concentrations (2 and 8%) or lose activity at lower ones [11].

A description of the metabolomic and phytochemical profile of LE 1.0%, ascertained in previous studies on plants bred and extracted according to the above procedure, is given below [11,15]. The LE phytochemical profile was determined by using untargeted metabolomics ultra-high-pressure liquid chromatography associated with a quadrupole-time-of-flight mass spectrometer (UHPLC-ESI/QTOF-MS), according to Del Buono et al. [11]. The results indicated a remarkable content of bioactives such as phenols (6714.99 mg kg^{-1}) and glucosinolates (4563.74 mg kg^{-1}). Also, flavonoids and phenolic acids were found in significant amounts and similar concentrations of 1829 and 1733 mg kg^{-1}, respectively [11]. The most abundant flavonoids were kaempferol and quercetin and their glucosides, followed by myricetin. Furthermore, hesperidin was the most abundant flavone, while caffeic acid was the most abundant of the phenolic acids (812 mg kg^{-1}). In addition, the following low molecular weight phenols were detected: mainly 5-nonadecenylresorcinol, hydroxytyrosol and 4-hydroxycoumarin. Phytohormones (auxins, cytokinins, gibberellins, jasmonate-related metabolites and brassinosteroids) were also found in the LE [15]. The metabolomic profile also revealed the presence of amino acids, phenylpropanoids and alkaloids [15]. Isoprenoids, including triterpenoids, sesquiterpenes and terpene hormones (gibberellins and their precursors, abscisic acid derivatives and brassinosteroids) were well represented. Finally, antioxidant and plant-to-stress response-related compounds were identified (ascorbates and glutathione) [15].

2.2. Growth Conditions of Tomato Plants and LE Treatments

The experiments were conducted on tomato plants (*Lycopersicon esculentum* Mill.) cv. Rio Grande, a variety widely cultivated in Italy that produces large and pear-shaped tomatoes, suitable, for instance, for processing to obtain peeled tomatoes and preserves. The seeds were directly sown in plastic pots containing commercial peat and germinated in the dark for 5 days before light exposure. Tomato seedlings were cultivated in a growth chamber, with a photoperiod of 12/12 h (day/night), light intensity at 300 μmol m^{-2} s^{-1}, at a constant temperature of 24 ± 2 °C, and irrigated daily with water up to 75% field moisture capacity.

The leaves of tomato plantlets were sprayed, using a domestic sprinkler, at 4 (third true leaf stage) and 5 weeks after sowing. The temporal sequence of the treatment was chosen based on the development stage of the seedling, as the third true leaf was well formed. In detail, 2.5 mL per plant of water (control) or LE 0.1, 0.5 or 1.0% were applied, depending on the experimental group. For each treatment, 5 replicates were carried out, according to a completely randomized experimental design. Six-week-old plants were harvested for the physiological, morphological and biochemical determinations, as indicated in the following sections.

2.3. Effects of LE on Tomato Photosynthetic Activity

Some aspects of the photosynthetic processes were monitored on intact and fully expanded leaves in the early morning after 2 h of light exposure. To this end, the MultispeQ device (PHOTOSYNQ INC., East Lansing, MI, USA) linked to the web platform PhotosynQ (http://www.photosynq.org) was used [22]. In particular, the following parameters were studied: the quantum yield of PSII (Phi2), the fraction of light that can be lost via non-regulated processes (PhiNO) or released as non-photochemical quenching (PhiNPQ), the fraction of PSII centers which are in the open state (qL), the maximal quantum efficiency of PSII (Fv/Fm), the dark-interval relaxation kinetics of P700 (P700 DIRK), PSI photosynthetic reaction center proteins in open state (PSI open centers) and oxidized state (PSI oxidized centers), the total non-photochemical quenching (NPQt), the linear electron flow between photosystems (LEF), the total electrochromic shift (ECSt) and the proton conductivity of the thylakoid membrane (gH^+).

2.4. LE Treatment Effect on Tomato Growth at the Shoot and Root Level

Shoot development was evaluated by measuring shoot height and number of leaves. Furthermore, the leaf thickness was recorded. At the root level, biomass production was investigated, and the phenotyping was carried out on the scanned root using RhizoVision Explorer v2.0.3.0, according to Seethepalli et al. [23], measuring the total root length (cm), number of root tips, diameter (mm), surface area (cm^2) and volume (cm^3). Finally, the fresh mass of shoots and roots was recorded, and the dry weight was determined after oven-drying the samples at 60 °C to constant weight.

2.5. Leaf Biochemical Analysis (Chlorophyll and Carotenoid Contents, TPC, TFC and Soluble Carbohydrates)

Chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoid contents were ascertained by extracting 0.5 g of fresh leaf samples in 5 mL of methanol. This suspension was then centrifuged (20,000 rpm, 5 min). According to Venkatachalam et al. [24], the resulting supernatant was analyzed spectrophotometrically.

Finally, the total phenolic content (TPC), total flavonoid content (TFC) and soluble carbohydrates were determined by extracting 0.25 g of fresh leaf samples in 2.5 mL of methanol, then centrifuging at 6000 rpm (20 min). The Folin–Ciocalteu method was adopted for TPC, and the phenols content was referred to as the gallic acid equivalent (GAE) g^{-1} [25]. TFC was determined spectrometrically, according to Atanassova et al. [26], and was expressed as mg of catechin equivalents (CE) g^{-1}. Soluble carbohydrates were evaluated using the anthrone method, according to Al Murad and Muneer [27]. The supernatant (50 µL) from the methanolic extract was transferred to the solution with 950 µL of distilled water, and 2.5 mL of 0.2% anthrone reagent was added. The solutions were heated (100 °C, 10 min) to complete the reaction with anthrone, and, after cooling, the absorbance of the samples was measured spectrometrically at 620 nm. The total soluble carbohydrates were expressed as mg g^{-1} fresh weight (FW).

2.6. Statistical Analyses

The experiment was carried out according to a completely randomized design with four treatments (control, LE 0.1, 0.5 and 1.0%) and five replicates per treatment. The full dataset was subjected to statistical analysis through a one-way analysis of variance (ANOVA), and significant differences were assayed using Duncan's test at the $p < 0.05$ probability level [28]. The data presented in the tables represent the mean value ± standard deviation.

3. Results

3.1. Effects of LE on Tomato Photosynthetic Activity

LE improved photosystem II in tomato plants. In particular, the quantum yield (Phi2) increased significantly in all plants treated, compared to the untreated ones, with the LE 0.1 and 0.5% concentrations being the most effective (Figure 1). In parallel, the light energy dissipated through non-regulated mechanisms (PhiNO) was reduced by LE, with the highest difference recorded for the dosage of 0.5%. Non-photochemical photoprotective quenching (PhiNPQ) was also significantly decreased in treated plants proportionally to the LE dosage applied. LE applications affected the open state rate of photosystem II centers (qL), particularly for LE 0.5 and 1.0%. Differently, LE treatments did not affect the Fv/Fm ratio. Concerning photosystem I, the dark-interval relaxation kinetics of P700 (P700 DIRK) underwent a considerable reduction in all samples on which the LE was applied. In addition, the centers of the photosystem I found in an open state (PSI open centers) were significantly higher in plants treated with LE 0.5% than the control samples. As for the oxidized state of the photosystem I (PSI oxidized centers), LE caused a dose-dependent increase, with the 1.0% concentration being the most effective.

Compared to control samples, the linear electron flow (LEF) increased for all the LE treatments, with the highest values reached by 0.5 and 1.0%. In addition, the total amount of non-photochemical quenching (NPQt) was lowered by LE 1.0% (Figure 2).

The LE reduced the total electrochromic shift (ECSt), regardless of the dosage applied, while no differences were detected for the proton conductivity of the thylakoid membrane (gH^+) (Figure 2).

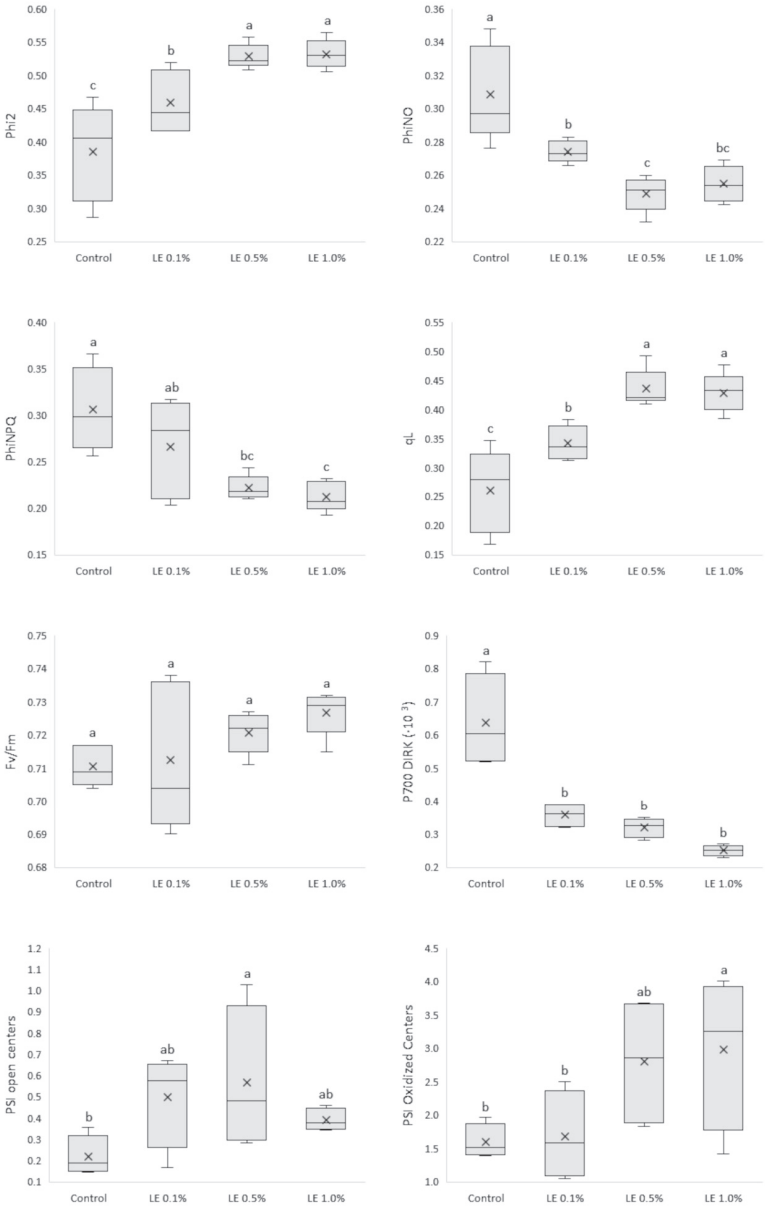

Figure 1. Effect of different duckweed extract concentrations (LE 0.1, 0.5 and 1.0%) on Phi2 (the efficiency of PSII), PhiNO (the non-regulated dissipation of light energy), PhiNPQ (the photo-protective non-photochemical quenching), qL (the open state of PSII), Fv/Fm (the photochemical efficiency of PSII), P700 DIRK (the dark-interval relaxation kinetics of P700), PSI open centers and PSI oxidized centers. Different letters indicate statistically different values, according to Duncan's multiple comparison test ($p < 0.05$).

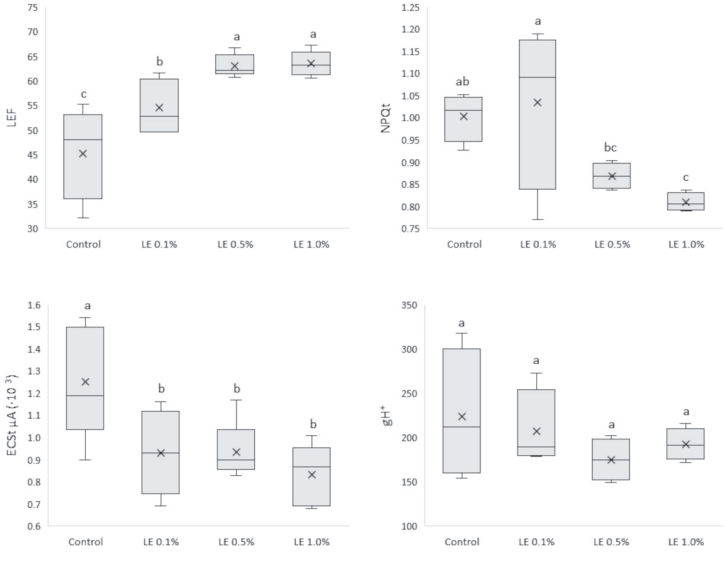

Figure 2. Effect of different duckweed extract concentrations (LE 0.1, 0.5 and 1.0%) on LEF (linear electron flow), NPQt (total non-photochemical quenching), ECSt (total electrochromic shift) and gH^+ (proton conductivity of the thylakoid membrane). Different letters indicate statistically different values, according to Duncan's multiple comparison test ($p < 0.05$).

3.2. LE Treatment Effect on Tomato Growth at the Shoot and Root Level

LE treatments affected the growth of tomato seedlings at both aerial and root levels. Plants treated with 1.0% concentration showed more leaves than those untreated (Table 1). Shoot height and leaf thickness were not influenced by any of the treatments. On the other hand, analyzing the shoot fresh and dry weights, it can be pointed out that all LE applications prompted tomato plants to produce more biomass than the control.

Table 1. Shoot analyses of tomato plants untreated (control) and treated with different duckweed extract concentrations (LE 0.1, 0.5 and 1.0%).

	Shoot Height (cm)	Number of Leaves (number)	Leaf Thickness (mm)	Fresh Weight per Plant (g)	Dry Weight per Plant (g)
Control	13.83 ± 0.50 a	33.5 ± 1.7 c	0.40 ± 0.07 a	6.25 ± 0.37 b	0.84 ± 0.14 b
LE 0.1%	14.65 ± 0.59 a	38.3 ± 1.7 a	0.62 ± 0.30 a	7.13 ± 0.31 a	1.07 ± 0.06 a
LE 0.5%	14.70 ± 0.59 a	38.8 ± 0.5 a	0.53 ± 0.10 a	7.21 ± 0.14 a	1.15 ± 0.02 a
LE 1.0%	14.48 ± 1.06 a	35.8 ± 1.0 b	0.53 ± 0.14 a	6.97 ± 0.34 a	1.02 ± 0.03 a

Different letters indicate statistically different values, according to Duncan's multiple comparison test ($p < 0.05$).

As for root phenotyping, the data highlighted that the number of root tips increased proportionally with the LE concentration, with all treatments being significantly higher than the control samples (Table 2). In addition, the extract increased the surface area and volume. Regarding the former, plants treated with LE 0.1 and 0.5% performed better than the control samples, while those belonging to LE 1.0% did not differ from the untreated samples. As for the root volume, the extract effectively increased this trait at 0.5 and 1.0% concentrations, and 0.1% was in line with the control. No treatment influenced the total length and average diameter of the roots. Finally, the root fresh weight per plant was higher than that shown by the control samples for all the samples treated with LE, regardless of the concentration applied. Regarding the dry weight per plant, it can be noted that only the LE 0.5% differed from the control.

Table 2. Root analyses of tomato plants untreated (control) and treated with different duckweed extract concentrations (LE 0.1, 0.5 and 1.0%).

	Total Length (cm)	Root Tips (Number)	Diameter (mm)	Surface Area (cm^2)	Volume (cm^3)	Root Fresh Weight (g)	Root Dry Weight (g)
Control	5094 ± 792 a	727 ± 91 c	0.73 ± 0.07 a	95 ± 20 b	2.46 ± 0.66 b	1.33 ± 0.10 b	0.25 ± 0.03 b
LE 0.1%	6051 ± 710 a	994 ± 130 b	0.69 ± 0.03 a	132 ± 14 a	3.46 ± 0.52 ab	2.93 ± 0.26 a	0.29 ± 0.02 ab
LE 0.5%	5806 ± 265 a	1061 ± 65 ab	0.72 ± 0.06 a	132 ± 6 a	3.74 ± 0.39 a	3.24 ± 0.13 a	0.32 ± 0.01 a
LE 1.0%	6110 ± 782 a	1216 ± 89 a	0.71 ± 0.03 a	120 ± 6 ab	3.73 ± 0.86 a	3.19 ± 0.27 a	0.30 ± 0.03 ab

Different letters indicate statistically different values, according to Duncan's multiple comparison test ($p < 0.05$).

3.3. Leaf Biochemical Analysis (Chlorophyll and Carotenoid Contents, TPC, TFC and Soluble Carbohydrates)

Some biochemical aspects were investigated in plants treated with LE (Table 3). For chlorophyll a, LE increased its content in the treated samples at the dosages of LE 0.5 and 1.0%. Regarding chlorophyll b, LE-treated plants showed higher values than the control for all dosages applied. Carotenoids did not differ in LE-treated samples, while the flavonoids (TFC) content increased in LE 0.5%-treated plants. Finally, the contents of phenols and soluble carbohydrates were not affected by treatments with the extract.

Table 3. Chlorophyll a (Chl a), chlorophyll b (Chl b), carotenoid content, total phenols (TPC), total flavonoids (TFC) and soluble carbohydrates in tomato plants untreated (control) and treated with different duckweed extract concentrations (LE 0.1, 0.5 and 1.0%).

	Chl a (mg g^{-1} FW)	Chl b (mg g^{-1} FW)	Carotenoids (mg g^{-1} FW)	TPC (mg GAE g^{-1} FW)	TFC (mg CE g^{-1} FW)	Soluble Carbohydrates (mg g^{-1} FW)
Control	0.99 ± 0.06 c	0.26 ± 0.03 b	0.25 ± 0.02 a	1.95 ± 0.47 a	1.42 ± 0.14 b	1.22 ± 0.29 ab
LE 0.1%	1.07 ± 0.06 bc	0.39 ± 0.05 a	0.27 ± 0.03 a	1.79 ± 0.11 a	1.42 ± 0.10 b	0.94 ± 0.07 b
LE 0.5%	1.24 ± 0.05 a	0.44 ± 0.08 a	0.30 ± 0.02 a	2.02 ± 0.09 a	1.64 ± 0.04 a	1.32 ± 0.24 a
LE 1.0%	1.13 ± 0.05 b	0.38 ± 0.02 a	0.30 ± 0.03 a	1.74 ± 0.08 a	1.49 ± 0.03 b	1.07 ± 0.03 ab

Different letters indicate statistically different values, according to Duncan's multiple comparison test ($p < 0.05$).

4. Discussion

Finding natural and functional biobased solutions, such as biostimulants, can decisively improve cropping systems and increase crop performance in normal conditions and their tolerance to environmental stress. In addition, biostimulants can allow for the reduction in use or replacement of synthetic chemical compounds, which can have a high environmental impact [29]. Biostimulants have gained prominence and are considered an innovative agronomic tool because they can improve crop performance, help plants cope with environmental pressure and have economic and environmental benefits [7]. This becomes particularly important when considering the effects of climate change on cropping systems and the challenges of meeting growing food demand on a global scale.

Biostimulants can be used in both horticultural and cereal crops, and to date, research is increasingly focusing on new biostimulants obtained from plants, such as simple extracts. In fact, in cases where they are rich in bioactive compounds, they can effectively promote the growth and traits of the crops to which they are applied. Furthermore, if these biostimulant materials can be obtained from non-food and invasive plant species, this solution becomes relevant, cheap and smart, and aligns with the main concepts of the circular economy [30].

In this frame, some studies have highlighted the richness of duckweed, a free-floating aquatic invasive species, in terms of substances that can promote the development of crops, such as maize and olive, under normal and abiotic stress conditions [11,14–16]. However, despite their agronomic and food importance, the LE has never been tested for horticultural crops. For the above, this study reports the results of experiments on tomato plants grown

under normal conditions and treated with different concentrations of LE. Our research has shown that according to a general dose–response type trend, LE promoted and stimulated a range of beneficial effects in tomato plantlets. In particular, the photosynthetic machinery was affected by the treatments (Figures 1 and 2). This benefit is worth mentioning as it enables plants to more efficiently utilize light energy and transform it into chemical energy, thus impacting biomass production and crop productivity [31]. LE improved the efficiency of PSII and PSI, which enhanced the ability of the photosystems to intercept light for biosynthetic purposes. The increase in Phi2 (the amount of light used for photochemical biosynthesis) indicates a higher ability of PSII to absorb electromagnetic radiation for photosynthesis. PSII is considered an indicator of the efficiency of plants in utilizing light for carbon dioxide assimilation by crops [32]. This benefit was associated with a decrease in PhiNPQ, the energy that plants do not use for photochemical reactions and disperse mainly as heat, and PhiNO, which represents the fraction of energy that can give rise to oxidative stress, thus negatively impacting the crop [33]. It has been documented that a marked decrease in photosystem II efficiency and an increase in PhiNPQ and PhiNO can occur due to abiotic environmental stresses and are associated with reductions in crop yield [33]. In addition, in support of the above beneficial effects, LE induced increases in PSII active centers (qL) without damaging the photosystems, as indicated by the Fv/Fm ratio, representing the integrity of PSII [34]. LE also had a positive impact on PSI function and activity, as revealed by P700 DIRK (i.e., PSI relaxation kinetics) and the increase in PSI open centers and P700 oxidized, thus indicating an enhanced ability of PSI to proceed with the transport of electrons transmitted by PSII, and then used for the reduction of $NADP^+$ to NADPH [35].

Thanks to a highly regulated mechanism, the linear electron flow (LEF) through the photosystems is associated with the formation of a proton electrochemical gradient across the thylakoid membrane that results in a proton motive force (*pmf*), which is then used for ATP synthesis [36]. The improved activity of the two photosystems in LE-treated tomato plants resulted in increased LEF (Figure 2), reflecting LE capacity to improve the ability of the plants to extract electrons from H_2O and transfer them through the PSs, reaching a higher NADPH production.

The overall decrease in NPQt, the total energy dissipated as heat by the photosynthetic machinery, aligns with the increased photosynthetic efficiency (Figure 2). Increasing NPQt attenuates energy transmission between photosystems in stress situations, but its reduction is worth mentioning under normal conditions as it assumes positive significance [37]. Photosynthesis is a highly regulated and coordinated flow of electrons associated with the translocation of protons from the stroma to the lumen, thus forming an electrochemical gradient exploited by ATP-synthase to produce chemical energy as ATP [36,38]. Indeed, the decrease in electrochromic shift (ECSt) (Figure 2) reveals that LE stimulated ATP production, as the amplitude of ECSt is proportional to the proton motive force (*pmf*), and its decrease indicates a concomitant consumption of the electrochemical energy by ATPase to synthesize ATP [36]. Accordingly, when plants suffer from environmental stressors or, for instance, there is a depletion in phosphate, the ATP synthesis can slow down with a *pmf* accumulation across the membrane and ECSt increase [39]. Finally, the gH^+, the thylakoidal membrane conductivity, did not change compared to the control samples. This parameter can slightly increase during high-intensity radiation but is relatively stable over a wide light-intensity range [37]. On the other hand, gH^+ may vary mainly in conditions such as decreasing CO_2 levels or during different light treatments, and it may also reflect metabolic alterations due to environmental stress [37].

Biostimulants can activate multifaceted aspects in the treated plants, but photosynthesis enhancements and biomass production have been very often recorded, both under normal and biotic and abiotic stress conditions [40]. In particular, bioactive compounds can improve plant efficiency by enhancing specific aspects of the photosynthetic machinery, improving its efficiency in capturing light and modulating electron and proton transfer in chloroplasts [41]. For instance, it has been found that biostimulant seed pretreatment

ameliorated the photochemistry of PSII in soybean. In particular, the biostimulant treatment resulted in a more efficient use of light energy in photochemical reactions rather than the induction of photoprotective processes (decreases in NPQ) [42]. Regarding LE, it has already been observed that this species can prompt general benefits to photosynthesis, mainly affecting the stomatal aperture, due to its wide range of bioactive, signal and regulatory molecules capable of influencing metabolic processes [14]. Our previous studies have shown that the phytochemical profile of LE reveals the presence of molecules with biostimulatory activity, such as auxins [11,15,16]. The presence in LE of such compounds explains the effects on the photosynthetic traits mentioned above; it is well known that the exogenous application of auxins induces photosynthetic activity [15]. Auxins can also positively influence transpiration and stomatal conductance [43]. In addition, Lemnaceae have a significant content of antioxidant metabolites [44], which can also induce photosynthesis, such as phenolic compounds [15], as evidenced by the extract used in this research.

Regarding aerial biomass production, data showed that all the LE concentrations generally promoted the stimulating effects. Indeed, the treatments increased the number of leaves and shoot fresh and dry weight (Table 1). Root phenotyping also showed an inductive effect in response to all concentrations investigated, with a consistent increase in root tip number and root fresh weight (Table 2). In addition, LE generally affected root surface area, volume, and for LE 0.5%, even dry weight. The phytochemical profile of LE should be considered to explain these effects in connection with the activation found in the functionality of the photosynthetic machinery [11,15,16]. In particular, in a previous study, we ascertained in LE, as already mentioned above, a significant number of auxins and related compounds that can activate root and aerial biomass production and photosynthesis [15,43]. In addition, the high content in LE of antioxidants, mainly phenolic compounds [15], can promote positive responses in the plant by improving crop functional traits and photosynthesis [14]. Our data also showed that LE modulated and improved root architecture. All these effects agree with the bibliography that documents that biostimulants can generally improve root tissues and their architecture and organization [45,46]. Also, in agreement with the effects recorded, the high content of phytohormones and glucosinolates found in LE [11,14–16], given their stimulating activity on the root system, justifies what was observed in this study. Finally, LE has a noticeable proline content, which can stimulate biomass production at shoot and root levels and crop resistance to biotic and abiotic stresses [14–16].

In addition to the above determinations, the content of chlorophylls, total phenols and flavonoids and soluble carbohydrates were analyzed in tomato plants treated with LE (Table 3). Regarding the pigments, the two highest dosages of LE significantly increased the content of chlorophylls a and b, while it did not affect carotenoids. Chlorophyll is a key pigment that plays a crucial role in photosynthesis, as it absorbs light in the visible region and uses it in reaction centers to support this anabolic process of chemical energy production [47]. This result is in line with what was found for photosynthesis and can be justified as an effect attributable to the bioactives in LE [11]. The induction in photosynthetic pigment content aligns with other studies in which crops treated with different plant extracts manifested significantly higher chlorophyll values than control untreated samples [48,49].

The treatments generally had no significant effects on phenol and flavonoid content, except for the LE 0.5% dosage, which enhanced TFC (Table 3). An increase in flavonoid content is relevant since these molecules play molecular regulatory roles in the cell and are involved in the defensive response to biotic and abiotic stresses and plant acclimatization [50,51]. In addition, there is a growing interest in increasing the content of these biomolecules in crops, given their protective action for human health. Flavonoids can exert numerous benefits, as they can exhibit antioxidant, anti-inflammatory, anticancer and antiviral properties, as well as neuroprotective and cardioprotective action [52]. The last aspect investigated in our experimentation was the content of soluble carbohydrates, as a variation in them may indicate possible treatment-related stress responses, as soluble

carbohydrates are involved in protective osmoregulatory processes. The results reveal that LE did not affect their content.

5. Conclusions

High environmental impacts characterize current agricultural systems. Therefore, solutions need to be found to improve their sustainability and, at the same time, increase their productivity to meet the growing global demand for food in the context of climate change. A smart and suitable way to increase the performance of crops is the development of new biostimulants, as these are ecological tools that can also help crops cope with challenging climatic conditions. In this context, one strategic way is to obtain plant extracts rich in bioactive compounds from non-food and/or invasive species. This study showed that it is possible to obtain a biostimulant from LE, a widespread free-floating aquatic species that can promote many benefits in tomato plants. LE stimulated photosynthesis by increasing the ability of photosystems I and II to intercept light and reducing the amount lost as heat or potentially toxic to chloroplasts. Furthermore, LE stimulated some physiological and biochemical aspects correlated with it, such as linear electron flow, ATP synthesis and pigment content. All these inductive effects resulted in increased biomass production and improved root traits. The results indicated that all concentrations of LE promoted substantial benefits in treated tomato samples, but 0.5% was the most effective in influencing the crop. In light of the above, this study demonstrated how natural resources such as duckweed can be obtained and developed with a convenient application in agriculture to increase the productivity of cropping systems, making them more sustainable. Nonetheless, studies like ours conducted on a laboratory scale must necessarily be followed by others in the field to verify the beneficial effects on crops throughout their life cycle. However, it is well known that positively conditioning the early stages of the plants has essential effects on their entire life cycle.

From a future perspective, we emphasize the strategic importance of the research as a key and crucial step that should help more attention be paid to identifying and obtaining useful materials to be applied in agriculture, with low or absent environmental impact and the characteristic of eco-sustainability. In this sense, an intelligent path is the development of innovative and effective biobased materials from unexploited biological resources. This will reduce the use of synthetic chemicals in agriculture that strongly impact the environment and ecosystems and mitigate the emission of greenhouse gases. Conversely, a not-so-easy substantial paradigm shift is needed in the manufacturing world for a real ecological transition, based on abandoning the current linear economy approach in favor of a circular one.

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Article

Role of an Aqueous Extract of Duckweed (*Lemna minor* L.) in Increasing Salt Tolerance in *Olea europaea* L.

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Abstract: Salt stress is one of the preeminent abiotic stressors capable of strongly impacting crop productivity and quality. Within the array of strategies garnering interest in safeguarding crops against abiotic stresses, the use of plant biostimulants is emerging as a noteworthy avenue. For the above, there is an increasing interest in finding new plant extracts showing biostimulating effects in crops. In the present study, the efficacy of an aqueous extract from an aquatic species, the duckweed (*Lemna minor* L.), was assessed in olive plants (cv. Arbequina) grown in hydroponics and exposed to severe saline stress (150 mM NaCl). Salt stress caused considerable diminutions in biomass production, leaf net photosynthesis (Pn), leaf transpiration rate (E), and stomatal conductance (gs). The application of the duckweed extract resulted in a notable plant functionality recovery and counteracted the detrimental effects of the NaCl stress. Indeed, the plants stressed with NaCl and treated with the extract showed enhanced physiological and biometric traits compared to samples treated with NaCl alone. In particular, the duckweed extract improved photosynthetic activity and stomatal conductance, reduced the intercellular CO_2 concentration, and ameliorated other physiological and morphological parameters. All these benefits influenced the whole plant growth, allowing samples treated with the extract to maintain a similar performance to that exhibited by the Control plants.

Keywords: biostimulants; aquatic species; photosynthesis; NaCl stress; olive

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1. Introduction

Due to ongoing climate change, soil salinity stress is an increasing worldwide issue for cropping systems and its impact is particularly detrimental for most fruit-tree crops cultivated in the countries of the Mediterranean basin [1,2]. In these zones, many agricultural coastal areas are experiencing the harmful effects of extreme events caused by climate change, such as flooding and rising sea levels [3–5], which are provoking the progressive degradation of the primary natural resources, soil and water. This is due to the uncontrollable land salt intrusion, which determines the gradual salinization of soils and saltwater dispersion into freshwater aquifers [3]. In addition, it should be considered that salinity stress often occurs along with high temperature and drought stress. Nowadays, it has been estimated that salinity is affecting about 800 million hectares of arable land worldwide, strongly decreasing crop yields. The situation has worsened over the last 20 years due to increased irrigation requirements in arid and semi-arid regions [4]. In this context, it should also be considered that the global demand for food is constantly increasing due to the continuously growing world population. These issues are particularly challenging to address, but they require effective solutions that could be implemented quickly.

Salinity severely impairs crop productivity because its harmful effects can cause a variety of different physiological, morphological, and biochemical changes [5]. In particular, salt stress can influence plant establishment, cause stunted growth, and give rise to oxidative perturbations, hindering pivotal metabolic processes such as photosynthesis

and, in turn, biomass production [6]. In addition, it has often been reported that salinity impacts plants by provoking osmotic and ionic alterations [7], negatively affecting nutrient acquisition and translocation, and interfering with enzyme activities [6]. Salinity can also cause the death of the leaves due to salt accumulation in the cell wall or cytoplasm since the vacuole cannot sequester high amounts of salt [8]. Under salt stress, plants usually show restricted root extension, a higher root-to-shoot ratio, as well as altered root morphology [9]. Moreover, reductions in the total leaf area have been recorded that decrease the photosynthesizing surface, which probably represents an attempt by the plant to minimize water loss by transpiration, or could be a consequence of hampered plant nutrition [10].

Olive is generally considered mildly tolerant to salinity [11], but different cultivars showed great differences in salt tolerance [12]. All the researchers agree on the negative influence of moderate and high salinity stress on plant growth (mainly due to the leaf area reduction), even if the extent of the latter varies with the length of salt exposure and the cultivar [13].

The cv. Arbequina, investigated in the present study, has been described in the literature as a plant species that is medium-tolerant to salt stress [14]. However, Arbequina is of great interest for cropping systems since it is one of the most suitable species for very high-density planting systems thanks to its low vigor, high branching density, and high fruit-bearing capacity [15,16]. For its adaptability to super-intensive planting systems, Arbequina cultivation is spreading rapidly in most of the olive-growing areas of the world.

Given the strong impact of biotic and abiotic stress on olive and other crops, there is an ever-increasing focus on finding, developing, and implementing eco-friendly solutions to help plants counteract the detrimental effects of salinity and other environmental stressors. Among the strategies that can be adopted to increase plant resistance to salt stress, biostimulants are gaining increasing attention for their capacity to prompt benefit in crops [17,18]. In general, biostimulants increase crop productivity by inducing plant nutrition and nutrient use efficiency, improving photosynthetic machinery and increasing crop resistance to various abiotic stresses [7]. In addition, they can also positively affect plants by stimulating primary and secondary metabolism [7]. Biostimulants can be natural products, but given the wide range of raw materials from which they can be obtained, they have been grouped into two main classes according to their origin [19]. The first classification concerns the distinction between biostimulants of microbial and non-microbial origin [19]. Thus, the latter include plant and algae extracts, protein hydrolysates (mainly plant-derived), fulvic and humic substances, chitosan, and some inorganic compounds [20].

In recent years, researchers have been directed at finding new natural substances with biostimulant effects. Particular attention is being paid to finding plant extracts with relevant bioactive properties and also characterized by eco-compatibility [21,22]. Indeed, biostimulants deriving from plant extracts can contain active molecules (protein, small peptides, amino acids, phytohormones, and antioxidants) that can activate biochemical, physiological, and metabolic responses in crops, thus improving their productivity in normal conditions and their ability to cope with some abiotic or biotic environmental stressors.

In this perspective, very recent studies have been conducted on the biostimulatory effects of extracts obtained from the freshwater aquatic species *Lemna minor* L. (duckweed) [23–25]. Duckweed is widespread in freshwater basins, found on several continents, and is characterized by fast growth and a remarkable ability to adapt to even very different and unfavorable environmental conditions [23]. It is also easy to grow under controlled conditions and has a high content of metabolites with stimulatory properties [23]. Concerning the extracts obtained from this species, it has been shown that they can promote benefits in maize [23,24] and olive plants [25], even when the former crop was grown on a copper-polluted substrate [24]. Metabolomic studies have revealed that this species contains a broad spectrum of bioactives, including signaling compounds, phenolics, flavonoids, and many different antioxidants that can be responsible for the beneficial effects recorded [23–25]. To date, no studies have been conducted on using duckweed aqueous extracts to ascertain their eventual beneficial effect on plants grown under salt-stress condi-

tions. Therefore, this work aims to evaluate the ability of an aqueous duckweed extract to increase the tolerance of olive trees to salt stress (cv. Arbequina). The experimentation was conducted in a hydroponic growing system since, even though it is different from the open field conditions, it allowed the study of the effects of the duckweed extract on olive plants, avoiding the interferences of the types of soil and microbiota.

2. Materials and Methods

2.1. Olive Material and Growing Conditions

Olive plantlets of the cv. Arbequina, approximately 20 cm in height, were transplanted in 200 mL pots containing rock wool for an acclimatization period of 60 days and were grown in a hydroponic system under controlled conditions. The olive plants were allocated in PVC containers comprising five plastic pots for hydroponics, with one plant for each pot. A tank containing the nutrient solution (3.5 L of half-strength Hoagland solution, pH 7.5) was connected to each container. The flux of the nutrient solution from the tank to the PVC containers containing the olive plants was ensured by an automated system three times per day. The nutrient solution was replaced twice per month, while the water lost due to evapotranspiration was replenished every 2 days.

The plants were exposed to light provided a system equipped with PHILIPS SON-T AGRO 400 W (Koninklijke Philips N.V., Amsterdam, The Netherlands) delivering a photon flux density of 200 μmol m^{-2} s^{-1}, with a photoperiod of 16 h d^{-1}. The temperature was maintained constantly at 23 °C (±1 °C) and relative humidity was maintained at about 60%.

2.2. Lemna Minor Growth Conditions and Preparation of the Aqueous Extract

Duckweed was harvested from a freshwater basin near Perugia (Italy). Initially, the plants underwent sterilization with a 0.5% sodium hypochlorite solution for 2 min. Following the sterilization, the plants were copiously rinsed twice with distilled water. Subsequently, duckweed plants were transferred to polyethylene trays (35 × 28 × 14 cm) and cultivated following a previously published protocol [26]. The culture media was replaced every two weeks.

Ten grams of duckweed were collected, washed, and dried at 40 °C until a constant weight was achieved. After that, 1 g of dried plant material was ground with a mortar and pestle and mixed with 100 mL water (pH 7). The resulting suspension was maintained in an orbital shaker (100 rpm) for 24 h. After this time, the extract was filtered under vacuum using a Buchner filter, and the liquid phase was brought to a volume of 100 mL, resulting in a 1.00% concentration of duckweed extract.

2.3. Salt Stress and Treatments with Duckweed Extract

Following the 60-day adaptation period to hydroponic conditions, 30 plants were exposed to salt stress by introducing 150 mM NaCl into the solution (Stress), whereas 15 olive plants continued to grow in the same nutrient solution but without NaCl (Control). The NaCl concentration was chosen because for the cultivar Arbequina can be considered a sublethal dosage. Furthermore, at the dosage of 150 mM NaCl for the cultivar Arbequina using a commercial biostimulant, good results were obtained in terms of enhanced tolerance [17]. Among the thirty stressed plants, fifteen were treated through foliar application twice (at 7 and 14 days after the beginning of salt stress) with 2.0 mL per plant of the duckweed extract at a concentration of 1% in volume (Stress + Bio). The dosage of the duckweed used in a previous study [25] on olive plants under non-stress conditions was effective in improving leaf gas exchange, chlorophyll content, plant biomass (leaf fresh and dry weight), and uptake of nitrogen (N), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), and zinc (Zn).

2.4. Olive Leaf Gas Exchanges and Plant Growth

Leaf net photosynthesis (Pn), leaf transpiration rate €, stomatal conductance (gs), and sub-stomatal CO_2 concentration (Ci) were assessed for each treatment at 7, 15, and 30 days

after the treatment with the duckweed aqueous extract (DAT). Leaf gas exchange rates were measured utilizing a portable IRGA (ADC-LCA-3, Analytical Development, Hoddesdon, UK) coupled with a Parkinson-type assimilation chamber. Leaves were enclosed within the chamber and exposed to the light of the hydroponic system. The airflow through the chamber was maintained at a rate of 5 cm^3 s^{-1}. During the gas exchange measurements, the external CO_2 concentration was approximately 375 cm^3 m^{-3}, and the temperature of the air inside the leaf chamber was around 1 °C higher than the temperature in the hydroponic room.

At 45 DAT (end of the experiment), six plants from each treatment were selected. The number of leaves, lateral shoots, and total lateral shoot lengths were assessed. Additionally, destructive measurements were carried out on the selected plants. In particular, the roots, shoots, stems, and leaves of each plant were weighed fresh (FW). Finally, root analysis was performed on root-scanned images using RhizoVision Explorer v2.0.3.0 to investigate root length, number of root tips, diameter, surface area, and volume [27].

2.5. Statistical Analysis

The experimental design was organized according to a randomized block design, with 3 treatments (Control, Stress, and Stress + Bio), 3 replicates, and 15 plants for each treatment. Statistical analysis was performed by analysis of variance (one-way ANOVA) and significant differences were determined according to the Tukey HSD test ($p \leq 0.05$). The statistical environment R-4.3.2 was used to perform the analysis [28].

3. Results

3.1. Leaf Net Photosynthesis (Pn), Leaf Transpiration Rate (E), Stomatal Conductance (gs), and Sub-Stomatal CO_2 Concentration (Ci)

At 7 DAT, Pn reductions were observed in Stress and Stress + Bio plants (Figure 1). However, at 15 and 30 DAT, the Pn values recorded for the Stress + Bio plants increased, reaching those shown by the Control plants, while the values of the Stress samples remained significantly lower. Furthermore, severe decreases in E and gs were recorded in plants stressed with NaCl alone throughout the experimental time. On the contrary, the plants biostimulated with the duckweed extract showed significant increases in the above parameters until reaching values similar to those observed for the Control plants at 30 DAT. Finally, at 7 DAT, Ci was significantly higher in the Stress and Stress + Bio plants compared to the Control samples. Despite this, at 15 and 30 DAT, the Ci values of the Control and Stress + Bio samples were lower than those of the Stress plants. Therefore, the values recorded for the plants treated with the biostimulant tended to reach the values observed for the Control plants, as further observed for the other parameters.

3.2. Plant Growth and Biomass Development

Salt stress caused a severe decline in plant growth (Table 1). Indeed, at 45 DAT, the samples treated with salt alone showed significant decreases in the number of leaves, lateral shoots, and length. On the contrary, the treatment of plants grown in salt stress conditions with the duckweed extract counteracted the reduction in plant growth caused by NaCl, and the values recorded were not statically different from those found for the Control plants (Table 1). In particular, in the plants treated with the extract, the number of lateral shoots and their length were not significantly different from the Control plants.

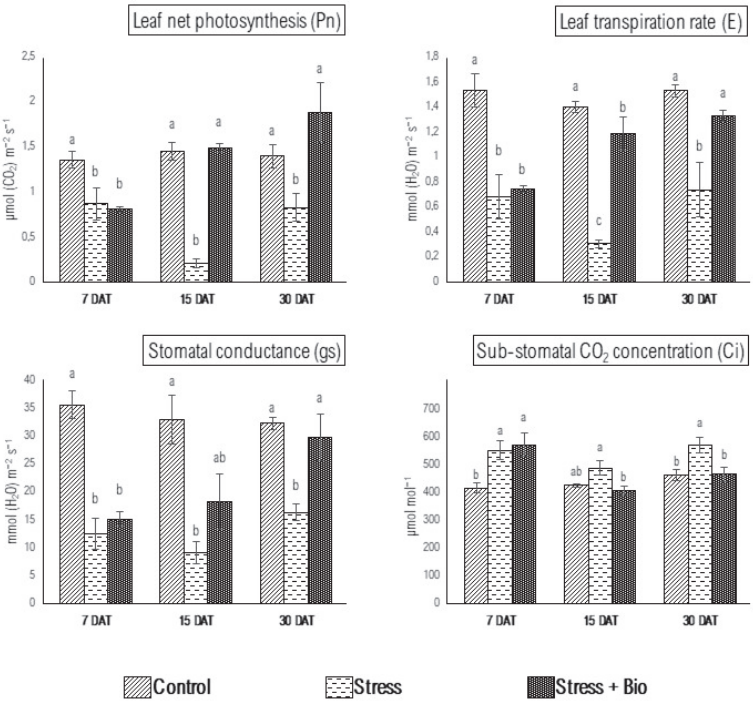

Figure 1. Leaf net photosynthesis (Pn) (µmol (CO_2) m^{-2} s^{-1}), stomatal conductance (gs) (mmol (H_2O) m^{-2} s^{-1}), sub-stomatal CO_2 concentration (Ci) (µmol mol^{-1}) and leaf transpiration rate (E) (mmol (H_2O) m^{-2} s^{-1}) measured at 7, 15 and 30 days after duckweed extract treatment (DAT). For each DAT and each parameter, means with different letters are significantly different ($p = 0.05$), as indicated by one-way ANOVA followed by Tukey's HSD multiple comparison test. The bars report SE (standard error).

Table 1. Number of leaves, lateral shoots, and total length of lateral shoots.

Treatment	Number of Leaves (n)	Number of Lateral Shoots (n)	Lateral Shoots Length (cm)
Control	31 ± 1.4 [a]	2.0 ± 0.5 [a]	2.80 ± 0.72 [a]
Stress	15 ± 1.8 [b]	1.0 ± 0.2 [b]	0.87 ± 0.33 [b]
Stress + Bio	26 ± 1.0 [a]	3.0 ± 0.3 [a]	3.91 ± 0.72 [a]

In each column, mean values ± SE followed by different letters are significantly different ($p < 0.05$) as indicated by one-way ANOVA followed by Tukey HSD test.

Moreover, as a consequence of the reduced growth (Table 1), the fresh weight of the plant components of the stressed plants, treated with NaCl alone, was lower than that observed for the Control samples (Table 2). In contrast, the plants treated with the duckweed extract and subjected to salt stress showed biomass values that did not statistically differ from those of the control samples (Table 2), marking the same trend as for the parameters in Table 1.

The root biomass analysis and morphology showed that salt stress caused a severe decrease in the number of root tips, length, diameter, surface, and volume. The duckweed extract prevented these detrimental effects. In fact, duckweed extract induced in samples under salt stress higher values for the number of root tips, root length, diameter, surface, and volume (Table 3). In addition, the Stress + Bio plants showed values similar to the Control samples for the abovementioned parameters.

Table 2. Fresh weight (FW) of leaves, roots, and stems and shoots.

Treatment	Leaf FW (g)	Stem and Lateral Shoots FW (g)	Root FW (g)
Control	3.81 ± 0.17 [a]	1.33 ± 0.08 [a]	3.23 ± 0.35 [a]
Stress	1.49 ± 0.20 [b]	0.83 ± 0.04 [b]	2.29 ± 0.16 [b]
Stress + Bio	3.36 ± 0.21 [a]	1.21 ± 0.06 [a]	4.54 ± 0.38 [a]

In each column, mean values ± SE followed by different letters are significantly different ($p < 0.05$) as indicated by one-way ANOVA followed by Tukey HSD test.

Table 3. Root analyses.

Treatment	Root Tips (n)	Total Length (cm)	Diameter (mm)	Root Area (mm^2)	Volume (mm^3)
Control	434 ± 19 [b]	138.1 ± 12.5 [a]	0.68 ± 0.02 [a]	3026 ± 342 [a]	850 ± 65 [b]
Stress	348 ± 10 [c]	88.1 ± 6.3 [b]	0.57 ± 0.03 [b]	1612 ± 151 [b]	469 ± 210 [c]
Stress + Bio	503 ± 32 [a]	146.3 ± 11.9 [a]	0.72 ± 0.04 [a]	3225 ± 328 [a]	1294 ± 108 [a]

In each column, mean values ± SE followed by different letters are significantly different ($p < 0.05$) as indicated by one-way ANOVA followed by Tukey HSD test.

4. Discussion

Plant biostimulants are recognized as an innovative agronomic tool due to their proven effectiveness in enhancing crop performance [29]. Numerous studies [30,31] have extensively documented their positive impacts on plant biomass production, nutrient utilization efficiency, flowering, and overall growth. Furthermore, the use of these substances is a common practice for boosting crop resistance against various detrimental biotic and abiotic environmental stresses [32,33]. In this context, a growing body of research aims to identify new bioactive substances and plant extracts to promote positive effects in crops cultivated under normal conditions or subjected to abiotic stressors. For these reasons, the present work investigated the capacity of an aqueous extract obtained from an aquatic species, the duckweed (*L. minor* L.), for its richness in bioactive compounds [23–25,34,35], to increase the salt tolerance in *Olea europaea* L. cv. Arbequina.

It is well known that salt stress, among other things, can affect plant photosynthesis, and the extent of this impairment is closely related to the duration and severity of the stress and salt concentration [1,36–38]. However, it is necessary to point out some variability in the ability of crops to tolerate or resist salt stress that also depends directly on the cultivar.

Our experiments showed that salt stress determined decreases in photosynthetic activity which were accompanied by increases in sub-stomatal CO_2 concentration. Such an effect can be considered the cause of the stomatal closure with a consequent decrease in stomatal conductance, as already observed by other authors [39]. The increase in sub-stomatal CO_2 concentration indicates that non-stomatal effects mainly caused a reduction in photosynthesis. Such CO_2 accumulation could result from damage to photosystems due to salt stress, as documented for other abiotic stresses, which can no longer sustain the light phase with a consequent decrease in the dark phase, which uses carbon dioxide to synthesize carbohydrates [40,41]. In general, the most significant inhibition in the photosynthesis rate occurs in olive cultivars characterized by inherently high photosynthesis and stomatal conductance [36]. For instance, six one-year-old olive cultivars subjected to salt stress (200 mM NaCl) for five months exhibited a notable reduction in the carbon assimilation rate by the end of the experiment [37]. In general, a decline in stomatal conductance can precede alterations in photosynthesis in salt-stressed olive plants. A marked reduction in photosynthesis in olive plants treated with some different NaCl concentrations was also observed by other authors [42]. Despite this, they reported a complete recovery of photosynthesis in plants subjected to 50 and 100 mM NaCl concentrations, especially in the salt-tolerant cultivar 'Frantoio,' accompanied by increased stomatal conductance and transpiration. These results suggest that during the initial stages of salinity stress, the plant experiences stomatal limitations that affect the entire photosynthetic process. More recently,

Loreto et al. [36] demonstrated that the primary limitations of photosynthesis in moderately salt-stressed olive plants result from the low chloroplast CO_2 concentration due to both low stomatal and mesophyll conductances.

A reduction in photosynthetic activity in NaCl-stressed crops generally hampers plant growth, as this effect adversely affects the plant's capacity to acquire nutrients, resulting in inadequate plant development, among other things [37,43,44]. In addition, some scientific evidence has demonstrated that plants may decrease biomass production to counteract the impact of certain stresses, and this to reprogram metabolism and activate defensive mechanisms [45]. In particular, in order to cope with oxidative stress due to salinity, some species increase the content of molecules and enzymes with antioxidant activity, regulate ion uptake and distribution, and maintain osmotic balance [45]. Our experiments corroborate the strong impact of salt stress on olive growth, and stressed plants displayed a lower fresh weight than the Control samples due to the reduced development of leaves, shoots, stems, and roots. However, the application of the duckweed extract reverted the detrimental effects on olive samples due to NaCl treatment, especially contrasting the impairments on photosynthesis and plant growth. In particular, our experiments indicated that the extract stimulated a significant recovery of Pn, associated with an increase in gs and a decrease in Ci. This suggests that the bioactives in the extract positively affected photosynthesis and stomatal aperture, although the plants were raised in salinity. Indeed, duckweed extract has been found to exhibit a range of bioactive metabolites and the presence of regulatory and signal molecules that can trigger changes in plant metabolic processes [23]. Our results align with previous studies that have highlighted the benefits of biostimulants in reducing the impact of salt stress. In particular, similar effects on NaCl-stressed olive plants were observed in response to Megafol treatment, a commercial plant biostimulant. In fact, olive plants subjected to salt treatment without Megafol exhibited substantial reductions in biomass production, leaf gas exchange, and relative water content (RWC) [17]. Differently, when the plants were subjected to the biostimulant treatment, they significantly improved despite salt stress.

In addition, a general negative effect of salinity on the number of leaves, lateral shoots, total length of lateral shoots, and main root tissue characteristics was observed, in line with what has already been found for photosynthetic activity. In contrast, olive samples treated with the duckweed extract showed a complete recovery at the shoot and root level for all the characters studied. Regni et al. [25] have already demonstrated the efficacy of an aqueous extract derived from duckweed in enhancing the vegetative activity of olive plants under non-stressing conditions. The phytochemical profile of duckweed showed the presence of compounds with biostimulatory activity, particularly a high content of auxins and related compounds [25] that can explain the benefits we found on photosynthesis, shoot biomass, and root development. In fact, the addition of auxins to stressed plants (e.g., indoleacetic acid) was shown to promote the photosynthetic activity of *Zizania latifolia* [46], thus resulting in increased biomass production. In addition, auxins can help increase stomatal conductance and transpiration, and these results align with what we ascertained in our experiments [46].

Finally, it is to be remarked that the *Lemnaceae* species has a very high content of metabolites with antioxidant properties [47]. For instance, the duckweed extract employed in this study showed a significant content of phenolic compounds [25]. Therefore, the treatments of the olive samples with this extract resulted in the exogenous application of phenolics, which promoted beneficial responses, improving the plant tolerance to salinity. In fact, some phenolic compounds can counteract the impact of salt stress by stimulating photosynthesis and modulating the functional traits of the crops [25]. In this context, the duckweed extract showed abundant amounts of hesperidin [25], which may benefit photosynthesis and shoot and root biomass production in plants grown in salinity [48]. Finally, it is worth mentioning that this extract also has a high content of proline, an amino acid that can effectively improve the tolerance of olive plants to salinity by stimulating the activity of some antioxidant enzymes and biomass production and improving the water

status [49]. In light of the above, the results of our experiment can also be related to the possibility that the duckweed extract could have activated the antioxidant machinery [24], thus resulting in a protective effect against the abiotic stressor.

Finally, regarding the roots, the inductive effect exerted by the extract should be considered of pivotal importance since a functional root system, especially under stress conditions, allows the crop to carry out adequate plant nutrition. Therefore, these results indicate that duckweed extract modulated root development and architecture, thus improving the plant's adaptability to cope with the abiotic stress. In agreement with the above, it has indeed been demonstrated that substances with biostimulatory properties can also improve root biomass production or modify root architecture and organization, thus resulting in more efficient plant productivity and nutrition, water acquisition, and resistance to abiotic stresses [50–53]. In line with such an effect, in addition to the already mentioned high phytohormone content, a considerable presence of glucosinolates is present in duckweed extracts [26,27], and these substances may exert a positive effect on the development of the root portion of the plant.

5. Conclusions

In conclusion, this study demonstrated for the first time the potential of an extract obtained from an aquatic species, duckweed (*Lemna minor* L.), to counteract the detrimental effect of salt stress in olive plants. Indeed, the duckweed extract improved photosynthetic activity and whole plant growth in olive plants exposed to NaCl stress, allowing them to maintain values of the studied parameters similar to those of Control plants not subjected to salt stress. However, further investigations are needed to reach a deeper understanding of the stimulatory potential of the duckweed extract on olive and other crops in salt stress conditions, as well as test this extract against other environmental stressors. In light of the above, this research demonstrated that resources readily available in nature could be sources of bioactive or biostimulant substances effective in increasing the plant's capacity to face salinity, one of the main abiotic stresses, and their eco-friendly properties pave the way towards more sustainable ways to maintain high crops productivity.

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Vegetative and Reproductive Responses Induced by Organo-Mineral Fertilizers on Young Trees of Almond cv. Tuono Grown in a Medium-High Density Plantation

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Abstract: Field experiments were conducted in three successive seasons (2019–2021) to evaluate the effects of four commercial organo-mineral fertilizers with biostimulating action (Hendophyt®, Ergostim®, and Radicon®) on the vegetative and productive performance of young almond trees (*Prunus dulcis*, cv. Tuono) grown in a semiarid climate in Southern Italy. Foliar treatments were applied three times during each season (at the swollen bud, beginning of flowering, and fruit set-beginning of fruit growth stages). Both 2020 and 2021 were adversely affected by late frosts, resulting in damage to the flowers and small fruits without any positive effect of the biostimulant applications. In contrast, the results obtained during the normal climate year (2019) indicated that the growth of trunk diameter and shoot length of trees tended to increase in biostimulant treatments compared to those of the control. The number of buds and flowers per unit length of the branch revealed no significant differences among years and all compared treatments. However, in 2019, the fruit set percentage, number, and weight of kernels per tree were significantly higher in the biostimulant treatments compared to those of the control. To this regard, the use of biofertilizers is suitable for maintaining soil fertility and improving crop productivity This information holds significance for almond tree growers.

Keywords: almonds; foliar spray; humic and fulvic acids; carboxylic acids; polyglucosamine; flowering; yield

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1. Introduction

The almond tree (*Prunus dulcis* [Mill.] D.A. Webb) is cultivated in the Northern hemisphere between 30° and 44° latitude and in the Southern hemisphere between 20° and 44° latitude. This cultivation spans over 40 countries, covering a total area of 2,283,414 hectares and yielding a production of 3,993,998 tons of almonds in their shells [1]. Italy holds the seventh position among the world's largest almond producers, with 54,939 Ha cultivated and a total production of 77,677 tons in 2023 [2,3]. The majority of almond cultivation in Italy is concentrated in the Southern regions, particularly in Sicily (52,185 tons on 32,905 hectares) and Puglia (18,445 tons on 18,891 hectares) [3]. There is currently a growing interest in this cultivation even in emerging regions with climates and temperatures favorable to the development and fruiting of the almond tree [4,5].

Over the past 10 years, new almond cropping systems, inspired by the Californian model, have been emerging worldwide in irrigated areas. These systems, including medium-high density (MHD, about 300 to 1000 trees ha^{-1}) and super-high density (SHD, resulting more than 2000 trees ha^{-1}) of trees, prioritize mechanization and sustainability to enhance efficiency and productivity [6,7]. In Italy, a significant portion of the new almond acreage is dedicated to the cultivation of both Lauranne® Avijor (constituting 48% of the total) and Guara-Tuono (comprising 39% of the total). The choice of Rootpac-20® as rootstock is crucial for controlling tree vigor, promoting early production, and improving

adaptation to specific soil conditions [7]. A new cultivation system requires the definition of efficient agronomic techniques, with fertilization as a crucial management tool to enhance both growth and quantitative–qualitative yield parameters. In this context, the goal of modern agriculture is to employ a sustainable fertilization strategy that complements chemical fertilizers. In this regard, the use of biofertilizers is suitable to improve nutrient use efficiency and ensure the stability of crop yields under both optimal and suboptimal conditions [8,9]. In this context, agricultural biostimulants (ABs), composed of organic and inorganic materials of various origins, many of which are still unknown, constitute an important category of agricultural inputs with multiple functions [10–12]. These products are utilized to support crop growth, enhance yield, and improve the final quality of produce [13–17]. Specifically, they aim to mitigate nutritional stresses arising from abiotic factors such as drought, soil salinity, and various climatic parameters to which crops may be exposed [18,19]. In almonds, buds are regarded as a crucial yield component [20], and their development is significantly influenced by environmental and management factors [21]. Furthermore, flowering is a fundamental phase in the plant's life cycle, as the overall yield is contingent upon both the number of flowers on a tree and the percentage of flowers that ultimately result in fruit formation [22]. For this reason, agronomic practices should strive to maintain the highest and consistent number of flowers throughout the growing season of the orchard. Regarding climatic conditions, the almond tree's early flowering (in February in Italy) compared to that of other fruit trees makes it susceptible to damage from frosts, occurring at temperatures as low as −2 °C during this period. Late frosts, in particular, constitute the primary limiting factor in the cultivation of this species in the Mediterranean basin [5,23,24]. Furthermore, during flowering, leaves are either absent or still too small to provide the necessary nutrients at the time of setting and in the immediate post-setting moments. As a result, plants can benefit from the foliar absorption of nutrients, such as the small organic molecules contained in biostimulants. Although the effect of foliar-applied biostimulant substances on plant growth, yield, and fruit quality has been studied in various fruit tree species [16], the availability of information on this effect on almonds is relatively limited. Existing studies refer to research conducted in pots [25] or in fields using traditional low-density systems [26–30]. Notably, no research has been published on almond trees in the medium-high density (MHD; about 300 to 1000 trees/ha) and super-high density (SHD; resulting in more than 2000 trees/ha) systems. The results of research on potted plants [25] indicate that foliar applications of two biostimulants derived from microbial fermentation and algae extraction, respectively, demonstrate a substantial positive effect on the total leaf shoot area. There was also a significant increase in shoot length and biomass. Regarding the nutritional content of almond fruit, various types of plant biostimulants, in general, led to elevated levels of important bioactive compounds, particularly concerning γ-tocopherol and β-tocopherol [27]. The use of biostimulants under drought conditions improves the almond yield response of three varieties (Guara, Marta, and Lauranne), demonstrating higher leaf water potential values [28]. In another study [29], the results indicate that certain treatments involving foliar fertilization with urea and humic acid at different concentrations lead to a significant increase in components of vegetative growth, including the length and diameter of the stem, leaf area, fresh weight, and dry weight. In an experimental test conducted in Egypt [30], the foliar application of humic acid and milagro enhanced the vegetative growth of the seedlings. This improvement was evident in the length of the stem, diameter, number of branches and leaves, leaf area, fresh and dry leaf weight, and specific weight of dry leaf. Additionally, there was an increase in chlorophyll and leaf mineral content compared to those of untreated young trees.

Taking into account all the considerations mentioned above, the objective of this study was to assess, in the semiarid environment of the Apulia region in Italy, the effects of foliar applications of four commercial organo-mineral fertilizers (Hendophyt® PS, Iko-Hydro, Rutigliano BA, Italy; Ergostim® XL, Isagro SpA, Sumitolo Chemical, Italy; and Radicon®, Fertek, Cavizzano NA, Italy) on the vegetative growth (shoot and trunk growth), bud production, flowering, fruit set, and yield of almond cv. Tuono in the SHD system.

2. Materials and Methods

2.1. Trials Site and Bioastimulant Treatments

A three-year study was conducted in an irrigated almond orchard using the medium-high density (MHD) system from 2019 to 2021, corresponding to the 3rd, 4th, and 5th years after planting (YAP). The commercial orchard is situated in the Foggia countryside, located in the Apulia region of southern Italy at coordinates 41°27′08″ N, 15°31′56″ E and an elevation of 54 m above sea level. The orchard consists of Tuono variety almond trees (synonymous with Guara) [6,31], grafted on a hybrid Rootpak 20® of *Prunus besseyi* × *Prunus cerasifera* L-H. Bailey and Ehrh. The trees are spaced 4 × 1.5 m^2 apart (1666 trees ha^{-1}) and are grown in a vase shape with three production axes, oriented in rows from North to South. Tuono is a native variety from Apulia and is currently cultivated in the primary Italian almond cropping areas and other European regions due to its self-fertility and favorable fruit characteristics [32].

The soil texture is a silty-clay vertisol of alluvial origin (1.20 m depth) (Typic Chromoxerert, fine, thermic, according to the Soil Taxonomy-USDA-NRCS 1999 [33]). The soil composition includes sand (36.8%), silt (32.7%), and clay (30.5%), with various essential parameters: total N (Kjeldahl) = 1.5‰, assimilable P_2O_5 (Olsen) = 56 mg kg^{-1}, exchangeable K_2O (Schollemberger) = 1390 mg kg^{-1}, exchangeable Ca = 3128 mg kg^{-1}, electrical conductivity (ECe) = 0.68 dS cm^{-1}, pH (soil: water 1:2.5) = 8.0, and organic matter = 1.6%. In this study, four water-soluble commercial organo-mineral fertilizers with biostimulant action—Hendophyt® PS (Iko-Hydro), Ergostim® XL (Isagro), and Radicon® (Fertek)—were applied through foliar spraying and compared to a control (sprayed with water). Table 1 presents the composition, including the main active compounds and the dosage of different products used in the trials. Specifically, these formulations include polysaccharide biopolymers (polyglucosamine), carboxylic acids (N-acetylthiazolidin-4-carboxylic acid—AATC and triazolidinecarboxylic acid—ATC), as well as humic and fulvic acids. These substances contribute to the biostimulating action in plants [34]. The products were applied three times during each growing season, specifically at the swollen bud, beginning of flowering, and fruit set–beginning of fruit growth stages. The application dates were 5 March, 12 April, and 8 May in 2019; 3 March, 10 April, and 4 May in 2020; and 26 February, 16 March, and 20 April in 2021. All treatments were administered between 10:00 and 11:30 am, with a total volume of 550 L ha^{-1}. Each tree was sprayed using a pulled sprayer under favorable weather forecasts, ensuring no rainfall was expected in the following 24 h. The experimental setup followed a completely randomized block design, with three replications per treatment and five trees per plot. The trial was inserted in an orchard with a surface area of approximately 2 hectares. One buffer row was located between replicates and blocks, and two or more buffer rows were around the perimeter of the experimental field. Each replicate had 15 plants, and three centrally located plants per plot were used to collect vegetative and reproductive parameters.

Table 1. Formulations and doses of foliar application of agricultural biostimulant (AB) commercial products used in the experiment.

ABsTreatment
HENDOPHYT PS (Iko-Hydro): a fully water-soluble powder comprising biopolymers of polysaccharides (polyglucosamine), 60%; carbon, 35%; organic nitrogen, 4%; boron, 0.25%; applied at a dose of 150 g 100 L^{-1} of water.
ERGOSTIM XL (Isagro): a concentrated water-soluble liquid N-acetiltiazolidin-4-carboxylic acid (AATC), 2.5%; and triazolidine-carboxylic acid (ATC) 2%; applied at a dose of 200 mL 100 L^{-1} of water.
RADICON (Fertek): a suspension–solution of humic and fulvic acids, obtained from worm compost (night crawled). Dry composition: total organic matter, 60%; extractable organic substance of organic matter, 4%; humified organic substance extractable organic matter, 90%; organic substance of extractable organic nitrogen, 1.0%; C/N ratio = 4; applied at a dose of 500 g 100 L^{-1} of water.

To prevent contamination between treatments, a buffer row was positioned between replicates and blocks, and two or more buffer rows were established around the perimeter of the experimental field. In each replicate, three centrally located plants per plot were selected for the collection of vegetative and reproductive parameters. Trees were chosen to be healthy and as uniform as possible. The same set of trees was consistently selected for the experiment across the three growing seasons under consideration.

2.2. The Climate

The research site was situated in a typical semi-arid zone, characterized by a Mediterranean climate classified as an accentuated thermomediterranean climate [35]. The temperatures in this region may fall below 0 °C in the winter and exceed 40 °C in the summer. Rainfall is unevenly distributed throughout the year, with the majority concentrated in the winter months, resulting in a long-term annual average of 559 mm [36]. Daily climatic parameters, including maximum and minimum temperatures, air humidity, wind speed, and total precipitation, during the three growing seasons were recorded by the meteorological station nearest to the experimental area, supplied by Syngenta [37]. The weather conditions varied significantly among the three years, particularly in terms of air temperature and rainfall (Table 2). A notable difference in air temperatures was observed during the flowering and fruit set period in the frost-heavy seasons of 2020 and 2021. During these seasons, trees and flowering plants were affected by actual ice stalactites (Figure 1), in contrast to the more favorable temperature trend for almond growth observed in 2019.

Figure 1. Ice stalactites on almond trees.

Specifically, in 2020, frosts were recorded on 24 and 25 March (−0.24 and −1.43 °C, respectively), occurring after the first biostimulant treatments. In 2021, the frosts occurred very late, on 8, 9, and 10 April (−0.6, −2.6, and −0.9 °C, respectively), after both the first and the second biostimulant treatments had taken place (Table 3). Consequently, the average maximum and minimum temperatures in March 2020 were colder, with averages of 15.6 °C and 2.1 °C, respectively, compared to those in 2019, which had averages of 18.6 °C and 8.2 °C, respectively. Similarly, in April 2021, the average maximum and minimum temperatures (15.4 °C and 3.4 °C, respectively) were lower than those recorded in April 2019. Furthermore, the annual precipitation was higher in 2021, reaching 627.8 mm, compared to 527.1 mm in 2020 and 461.7 mm in 2019.

The orchard was managed using common practices prevalent in the area. Drip lines with 2 L h^{-1} drippers spaced 40 cm apart were positioned 50 cm from the ground along the tree rows. Controlled irrigation was implemented, with a mean seasonal irrigation volume of 3500 m^3 ha^{-1}. Fertilization was conducted annually through the fertigation system, involving 100 kg ha^{-1} of N, 60 kg ha^{-1} of P, and 80 kg ha^{-1} of K. Protection against fungal diseases primarily occurred in the autumn–winter period using copper-based products compliant with phytosanitary regulations outlined in the Integrated Production Regulations of the Puglia Region [38].

Table 2. Monthly mean maximum and minimum temperatures (T_{max}, T_{min}) and relative air humidity (RH_{max} and RH_{min}), wind speed (W_s), and total precipitation (P) in 2019, 2020, and 2021.

Month	T_{max} (°C)	T_{min} (°C)	RH_{max} (%)	RH_{min} (%)	W_s (m s^{-1})	P (mm)
2019						
Jan	10.6	1.6	99.2	63.3	3.4	61.0
Feb	14.6	2.6	95.1	51.2	4.3	21.2
Mar	18.6.	4.5	98.8	44.2	4.4	32.0
April	20.6	8.2	94.4	51.0	3.7	40.3
May	21.3	10.2	95.3	56.3	4.0	86.7
June	33.2	17.5	85.9	35.1	3.7	9.2
July	33.7	19.5	84.0	33.9	3.7	30.0
Aug	34.8	20.3	79.9	33.9	3.6	5.7
Sept	29.5	16.8	88.7	42.6	3.6	3.8
Oct	25.5	11.5	93.2	43.9	2.6	29.2
Nov	19.3	9.4	98.5	62.2	5.2	112.6
Dec	14.7	5.0	99.0	65.2	6.5	30.0
Mean	23.4	10.6	92.7	48.6	4.1	
Total						461.7
2020						
Jan	10.5	1.6	98.3	55.1	4.8	3.6
Feb	14.6	2.9	94.8	42.6	5.1	51.0
Mar	15.6.	2.1	96.4	60.8	3.3	83.0
April	18.8	6.1	94.1	53.2	3.4	48.9
May	27.5	14.7	90.8	43.1	3.8	25.8
June	28.8	17.7	80.5	48.3	4.0	19.7
July	31.0	21.2	79.7	40.6	3.9	20.4
Aug	31.5	21.8	83.7	44.3	3.9	40.0
Sept	22.2	17.4	72.8	58.4	4.0	38.5
Oct	25.5	9.7	97.1	47.6	3.9	44.6
Nov	19.3	7.7	99.5	72.8	4.2	68.6
Dec	14.7	5.2	99.6	71.9	4.3	83.0
Mean	21.7	10.9	90.6	53.2	4.1	
Total						527.1
2021						
Jan	12.2	2.4	99.5	63.3	5.8	58.2
Feb	15.5	3.4	99.6	56.0	5.1	35.2
Mar	15.4	3.4	98.9	52.3	4.7	57.8
April	19.9	4.7	99.5	44.7	4.3	40.4
May	26.5	10.8	95.7	30.3.	3.5	26.0
June	33.2	15.9	85.1	24.7	3.3	8.6
July	35.4	19.3	83.8	26.1	3.7	100.8
Aug	34.9	19.4	92.3	28.3	3.8	29.2
Sept	29.5	15.4	94.8	35.6	3.5	19.4
Oct	21.2	10.9	98.5	54.9	3.5	70.2
Nov	17.2	10.8	99.6	80.0	3.1	135.4
Dec	13.7	4.8	99.0	64.9	4.7	46.6
Mean	22.9	10.1	95.5	48.3	4.1	
Total						627.8

Table 3. Daily mean maximum and minimum temperatures (T_{max}, T_{min}), relative air humidity (RH_{max} and RH_{min}), wind speed (W_s), and total precipitation (P) for the frosty days: 24 and 25 March, 2020 and 8, 9, and 10 April, 2021.

Date	T_{max} (°C)	T_{min} (°C)	RH_{max} (%)	RH_{min} (%)	W_s (m s^{-1})	P (mm)
			2020			
24 March	5.8	−0.3	99.5	67.0	5.0	10.8
25 March	6.5	−1.4	99.6	81.2	1.1	14.4
Mean	6.1	−0.8	99.5	74.1	3.05	
Total						24.4
			2021			
8 April	13.9	−0.6	99.4	23.2	1.7	1.0
9 April	18.1	−2.6	99.3	23.4	2.7	0
10 April	21.1	−0.9	99.4	15.9	3.7	0
Mean	17.7	−1.4	99.4	20.8	2.7	
Total						1.0

2.3. Plant Measurements

2.3.1. Vegetative Growth

The shoot length (SL) and trunk diameter (TD) were determined at the beginning (in February) and the end (in September) of each season on three central plants in each treated plot. SL was determined on two well-lit one-year-old shoots (subsamples) randomly selected from opposite sides (east and west) of the outer canopy of each plant. The selected shoots were marked and measured using a tape, with the measurements expressed in centimeters. TD was measured at a marked point 50 cm above the ground level using a Vernier digital caliper, and the measurements were expressed in millimeters. Annual shoot growth (ASG) and annual trunk growth (ATG) were calculated based on the difference between the measurements taken in February and those in September for each year.

2.3.2. Bud, Flower, and Fruit Counting

Throughout each year, on the previously mentioned three central trees, four branches per tree were randomly selected for measurements, including 1-year-old shoots and spurs. All selected branches were chosen as homogeneously as possible, originating from opposite sides of the canopy and being of the same order of branching, with an approximate length of 1 m and positioned ≈1.7 m above the ground. Approximately 150–200 buds (both flower and vegetative) were counted and recorded at the pre-blossom phase on each of these selected branches. Thus, 600–800 buds were found on each tree. Subsequently, the length of all the branches was measured, and the count of all buds was conducted on each of the four branches [39]. Measurements were taken when flower buds were just before bloom, at phenological stage B [40] (on 1 March 2019, 24 February 2020, and 20 February 2021) and when the flowers were completely open at stage F [40] (on 11 March 2019, 4 March 2020, and 10 March 2021). The parameters considered for analysis were bud density (buds cm^{-1}) and flower density (flowers cm^{-1}). Finally, the final fruit set, expressed as a percentage of fruit per total open flowers, was evaluated at a later date (on 10 July 2019, 17 July 2020, and 15 July 2021).

2.4. Harvesting, Fruit Collection and Yield

In each year, almond fruits for each treatment were hand-harvested at the commercial maturity stage (on 20 September 2019, 24 September 2020, and 30 September 2021), and the number and weight of fresh almond fruits per tree were measured. Samples of 2 kg of almonds with hulls were taken from each replicate, stored in plastic bags, and transported to the laboratory. Each fruit in the samples was separated from the hull, and the nuts were left to dry on the ground in the sun for 5 days, bringing the humidity to about 10% of the weight. The results were expressed as hull per fruit (% of the total fresh weight), kernel dry

yield (in % of kernel per nut), and double seeds (%). Furthermore, 10 fruits were randomly collected from each replication and subjected to the following morphological analyses: weight, length, width, thickness of the nuts, and kernels.

2.5. Statistical Analysis

The results were assessed using one-way ANOVA with JMP® software version 8 (SAS Institute Inc., Cary, NC, USA), and average values were compared using Tukey's test. Standard deviations (SD) were calculated using Excel from the Office 2007® suite (Microsoft Corporation, Redmond, WA, USA). Percentage values were transformed to arcsine before conducting the analysis of variance.

3. Results

3.1. Trunk and Shoot Development

Annual trunk growth (Figure 2) exhibited no significant differences among treatments over the years. However, overall, it tended to increase in the biostimulant treatments (average 20.5 mm) compared to that of the control (18.9 mm). Additionally, there was a decreasing trend from the first to the third year, with average values ranging from 22.4 to 20.8 and 17.0 mm, respectively.

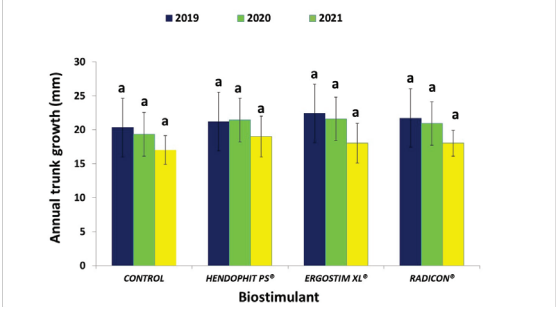

Figure 2. Annual trunk growth in different biostimulant and control treatments. The data are average values ± SD in three subsequent years (2019–2021). Similar letters per year and treatment indicate no significant differences according to Tukey's test ($p < 0.05$).

Additionally, shoot development (Figure 3) exhibited no significant differences among treatments and the control. However, the average shoot length each year tended to be higher under the biostimulant treatments (59.4 cm in 2019, 24.2 cm in 2020, and 23.3 cm in 2021) compared to that of the control (50.1 cm in 2019, 11.5 cm in 2020, and 15.3 cm in 2021).

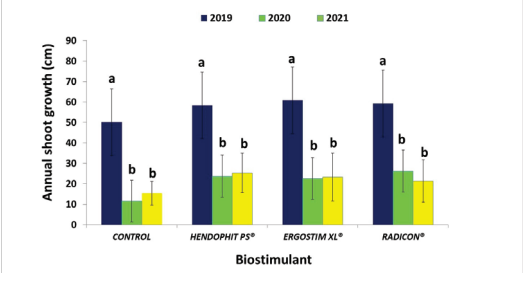

Figure 3. Annual shoot growth in different biostimulant and control treatments. The data are average values ± SD in three subsequent years (2019–2021). Different letters among years indicate significant differences according to Tukey's test ($p < 0.05$).

A significantly higher shoot length was observed in 2019 (ranging from 50.1 to 59.2 mm) compared to that of both 2020 and 2021 (ranging between 21.5 and 26.2 mm, respectively). The lower vegetative growth recorded in 2020 and 2021 could be attributed to the frosts that occurred in the respective months of March during these years, indicating the susceptibility of almond trees to climatic conditions.

3.2. Agronomical Characteristics: Bud, Flower, and Fruit Productivity

In Table 4, bud, flower, and fruit productivity is reported. Regarding total bud density, no statistical differences were found among treatments and years. However, it tended to be higher in both 2019 and 2020 (averaging 1.00 and 1.08 buds cm^{-1}, respectively) than in 2021 (averaging 0.89 buds cm^{-1}). Similarly, flower density showed no significant differences among years and treatments (averaging 0.50 flowers cm^{-1}), corresponding to flowering in 49% of the total bud population.

Table 4. Agronomical characteristics of almond trees in different biostimulant treatments and control in three subsequent years (2019–2021).

Parameter	Year	Treatment				
		Control	**Hendophit PS®**	**Ergostim XL®**	**Radicon®**	**Average**
Bud density (No cm^{-1})	2019	1.02 ± 0.30	0.99 ± 0.17	1.02 ± 0.17	0.97 ± 0.12	1.00 ± 0.19
	2020	1.10 ± 0.30	0.97 ± 0.28	1.08 ± 0.25	1.15 ± 0.16	1.08 ± 0.22
	2012	0.88 ± 0.18	0.89 ± 0.21	0.85 ± 0.26	0.94 ± 0.16	0.89 ± 0.20
Flower density (No cm^{-1})	2019	0.51 ± 0.10	0.43 ± 0.07	0.54 ± 0.10	0.44 ± 0.08	0.48 ± 0.08
	2020	0.45 ± 0.11	0.44 ± 0.18	0.50 ± 0.11	0.40 ± 0.09	0.45 ± 0.12
	2021	0.53 ± 0.19	0.56 ± 0.11	0.54 ± 0.15	0.65 ± 0.21	0.57 ± 0.20
Final fruit set incidence (%)	2019	21.5 ± 4.5 b	28.3 ± 1.3 a	28.4 ± 4.5 a	34.4 ± 5.4 a	28.4 ± 4.9 A
	2020	5.8 ± 7.8	2.4 ± 4.7	1.6 ± 5.2	2.8 ± 4.3	3.1 ± 5.5 B
	2021	9.8 ± 9.2	6.2 ± 5.1	6.9 ± 6.8	15.9 ± 9.9	9.7 ± 7.7 B
Fruit set per tree (No $tree^{-1}$)	2019	66.3 ± 8.5 b	85.7 ± 4.6 a	96.7 ± 5.3 a	81.6 ± 6.5 a	82.6 ± 6.2 A
	2020	55.3 ± 12.3	65.7 ± 11.4	55.0 ± 9.0	71.9 ± 8.2	65.0 ± 10.2 B
	2021	44.3 ± 8.0	48.5 ± 9.4	46.6 ± 10.0	46.9 ± 11.3	46.6 ± 9.7 B
Fresh kernel yield per tree (g)	2019	359.5 ± 74.5 b	460.2 ± 14.9 a	477.3 ± 40.0 a	405.7 ± 32.8 a	425.7 ± 56.3 A
	2020	298.2 ± 29.6	420.6 ± 57.9	333.6 ± 29.6 a	296.4 ± 65.1	337.2 ± 40.5 A
	2021	221.4 ± 16.1 c	242.0 ± 15.3 c	251.0 ± 18.4 c	235.2 ± 16.1 b	237.4 ± 16.5 A

The data are averages ± sd of different treatments in each year and averages ± SD across all seasons. Different lowercase letters on the lines indicate significant differences among biostimulant treatments, while lines followed by no letter are not significantly different (Tukey's test, $p < 0.05$). Different capital letters among year averages indicate significant differences at $p < 0.05$. The absence of letters indicates no significant differences among the years.

Furthermore, the observations in Table 4 itself indicate that only in 2019 the fruit set percentage was statistically higher in the biostimulant treatments than in the control. This parameter was the highest in the Radicon® treatment (34.4%), although it was not significantly different from that in both Hendophyt® and Ergostim XL® treatments (28.3 and 28.4%, respectively), and was significantly higher than that in the control (22.5%). A remarkably low percentage of fruit set was detected in both 2020 (ranging from 2.6 to 5.8%) and 2021 (ranging from 6.2 to 15.9%), with no discernible differences among the treatments. These results could be explained by the aforementioned adverse weather conditions that occurred in these last years.

The final number and weight of fruits per tree, parameters related to fruit set, indicated significantly higher average values in 2019 (82.5 No $tree^{-1}$ and 425.7 g, respectively) than in both 2020 (65.0 No $tree^{-1}$ and 337.2 g) and 2021 (46.6 No $tree^{-1}$ and 237.4 g). It should be noted that considering the data reported above, the reductions in fruit set percentage that occurred in 2020 and 2021 compared to that in 2019 were higher than the relative reductions detected in the same years in both the total number and weight of fruits per tree. This, of course, was due to the increase in plant canopy that certainly occurred over the years.

The positive effects of biostimulant treatments were noticed only in the 2019 season when the number and weight of fruit per plant were significantly higher (on average 88.0 No tree^{-1} and 447.3 g, respectively) compared to those of the control (66.3 No tree^{-1} and 359.5 g, respectively). On the contrary, no significant differences were found among treatments in both 2020 and 2021 when the late frosts occurred.

3.3. Yield-Related Variables

The fruit quality parameters reported in Table 5 showed no statistical differences both among the years and biostimulant treatments. Mean percentage values for hull per fruit, kernel per nut, and double seeds in the three years ranged from 39.6 to 52.3, from 28.1 to 31.4, and from 6.9 to 10.4%, respectively.

Table 5. Fruit quality parameters of almonds in different biostimulant treatments and control in three subsequent years (2019–2021).

Parameter	Year	Treatment				
		Control	**Hendophit PS®**	**Ergostim XL®**	**Radicon®**	**Average**
Hull per fruit (% of total fresh weight)	2019	52.3 ± 7.1	48.4 ± 8.9	44.9 ± 0.9	44.8 ± 3.4	47.6 ± 5.1
	2020	44.6 ± 9.4	43.7 ± 2.1	44.3 ± 7.6	39.6 ± 2.7	43.0 ± 5.4
	2021	44.1 ± 3.1	45.0 ± 4.1	43.9 ± 3.7	43.3 ± 5.0	44.1 ± 4.0
Shelling: Kernel per nut dry (%)	2019	28.1 ± 4.5	30.1 ± 4.9	31.4 ± 5.2	29.8 ± 6.1	29.8 ± 5.2
	2020	31.3 ± 0.9	30.3 ± 0.8	30.8 ± 0.8	30.2 ± 2.9	30.6 ± 1.3
	2021	30.2 ± 1.3	30.7 ± 0.9	29.9 ± 1.0	29.5 ± 1.4	30.1 ± 1.1
Double Seeds (%)	2019	6.4 ± 2.4	7.3 ± 3.5	10.4 ± 4.4	9.3 ± 2.5	8.3 ± 3.2
	2020	7.5 ± 1.3	8.1 ± 2.9	7.2 ± 2.4	6.9 ± 1.0	7.4 ± 1.9
	2021	6.9 ± 3.5	7.1 ± 2.4	6.9 ± 1.2	8.0 ± 3.5	7.2 ± 2.6

The data are averages ± sd of different treatments in each year and averages ± SD across all seasons. The absence of letters indicates no significant differences both among treatments and years.

3.4. The Nut and Kernel Morphological Traits

The morphological characteristics of the nuts, such as weight, length, width, and thickness reported in Table 6, showed no statistical differences between the biostimulant treatments and the control in each year, but they were higher in 2019 (on average 6.1 g, 42.7, 32.7, and 23.2 mm, respectively) than in both 2020 (on average 5.2 g, 34.9 mm, 27.1 mm, and 17.4 mm, respectively) and 2021 (5.5 g, 36.3 mm, 28.5 mm, and 18.2 mm, respectively).

Table 6. Morphological characteristics of almond nuts in different biostimulant treatments and control in three subsequent years (2019–2021).

Parameter	Year	Treatment				
		Control	**Hendophit PS®**	**Ergostim XL®**	**Radicon®**	**Average**
Nut dry weight (g nut^{-1})	2019	6.8 ± 0.8	5.7 ± 0.9	5.8 ± 0.9	6.1 ± 0.8	6.1 ± 0.8
	2020	5.1 ± 0.7	5.1 ± 1.0	5.4 ± 0.8	5.1 ± 0.9	5.2 ± 0.7
	2021	5.8 ± 0.6	5.4 ± 0.7	5.3 ± 0.8	5.6 ± 0.7	5.5 ± 0.7
Nut length (mm)	2019	41.6 ± 2.0	41.9 ± 2.0	44.3 ± 1.9	42.9 ± 2.1	42.7 ± 2.0 A
	2020	33.1 ± 2.3	35.5 ± 3.9	35.4 ± 2.8	35.6 ± 2.1	34.9 ± 2.8 B
	2021	34.0 ± 2.3	36.1 ± 2.7	37.2 ± 2.5	37.9 ± 2.3	36.3 ± 2.8 B
Nut width (mm)	2019	32.0 ± 1.4	32.0 ± 3.7	33.8 ± 1.8	32.9 ± 2.0	32.7 ± 2.2 A
	2020	27.1 ± 1.4	27.0 ± 2.2	27.6 ± 1.6	26.7 ± 2.3	27.1 ± 1.9 B
	2021	28.2 ± 2.3	28.9 ± 2.0	28.3 ± 2.2	28.5 ± 1.8	28.5 ± 2.1 B
Nut thickness (mm)	2019	22.5 ± 0.8	22.5 ± 5.4	24.1 ± 1.5	23.7 ± 0.9	23.2 ± 2.1 A
	2020	17.4 ± 0.5	17.3 ± 1.0	17.7 ± 0.9	17.1 ± 1.0	17.4 ± 0.8 B
	2021	18.0 ± 0.9	17.9 ± 0.8	18.4 ± 0.5	18.5 ± 0.8	18.2 ± 0.7 B

The data are averages ± sd of different treatments in each year and averages ± across all seasons. The absence of letters indicates no significant differences both among treatments and years. Different capital letters among year averages indicate significant differences (Tukey's test, $p < 0.05$). The absence of capital letters indicates no significant differences among years.

Likewise, the weight, length, width, and thickness of the kernels (Table 7) showed no statistical differences both among years and biostimulant treatments, with average values ranging from 1.5 to 1.7 g, from 24.3 to 26.6 mm, from 15.5 to 16.6 mm, and from 7.3 to 8.3 mm, respectively.

Table 7. Metric traits of almond kernel in different biostimulant treatments and control in three subsequent years (2019–2021).

Parameter	Year	Treatment				
		Control	**Hendophit PS®**	**Ergostim XL®**	**Radicon®**	**Average**
Kernel dry weight (g kernel^{-1})	2019	1.6 ± 0.3	1.6 ± 0.2	1.5 ± 0.2	1.6 ± 0.2	1.6 ± 0.2
	2020	1.7 ± 0.3	1.5 ± 0.2	1.6 ± 0.2	1.7 ± 0.2	1.7 ± 0.2
	2021	1.5 ± 0.2	1.6 ± 0.2	1.5 ± 0.2	1.6 ± 0.2	1.5 ± 0.2
Kernel length (mm)	2019	26.6 ± 2.4	25.4 ± 1.4	25.7 ± 1.3	26.0 ± 1.1	25.9 ± 1.5
	2020	26.3 ± 1.9	25.9 ± 1.0	26.0 ± 1.0	26.3 ± 1.6	26.1 ± 1.4
	2021	24.3 ± 2.3	24.3 ± 2.3	24.3 ± 2.3	26.3 ± 1.98	24.8 ± 2.2
Kernel width (mm)	2019	15.9 ± 1.5	15.8 ± 1.2	16.0 ± 1.0	16.0 ± 1.2	15.9 ± 1.2
	2020	16.6 ± 1.2	15.5 ± 1.4	16.4 ± 1.4	16.6 ± 1.1	16.3 ± 1.3
	2021	15.9 ± 1.1	16.1 ± 1.1	15.7 ± 1.2	16.3 ± 1.3	16.0 ± 1.2
Kernel thickness (mm)	2019	7.3 ± 1.5	7.9 ± 0.5	7.3 ± 0.7	7.6 ± 0.4	7.5 ± 0.8
	2020	8.1 ± 0.6	8.0 ± 0.7	8.1 ± 0.6	7.9 ± 0.8	8.0 ± 0.7
	2021	8.3 ± 0.6	8.0 ± 0.5	7.9 ± 0.7	7.7 ± 0.6	8.0 ± 0.6

The data are averages ± sd of different treatments in each year and averages ± sd across all seasons. The absence of letters indicates no significant differences both among treatments and years.

4. Discussion

This study aimed to evaluate the influence of biostimulant treatments on the vegetative growth and reproductive behavior of young almond trees. The products were applied three times during each growing season—at the swollen bud, beginning of flowering, and fruit set–beginning of fruit growth stages. The impact of the tested biostimulants on the vegetative system primarily focused on the growth of trunk diameter and shoots. Specifically, long shoot growth during the early years of orchard establishment is the main component of vegetative development in almonds [41]. Our results, indicating a slight positive effect of biostimulants on the increase in trunk diameter and shoot length, align with previous studies [25,30]. This increase in vegetative shoot growth can result in more buds that will support future production. Growers should expect the mainstay of vegetative growth to be the production of long vegetative shoots. Regarding the number of buds per unit of branch length, mostly detected before or during the application of the biostimulant products, no statistical differences were found among all treatments. Overall, the average total bud density in each year (ranging from 0.89 to 1.08 buds cm^{-1}) was close to the range (0.46 to 1.02 buds cm^{-1}) reported in other research [42]. However, in the last year of this study (2021), our data tended to be low, likely due to the impact of the spring frost the previous year (2020), which negatively affected tree performance and also the formation of buds, which occurred during the prior season [41]. This dynamic of both the vegetative growth of the shoots and of all the buds (vegetative and floral) are key components for the development of an economically sustainable and productive orchard. Even the density of the flowers (varied between 0.40 and 0.65 flowers cm^{-1}) did not highlight significant differences either between years or between biostimulant treatments and fell within the wide range (from 0.03 to 1.52) detected in different almond genotypes in previous research [43]. The percentage of fruit set in 2019 was significantly higher in the biostimulant treatments than in the control. This phenological stage is delicate for the tree, and the application of external energy sources plays a vital role in ensuring the quality of pollen and nectar in the flowers [44]. Among the three types of organo-mineral fertilizers used, the best result was observed for Radicon®, which contains humic

and fulvic acids, and similar positive effects have been detected in another study [45]. Furthermore, overall, our range of relative fruit set values (varying from 22.5% to 34.4%) is in agreement with previous reports on several almond cultivars (ranging between 15% and 40%) [5,22,46]. In both 2020 and 2021, significantly lower fruit set percentages than in the previous season were observed, with no significant differences among biostimulant treatments and the control. The decreases in fruit set percentage in these last years were undoubtedly due to the frosts that occurred during and after the flowering period (almond phenological states from B, "Swollen bud", to I, "Young fruit, Jacket stage", of the Felipe classification), as previously reported in paragraph 2.1. These results align with those of previous studies [23,47], which demonstrated that almond flowers and young fruits are extremely sensitive to frost, suffering damage at temperatures below 0 °C (−1 or −2 °C), depending on the exposure time. In these phenological states, a couple of hours at these temperatures can cause serious damage and even ruin the year's production [47]. To this regard, the foliar application of biostimulants did not produce any effect on crops subject to frost, due to the formation of tiny ice crystals outside and inside the plant cell, which are lethal for them. In general, the ability of crops to defend themselves from frost is determined by the cultivar's ability to escape freezing temperatures over time. However, a possible positive action of biostimulants to alleviate non-excessive thermal stress from cold in plants is to improve the absorption of nutrients, increasing their concentration within the plant tissues, making them more resistant to low temperatures [48].

In consonance with the fruit set incidence, in the 2019 season, the yield, in terms of the number and weight of fruit per tree, was significantly higher in the biostimulant treatments than in the control. On average, an increase in the number of fruits per tree and fruit yield per tree achieved with the application of biostimulants relative to the control was 24.7% and 19.7%, respectively. These results are in accordance with some previous research [49]. The smaller increase in weight per tree compared to the number of fruits in this season could be due to the slightly higher weight values of the fruits recorded in the control (Table 6). The fruit incidence characters, such as the hull, shelling, and double seeds percentage, showed no statistical differences both among the years and biostimulant treatments. Our hull percentage data (on average 44.9%) are consistent with data previously reported in the literature [50], as is the percentage of shelling (on average 30.1%), which fells within the 30–40% range reported in previous research [5,7,24,51–55]. On the contrary, our data regarding the percentages of double seeds (on average 7.6%) are lower than those obtained for the same Tuono cultivar (between 15% and 31%) by other authors [7,53,56,57]. Indeed, as for the incidence of each single part of the fruit, they are primarily determined by genotype but also by environmental factors [49,58–60]. Therefore, in this regard, our data showed distinctive and commercially interesting agronomic characteristics.

Based on the use of almond components, the following information is known: Almond hull is a by-product that can be used as supplemental livestock feed or, due to its beneficial properties (mainly caused by polyphenols and unsaturated fatty acids), in the food, cosmetic, and pharmaceutical industries [61]. The shelling percentage parameter is used to obtain a quantitative measure of shell density and is utilized commercially to calculate kernel yield [56]. Finally, a high presence of double-seeded nuts significantly reduces their commercial value, as having a flat or concave face is undesirable both for the industry (since they present difficulties for confectionery use) and for consumers (because they are less attractive than single-seeded nuts) [62]. Regarding the morphological traits of nuts, such as weight, length, width, and thickness, there were no statistical differences between biostimulant treatments and the control (on average 5.6 g, 38.0 mm, 29.4 mm, and 19.6 mm, respectively), but significantly higher values were observed in 2019 (on average 6.1 g, 42.7 mm, 32.7 mm, and 23.2 mm) compared to both 2020 and 2021 (on average of the two years 5.3 g, 35.6 mm, 27.8 mm, and 17.8 mm, respectively). Regarding the weight, length, width, and thickness of the kernels, there were no statistical differences both among years and biostimulant treatments, with average values of 1.5 g, 25.6 mm, 16.1 mm, and 7.8 mm, respectively. Overall, our data on the characteristics of the nuts and

kernel morphological traits were somewhat superior to those of the same Tuono cultivar reported in other research [7,53,56,63,64], in which for the nuts they varied between 3–4 g, 28–34 mm, 21–23 mm, and 15–20 mm, respectively, and for the kernel they varied between 1.2 and 1.4 g, 23.4 and 23.9 mm, 12.2 and 14.9 mm, and 6.3 and 7.2 mm, respectively. Socias i Compañy et al. [65] commented that the general trend in the industry is the preference for large kernels in order to facilitate and cheapen the processes of cracking and blanching. Nonetheless, for some special confectioneries, very small sizes are chosen, as well as those with definite shapes. For sugared almonds (peladillas or dragées) and for chocolate almonds, large kernels are selected, preferably round to reduce the layer of sugar or chocolate covering the kernel

5. Conclusions

In the three years of experimentation (2019, 2020, and 2021), only in the first year, characterized by a normal climate trend, did the biostimulant treatments show a slight positive effect on the growth of the trees and on the percentage of total fruit set. Furthermore, a significantly higher fruit load and weight per plant were observed. Therefore, the use of biostimulants proved to be crucial during the flowering of almond trees. On the contrary, the second and third experimental years were affected by late frosts, causing damage to the flowers and small fruits. This resulted in reduced growth of the trees, a lower percentage of fruit set, and diminished yield. Furthermore, during these years, no significant effect of the biostimulant treatments on tree crops was observed. Additionally, the study's findings highlight that the frequent occurrence of late frosts, likely influenced by climate change, poses a greater risk to almond production than anticipated. Therefore, further research on the use of extra and ultra-late cultivars is needed to address this challenge. Furthermore, characteristics such as fruit, nut, and kernel quality were not significantly affected by the foliar application of biostimulants, probably because they could have reached their maximum quality potential in this growing environment. However, considering the positive results in terms of yield mentioned above, the foliar application the biostimulants Hendophyt®, Ergostim®, and Radicon® could be recommended to enhance the performance of almond tree cv. Tuono under normal climatic conditions in arid and semi-arid areas, similar to those covered by this study, such as Southern Italy. Finally, further research is needed on different almond cultivars and application methods, as well the specific mechanisms of action of the biostimulant treatments.

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Physiological Response of *Miscanthus sinensis* (Anderss.) to Biostimulants

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Abstract: Soil salinity stress is a serious problem in plant cultivation. The effect of this stress is to disrupt the photosynthetic process, which can cause growth restrictions and a decrease in plant productivity. The use of biostimulants can be one of the stress mitigation strategies in plant cultivation. Biostimulants increase the tolerance of plants to abiotic stresses, thus mitigating their adverse effects. In the present study, based on a pot experiment, the effect of foliar application of biostimulants differentiated in terms of chemical composition (Bombardino (B1), Quantis® (B2), Biofol Plex (B3) and Megafol (B4)) on the physiological properties of Chinese silver grass (*Miscanthus sinensis* (Anderss.)) plants growing under salt stress conditions was determined. Salt stress was induced by soil application of NaCl at concentrations of 200 and 400 mM. The application of salt solutions was followed by spraying *Miscanthus* plants with biostimulants using a hand-held sprayer. Physiological investigations (chlorophyll content, chlorophyll fluorescence and gas exchange) have been carried out twice: on the 1st (Term I) and 7th (Term II) day after spraying with biostimulants. It was shown that salt stress causes a decrease in the values of most of the physiological indicators tested (except Ci). On both measurement dates, the application of biostimulants, especially B2, caused an improvement in the values of the physiological indices studied, both for plants growing under optimal conditions and under salt stress. Term II showed an upward trend in most of the analyzed parameters compared to Term I, indicating plant acclimatization to stress conditions. Conducted studies have shown that using biostimulants contributes to the alleviation of the effects of soil salinity stress. The implementation of these practices can contribute to the advancement of sustainable farming.

Keywords: Chinese silver grass; salt stress; photosynthesis; chlorophyll content; chlorophyll fluorescence; gas exchange

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1. Introduction

The growing demand for fossil energy has contributed to the increase in the global warming effect that threatens the ecosystem. Therefore, it is essential to substitute fossil fuels with alternative, renewable sources of energy [1]. Biofuels are considered sustainable energy options because they can mitigate CO_2 emissions and reduce dependence on fossil fuels [2]. Bioenergy is renewable energy that comes from the processing of several types of organic sources called biomass, which can be wood, forestry waste, harvest residues, manure, urban waste, food industry residues, and many other by-products of farming processes [3–5]. Energy crops, in addition to wood, are a raw material commonly used for biofuel production through high biomass yields high biomass yield, high calorific value, and low agronomic inputs. Biofuel production is carried out both through direct burning, and bio-fermentation, i.e., biogas and bioethanol production [6]. However, the production

of biomass for non-food uses should not be based on competition for agricultural land. This has become the cause for the use of marginal soils with disadvantageous agronomic characteristics for non-food cultivation [7,8]. Energy crop plantations have been promoted for many years as renewable source of energy within the policies of the European Union and United States of America [9]. Perennial energy grasses are a good option because they require relatively low nutrient levels and show high yields on marginal land. With regards to the beneficial environmental aspects, energy grasses absorb CO_2 and provide valuable shelter and food for wildlife [10]. Chinese silver grass (*Miscanthus sinensis* (Anderss.)) is a perennial grass species commonly grown as an ornamental and for bioenergy production. Due to its ability to be grown from seed and tolerance to low temperatures, this species has advantages over the high-yielding hybrid species such as *M. x gigantheus*. *M. sinensis* is grown for energy purposes mainly due to its high genetic variability, tolerance to stress, and biotic interactions with fauna. The species is a good candidate for C4 bioenergy crop development for marginal lands. In addition, it is used as a fodder crop and building material [11–13]. Studies have shown that *M. sinensis* can be grown on marginal land and land polluted with heavy metals [14]. *M. sinensis* belongs to C4 species, which are better adapted to abiotic stress conditions under some environmental stresses than C3 species. These crops not only have better photosynthetic efficiency and CO_2 fixation rates but also improved water use efficiency and transpiration, suggesting their superiority to C3 plants [15,16].

Salts are common in soil and are counted among the compounds necessary for plant growth. Their content at optimal concentrations has an important role in determining the maintenance of physiological plant functions [17]. However, excessive salt concentrations in the soil can contribute to osmotic stress and ionic toxicity by disrupting the ionic balance of nutrients, which ultimately affects the functioning of physiological processes and yield [18]. Salt stress is a serious abiotic stress occurring in many areas of the world mainly due to the use of poor-quality water for hydration, as well as soil salinity and inappropriate agricultural practices. The effect of salt stress is to reduce the growth and productivity of crop plants [19,20]. Shahid et al. [21] report that according to various estimates, about 10% of the total agricultural area is affected by salinity and sodicity, a billion hectares are covered with saline and/or sodic soils, and between 25% and 30% of irrigated land is saline and essentially economically unproductive. According to Singh [22], soil salinity is a widespread problem, involving more than one billion hectares in 100 countries. Salt stress in plants results in a combination of osmotic stress caused by dehydration and damage associated with Na^+ ion accumulation, which causes premature aging, chlorosis, and necrosis of leaves. These changes adversely affect protein synthesis and photosynthetic activity [23]. Deskoy et al. [24] on the example of cowpea showed that fennel and ammi seed extracts modulate the antioxidant defense system and alleviate salinity stress. In the studies of these authors, extracts of *Foeniculum vulgare* and *Ammi visnaga* seeds, applied foliarly, significantly increased the content of osmoprotectants and the activity of components of the antioxidant system. This was reflected in a decrease in Na^+, electrolyte leakage, and biomarkers of oxidative stress, and an increase in growth and yield traits, relative leaf water content, membrane stability index, photosynthetic efficiency, nutrient content, and K^+/Na^+ ratio. In another study [25], the application of a microbiological biostimulant including *Rhizophagus intraradices* and *Trichoderma atroviride* individually or in conjunction with plant-derived protein hydrolysates resulted in an increase in chlorophyll content and photochemical yield of PSII, as well as a better nutritional status of lettuce leaf tissue. The improved crop yield was due to better architecture of the root system, enhanced chlorophyll synthesis, and improved proline storage.

According to Ahmad et al. [26], one of the strategies for alleviating salt stress is to apply natural extracts of plants in place of artificial fertilizers, thus reducing water, soil, and environmental pollution. Plant biostimulants are substances that have positive effects on plant growth and nutrition and enhance tolerance to both biotic and abiotic stresses. A biostimulant can be an organic material and/or microorganism used to increase

nutrient uptake, stimulate growth, and improve stress tolerance or yield quality [27]. Since these substances are rich in bioactive compounds including carotenoids, flavonoids, and phenols, they effectively regulate redox metabolism thus developing plant growth and yield. Biostimulants enhance plant tolerance to salinity mainly through the modulation of signaling signatures and pathways and regulation of redox machinery [26].

The objective of this research is to identify of the impact of foliar application of biostimulants on the physiological processes (relative chlorophyll content, chlorophyll *a* fluorescence, and gas exchange parameters) in *M. sinensis* plants exposed to soil salinity stress. It is hypothesized that the use of biostimulants will have an impact on alleviating the effects of salt stress.

2. Materials and Methods

2.1. Pot Experiment Design

Pot experiments on *M. sinensis* plants were conducted under laboratory conditions. *Miscanthus* seeds were sown into multi pots, and, after germination, the seedlings were transplanted into 15 × 15 cm plastic pots, in which soil with a slightly acid reaction and a granulometric composition of loamy sand with (pH: KCl 6.35; H_2O 6.52) was placed [28]. The experiment was conducted in four replicates in a phytotron (Model GC-300/1000, JEIO Tech Co., Ltd., Seoul, South Korea) at a temperature of 22 ± 2 °C, humidity of 60 ± 3% RH, photoperiod of 16/8 h (L/D), and a maximum light intensity of 300 µE m^{-2} s^{-1}. In the experiment, the position of the pots was changed weekly. After the plants reached the tillering stage (approx. 8 months after sowing *M. sinensis* seeds), they were watered with aqueous solutions of neutral salt (NaCl) at concentrations of 200 and 400 mM at a rate of 100 mL per pot. In the control trial, the plants were watered with demineralized water of the same volume (100 mL). The application of salt solutions was followed by spraying *M. sinensis* plants with various biostimulants: BioFol Bombardino, Quantis®, Biofol Plex, and Megafol using a hand sprayer. The characteristics of biostimulants are specified in Table 1.

Table 1. The characteristics of biostimulants.

Foliar Fertilizers	Producer	Fertilizer Characteristics	Dose (per 1000 mL of Water)
BioFol Bombardino	Biostyma Sp. z o.o. (Poland)	70.0% organic matter content, 35.0% seaweed concentrate, 30.0% organic carbon, 5.0% free L-amino acids, polysaccharides, phosphorus, potassium, magnesium, iron, calcium, copper, vitamins B1, B2, B3, B6, B9;	5 mL
Quantis®	SAF Argentina S.A. (Argentina)	1% total nitrogen (N), 0.9% organic nitrogen (N_{org}), 9.3% potassium (in conversion to K_2O), 4.6% calcium (in conversion to CaO), dry matter 52%, organic matter 26%	10 mL
BioFol Plex	Biostyma Sp. Z o.o. (Poland)	2.0% N_{tot}; 0.3% Mg; 5.0% S; 0.15% B; 0.05% Cu; 0.20% Fe; 0.10% Mn; 0.50% Zn; 1.25% C; 5.0% extract from algae; traces of plant hormones, betaine ($C_5H_{11}NO_2$), amino acids, thiamine	7.5 mL
Megafol	VALAGRO (Italy)	amino acids (proline and tryptophan), glycosides, polysaccharides, organic nitrogen and organic carbon	10 mL

The variants of the experiment were:

Control
S1—200 mM NaCl
S2—400 mM NaCl
B1—Bombardino
B2—Quantis
B3—BiofolPlex
B4—Megafol
S1 + B1—200 mM NaCl + BioFol Bombardino
S2 + B1—400 mM NaCl + BioFol Bombardino
S1 + B2—200 mM NaCl + Quantis®
S2 + B2—400 mM NaCl + Quantis®
S1 + B3—200 mM NaCl + BiofolPlex
S2 + B3—400 mM NaCl + BiofolPlex
S1 + B4—200 mM NaCl + Megafol
S2 + B4—400 mM NaCl + Megafol

2.2. Physiological Measurement

Physiological measurements were taken twice on fully expanded leaves on the first and the seventh day after spraying: Term I—the 1st day after biostimulant application, Term II—the 7th day after biostimulant application.

2.2.1. Relative Chlorophyll Content

A CCM-200plus portable chlorophyll meter (Opti-Sciences, Hudson, NH, USA) was used to determine relative chlorophyll content. Measurements were performed on 5 fully expanded leaves per pot.

2.2.2. Chlorophyll Fluorescence

A Pocket PEA portable fluorimeter (Pocket PEA, Hansatech Instruments, King's Lynn, Norfolk, UK) was used to measure chlorophyll fluorescence parameters. Specialized leaf clips were used to adapt the plants to darkness for a period of 30 min [29]. The fluorescence signal has been collected in red actinic light with a light source peak wavelength of 627 nm and was used for 1 s at the maximum intensity available of 3500 µmol photosynthetically active radiation (PAR) m^{-2} s^{-1}. Chlorophyll fluorescence was measured on 4 fully developed leaves per pot. The following parameters were determined during the measurements: the maximum quantum yield of primary photochemistry (Fv/F0), the photochemical efficiency of PS II (Fv/Fm), and the performance index of PS II (PI).

2.2.3. Gas Exchange

Gas exchange was metered using an LC pro-SD apparatus (ADC Bioscientific Ltd., Herts, UK) on two fully developed leaves per pot. During the measurement, the intensity of light inside the measurement chamber was 1500 $mol \cdot m^{-2} \cdot s^{-1}$, while the temperature was 22 °C. During gas exchange measurements, the following parameters were determined: net photosynthetic rate (PN), transpiration rate (E), stomatal conductance (gs), and intercellular CO_2 concentration (Ci).

2.3. Statistical Analysis

The results obtained in the experiment were tested to statistical analysis using Statistica 13.3.0 (TIBCO Software Inc., Palo Alto, CA, USA). The Shapiro–Wilk test was performed to check the normality of the distribution at $p = 0.05$, followed by a two-factor (two-way) ANOVA with repeated measurements (time assessment as a factor). Tukey's post hoc test was used to determine and verify the relationship at a significance level of $p \leq 0.05$.

3. Results

3.1. Relative Chlorophyll Content

The application of salt stress to S1 and S2 in Term I reduced chlorophyll content compared to the control by 18.3 and 49.0%, respectively (Figure 1). Term II, on the other hand, showed no significant difference ($p = 0.000$) between S1 and the control. There were no differences in the value of the studied parameter between B1 and B2 and B3 and B4 variants. However, higher chlorophyll content was shown between B2, B3, and B4 in Term I ($p = 0.000$), and no differences between the biostimulants in Term II. The use of biostimulators in variants in which salt stress occurred alleviated its effects and increased the value of the tested parameter. In Term I, in the case of variants S1 + B1, S1 + B2, and S1 + B3, the chlorophyll content was at the control level. However, in Term II, such a relationship was found in variants S1 + B2, S1 + B3, and S1 + B4. Most of the analyzed variants showed an increasing tendency in the chlorophyll content in Term II. However, a significant increase about Term I was demonstrated only in the variants: S2, S2 + B1, S2 + B2S1 + B3, and S1 + B4. In Term II, an increase in chlorophyll content was observed in the variants with biostimulants compared to the S2 variant, but a significant difference was demonstrated only in S2 + B1 (5.5% increase).

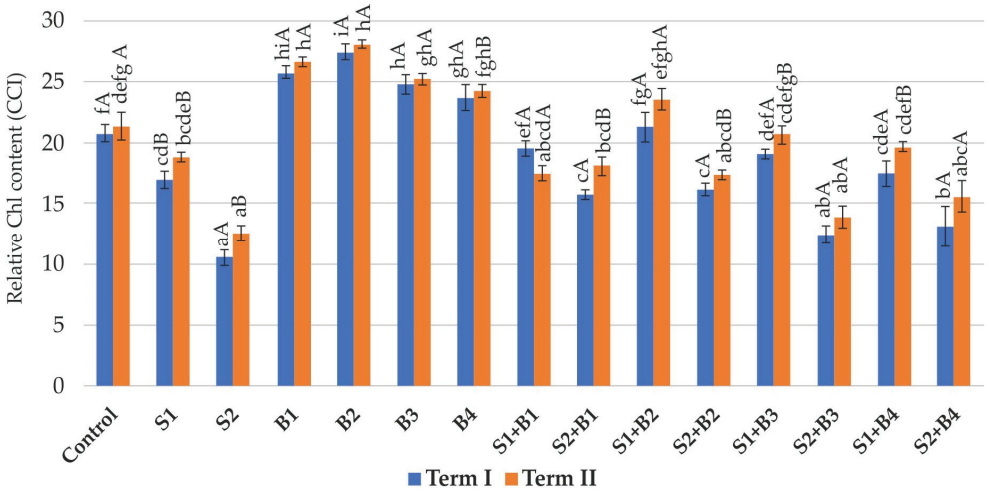

Figure 1. Effect of salt concentrations, biostimulants treatment, and terms of measurement on relative chlorophyll content (Term I—the 1st day after biostimulant application, Term II—the 7th day after biostimulant application). Lowercase letters indicate significant differences among means of the variants within the respective measurement term. Capital letters indicate significant differences among means of individual measurement terms within each experiment variant. As determined by ANOVA and followed by Tukey's HSD test ($n = 30$, $p = 0.05$).

3.2. Chlorophyll Fluorescence

Salt stress in both Term I ($p = 0.000$) and Term II ($p = 0.000$) caused a significant decrease in Fv/Fm values (Figure 2a). Relative to the control, the decrease was 32.9% (Term I), 19.5% (Term II) with the S1 variant, 58.5% (Term I), and 48.6% (Term II) with the S2 variant. The application of biostimulants demonstrated a significant increase in Fv/Fm values in comparison to the control only in Term II, after spraying with biostimulants B1, B2, and B3. After biostimulants on plants growing under salt stress, the Fv/Fm value was at the control level only in the case of the S1 + B2 variant (Term I and II). In variants, S1 + B3, S1 + B4 (Term I and II) and S2 + B4 (Term II). The use of the biostimulator did not result in a significant increase in the Fv/Fm value compared to the variants in which the same concentration of salt was applied. In Term II, variants S1, S1 + B1, S2 + B1, S1 + B2, S2 + B2,

S2 + B3, S1 + B4, and S2 + B4 showed a value increase in the tested parameter compared to Term I.

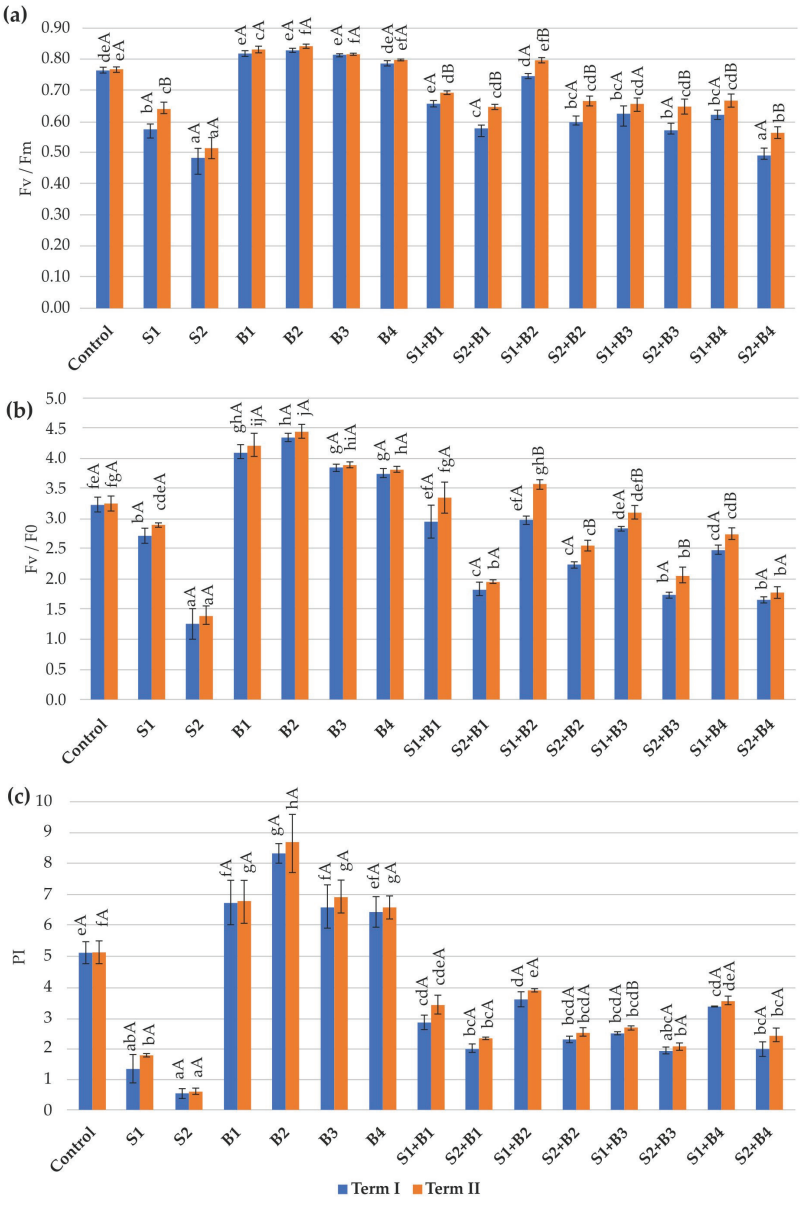

Figure 2. Effect of salt concentrations, biostimulants treatment, and terms of measurement on chlorophyll fluorescence parameters: (**a**) the photochemical efficiency of PS II (Fv/Fm); (**b**) the maximum quantum yield of primary photochemistry (Fv/F0); (**c**) the performance index of PS II (PI). (Term I—the 1st day after biostimulant application, Term II—the 7th day after biostimulant application). Lowercase letters indicate significant differences among means of the variants within the respective measurement term. Capital letters indicate significant differences among means of individual measurement terms within each experiment variant. As determined by ANOVA and followed by Tukey's HSD test (n = 30, p = 0.05).

Soil salinity resulted in a decrease in Fv/F0 values compared to the control, except for the S1 variant in Term II (for both Terms $p = 0.000$) (Figure 2b). The lowest decline, which amounted to 157.9% in Term I and 133.1% in Term II, was demonstrated in the S2 variant. The use of biostimulants resulted in an increase in the Fv/F0 value compared to the control in both Term I and Term II. After the application of biostimulants, in the variants with saline soil (S1 + B1 and S1 + B2 in Term I and II and S1 + B3 in Term II), an increase in the Fv/F0 value to the control level was shown. In Term II, variants S1 + B3 and S1 + B4 showed no improvement in Fv/F0 values due to spraying with biostimulants. Measurements carried out in Term II generally showed an enhancement in the value of the tested parameter compared to Term I. However, significant differences were observed only in the variants with saline soil and biostimulants (S1 + B2, S2 + B2, S1 + B3, S2 + B3, and S1 + B4).

Salt stress caused a significant decrease in PI values, compared to the control (for both Terms $p = 0.000$) (Figure 2c). With salt S1 application, the PI value was only 1.355 (Term I) and 0.530 (Term II), while salt S2 was 0.532 (Term I) and 0.600 (Term II). The application of biostimulants resulted in a significant increase in PI values compared to the control, except for biostimulant B4 applied in Term I. In the variants with saline soil, spraying with biostimulants increased PI values, but did not reach the level of the control in any of the analyzed variants. In the case of variants S1 + B3 (Term I and II) and S2 + B3 (Term I), no increase in values was observed compared to variants without biostimulants. Most of the analyzed variants showed an increasing tendency measured during Term II compared to Term I. However, only in the case of variants S2 + B1 and S1 + B3, this increase was statistically significant.

3.3. Gas Exchange

For the parameter Ci, a significant increase in its value was demonstrated in comparison to the control due to salt stress (Figure 3a). This increase was 86.5% (S1) and 118.2% (S2) in Term I ($p = 0.000$) and 115.8% (S1) and 144.2% (S2) in Term II ($p = 0.000$). As a result of spraying with biostimulants, there was a decreasing tendency in the value of the tested indicator compared to the control, but these values were not statistically significant. After treatment of plants growing under salt stress conditions with biostimulants, all variants showed a decrease in Ci values compared to variants without biostimulants. However, the control level was reached only in the S1 + B2 variant (Terms I and II). In Term I, higher Ci values were shown for the tested variants compared to Term II. However, they were statistically significant only for the control, S2, S1 + B1, S1 + B2, and S1 + B3.

In comparison to the control, a significant decrease in E values was observed in plants growing in salt stress conditions (for both Terms $p = 0.000$), which was 53.8% (S1) and 62.2% (S2) in Term I and 45.4% (S1) and 57.3% (S2) in Term II (Figure 3b). After spraying with biostimulants, there was an upward trend in E values compared to the control. However, only the biostimulant B2 in Term II showed a significant increase of 21.1% in E values compared to the control. Spraying with biostimulants also increased the value of the parameter under study and reached the control level in the S1 + B2 and S1 + B4 variants in Terms I and II. Variants S1 + B1, S2 + B2, and S2 + B3 in Term II and variant S2 + B4 in Terms I and II showed a significant increase in E values compared to variants with salt stress without biostimulant application. Only in the case of variants S1 + B2 and S2 + B2, no significant increase in the value of the studied parameter was observed in Term II.

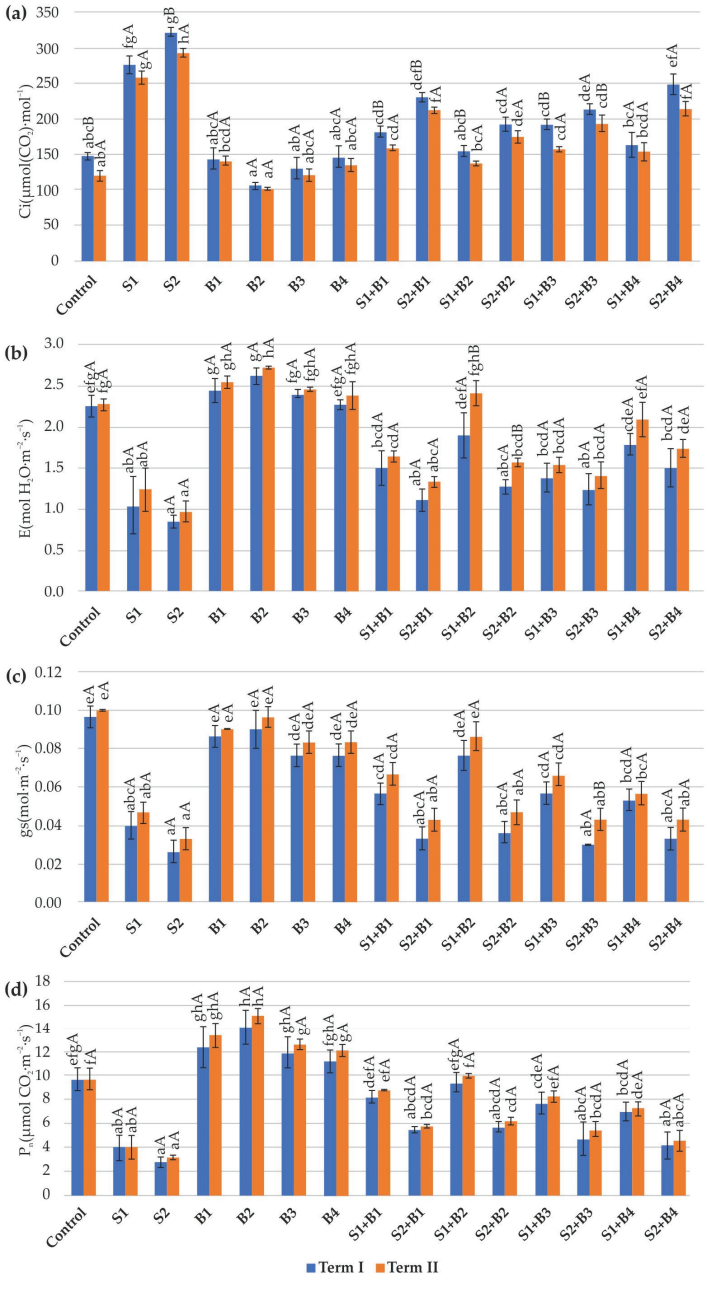

Figure 3. Effect of salt concentrations, biostimulants treatment, and terms of measurement on chlorophyll fluorescence parameters: (**a**) intercellular CO_2 concentration (Ci); (**b**) transpiration rate (E); (**c**) stomatal conductance (gs); (**d**) net photosynthetic rate (PN). (Term I—the 1st day after biostimulant application, Term II—the 7th day after biostimulant application). Lowercase letters indicate significant differences among means of the variants within the respective measurement term. Capital letters indicate significant differences among means of individual measurement terms within each experiment variant. As determined by ANOVA and followed by Tukey's HSD test (n = 20, p = 0.05).

As a consequence of soil salinization, a significant decrease in gs values was shown in relation to the control (for both Terms $p = 0.000$) (Figure 3c). The application of salt S2 resulted in a decrease in the value of the studied indicator by 72.2% (Term I) and 67.0% (Term II). Spraying M. sinensis plants with biostimulants did not result in an increase in Ci values compared to the control. However, under salt stress conditions after the application of biostimulants, an increase in gs values to the control level was shown for the S1 + B2 variant compared to the variant without biostimulants. On the other hand, the rest of the variants did not show a significant increase in the value of the studied indicator except for S1 + B1 and S1 + B3 (Term II). Compared to the variants in which no biostimulant was applied, the increase was 100.0% (S1 + B1) and 103.0% (S1 + B3). Although in most of the analyzed variants, an increase in gs values was observed in Term II, but only in the case of the variant S2 + B3 it was statistically significant.

The salt stress application caused a decrease in Pn values relative to the control (for both Terms $p = 0.000$) (Figure 3d). It amounted in Term I to 59.1% (S1) and 71.6% (S2), while in Term II to 58.9% (S1) and 68.0% (S2). The treatment of biostimulant spraying resulted in a significant increase in the value of the studied indicator compared to the control except for biostimulant B4 (Term I). Spraying with biostimulants promoted an increase in Pn values in the variants where salt stress was applied. In the case of variants S1 + B1, S1 + B2, and S1 + B3 (Term I and II) and variant S1 + B4 (Term II), the Pn value was at the control level. In contrast, the S2 + B1, S2 + B2, and S2 + B3 variants (Term I) and the S2 + B4 variants (Term I and II) did not demonstrate significant differences between the variants without the biostimulant. No significant changes were shown in the value of the studied indicator between Terms I and II.

4. Discussion

Salt stress is a significant problem in plant cultivation. In the initial stage, salt stress is seen by the root system as causing osmotic stress due to reduced water availability. At a later stage, salt stress induces ionic toxicity caused by nutrient imbalances in the cytosol [30].

In the conducted experiment, there was a decrease in the values of the studied parameters of chlorophyll content and fluorescence (Fv/Fm, Fv/F0, and PI) and gas exchange (E, gs, and Pn) caused by soil salinity.

Photosynthesis is an important biological process in plants that determines life on Earth. Soil salinity significantly affects the photosynthetic process. As a consequence of salt stress, the photosynthetic pigments, photosystems, and enzymes engaged in carbon metabolism can be damaged [31,32]. The decrease in chlorophyll content, on the other hand, can be explained by the inhibition of several steps in porphyrin formation and a decrease in chlorophyll-binding proteins [33].

Analysis of chlorophyll *a* fluorescence parameters is an important tool used in plant physiological research, which can provide valuable information about the state of PSII [29]. Such studies can be particularly useful for quantifying injury to the photosynthetic apparatus as a result of various stress factors, which allows us to determine photosystem II (PS II) damage [34,35]. In salt stress treatment, the decline in chlorophyll fluorescence parameters was noted in the present study. In particular, in the case of the PI parameter, which is a very significant and responsive index of photosynthesis, a significant decrease in its values was recorded about the control. A similar relationship was also obtained in the studies of Metha et al. [35] and Jańczak-Pieniążek et al. [36] in which salt stress was applied to wheat plants. Salt stress causes restrictions in the conductivity of stomata as a result of their closure, leading to inhibition of CO_2 absorption and stimulation of huge energy levels. This is the cause of an increase in the amount of reactive oxygen species (ROS) [37], which leads to oxidative stress due to their overproduction and lack of balance between defense mechanisms. The decrease in chlorophyll content and the value of chlorophyll fluorescence parameters may result from disturbances in the cell membrane permeability and the functioning of thylakoids in chloroplasts. This leads to a gradual decline in the

activity of photosystems [38,39]. Physiological process inhibition associated with overaccumulation of Na^+ and Cl^- ions, which reduces photosynthetic electron transport and photosynthetic efficiency [40]. As a result, this leads to a significant inhibition of plant growth and reduces the yield level. Under salt stress, plants have developed several cellular and tissue level mechanisms to avoid its effects. These mechanisms involve alterations in stomatal conductance, hormonal balance, antioxidant defense system, osmotic regulation, and ion exclusion [37,41,42].

In the short term, salinity causes a reduction in stomatal restrictions resulting in a decrease in CO_2 assimilation. On the other hand, in the long term, salt stress leads to a decrease in chlorophyll and carotenoids due to salt storage in young leaves [30,43]. In addition, as a consequence of the decrease in CO_2 assimilation, the activity of the Rubisco enzyme that converts CO_2 into high-energy substances decreases [44]. In the presented studies, an increase in substrate salinity increased the intercellular CO_2 concentration (Ci), which shows a decline in the CO_2 attachment capacity in the Calvin–Benson cycle [45]. A similar plant response to stress was observed in other crop species. For example, an increase in Ci with a concomitant decrease in Pn, gs, and E was observed in potato under stress conditions caused by plant exposure to ozone (O_3) [46] and spraying with hydrogen peroxide (H_2O_2) [47].

Plants, due to constant exposure to biotic and abiotic stresses, have adapted and remodeled their defense system, which helps them respond to constantly changing environmental conditions [48]. In the study, most of the analyzed cases showed an enhancement in the values of chlorophyll content and fluorescence indicators and gas exchange in Term II. A comparable relation was achieved in the case of study of Jańczak-Pieniążek et al. [36] conducted on wheat seedlings. A higher value of these indices was obtained at successive measurement dates. This demonstrates the activation of defense mechanisms that counteract the effects of stress by reducing the production of ROS and scavenging them [41,49]. The antioxidant system is then activated, consisting of enzymatic (including superoxide dismutase, ascorbate peroxidase, guaiacol peroxidase, and catalase) and non-enzymatic (including flavonoids, carotenoids, tocopherols) antioxidants [50,51].

The yield losses are mainly due to drought and soil salinization caused by climate change and agricultural intensification leading to soil degradation. Plant defense strategies can be improved and sensitized using chemical and biological treatments. As a result of this process, the plant-based immune system and defense mechanisms are pre-conditioned, which results in faster and more efficiency defense and resistance mechanisms to later biotic and abiotic stresses. Some substances of natural origin have positive effects on plant development [52–55]. To this end, the use of biostimulants is recommended as a means of protecting plants from environmental stresses [56]. The function of biostimulants is to promote growth and development of plant by improvement of plant metabolism efficiency to increase crop growth and improve yield quality, increase tolerance to abiotic stresses, facilitate assimilation, translocation, and utilization of nutrients, etc. Biostimulants are divided into categories: microbial modifiers, humic acids, fulvic acids, protein hydrolysates and amino acids, and seaweed extracts [56–58]. The use of plant biostimulants is fast becoming popular in agriculture. In the past decade, the plant biostimulant domain has been growing steadily and has become one of the key strategies for increasing crop production and immunity to a changing climate. In addition to increasing stress tolerance, biostimulants effectively regulate several plant physiological processes [59]. This was also demonstrated in this study, which found an increase in the values of the physiological indicators tested as a result of the application of biostimulants both in the case of variants without salinity and in which salt stress was applied. The effects of salt stress were best alleviated by foliar spraying of plants with Quantis, which contains, among others, potassium (K) and calcium (Ca). K is a crucial macronutrient that controls growth and development by changing physiological and biochemical indicators. This element influences the osmolyte accumulation and increases antioxidant components in plants subjected to water and salt stress [60]. Soil salinity stress causes rapid depolarization of the cell membrane, activating

voltage-gated GORK channels and causing K^+ efflux. ROS accumulation under salinity conditions may subsequent mobilization $GORK^-$ and ROS-activated NSCC channels, inducing greater K^+ efflux. This, in turn, results in fast loosing of K^+ from the cytosol, which impairs the homeostasis of the cytosolic Na^+/K^+ ratio [61]. Ca is also fundamental to plant physiology. It affects the maintenance of ionic homeostasis on an intracellular scale [62]. Of the biostimulants used in the experiments, in most of the analyzed physiological parameters, in general, their lowest values were obtained after Megafol application. It is hard to say exactly why. This biostimulator contains proline, which ensures the appropriate rate of photosynthesis under various stress conditions. It helps maintain the water content in the cell, protects photosynthetic units against the harmful effects of high-energy free electrons, protects the cell membrane by lipid peroxidation inhibition, and increases the level of various antioxidant enzymes and non-enzymatic compounds [63]. Extended research is necessary to understand the basic mechanisms responsible for these effects.

5. Conclusions

The study showed that soil salinity stress is resulting in a decrease in the values of most of the tested physiological indicators (except Ci). The application of spraying with biostimulants, especially Quantis (B2), caused in an enhancement in the values of the studied physiological indices both for plants being grown in optimal conditions and under salt stress. The second measurement term (Term II) showed an increasing trend for most of the analyzed parameters compared to the first measurement term (Term I), suggesting plant acclimatization to stress conditions. Based on the experiment, it was proved that the use of biostimulants can be an innovation in crops and allows to alleviate the negative impacts due to salt stress. This knowledge can contribute to the implementation of sustainable practices in crop production in the future. However, further investigations are needed on the effects of biostimulants on different plant species grown under different environmental conditions and/or different degrees of salinity stress.

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Description of Meteorological Indices Presented Based on Long-Term Yields of Winter Wheat in Southern Germany

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Abstract: This study had three main objectives. First, weather indices were listed and their derivations were described to show which weather parameters could be used to describe the influence on agricultural yields. Second, farmers and agricultural scientists should be given the opportunity to evaluate the weather of the observation years in the study region. Furthermore, significant fluctuations in winter wheat yields were compared with weather events. As weather variables, 45 meteorological indices were used, such as precipitation-, temperature-, precipitation-temperature-, growing-period-, and radiation-related indices. In the case of winter wheat, heat waves and dry periods were the most important factors that affected the yields. For the past 20 years, in particular, there have been recurrent spring and summer months with low precipitation and, in some cases, significantly too warm periods, such as in 2003 and 2018 (April to October 2003: +16% °C, 2018: +27% °C, 2003: −38% mm, 2018: −12% mm in relation to 1978 to 2020), which were associated with particularly high yield losses. The qualitative assessments illustrate that in the observation period, years with reduced yield compared with the multiannual trend were frequently well explainable by extreme weather events.

Keywords: climate indices; crop production; long-term yield; plant growth; fertile site; weather anomaly

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1. Introduction

1.1. Background

Approximately 80% of the yield variability of crops can be explained by prevailing weather conditions [1]. Extreme weather events, such as heatwaves, dry periods, heavy precipitation, or unusual frost events, have a particularly significant impact on agricultural yields. These can occur either as individual events, in combination with each other, or with a time lag and result in a wide variety of effects depending on the preceding weather. Thus, weather extremes relevant to agriculture can trigger damage within a few hours, days, or weeks [1]. The temporal occurrence of extreme weather conditions plays a decisive role, as crops react differently to weather extremes during the various stages of development. Depending on the duration, extent, and geographical coverage, damage can ultimately be observed in local, narrowly defined areas or on a supra-regional scale.

Wittchen et al. [2] and Bernhofer et al. [3] provided important parameters for measuring, classifying, and evaluating extreme agrometeorological events and showed which indices are of particular relevance for arable farming.

In recent decades, extreme weather events have mainly been discussed in combination with climate change (Table 1). A selection of research works to better understand the fluctuations of yield in experimental areas show that approaches to the evaluation of certain weather anomalies, such as heat, drought, waterlogging, and frosts, are widely

available. However, the combination of several successive extreme events and their concrete impact on agriculture that occur in reality has not yet been adequately investigated.

Table 1. Literature overview of various studies that investigated the effect of climate on barley, wheat, and maize grain yields.

Author	Year	Location	Crop	Factors and Effects
Weigand [4]	2014			Agricultural meteorology and the significance of certain weather anomalies for arable farming
Gömann et al. [1]	2015			Thresholds for agrometeorological extreme weather events and impacts on different agricultural crops
Barlow et al. [5]	2015	Germany	Wheat	Effects of extreme heat and frost events on wheat
Kristensen et al. [6]	2011	Denmark	Wheat	Summer temperature has the strongest effect, resulting in lower yields with increasing temperature, while yields increase with increasing radiation in summer and spring
Gobin [7]	2012		Cereals	Effects of heat stress and drought on cereal development
Ontel, Vladut [8]	2014		Maize	Correlation between drought indices and yield in maize
Wu et al. [9]	2014		Wheat	Influence of late frosts on the development of wheat
Seidel [10]	2016		Wheat, barley, maize	Extreme weather events and their role in the development of pests in wheat, barley, and maize
Ren et al. [11]	2014		Maize	Effects of heavy precipitation and waterlogging on maize cultivation
Wollmer [12]	2016	Germany	Wheat	Temporary waterlogging causes reduced growth, nutrient concentration, and yield of wheat
Heil et al. [13], Heil et al. [14]	2020, 2021	Germany	Wheat	In more fertile locations, the yield is determined, to a considerable extent, by climatic conditions in winter and the transition periods from winter to the warmer season and vice versa, and less by climatic conditions during the main growing season

Barnabas et al. [15] and Gobin [7] investigated the effects of drought and heat stress on the productivity of cereals. They pointed out that the consequences of this combination of extreme events are still insufficiently known. Seidel [10] addressed extreme weather events and their role in the development of pests in wheat, barley, and maize. According to this study, we can expect more frequent unusual weather anomalies and increased pest pressure to have a negative impact on yields.

Several authors, such as Sivakumar et al. [16], Rippel [17], and Frühauf [18], already highlighted the consequences of climate change for agriculture, also in connection with extreme weather events. They investigated the extent to which unusual weather anomalies, such as heatwaves and drought, will continue to develop in terms of their frequency and intensity. In addition, the opportunities and risks for arable farming in the wake of rising temperatures and increased precipitation variability as a result of climate change are being researched. Weigand [4] presented basic points on agricultural meteorology and the significance of certain weather anomalies, such as drought, waterlogging, and heat, with possible adaptation strategies. However, the interaction between extreme weather conditions relevant to agriculture and, ultimately, their impact on agricultural production still poses a particular scientific challenge [19].

Osborne and Wheeler [20] analyzed changes in the variability of wheat, maize, and rice in major producing countries by calculating 23 years of deviations of yield residuals

from the average trend. They concluded that yield variability has decreased rather than increased since 1961, particularly for wheat and rice.

Last but not least, the Thünen Report 30 by Gömann et al. [1] on the effects of extreme weather conditions relevant to agriculture, which was commissioned by the Federal Ministry of Food and Agriculture, shows the importance of such meteorological anomalies for arable farming and the need for further research into the interactions between the extremes. They provided an overview of general extreme weather events with corresponding threshold values for them depending on their relevance for various agricultural crops during the different stages of development. Accordingly, weather situations that deviate particularly strongly from the long-term reference period and those with economic damage that exceed a certain threshold value are classified as extreme.

1.2. Objectives of This Study

This study aimed to (i) identify years with significant yield reductions; (ii) describe the relationships between these years and weather events, as well as (iii) which periods are essential for yields; and (iv) identify indices that indicate the severity of a reducing impact on yield.

2. Materials and Methods

These relationships were derived and classified based on weather indices of the climate station Freising Weihenstephan-Dürnast of the German Weather Service [21] and winter wheat yield data from the district of Freising. For this purpose, the period from 1978 to 2020 was considered and comparative values from the 30-year reference period 1950 to 1979 were used to compare the climatic indicators.

2.1. General Description, Soil, and Physiography of the Freising District

The district of Freising is divided into two main parts in terms of geology, pedology, and landscape.

The northern part is partly covered with Pleistocene loess, partly waterlogged brown earth (Cambisol), and pseudogleys (Planosol and Luvisol). The other soil types are pelosols (Vertisol) in clay lenses and para-brown earth (Luvisol) in small loess areas. On eroded hills, regosols (Leptosols, Arenosols) are often accompanied by kolluvisols (Anthrosol) in the valleys. At the bottoms of valleys, waterlogged soils dominate (Gleysol) [22,23]. Holocene deposits with small-scale changes of partly very different soil types (Phaeozem, Chernozem partly gleyic, Leptosol, and Histosol) are further observed [23]. In contrast with the northern part, the area in the south consists of Holocene deposits (dominated by flat accumulated gravel material).

The climate of the Tertiärhügelland (Tertiary Hill Country) is characterized by an annual average precipitation of 765 mm (1990–2019). The average annual temperature is 8.7 °C (1990–2019).

The location of the weather station is latitude 48.4022° N and longitude 11.6944° E, and has an elevation a.s.l. of 477 m (Figure 1 [13,14]).

Cool, humid, and, therefore, good growing conditions for agricultural plants usually prevail during the year.

Winter wheat is the cereal with the highest soil requirements. Potential evaporation from emergence to harvest is about 500 mm in the main growing season. From the beginning of May to mid-July, it is 300 to 350 mm, with high evaporation demands (radiation, temperature) up to 400 mm, with correspondingly higher yields (approx. 70–100 dt/ha grain) if this water requirement can be met. Due to its early root penetration and high root formation, winter wheat is better able than many other crops to exploit the moisture reserves of deeper soil layers (up to approx. 1.8 m on deep loamy soils, approx. 120 mm soil water). Therefore, it has deep soils with good storage capacity, even in areas with low precipitation (<600 mm annual precipitation), and has high yield stability (Bavarian State Office for Statistics, 2020). For winter wheat, the increasingly dry early summer periods

present particular challenges. Shortly before flowering in May, wheat is particularly sensitive to high solar radiation, which can lead to the sterilization of pollen and prevent fruit sprouting; just before maturity in July, on the other hand, wheat is particularly sensitive to precipitation, as it can prevent the main ear from maturing by forming smaller spikelets.

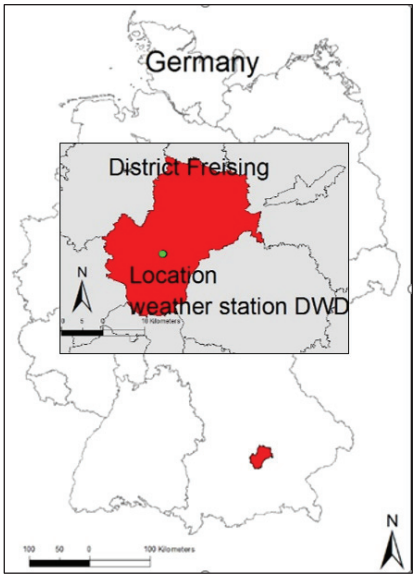


Figure 1. Location of Freising district and the weather station "Weihenstephan-Dürnast".

In a Bavarian comparison, the district has slightly above-average yields for winter wheat and winter barley and slightly below-average yields for grain maize (Bavarian State Office for Statistics, 2020).

2.2. *Description and Classification of the Weather Indices*

The basic data set contained daily data of the following:

- Maximum temperature (°C);
- Minimum temperature (°C);
- Temperature amplitude;
- Average air temperature (°C);
- Precipitation (mm);
- Relative humidity (%);
- Sunshine duration (h);
- Global radiation (Wh/m^2).

From these data, the indices were calculated and are presented in Table 2.

In the first step, it is important to define what an extreme event is. This term is not based on a precise definition. An extreme event describes an "extraordinary" event, i.e., an event that deviates from certain average values compared with other events of its kind and has a very long, irregular return period. This means for the place where the event occurs, it is rather a rarity. By definition, the characteristics of so-called "extreme weather" can vary in absolute terms from place to place. If a pattern of extreme weather persists over a period, e.g., a season, it can be classified as an "extreme climate event", especially if it has a mean or sum that is itself extreme (e.g., drought or heavy rainfall over an entire season) [25].

Table 2. Overview of the climate variables used in this study (compiled according to Bernhofer et al. [3], Wilhite [24], and Heil et al. [13,14]).

	Variable	Definition/Time Range		The Formula for the Derivation of Indices
Precipitation-related indices	Precipitation sum (Pm)	The sum of precipitation (yearly, April–October, monthly, and daily)		$P_m = \sum_{i=1}^{n} P_d$ where P_d is the precipitation per day
	Precipitation intensity (PI)	Sum of days on which a certain amount of precipitation occurred	PI1: >0–1 mm per day	$PI1 = \sum_{i=1}^{n} P > 0\ mm + P \leq 1\ mm$
			PI2: >1–10 mm per day	$PI2 = \sum_{i=1}^{n} P > 1\ mm + P < 10\ mm$
			PI3: ≥10 mm per day	$PI3 = \sum_{i=1}^{n} P \geq 10\ mm$
			Heavy precipitation, number of days	$PI4 = \sum_{i=1}^{n} P \geq 30\ mm$
			Vegetation-favorable precipitation, number of days with 2–4.9 mm	$PI5 = \sum_{i=1}^{n} P \geq 2\ mm + \leq 4.9\ mm$
		Daily, where P is the precipitation (mm) and n denotes the number of days		
	Rain-free days (P0)	Sum of days without precipitation (P0); monthly		$P0 = \sum_{i=1}^{n} N = 0\ mm$ where N is the height of the precipitation
	Number of precipitation-free pentads (P0_5 days)	The sum of the number of pentads (moving 5-day period) without precipitation		$P0\_5\ days = \sum_{i=5}^{n} N = 0\ mm$ where N is the height of the precipitation
	Meteorological dry periods (PD)	At least 11 consecutive days with daily precipitation less than or equal to 1 mm during the growing season		$PD = \sum_{i=11}^{n} N =< 1\ mm$ where N is the height of the precipitation
	Percent-from-normal ($P_y\% - normal$) ($P_m\% - normal$)	Current annual/monthly precipitation in relation to the 30-year mean from 1950 to 1979		$P_y\% - normal = \frac{P_y}{P_{(1950to1979)-year}}$ $P_m\% - normal = \frac{P_m}{P_{(1950to\ -1979)-month}}$ where P_y, P_m: precipitation per year, per month, respectively
	Cumulative precipitation deficits/surpluses (CPD)	Summation of precipitation anomalies annually/over the growing season/monthly		$CPD = \sum(P_{1950–1979} - P_{actual})$
	Precipitation (rainfall) anomaly index ($RAI_{positive/negative}$)	Relation of precipitation to extreme precipitation events from 1950 to 1979		$RAI_{positive} = 3 \times \frac{P_{actual} - P_{1950–1979}}{E_{1950–1979} - P_{1950–1979}}$ $RAI_{negative} = -3 \times \frac{P_{actual} - P_{1950–1979}}{E_{1950–1979} - P_{1950–1979}}$ P_{actual}: current precipitation per month; $P_{1950–1979}$: mean per month; $E_{1950–1979}$: mean of the 10% most extreme precipitation sums (10% percentile for positive/negative anomalies) of the validation period 1950 to 1979 for the observed month (e.g., January, then E is the mean of the 10% most extreme January precipitation sums of the years 1950 to 1979)

Table 2. *Cont.*

	Variable	Definition/Time Range	The Formula for the Derivation of Indices
Temperature- and precipitation-related indices	de Martonne aridity/humidity index (M-AI)	Evaluates the effect of precipitation and temperature on plant physiology per year	$M-AI=\frac{P_y}{T_y+10}$ where P_y is the annual precipitation and T_y is the average annual temperature
	de Martonne–Reichel dryness index (MR-DI)	Evaluates the effect of precipitation and temperature on plant physiology and precipitation distribution per year	$MR-DI=\frac{P_y}{T_y+10}\times\frac{K}{120}$ where P_y is the precipitation, T_y is the temperature, K is the number of days with precipitation in the observed period with $\geq$1 mm; 120 is the annual average number of days with precipitation $\geq$1 mm in Germany; 10 indicates that negative values in the denominator should be avoided
	Hydrothermal Selyaninov coefficient (HTC)	The ratio of the sum of precipitation and the sum of temperature (mean of the day) for all days above 10 °C per year	$HTC=10\times\sum P_y/\sum T_d>10\ ^\circ C$ P_y is the precipitation per observed period and T_d is the mean temperature per day
	Rain factor (RF) after Lang	Relationship between precipitation and temperature per year (calculated for every year)	$RF=\frac{P_y}{T_y}$ where P_y is the annual precipitation and T_y is the average annual temperature
Temperature-related indices	Mean temperature	Mean temperature per year, vegetation period (April to October), month (T_y, T_{veg}, T_m, respectively)	$T_y, T_{veg}, T_m=\frac{(\sum_{i=1}^{n} Td)}{n}$ where T_d is the diurnal mean air temperature of the day and n is the number of days
	Temperature threshold (TT)	Sum of the days on which the threshold values of 5 or 10 °C are exceeded; monthly values	$TT1=\sum_{i=1}^{n} T_{max}\geq 5\ ^\circ C$, $TT2=\sum_{i=1}^{n} T_{max}\geq 10\ ^\circ C$, where n is the number of days and T_{max} is the daily maximum temperature
	Frost-alternating days ($FAD_{(Oct–Jul)}$)	Sum of days (October to July) with a change in temperatures above and below 0 °C within a day, between consecutive days	$FAD=\sum_{i=1}^{n} T_{max}>0+\sum_{i=1}^{n} T_{min}<0$ where n is the number of days, T_{max} is the daily maximum temperature, and T_{min} is the daily minimum temperature
	Frost index per Liu (FI_Liu)	Sum of the days on which the minimum air temperature is below −3 °C and the temperature difference is at least 8 °C from the mean value of the last 20 days; from September to May	$FI\_Liu=\sum_{i=1}^{n} T_{min}<=-3\ ^\circ C+\sum_{i=1}^{n=20}$ $Td<8\ ^\circ C$ where n is the number of days, T_{min} is the daily minimum temperature, and T_d is the daily mean temperature
	Summer cold per Liu (SC_Liu)	Sum of the days on which the minimum air temperature is below −3 °C and the temperature difference is at least 8 °C from the mean value of the last 20 days; from April to August	$SC\_Liu=\sum_{i=1}^{n} T_{min}<=-3\ ^\circ C+\sum_{i=1}^{n=20}$ $Td<8\ ^\circ C$ where n is the number of days, T_{min} is the daily minimum temperature, and T_d is the daily mean temperature
	Late frost index 1 (LFI 1)	Sum of the days on which the minimum air temperature falls below 0 °C; from April to June	$LFI1=\sum_{i=1}^{n} T_{min}<0\ ^\circ C$ where n is the number of days and T_{min} is the daily minimum temperature

Table 2. *Cont.*

	Variable	Definition/Time Range	The Formula for the Derivation of Indices
	Late frost index 2 (LFI 2)	Sum of days on which the temperature is <0 °C; from April to June	$LFI2 = \sum_{i=1}^{n} T_{min} < 0\ ^{\circ}C$ where n is the number of days with a temperature <0 °C and T_{min} is the daily minimum temperature <0 °C
	Early frost index 1 (EFI 1)	Sum of days on which the minimum air temperature falls below 0 °C; from July to October	$EFI1 = \sum_{i=1}^{n} T_{min} < 0\ ^{\circ}C$ where n is the number of days with a temperature <0 °C and T_{min} is the daily minimum temperature <0 °C
	Early frost index 2 (EFI 2)	Sum of days on which the minimum air temperature falls below 0 °C; from July to October	$EFI2 = \sum_{i=1}^{n} T_{min} < 0\ ^{\circ}C$ where n is the number of days and T_{min} is the daily minimum temperature
	Frost days (FT)	Sum of days on which the air temperature falls below 0 °C; monthly values; from October to July	$FT = \sum_{i=1}^{n} T_{min} \leq 0\ ^{\circ}C$ where T_{min} is the daily minimum temperature (°C)
	Ice days (ID)	Sum of days with a maximum temperature of <0 °C over the entire year	$ID = \sum_{i=1}^{n} T_{min} \leq 0\ ^{\circ}C$ where T_{min} is the daily minimum temperature
	Frost severity (FSev)	Annual minimum temperature	$FSev = T_{min} \leq 0\ ^{\circ}C$, where T_{min} is the daily minimum temperature
	Frost shock (FSh)	Sum of days on which the air temperature drops by 15 °C within 24 h and the minimum air temperature falls below −3 °C; annual values	$FSh = \sum_{i=1}^{n} T_{max}–T_{min} = 15\ ^{\circ}C + \sum_{i=1}^{n} T_{min} < -3$
	Summer days (SD)	Sum of days on which the air temperature exceeds 25 °C; monthly values per year	$SD = \sum_{i=1}^{n} T_{max} \geq 25\ ^{\circ}C$
	Hot days (HD)	Sum of days on which the air temperature exceeds 30 °C; monthly values per year	$HD = \sum_{i=1}^{n} T_{max} \geq 30\ ^{\circ}C$
	Maximum values (MVa)	Absolute maxima per year in °C	$MVa = T_{max}$
	Summer index (SI_y)	Sum of days with a daily maximum air temperature above 5 °C; yearly	$SI_y = \sum_{i=1}^{n} T_{max} \geq 5\ ^{\circ}C$
	Summer index (SI_{veg})	Sum of days with a daily maximum air temperature above 5 °C; from April to October	$SI_{veg} = \sum_{i=1}^{n} T_{max} \geq 5\ ^{\circ}C$
	Winter index (WI)	Sum of days with a daily maximum air temperature below 5 °C; from November to April	$WI = \sum_{i=1}^{n} T_{max} \leq 5\ ^{\circ}C$ where n is the number of days
	Sum of the active temperatures (SAT)	Sum of temperatures above 5 °C during the growing season	$SAT = \sum_{i=1}^{n} T_{veg} \geq 5\ ^{\circ}C$ where n is the number of days

Table 2. *Cont.*

	Variable	Definition/Time Range	The Formula for the Derivation of Indices
Growing-period-related indices	Beginning/end of the main vegetation period	The first week of the year on which the threshold value of 5 °C is permanently exceeded (at least 5 days)	
	Climatic vegetation time duration 1 (CD1)	Number of 5-day periods with a mean daily air temperature above 5 °C; values per year	
	Climatic main vegetation time duration 2 (CD2)	Number of days with the diurnal mean daily air temperature above 5 °C; values per year	
	Grassland temperature sum (GT-1)	Sum of the mean daily temperature until the value of 200 °C	
	Grassland temperature sum (GT-2)	Sum of the mean daily temperature until day 105	
Radiation-related index	Global radiation $GR_{(Oct–Jul)}$	Sum of radiation	$GR = \sum_{i=1}^{n} GR$ where n refers to the months

Additional explanations and interpretations of different levels are given in the Supplementary Materials.

Leser et al. [26] explained weather extremes as events that deviate in their occurrence from average values, trends, and experience and are characterized by extraordinary dimensions, special intensities, and a longer-term recurrence. The German Weather Service specifically describes an extreme weather event as a rare event that is rarer than the 10th or 90th percentile of the observed probability distribution. However, it should be taken into account that not only the severity but also the duration of an event is important. For example, the frequent occurrence of certain anomalies can only be classified as extreme by the sum of the deviations in a period, although the individual events are less unusual in themselves.

Using the example of the 2013 Elbe Flood, Gömann et al. [1] showed that at that time, as a result of recurring precipitation at the beginning of June and the preceding high soil moisture, the soil was no longer able to store precipitation, although the quantities that fell were not extremely high. Rather, the weather period, which in meteorology cannot be statistically classified as an extreme event, resulted in critical threshold values in ecological, physical, and social systems being exceeded, causing considerable damage. Thus, in addition to the duration, extent, and intensity, the preceding weather is also decisive.

However, it is possible that at the same time, extreme events are compensated for by favorable weather before and after the event and that damage is only slight or does not occur at all.

In this evaluation, only agriculturally relevant weather extremes that were accompanied by significant crop losses were considered. No distinction was made here as to whether the extreme events that occurred were regional events, such as droughts and heatwaves, or very local anomalies, such as heavy precipitation events, hailstorms, or topographically induced temperature extremes. Precipitation and temperature anomalies are primarily decisive for agriculture. The main focus is on the so-called drought indices. Dry periods or droughts as negative precipitation anomalies in combination with very high temperatures are some of the most important limiting factors in agriculture and, depending on their duration and severity, can lead to considerable yield losses [3].

The preceding explanations allow for a classification of weather events according to the following structure (compiled according to Bernhofer et al. [3] and Wilhite [24]).

2.3. Yield Data and Extreme Value Analysis

The yield data of winter wheat has been registered yearly by the Bavarian State Office for Statistics from all farmers and was provided by the Bavarian Office of Agriculture (Institut für Betriebswirtschaft und Agrarstruktur). The lowest level of the area of this recording is the district.

In the first step, the time series of the yield values were exponentially smoothed (with the trend after Holt). In the next step, the residuals between the measured yields and the smoothed yields were used as response variables to evaluate the weather influence.

This procedure is needed to remove any development trends in the time series. This smoothing filters out the effects of new varieties, herbicides, insecticides, fertilizers, technical equipment, crop rotation, tillage, and climate change. According to Sterzel [27], all quantifiable factors can thus be systematically removed from the yield. Weather effects remain implicitly embedded in detrended crop yield values (Table 3).

Table 3. Overview of the effects on the temporal yield development and the effects eliminated by calculating residuals (Sterzel [27]).

Effects	Effects Eliminated by Residuals	Effects Remaining in Residuals
Biological and chemical	New varieties Herbicides Insecticides Fertilizer and fertilization level	Diseases and pest infestation
Mechanical management	Technical equipment processing	
Management advancement	Crop rotation	
Atmospheric	Climate change	Weather deviations and extreme weather events

In the second step, the residual percentile levels were calculated. These levels were then the limits for the assessment where the yield was extreme. Statistical analysis was performed using SPSS v24.0. To carry out an extreme value analysis, and thus, clarify in which years extremely low or high yields could be observed, the 10th and 90th percentiles were considered. The 50th percentile was the average of the calculated residuals, and thus, the average deviation of the measured values from the predicted values. Furthermore, the 25th and 75th percentiles were calculated for the yield residuals to be able to identify further significant deviations in the yield patterns of individual years.

3. Results

3.1. Temporal Course of the Yields

From 1978 to 2019, the yields of winter wheat in the Freising district indicated a continuous increase, albeit with considerable fluctuations at times, from approximately 50 to approximately 80 dt ha^{-1}. This means nearly 0.5 dt ha^{-1} per year (Figure 2). The reason here was mainly the progress in breeding, but biological, chemical, mechanical, and management advancements were also influential.

An additional reason was indicated by the time course of the deviations. During the observation period, positive values predominated. Negative developments were observable in the years 1979, 1980, 1982, 1993, 2003, 2009, 2010, and 2018.

It is important to note that the weather is not a directly quantifiable factor but is nevertheless very relevant to yield.

In general, the more intensive and specialized the land management, the higher the risk. This is especially true for modern high-yielding varieties, which produce top yields under favorable conditions but offer less yield security under extreme conditions [28].

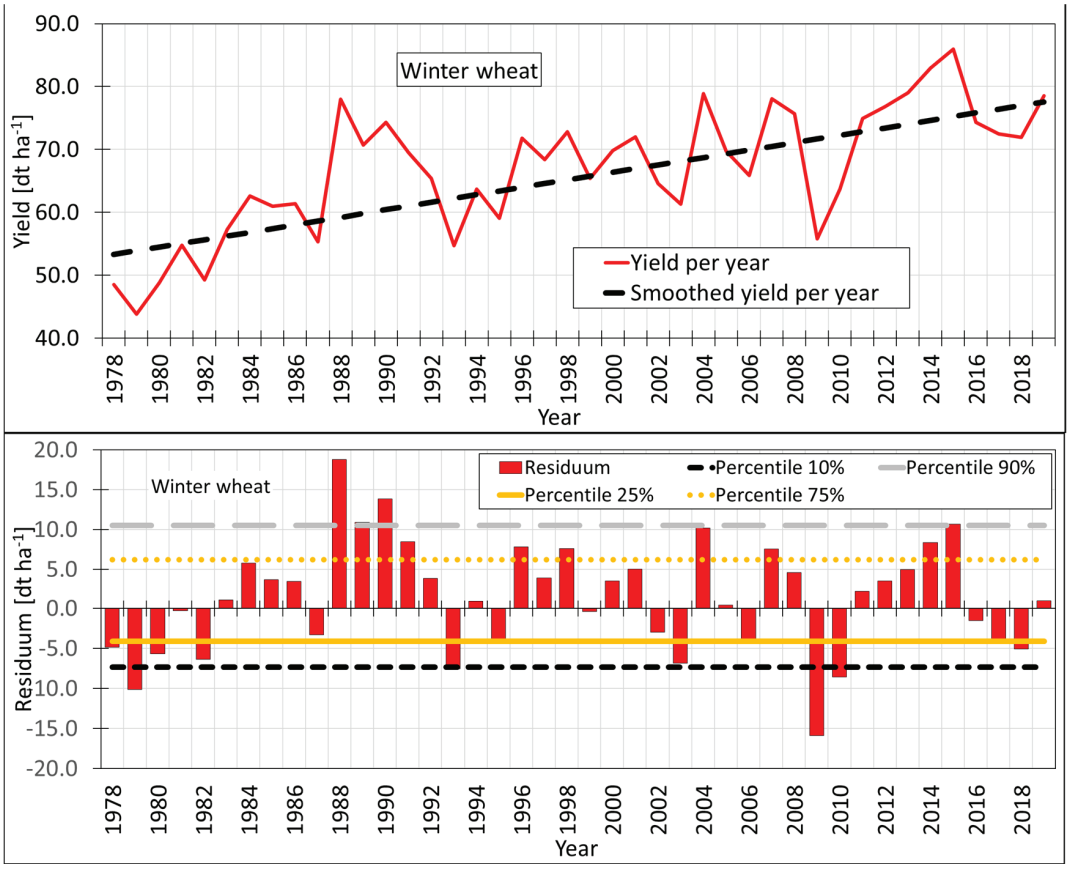


Figure 2. Annual yields of winter wheat between 1978 and 2019 in the district of Freising with the smoothing line (above) and the deviations from the smoothing line and the percentile levels (10%, 25%, 75%, and 90%).

3.2. Comparison of the Annual Variation of Yields with Weather Patterns

During the evaluation, the residuals were compared with the weather indices, and explanations for the low yields were worked out.

These comparisons were divided into the following stages: stock establishment, stock build-up, and production. The first stage began at sowing (October) and lasted until the beginning of shooting (May). During this period, the yield-bearing shoots/tillers were formed. The second phase began when the first node was visible and lasted until flowering (June). The production phase began after flowering and lasted until grain filling/ripening (June/August) and harvest (August).

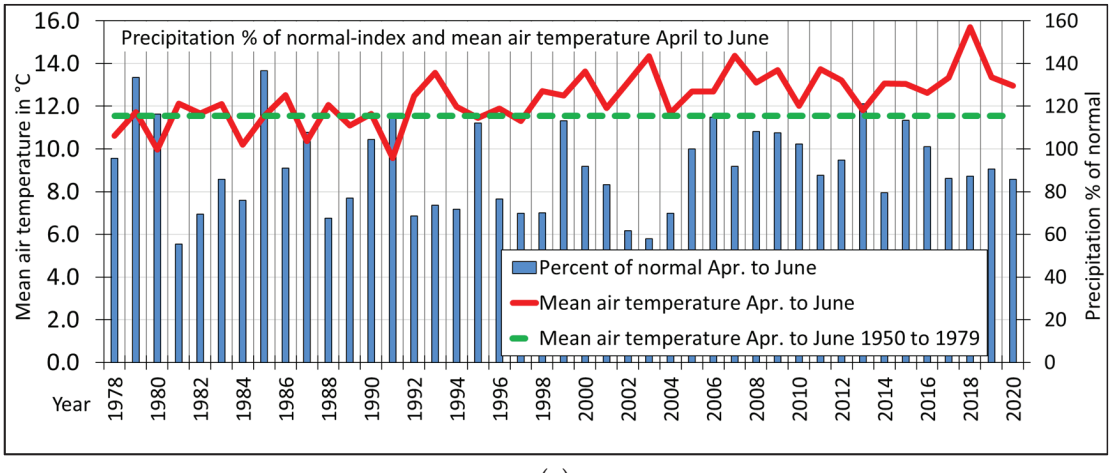
1979: Especially from mid-June 1979 onward, there were repeated heavy rainfalls, as well as continuous rainfall events. The total June precipitation was 243 mm, which could be classified as extremely wet, with an $RAI_{positive}$ of 4.32 (Figure 3). Other indices also confirmed this evaluation (CPD, P_m%-normal, precipitation summed, and M-AI). The highest individual precipitation was just under 80 mm per day. During other times of this month, the precipitation was more or less evenly distributed over the entire period. There were no other heavy precipitation events (Figure S4); it can thus be assumed that conditions of waterlogging prevailed in certain areas. Wollmer et al. [12] showed that temporary waterlogging in winter cereals, especially during grain filling, shortens this

phase through premature leaf senescence, and smaller grains form as a result. According to Marti et al. [29], waterlogging during the generative development phase is associated with impairments in flower formation and fertility, and thus, ultimately with a decline in grain number. In the case of increased silt content in the soils, as is the case in the district of Freising, persistent precipitation also leads to silting. The rainwater infiltrates insufficiently and a large part runs off superficially, which can lead to erosion damage [30]. Between the months of April and June, 134% of the normal amount of precipitation according to the climatological mean fell (Figure 2). For winter wheat, this was the second largest in the study period. It can be assumed that the wet weather also favored fungal infections, which could also have been responsible for the high crop losses. However, it must be taken into account that the data on the event are insufficient and there are hardly any reports on the 1979 harvest year.

1980: The mean yield decline in 1980 ranged between the 10th and 25th percentiles. Until April, the precipitation was higher than the 30-year average, but in May and June, the percent-of-normal reached only 0.67 and 0.93 (Figure S8). Since most of the sites have a high water storage capacity or are connected to groundwater, drought cannot be assumed. Moreover, the combined indices do not indicate plant stress (RAI April to June, 0.94; CPD May and June, −28.8 and −8.1; M-AI May and June, 34.0 and 50.0; MR-DI April and June, 37.4 and 80.1) (Figure 3). This also applies to the temperature indices. Additionally, the winter season delivered no indication of less favorable growing conditions (frost days, frost-alternating days, and frost shock).

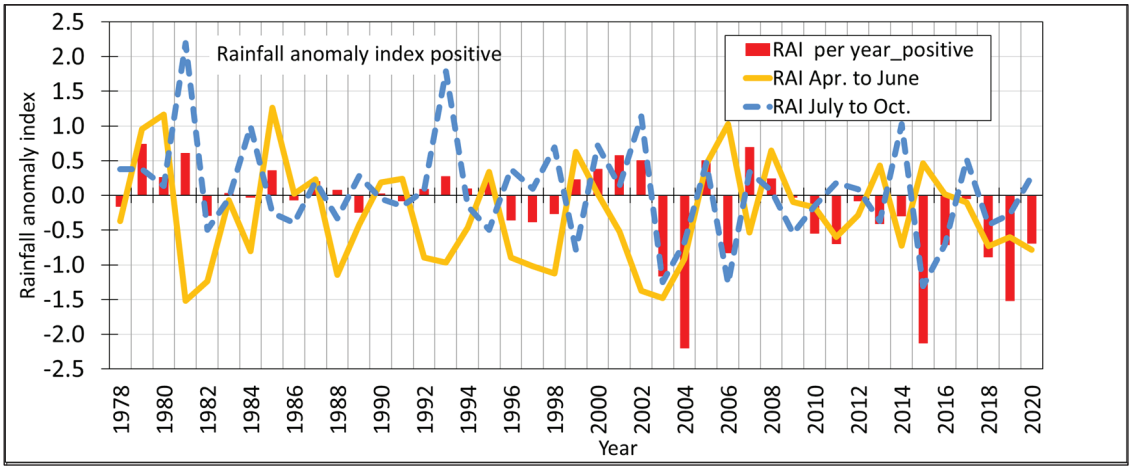
Therefore, no cause for this reduction in yield can be inferred from the available data.

1982: This year was characterized by lower precipitation from February to May, in July, and from September to November compared with the 30-year average (Pm%-normal: April, 0.45; May, 0.33; July, 0.71). The reduction was particularly pronounced in July, with only 74 mm (107 mm in the long-term mean). This was particularly evident in the CPD values (April, −28.9; May, −58.2; July, −30.4), HTC (whole year, 2.7), MR-DI (April to June, July to October, and April to October, approximately 22), and M-AI (April, 17.8; May, 15.2; July, 30.9).

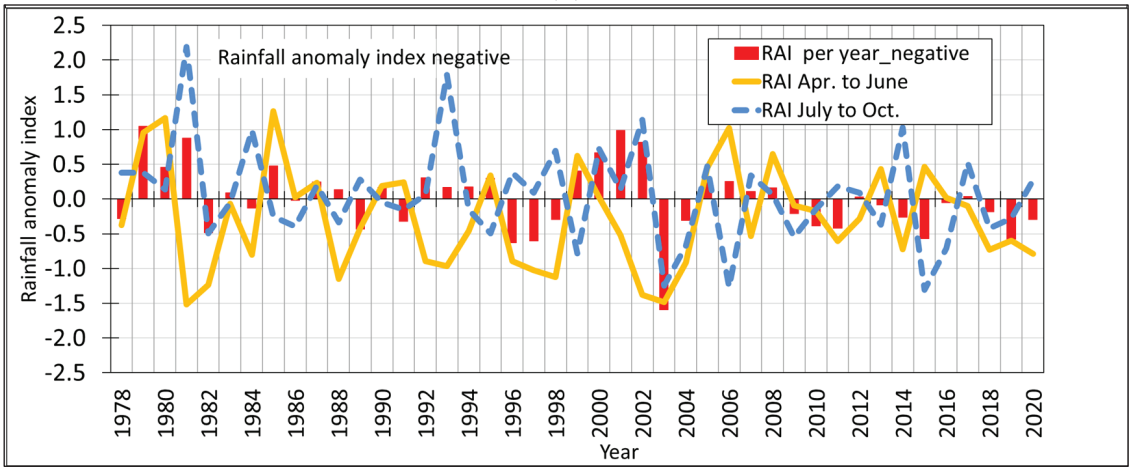


(**a**)

Figure 3. *Cont.*



(**b**)



(**c**)

Figure 3. (**a**) Time course of the mean air temperature of the years 1978 to 2020, multi-annual mean temperature of 1950 to 1979, and yearly precipitation about the multi-annual precipitation (1950 to 1979). (**b**,**c**) Rainfall anomaly indices per year from 1978 to 2020 and for April to June and July to October.

The fact that the decline in yield was not even more pronounced was most likely due to June. In this month, the precipitation level reached the level of the long-term average (1982, 121 mm; 30-year average, 112 mm). This is also evident from the other indices (CPD value, 10.3; MR-DI, 60.3; M-AI, 54.9).

In addition to insufficient rainfall, plant stress may have occurred due to higher temperatures. The described year indicated 38 summer days (with 25 days in June and July) and two hot days with elevated values (Figures 4 and 5).

1987: The moderate yield reduction was caused by severe fluctuations in the winter temperatures. From November, the minimum values oscillated around 0 °C, and on 13°C, the temperature dropped to −26.3 °C. This was the lowest temperature during the whole observation period; plant damage likely occurred here. The prolonged frost meant that the number of frost change days in early 1987 was comparatively low (Figure S17).

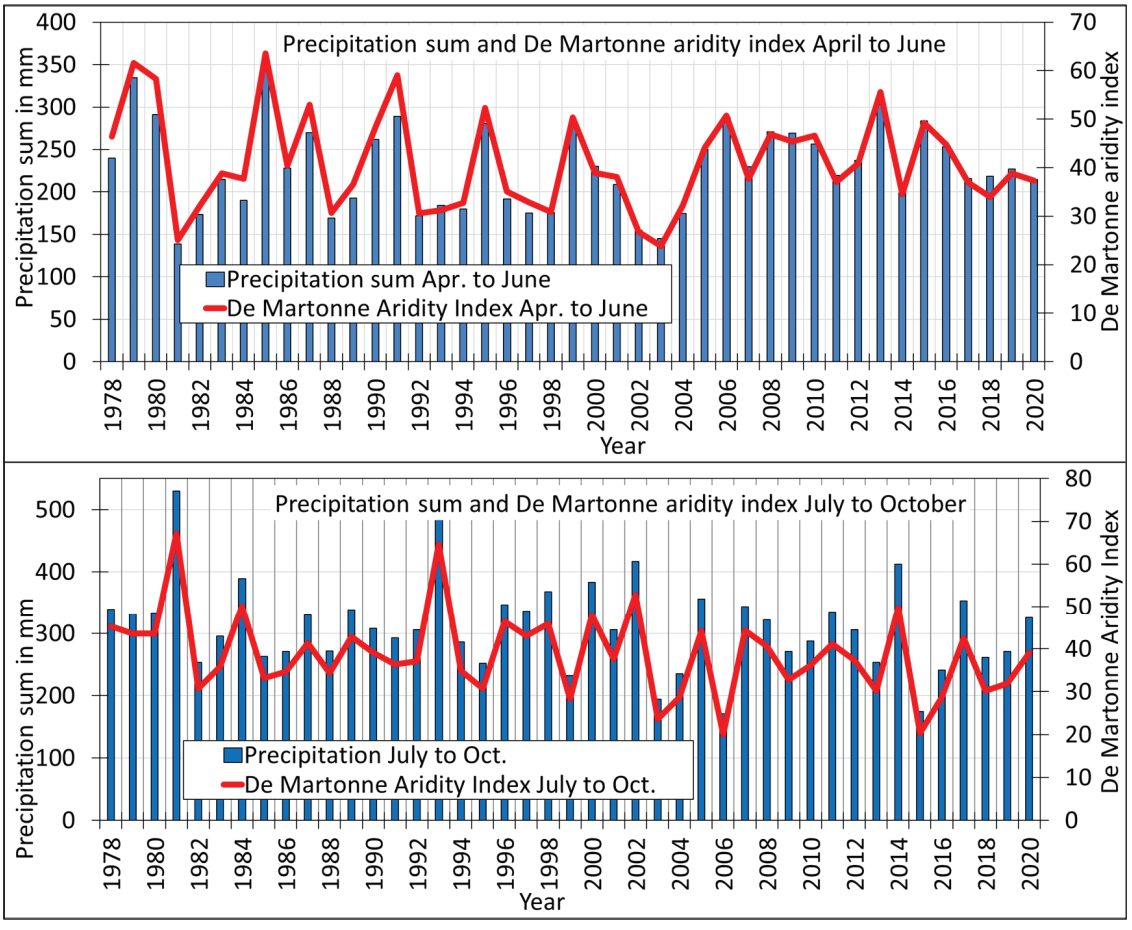


Figure 4. Time course of the precipitation sum and the de Martonne aridity index.

1993: The yield loss was ultimately considerable, with over 12%, thus belonging to the 10% of the worst yield years from 1978 to 2019.

Based on the meteorological data, two main observations were responsible for this decline.

The first five days of this year were characterized by a temperature of >5 °C in the second week. In the whole measurement period, this was the earliest beginning of the vegetation period. However, until April, 66 frost days, 44 frost-alternating days, and two frost shock days followed.

Severe drought-related crop failures had already occurred previously in 1993 when the entire first half of the year was characterized by precipitation deficits (January–May, 112 mm; January–June, 193 mm; 30-year average January–May, 268 mm; 30-year average January–June, 379 mm). The percent-of-normal precipitation indicated values from January to June of 0.9, 0.22, 0.5, 0.59, 0.83, and 0.73. These observations correspond with the number of rain-free days (January–May, 86 days; January–June, 102 days).

This is also evident from other indices from April to June (CPD values, −21.4, −14.5, and −30.3; MR-DI, 20.2, 7.0, and 61.0; M-AI, 18.4, 34.8, and 38.1) (Figures 4, 5 and S9).

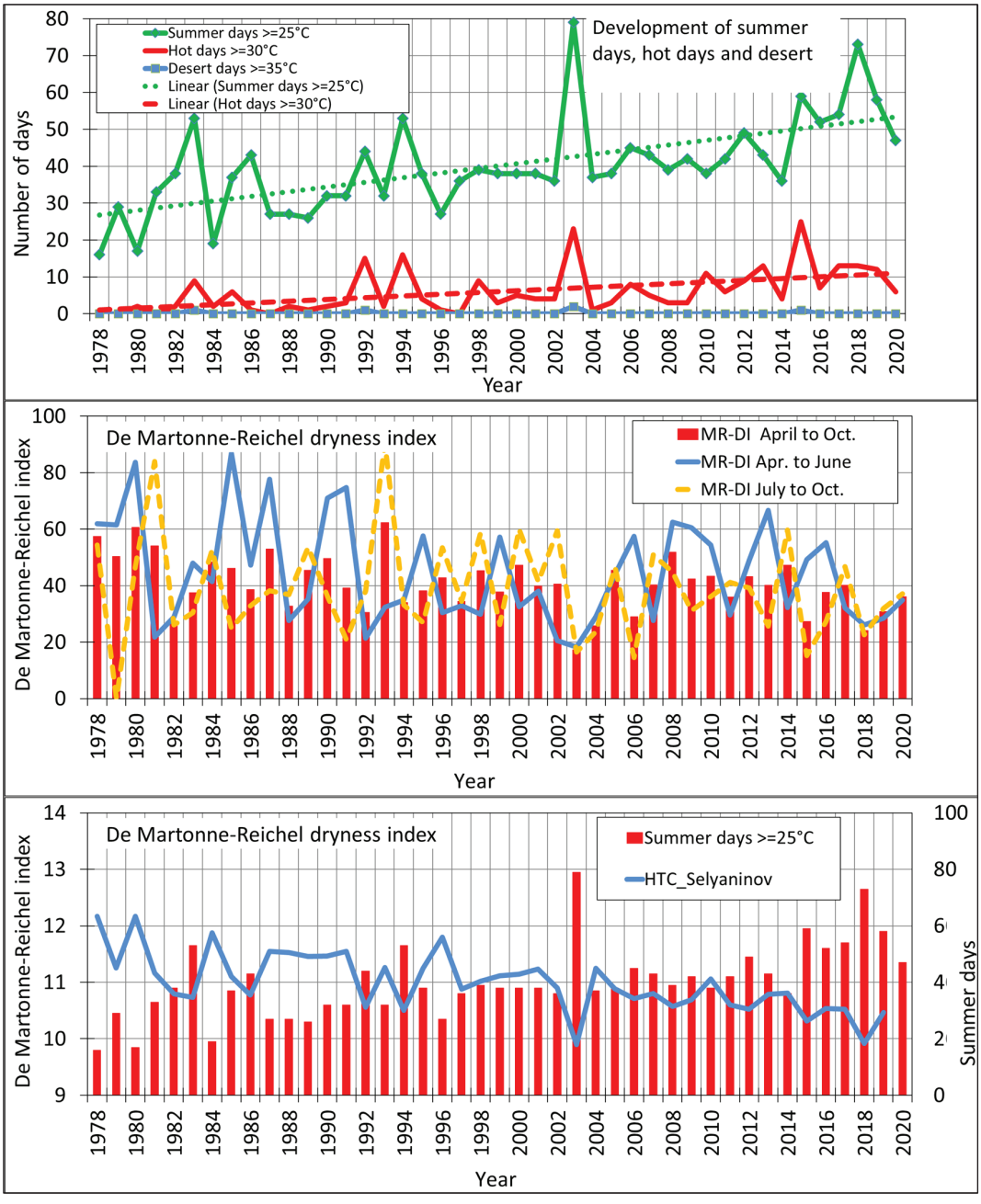


Figure 5. Time course of the de Martonne–Reichel dryness index, summer days, hot days, desert days, and HTC.

1995: The comparatively low yield reduction was likely caused by severe fluctuations in the temperature during the spring of this year. The temperature dropped on 15.05.1995 to a level of −1.3 °C at an altitude of 2 m after four weeks, with temperatures up to

11.3 °C. The last negative temperatures were recorded on 14 April, with −1.0 °C. This late frost event damaged the rapid plant development and caused a lower yield.

Additionally, a negative effect of the high precipitation in June during the phase of grain filling is imaginable. The total June precipitation was 153 mm, which means a CPD of 41.92 and a P_m%-normal of 1.38. Figure S5 indicates that the precipitation was more or less evenly distributed over the entire period. Wollmer et al. [12] and Marti et al. [29] described the influence of temporary waterlogging and wheat development. Especially during grain filling (June), waterlogging leads to premature leaf senescence, smaller grains, and a lower grain number.

2003: A very significant extreme weather period for the vegetation occurred in the year 2003, which, in comparison with the 30-year means, was too dry in February to April and June to September. Only the month of May showed a normal level of precipitation (Figure S2). Because the last five months of the preceding year (2002) were very rainy, the impact on vegetation was probably somewhat mitigated (Figure S5).

Low precipitation can also be seen in the corresponding indices (CPD, percent-of-normal, precipitation-free days and pentads, meteorological dry periods, number of summer days, and hot days).

In June, which is important for flowering and grain filling, approximately 19 summer days and 5 hot days were registered. With a mean air temperature of 20.26 °C, it was the warmest June since weather records began at the Weihenstephan-Dürnast site. In addition, only 38 mm of precipitation occurred. The unusual meteorological dry period could be documented using the de Martonne–Reichel aridity index, which showed a value of only 13.6. The very low $RAI_{negative}$ value of −3.06 is also an indicator that the month was too dry (Figure 3).

A cumulative precipitation deficit of −82.1 mm had already built up between February and April. From April to June, only 145 mm of precipitation occurred, which was approximately 58% of the usual amount of precipitation according to the 1950–1979 climatological mean. In the wake of high temperatures, the Martonne drought index was 23.8 during the period, lower than in any other year between 1978 and 2019 in the same time interval.

It can thus be assumed that a large proportion of the winter wheat stands suffered from water stress during June. This was reflected in the yield pattern, which was approximately 10% lower than expected. Since the decline was outside the 25th percentile and just above the 10th percentile, it can be considered a significant but not extreme loss. Because vegetative growth was almost complete at the beginning of the heatwave, the drought-related decline was thus less severe than for summer crops [31]. Nevertheless, the numerous days above 25 °C or above 30 °C from the beginning of June onward, precisely at the time of flowering and grain formation, led to a considerable proportion of the crop losses.

2006: This vegetation year showed strongly changing weather conditions. An extremely mild second half of October and the first half of November in 2005 likely promoted the development of infection.

In the long cold winter of 2005/2006, a persistent snow cover occurred, which repeatedly thawed and subsequently froze due to repeated severe frosts. In some places, the snow cover reached a record height of up to 50 cm for the northern foothills of the Alps, in early March 2006. In addition to an increasing lack of air under the hardened snow cover, the yield losses this year were likely to have been caused by increased snow mold infestation (*Gerlachia nivale* L.) in unfavorable areas [32]. This is the most important wintering disease of winter cereals, often originating from infected crop residues [33]. Particularly favorable infection opportunities are already offered by well-developed stands in the fall [31].

The growing period in 2006 started with unusually high precipitation in March and April. This filled possible deficits in the soil water reservoir.

The entire summer was characterized by very low precipitation. In particular, July was too dry, with 19.5 mm and only 19% of the long-term average of the years 1950–1979 (Figure S5). In addition, the highest temperature since records began was recorded for a July month at 21.06 °C. This combination resulted in a very low MR-DI of only 5.3. It should

be noted that precipitation deficits already occurred in May and June, which ultimately added up to approximately 110 mm by the end of July [21].

Between July and October, the MR-DI fell to 20, the lowest value in the entire observation period (Figure S5), although this was mainly due to the exceptionally dry and hot July. In addition, only 170 mm of precipitation fell in these four months, which was the lowest between 1978 and 2020.

The climate indices reflect these conditions well (CPD, percent-of-normal, RAI, and HTC).

Despite the drought, which can be classified as extreme, especially in July but also in May and June, the reduction in the observed yield was only comparably weak. This could have been because the spring precipitation prevented a sharper decline.

2009: The 2009 growth period was characterized by several negative impacts. The autumn of 2008 and the following winter were already too dry overall. At 11.82 °C, April was the warmest month since weather records began. The greatest damage was caused by a violent thunderstorm in the district of Freising with hailstones up to 3 cm in size (May 26). The northern parts of the district were particularly affected, with complete crop destruction as a result of the storm [34]. At the Weihenstephan-Dürnast weather station, 26.5 mm of precipitation was measured within one hour. However, as this was a local thunderstorm cell, the amount of precipitation was likely to have been much greater in some parts of the district. The other districts surrounding Freising were less affected by this storm, but here, the yields were also lower. With only 55.8 dt/ha in the Freising district, almost 16 dt/ha less winter wheat was harvested than was expected from the smoothed forecast values. The 10th percentile was again clearly undercut with the largest negative deviation in the observation period. According to Weigand (2014), hail can not only destroy entire plant stands in a short time, but the numerous wounds also favor fungal secondary infections, even in the case of small hailstones.

2010: Significant yield losses, although not quite as high as in 2009, were also recorded for 2010. The beginning of the main vegetation period was characterized by a drought in January to April. This was followed by a very wet period from May to mid-June. Around 230 mm of precipitation occurred within these six weeks. At the same time, the temperatures rose significantly in June. According to local media, numerous fungal infections occurred during this period [35]. According to Hatfield et al. [36], very humid and warm conditions, especially in May and June, cause an increased risk of infestation by plant pathogens in wheat. According to Jahn et al. [37], the most important disease for cultivated winter wheat in Germany, namely, Septoria leaf drought, as well as brown rust, may have spread as a result of the warm and humid conditions. The fungus Septoria tritici causes oval spots on the leaves and causes, on average, the highest yield loss of 7 dt/ha and peak losses of up to 30% [35]. Brown rust (*Puccinia triticina*) shows a similar disease pattern with the formation of oval, brown summer spore deposits and an average yield loss of 2.5 dt/ha [34]. Fusarium infections, such as Fusarium graminearum, may also have been widespread. In partial dew rot, the ear spindle axes are colonized by the fungus. As a result, the water supply is interrupted and the green color of the ear fades to whitish. In the process, the fungus produces, among other things, the Fusarium toxin deoxynivalenol, for which there are strict limits in food processing [38]. If the existing limits are exceeded, the harvested crop cannot be further processed and must be disposed of. According to West et al. [38], drought from autumn to spring can increase the probability of increased pest pressure from Fusarium. Accordingly, the conditions in 2010 were optimal for the strong spread of fungal infections. Also, for this year, the yield deficit was below the 10th percentile value, and thus, extremely high.

2017: In this year, the average yield reductions were around 5 dt/ha. The month of June was the third warmest since the beginning of weather records, with 18.54 °C, and it can therefore be assumed that the high water demand of winter wheat, especially in shallow soils, could not be fully met during this period and that it partly suffered from water stress. In addition, there was an unusually high number of summer days, with

16 days, as well as 3 hot days, associated with the negative effects described above during and in the days around the flowering period (Figure 5).

2018: In this year, the drop in yields due to drought was also pronounced. Thus, already in the late winter (February and March), as well as in the following spring (April, 11.3 mm), there was significantly too low precipitation. Much of the cumulative precipitation deficits of the 2018 growing season are shown in Figures S9 and S11.

May and June again showed normal precipitation compared with the long-term mean, but July and August were again too dry.

The April of 2018 was the warmest April, with 13.1 °C, since the beginning of weather records. The DI dropped to an extremely low value of 4.1, the RAI was −3.7, and the summer mark of 25 °C was exceeded on three days. This was immediately followed by the warmest May since records began (16.25 °C). Due to the two extremely warm months and further above-average temperate weather in the following months, it was the warmest vegetation period from April to October in the entire observation period (Figure S18). This is also shown by the summer days, with 40 days from April to July. The high-temperature totals in spring in particular are likely to have caused plant growth to be too rapid, to the detriment of the grain size and number, thus ultimately leading to lower yields. Over the course of the soil moisture deficit in early summer, the plant availability of nutrients decreased, and fertilization measures were only effective to a limited extent. In addition, the weather, which was also significantly too warm in the further course, accelerated a rapid maturation of the grain, which, in some cases, led to a stunting of the ears and a significant loss of mass in the grain yield (DWD 2018, 2).

Years with high yields: Evaluating years with high yields is much more difficult. These cannot be linked to individual events. Years with particularly good yields were those with adequate and well-distributed precipitation and moderately warm temperatures during the heat-sensitive development stages, such as 1988, 1989, 2012, and 2014. The site-specific water content must be included in the analysis of high-yield years.

3.3. Summary Evaluation of the Meteorological Indices

As per Döring et al. [39], there is no clear standard for evaluating such indices, and thus, several criteria are used:

- Agreement of the indices with yield data;
- Sensitivity of the indices to changes in the input values;
- Efforts to determine the indices.

When looking year-by-year, several indices could be identified that can be used as assessment variables for the annual weather. A visual assessment could, of course, only provide indications of meaningful variables, but explanatory patterns could be discerned in the temporal sequence. On the one hand, these were combined indices, such as the rain factor (RF) after Lang, precipitation (rainfall) anomaly index, de Martonne aridity/humidity index (M-AI), and the hydrothermal Selyaninov coefficient (HTC) (Figure 5). However, the precipitation indices percent-of-normal and the cumulative precipitation deficits/surpluses (CPD) also show parallels to the yield values. Additional indices that were also used as explanations were the summer index and the grassland temperature sum (GT-2).

When looking at the monthly values, the visual comparison also shows an influence of precipitation-free pentads and frost indices (early frost index 1 and late frost index 1).

According to the current state of the evaluation, the question of the most meaningful index for the investigated location cannot be answered. While it may be established that several indices together explain the yield declines, it was not possible to identify one or a few indices.

One reason for this was that none of the indices considered here adequately took into account the amount of water available to plants in the soil. This is important, however, because water stored in the soil can buffer a temporary precipitation deficit. Therefore, any drought index that does not or does not properly account for the amount of water stored in the soil is ultimately flawed.

4. Discussion

The results presented and discussed provide an important basis for the investigation of the question of which weather extremes are of particular importance for arable farming and in which context they lead to particularly high yield losses. Based on this, this overview can also contribute to finding out whether climate change will lead to increased yield variability in the future as a result of more frequent and more intensive occurrences of weather extremes relevant to agriculture. Thus, the time series from 1978 to 2020 was long enough to derive a certain trend development concerning the significance of special weather anomalies.

In general, it should be noted that no fixed percentage decrease or increase in yield could be determined as a result of certain extreme meteorological events. Weather conditions determine decisive components, such as the soil water balance, the development stage, and the degree of hardening of the arable plant at the time of the weather extreme, which is why the reactions of the plants in the respective stress situations can turn out to be completely different. In this respect, a further challenge is to clarify which extreme weather phenomena cause damage and to what extent. This was discussed and classified, but not precisely quantified, taking into account the respective development stages and their demands on climate and soil.

In addition, it should be noted that the entire analysis that was carried out was based on point-by-point weather data from the Weihenstephan-Dürnast weather station. In particular, in the case of locally occurring extreme events, such as violent thunderstorms with very high rainfall amounts in a short time or hailstorms, it must be taken into account that significant deviations could have occurred within the district of Freising.

In the case of winter wheat, heat waves and dry periods played the most important role in yields in the Freising district under consideration. In particular, for the last 20 years or so, there have been frequent spring and summer months with low precipitation and, in some cases, being significantly too warm, such as in 2003 and 2018, which were accompanied by particularly severe crop losses. The climatic conditions were also influential in the parameters of summer and hot days (Figure S5), as well as a lower de Martonne–Reichel dryness index (Figure 5). The same applies to the hydrothermal Selyaninov coefficient, which showed decreasing index values over time from 1978 to 2020, and this indicates increasing dryness (Figure 5). Over the course of climate change, an accumulation of heat and drought is thus to be expected [19,27]. It can therefore be assumed that the district will suffer more frequently from heat and water stress, and thus, be associated with an increased yield risk. According to this, a more frequent occurrence of spring and early summer drought is also to be expected. According to Semenov and Shewry [40], more hot days before and during the wheat flowering period are to be expected, which are associated with considerable yield losses. Accordingly, a greater yield risk is expected in the future, particularly from heat waves and less from dry spells.

Heavy precipitation events with large surface runoff or waterlogging during prolonged precipitation, which occur repeatedly due to the proximity to the Alps, are also of crucial importance for arable farming, especially for the moisture-sensitive maize. An increase in the observed 42 years could not be detected, but such precipitation events cause, in addition to plant damage, major erosion damage, as well as the washing away of nutrients [4]. In this context, according to Kornhuber et al. [41], a decrease in precipitation variability can be expected due to a change in circulation patterns. According to this, certain weather situations in Central Europe manifest themselves over significantly longer periods. The consequences are very wet phases with the danger of waterlogging and flooding due to persistent low-pressure influence, as well as heatwaves and dry periods lasting weeks with long-lasting high-pressure areas. These contrasting weather extremes sometimes follow one another directly, as was particularly the case in 2010. As a result, considerable yield losses are to be expected in some cases. In the district of Freising, a slight increase in the frequency of the occurrence of meteorological dry periods, during which less than 1 mm of precipitation occurred for at least 11 days (mainly 2000–2020),

as well as a decrease in days with precipitation amounts to the vegetation of 2–4.9 mm, could be observed in the period under consideration (Figure S5). Instead, heavy precipitation events are likely to be more frequent. However, the connection was not tested for statistical correlation.

For winter wheat, it can also be assumed that secondary infections caused by plant damage over the course of severe weather events, such as thunderstorms or hailstorms, will occur more frequently in the future [42].

5. Conclusions

A lack of precipitation and/or the presence of high temperatures cause significantly reduced yields in agriculture. To describe and quantify these conditions, so-called meteorological indices are often used in agrometeorological descriptions. There are a variety of such indices, of which in this work, those frequently described in the literature were used.

Using Freising, which has mostly fertile soils, as an example location, the yields of winter wheat were compared with these indices.

The correlations between unusual weather anomalies and yields serve as an important basis for investigating the question of which weather extremes are of particular importance for arable farming and in which context they lead to particularly high yield losses. Based on this, the resulting overview can also contribute to determining whether climate change will lead to increased yield variability in the future as a result of more frequent and more intensive occurrences of weather extremes relevant to agriculture.

Supplementary Materials: The following supporting information can be downloaded from https://www.mdpi.com/article/10.3390/agriculture13101904/s1. Figure S1: Monthly precipitation (observation period covers 1978–2020); Figure S2: Precipitation summed up monthly with daily values (observation period covers 1978–2020, with means of 30 years from 1950 to 1979); Figure S3: Number of days with precipitation intensities of <1 mm, 1–10 mm, and >10 mm per day summarized as monthly values; Figure S4: Number of days with heavy precipitation summarized as monthly values (PI4); Figure S5: Number of days with vegetation-favorable rainfall (2–4.9 mm) summarized as monthly values (PI5); Figure S6: Number of rain-free days and meteorological dry periods summarized as monthly values; Figure S7: Number of days with precipitation-free pentads and rain-free days summarized as monthly values; Figure S8: Precipitation percentage of the normal means over the 30 years calculated for every month; Figure S9: Cumulative precipitation deficits/surpluses calculated for every month; Figure S10: Yearly values of the rain factor after Lang; Figure S11: Temperature-related indices (images are not indicated in the main text); Figure S12: Summer index and sum of the active temperature (April–October); Figure S13: Number of summer days, hot days, and desert days; Figure S14: First week with a temperature < 0 °C and number of days with a temperature <3 °C during the day; Figure S15: Yearly values of the early frost indices 1 and 2; Figure S16: Grassland temperature sum and late frost index yearly values; Figure S17: Frost shock days and frost-alternating days; Figure S18: Annual values of the frost days and ice days; Figure S19: Annual values of the frost severity and frost index per Liu; Figure S20: Beginning and end of the vegetation periods with a mean temperature for >5 days of >5 °C and $\leq$5 °C calculated for every year; Figure S21: Number of days/pentads with means with >5 °C, summarized as annual values (CD1 and CD2); Figure S22: Sum of the temperature daily mean until the value of 200 °C (GT-1) and sum of the temperature daily mean until day 105; Figure S23: Global radiation monthly values; Table S1: Classification of drought conditions using the percent-of-normal approach for monthly and annual values relative to the long-term mean; Table S2: Classification of the monthly RAI values (Van Rooy, 1965, Bernhofer et al., 2015); Table S3: Compilation of all of the climate indices used in this publication.

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Bioactive Compounds of Endemic Medicinal Plants (*Cuphea* spp.) Cultured in Aquaponic Systems: A Short Study

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Abstract: Aquaculture waters can be associated with the modification of the phytochemical profile in plants when they are used for irrigation; thus, Integrated Agri-Aquaculture Systems such as aquaponics represent a strategy to improve the bioactive content of medicinal plants. This study aimed to analyze the effect caused by cultivation using aquaponics on the modification of the content of bioactive compounds such as phenols, flavonoids, and apigenin in *Cuphea hyssopifolia* and *Cuphea cyanea* irrigated with *Cyprinus carpio* waters. The results of each culture method showed unique differences ($p \leq 0.05$) in the concentrations of bioactive compounds and antioxidant activity in *Cuphea* spp. For *C. hyssopifolia* in aquaponics, 76% (61.08 ± 7.2 mg g^{-1} GAEq) of phenols and 50% (5.62 ± 0.5 mg g^{-1} CAEq) of flavonoids were maintained compared to 20% (16.99 ± 0.4 mg g^{-1} GAEq) of phenols and 76.5% (8.19 ± 1.6 mg g^{-1} CAEq) of flavonoids in conventional culture. For *C. cyanea* in aquaponics, 91% (15.36 ± 0.8 mg g^{-1} GAEq) of phenols and 47% (3.52 ± 0.6 mg g^{-1} CAEq) of flavonoids were maintained compared to 24% (14.11 ± 1.3 mg g^{-1} GAEq) of phenols and 82% (1.79 ± 0.1 mg g^{-1} CAEq) of flavonoids in conventional culture. An increase of more than 60% in the apigenin content of *C. hyssopifolia* in aquaponics confirms a eustress effect related to the use of *organically enriched waters*. The results indicate that aquaponics can promote the biostimulation/elicitation of medicinal plants and increase their bioactive compounds, but this effect does not occur in the same way between species.

Keywords: medicinal plants; aquaponics; biostimulation; sustainable food production systems; secondary metabolites

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1. Introduction

Aquaponics is part of a broader area, Integrated Agri-aquaculture Systems (IAAS), in which joins two of the most productive sectors in the field: aquaculture and hydroponics [1]. According to [2,3] vegetables produced in aquaponic systems show greater fruiting than those grown in hydroponics systems. In this way, aquaponics directly and positively impacts some goals of the 2030 agenda, such as zero hunger, good health and well-being, and climate action, because it increases productivity and protein diversification, and decreases waste of nutrients and water [4]. Moreover, aquaponic systems are an alternative for sustainable and organic production because they impact the environment to a lesser extent compared to aquaculture and traditional hydroponics [5]. Aquaculture water contains a wide variety of nutrients such as metabolic waste from fish produced via respiration and found in urine, faeces, and unconsumed dissolved food, dissolved organic molecules (DOM), and microorganisms such as bacteria, fungi, and protozoa [6]. This organically enriched water (OEW) can help activate secondary metabolism, defences,

and plant growth when used as irrigation, and can consequently increase the quality of vegetables by modifying their phytochemical profile [7,8]. According to [9], the antioxidant activity of aquaponic herbal crops (i.e., *Ocimum basilicum* and *Petroselinum crispum*) was significantly higher than that of crops grown organically in soil. [10] found the same effect in lowering blossom-end rot symptoms (BER); this was related to the microorganism and the DOM in the water acting as biostimulants in the crops. Therefore, aquaponics cultivation presents an alternative to biostimulation for enhancing the nutritional value and stress tolerance of species with a high content of bioactive compounds, such as medicinal plants.

Cuphea is a plant genus with approximately 260 species that has a significant role in Mexican ethnopharmacology [11,12] The species in this genus are known due to their content of medium-chain fatty acids in seeds, such as capric, lauric, and myristic acid, a profile comparable to that of *Cocos nucifera* [13,14]. However, the most significant potential is in its content of phytochemicals associated with antimicrobial, antiviral, and cytotoxic activities [15]. Other popular uses range from treating dermatological conditions like skin tumours, pain, inflammation, and wounds, to diarrhoea and stomach infections [16]. One promising species is *Cuphea hyssopifolia*, also called *Falso brezo* (false heather), which is a small shrub that does not reach more than 60 cm and is also commonly used for ornamental purposes [15]. Its content of tannins, flavonoids, and phenols has been described to some extent, along with its cytotoxic and antioxidant activities [17]. Another endemic species of North America, but less described, is *Cuphea cyanea*. It is popular for ornamental purposes as a vine, with flowers resembling Christmas lights, thanks to which it received its colloquial name *Serie de Luz* (light series). Descriptions of its secondary metabolism are limited. Similarly, the use of aquaponic systems to grow herbaceous medicinal plants, including identifying, quantifying, or characterising their phenolic contents and antioxidant activity, is scarce. According to this evidence, this article aims to analyze the effect of an Integrated Agri-aquaculture System with Koi carp (*Cyprinus carpio*) on the content of bioactive compounds such as phenols, flavonoids, and apigenin, and on the antioxidant activity of two medicinal plants, *Cuphea hyssopifolia*, and *Cuphea cyanea*.

2. Materials and Methods

2.1. Experiment Setup

The experiment was carried out in facilities at the Faculty of Engineering, Campus Amazcala of the Universidad Autónoma de Querétaro, and lasted 90 days (that is, 23 days for the acclimatization of the carp within the system, 7 days for acclimatization of both plants species in the IAAS, and the last 60 days for testing the integration of Koi with each medicinal plant in an independent IAAS). The independent aquaponic systems were installed with their controls in conventional cultivation under the same controlled conditions within a 504 m^2 multi-tunnel-type greenhouse. The experiment was set according to a full factorial design with the species and the cultivation method as independent variables. Three biological replicates were processed for the measurements of bioactive compounds, and three technical replicates were performed for each assay. The experimental unit consisted of 12 plants. Table 1A shows the climate conditions of the experiment.

As an initial step towards exploring the biostimulant properties of OEW in the production of biocompounds with IAAS, a vertical closed system was considered. This system was coupled with a nutrient film technique (NFT) unit without a growing medium for each plant species, as it is considered the most efficient hydroponic system [18]. For this approach, in place of a biofilter, biofilms were allowed to form on the available surfaces of plastic tubes including plant roots.

A metallic structure (2 m $\times$ 1 m) was used to support the system, which consisted of three plastic tubes to support 12 plants per level, for a total of 36 plants per system. Two submersible pumps (30 W; 1 Hp; 56 L min^{-1}) were used, one in each IAAS, and they were left on from the beginning until the end of the experiment (90 days). Two fish tanks (40 cm $\times$ 40 cm $\times$ 100 cm) were filled to 160 L each. Oxygenation was carried out by

returning the water to the tank via gravity. The flow within the NFT tubes of the system was 56 cm^3 s^{-1}. The three tubes together returned a total volume of 1.73 cm^3 s^{-1}, which is in the range of tolerance [19]. A 6% daily replacement with fresh water was carried out, according to [20].

Table 1. (**A**) Microclimate conditions of a greenhouse. (**B**) Water conditions in situ in a greenhouse at the Amazcala Experimental Campus. * The value was obtained from outside of the greenhouse.

Variables A	**Data**
Temperature greenhouse (°C)	29.02 ± 9.48
Radiation * (W/m^2)	163.6 ± 42.06
Relative Humidity (%)	53 ± 8.05
Variables B	Data
Temperature in the fish tank (°C)	24.62 ± 5.04
pH	8.95 ± 0.20
Oxygen (mg L^{-1})	7.23 ± 0.88
Conductivity (μS cm^{-1})	248.52 ± 14.22

The electric conductivity (EC), pH, and dissolved oxygen (DO) were monitored twice a week (Table 1B). The DO of the water in the systems was determined with a multiparametric meter HQ40D (RYE-HACH, CDMX, México) with the sensor LDO101-03 (°C and DO) and EC (series-H, °C, and μS cm^{-1}). The pH was measured with the waterproof pH tester 10 sensor (Thermo Fisher Scientific Inc., EUTECH, CDMX, México). An initial analysis of the water quality (ammonium, nitrate, nitrite, phosphorus, and potassium) was carried out with a DR/6000 spectrophotometer (RYE-HACH, CDMX, México) using the Hach 380 N method. The 23-day baseline for recirculation is described in Table 2.

Table 2. Nutrients in the water of the 23-days trial recirculation (hydraulic test) in an Integrated Agri-aquaculture Systems (IAAS) in a greenhouse at the Amazcala Experimental Campus; baseline fish water quality before the integration of Koi carp–*Cuphea* spp. * Concentrations in the aquatic phase.

Water Quality (mg L^{-1}) *	**Min**	**Max**
NH_4-N	0.15	1.00
NO_3-N	5.00	22.0
NO_2-N	0.04	0.045
PO_4-P	0.50	3.00
SO_4-S	1.50	24.0
Ca	10.0	90.5
Mg	11.0	41.0
Cl	0.02	0.024
K	26.0	28.0

2.1.1. Aquatic Species

An ornamental species at a juvenile stage, *Cyprinus carpio* L. var. Koi was obtained from a local provider. Commercial food was used with crude protein 31.0%, crude fat 5.0%, crude fibre 2.0%, moisture 7.0%, phosphorous 0.9%, and ascorbic acid (Vit. C) 100 mg kg^{-1}. The Koi carps were fed at a daily rate of 4% of the total biomass of each tank (78 g) divided into two servings per day described by [21].

2.1.2. Plant Species

The plants of *C. hyssopifolia* and *C. cyanea* were obtained from the greenhouse Red Viverista in Cuernavaca Morelos, México from the same batch. MSc Yolanda Pantoja carried out the authentication of the species endorsed by Dr Luis Hernandez-Sandoval, herbal curator, in the QMEX herbarium of the Faculty of Natural Sciences of the Universidad

Autónoma de Querétaro, México. These species are not listed under Official Mexican Standard NOM-059-SEMARNAT-2010; (Available online https://www.profepa.gob.mx (accessed on 8 August 2023)) as threatened or subject to special protection. The authentication code for *Cuphea hyssopifolia* Kunt (Figure 1A) was 00006843 (see Supplementary Materials). The authentication code for *Cuphea cyanea* Moc. and Sessé ex DC (Figure 1B) was 00006847 (see Supplementary Materials). The collection of plant material and the performance of experimental research on such plants complied with the national guidelines of México in the standards NOM-003-STPS-1999 and NOM-007-STPS-2000; (https://www.stps.gob.mx (accessed on 8 August 2023)).

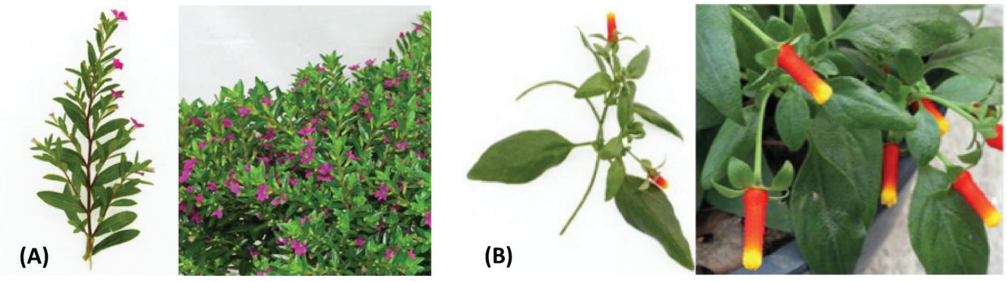

Figure 1. (**A**) *Cuphea hyssopifolia* Kunt; (**B**) *Cuphea cyanea* Moc. and Sessé ex DC; collection date February 2016, species acquired from "Red viverista" Located in Cuernavaca Morelos, Mexico (No collection number).

To integrate fish and plants into the system, 36 plants of each species were taken out of their transport pots and carefully transplanted, with their substrate removed, into 12 plastic tubes. Another 36 plants of each species were kept in their original pots, along with the substrate, which is a mixture of ground dry leaves that helps to maintain plant moisture without the need for soil.

For the *Cuphea* ssp. growth performance, maximum branch height and leaf area index were measured based on the methodology of [22]. The maximum branch height was recorded for time zero (T0) when the plants arrived; a second measurement was made one month before, another measurement was made at the beginning of April upon integration with the carp, and the last one was recorded at the end point of the trial time. The determination of the leaf area index was carried out as follows: for *C. hyssopifolia*, 5 leaves from 5 branches were measured randomly from the apex, in the middle, and at the end; for *C. cyanea*, 15 leaves from 2 branches were measured randomly from the apex, in the middle, and at the end.

2.2. Samples and Treatment of Cuphea spp.

2.2.1. Pre-Treatment of Samples and Monitoring

Sampling was carried out twice, in February (T0) and at the end point (April 2016), and maintaining the original proportion of the plant, the leaf, flower and dry stem were collected. Samples were collected randomly from the apex, in the middle, and at the end of each species. Once the samples were collected, they were weighed and placed in paper bags in an oven at 35 °C for four days. After grinding on a sieve with a 20 mm mesh opening, 500 mg was taken from here for extraction, and the rest was stored in amber plastic bottles at room temperature without exposure to light. Dry samples (500 mg) were added to 5 mL of a solvent mixture containing 80% methanol, 18% distilled water, and 2% formic acid. After 30 s of vortex agitation, the extracts were sonicated for 30 min at room temperature and centrifuged at 8500 rpm for 15 min at 4 °C. The supernatant was recovered, and 5 mL of the solvent mixture was added to the remaining pellet. It was stirred for 30 s in a vortex, sonicated for 30 min at room temperature, and centrifuged at 8500 rpm for 15 min. The

supernatant was recovered with the above. Once together, the final volume of the extracts was measured, filtered in an acrodisc then used in all determinations.

2.2.2. Determination of Total Phenolic Compounds

Total phenols were determined using the Folin–Ciocalteu colourimetry method [23], using gallic acid as standard and a 10-point calibration curve. In 2 mL tubes, 100 µL of the extract was added, 400 µL of the solvent (80% methanol + 20% distilled water) with 250 µL of Folin–Ciocalteu reagent (1 N), and after 5 min, 1.25 mL of Na_2CO_3 was added to neutralize. The samples were incubated for 2 h without stirring out of the reach of light, and then measured. Absorbance was measured at 765 nm using a Spectra Max reader (Molecular Devices Co., Sunnyvale, CA, USA). Concentrations are expressed as milligrams of gallic acid equivalents per g of dry weight of extract (mg g^{-1} GAEq DW). All assays were performed in triplicate in 2 mL Eppendorf tubes (Eppendorf North America, Inc., Enfield, CT, USA).

2.2.3. Determination of Total Flavonoids

Total flavonoids were determined according to the method proposed by Brand-Williams et al. (1995) [24], with catechin as standard and a 6-point calibration curve. A volume of 300 µL of the standard/extract + 120 µL of distilled water + 90 µL of a 5% $NaNO_2$ solution, and, after 5 min, 90 µL of 10% $AlCl_3.6H_2O$ was added and allowed to stand for 6 min. Afterwards, 600 µL of NaOH (1 M) was added, and the volume was increased to 2.5 mL using distilled water. The solution was mixed, and the absorbance was measured at 510 nm using a Spectra Max reader (Molecular Devices Co., Sunnyvale, CA, USA). Concentrations are expressed as milligrams of catechin equivalents per g of dry weight of extract (mg g^{-1} CAEq DW).

2.2.4. Determination of Antioxidant Activity,1-Diphenyl-2-picrylhydrazyl Radical (DPPH) Inhibition Assay

Determination of the antioxidant activity was carried out via the DPPH method [25] using DPPH (1,1-diphenyl-2-picrylhidrazil) reagent with methanol. Aliquots of 1.865 mL of the reagent were placed in 2 mL microtubes along with 0.135 mL of the methanolic extract of each sample. The mix was allowed to stand for 30 min, protected from light. Trolox was used for the 7-point calibration curve, and the reading was performed at a wavelength of 480 nm. The results were expressed as milligrams of Trolox equivalents per g of dry weight (mg g^{-1} TEq DW).

2.2.5. Determination of Antioxidant Activity Ferric-Reducing/Antioxidant Power (FRAP) Assay

To determine the antioxidant activity using the FRAP method [26], the reagent was prepared with a mixture of a 20 mM solution of iron trichloride ($FeCl_3$), acetate buffer with anhydrous sodium acetate, and sodium acetate trihydrate at pH 3.7. Finally, TPTZ (2,4,6-tripyridyl-2-triazine) was prepared at 10 mM dissolved in 40 mM HCL. A mix of 1.865 mL of the FRAP reagent and 0.135 mL of the methanolic extract of the samples were placed in 2 mL microtubes and allowed to react for 30 min, protected from light. Trolox was used for the 7-point calibration curve. The absorbance was read at 630 nm. The results were expressed as mg g^{-1} TEq DW.

2.2.6. Ultra-Performance Convergence Chromatography

Extraction for Identification and Quantification

For the analysis of phenolic compounds, 200 mg of dry and finely ground samples were weighed, and then 1 mL of methanol (HPLC grade) was added to each and stirred in a vortex for 30 s. Subsequently, the samples were placed in an ultrasonic chamber for 30 min at RT and protected from light. After this time, the samples were centrifuged at 9500 rpm for 5 min. The supernatant was recovered, and the solid residue was subjected

to the same extraction procedure four consecutive times. Finally, the supernatants were pooled, and the total volume was increased to 5 mL. The extract obtained was filtered with an acrodisc and stored in amber vials at −20 °C until analysis.

Analysis of Phenolic Compounds

The analysis of phenolic compounds was carried out via convergence chromatography (UPC^2: ultra-performance convergence chromatography). A Waters System HPLC chromatograph (Waters Corporation, Milford, MA, USA) was used, which consists of a quaternary pump, a diode array detector (model 996), an online vacuum degasser (MetaChem Technologies Inc., Freisenbergstraße, Germany) and a Rheodyne injector (4793). The control of the equipment, the process, and the management of the chromatographic information was carried out with the Millennium program (Waters). The previously prepared samples were injected into the UPC^2 according to analysis conditions to determine their chromatographic profiles (Table 3). Subsequently, the standard (Sigma-Aldrich, Productos Químicos del Sur, CDMX, México; purity $\geq$ 95%) apigenin, kaempferol, catechin, quercetin, caffeic acid, and p-coumaric acid were injected to decide the retention time and obtain their UV spectra. The retention times and UV spectra of the different peaks in the samples were compared with those of the standards. Coincident peaks were subjected to co-elution to confirm the correspondence of the compounds.

Table 3. Method for detecting the phenolic compounds of *Cuphea* spp. with UPC^2. The conditions in which the samples were introduced are as follows: injection volume: 10 µL, flow rate: 1.5 mL min^{-1}, column: Viridis BEH 5 µm, 4.6 × 100 mm, column temperature: 40 °C, ABPR: 1500 psi. * CO_2 Coleman grade.

Time (Min)	* CO_2 (%)	Methanol (%)
0	95	5
8	70	30
9	70	30
10	95	5
11	95	5

2.3. Statistical Analysis

The results are reported as the mean ± standard deviation (SD). A one-way ANOVA and a Tukey means comparison test ($p \leq 0.05$) were performed. Additionally, a multifactorial ANOVA was performed (see Supplementary Materials) for all the biochemical variables, and interaction graphics produced using the Statgraphics Centurion v. 19 software.

3. Results

3.1. Integrated Agri-Aquaculture System Performance

Per guidelines described by Palm et al. (2018) [1], due to its size, the system enters the first category of IAAS—aquaponics ($\leq$50 m^2), and according to its design, it can be used domestically, recreationally, or in a backyard. For this domestic vertical aquaponic system, the leakage and flow, drainage, and sedimentation tests of solids without aquatic organisms lasted 15 days (before the time zero, or T0). In the following 23 days, the aquatic organism was introduced, and tests related to the accumulation of food were carried out. The flow within the NFT tubes (56 cm^3 s^{-1}) and the return of water to the fish tank resulted in oxygen levels above 7 mg L^{-1}, an adequate level for the carp. Similarly, the 1-inch hoses used to recirculate water showed no blockages due to solids and sediment. The pump had enough power to carry water to the 36 plants on all three levels. No fish mortality occurred during the experiment.

3.1.1. Water Quality in the Integrated Agri-Aquaculture System

Water temperature, pH, DO, and EC concentrations varied between 17–32 °C, 8.6–9.3, 6–8.5 mg L^{-1}, and 225–280 µS $cm^{-1,}$ respectively. The average water hardness values in

the fish tank at the beginning were 50–90 mg L^{-1} of Ca, 10–30 mg L^{-1} of Mg, 30–35 mg L^{-1} of K; and for Cl, PO_4-P and SO_4-S, the values were <0.5 mg L^{-1} for each. However, once the 30-day test ended (only fishes in tanks) and the medicinal plants were added to the NFT tubes, the water showed a different nutrient dynamic. In the IAAS with *C. hyssopifolia* (IAAS-H), the K concentration decreased by almost 80%. At the same time, the Mg was almost three times higher, the SO_4-S was 3.7 times higher, the PO_4-P was four times higher, and the NO_3-N was the highest, with 6.5 times more concentration. The Cl, NH_4-N, and NO_2-N remained at <0.2 mg L^{-1}. At the end of the trial time, the released rate (mg L^{-1}) of nutrients in the water derived from fish feeding was in the following decreasing order: NO_3-N (130) > Mg (90) > SO_4-S (64) > PO_4-P (9) > K (2) > Cl (0.28) > NH_4-N (0.1) > NO_2-N (0.04).

For the IAAS with *C. cyanea* (IAAS-C), the concentration of K, Mg, Cl, NH_4-N, and NO_2-N had similar values to IAAS-H. At the same time, PO_4-P (1.58 mg L^{-1}) remained constant over time, and the SO_4-S decreased slightly (15–11 mg L^{-1}), whereas NO_3-N did not behave the same, dropping by 70%. At the end of the trial time, the released rate (mg L^{-1}) of nutrients for IAAS-C was Mg (80) > NO_3-N (6) > SO_4-S (11) > PO_4-P (1.50) ≈ K (1.58) > Cl (0.23) > NH_4-N (0.1) > NO_2-N (0.008).

3.1.2. Growth and Development of *Cuphea* spp.

There were no significant differences in the growth of the species in both IAAS-H and IAAS-C and each of their controls, conventional *C. hyssopifolia* cultivation (CCH) and conventional *C. cyanea* cultivation (CCC), respectively. Regarding the percentage humidity in the greenhouse, the samples from IAAS-H showed 59.67% humidity at the beginning of the test, while once the experiment was finished, this was 43.26%. The IAAS-C samples had 81.31% humidity at the beginning of the test, while at the end of the test period, they only had 51.92% humidity. According to descriptions of the geographical zones in which *Cuphea* grows, the temperature (29.02 ± 9.48 °C) and humidity (53 ± 8.05%) within the protected system were at their tolerable limits [11]. Table 4 shows the results observed for the length (cm) of their branches and their leaf index area (cm). The multifactorial ANOVA for growth variables showed no statistically significant interactions between species and cultivation method ($p = 0.59$). No statistical differences were found in the simple main effects of the cultivation method on growth performance during the trial period. For interaction graphics, tables, and the dynamics of the IAAS inside the greenhouse, see Supplementary Materials.

Table 4. Growth and development of *Cuphea* spp. measured at the time zero (T0) in Integrated Agri-aquaculture Systems with *C. hyssopifolia* (IAAS-H), and in Integrated Agri-aquaculture Systems with *C. cyanea* (IAAS-H), with its controls, Conventional *C. hyssopifolia* cultivation (CCH), and Conventional *C. cyanea* cultivation (CCC), respectively, at the final trial time. Data are means ± standard deviation for five replicates for each system in each level of NFT tubes. Different letters indicate statistically significant differences according to a multiple comparisons test (Tukey, $p < 0.05$).

Plant Species		Maximum Branch Height	Leaf Area
C. hyssopifolia [b]	T0	29.71 ± 8.1	1.42 ± 0.7
	IAAS-H	39.98 ± 10.3	1.86 ± 0.5
	CCH	30.05 ± 5.2	1.87 ± 0.2
C. cyanea [a]	T0	77.76 ± 40.1	19.16 ± 1.4
	IAAS-C	69.78 ± 32.9	22.07 ± 3.8
	CCC	65.04 ± 19.7	23.74 ± 8.5

3.2. Bioactive Compounds

The cultivation method generated unique differences in the concentration of bioactive compounds and the antioxidant activity in *Cuphea* spp. (Table 5). A multifactorial ANOVA revealed significant interactions between factors (species*cultivation method) for the total phenolic, flavonoids, and apigenin content as well as for DPPH ($p = 0.00$). Significant main

effects were observed for both factors (species and cultivation method) (p = 0.00) (Table 6). A simple main effects analysis showed statistically significant differences for all variables (p = 0.00) in relation to the cultivation method (Table 5, Figure 2). For *C. hyssopifolia* in its acclimatization stage (T0), the compound contents were 80.39 ± 9.9 mg g^{-1} GAEq and 10.71 ± 1.0 mg g^{-1} CAEq for phenolic and flavonoids, respectively. At the end of the trial period, approximately 76% of phenols and 50% of flavonoids remained in the dry basis of the plant cultivated in IAAS-H, with 20% of phenolics and 76.5% of flavonoids in CCH. For *C. cyanea* metabolites, their content at T0 was 17.06 ± 0.8 mg g^{-1} GAEq and 7.45 ± 0.8 mg g^{-1} CAEq for phenolic and flavonoids, respectively. At the end of the trial, 91% of phenols and 47% of flavonoids remained in IAAS-C, while 24% of phenolics and 82% of flavonoids remained in the dry basis of the plant cultivated in CCC. The antioxidant capacity in the methanol extract in *C. hyssopifolia* showed significant differences from T0 to the end of trial time, with higher antioxidant capacity; however, in *C. cyanea,* an increase of 4.11 mg g^{-1} TEq in the dry basis was observed, and remained in the integrated system.

Table 5. Bioactive compounds and antioxidant capacity of *Cuphea* spp. Concentrations are expressed as milligrams of gallic acid equivalents per g of dry weight of extract (mg g^{-1} GAEq DW), milligrams of catechin equivalents per g of dry weight of extract (mg g^{-1} CAEq DW), and milligrams of Trolox equivalents per g of dry weight (mg g^{-1} TEq DW) for phenolic, flavonoids, DPPH and FRAP, respectively. Data are presented as means ± standard deviation. Different letters indicate statistically significant differences for main and simple main effects (p = 0.00).

	C. hyssopifolia [a]			*C. cyanea* [b]		
	T0	**IAAS-H**	**CCH**	**T0**	**IAAS-C**	**CCC**
Total phenolic content (mg g^{-1} GAEq)	80.39 ± 9.9 [a]	61.08 ± 7.2 [b]	16.99 ± 0.4 [c]	17.06 ± 0.8 [a]	15.36 ± 0.8 [b]	14.11 ± 1.3 [c]
Total flavonoid content (mg g^{-1} CAEq)	10.71 ± 1.0 [a]	5.62 ± 0.5 [c]	8.19 ± 1.6 [b]	7.456 ± 0.8 [a]	3.52 ± 0.6 [b]	1.79 ± 0.1 [c]
DPPH (mg g^{-1} TEq DW)	125.73 ± 3.4 [a]	114.82 ± 6.0 [b]	96.92 ± 12.1 [c]	11.05 ± 0.9 [b]	15.16 ± 0.5 [a]	7.22 ± 0.6 [c]
FRAP (mg g^{-1} TEq DW)	133.05 ± 9.0 [a]	134.53 ± 14.1 [a]	114.878 ± 16.3 [b]	13.34 ± 0.9 [a]	13.35 ± 1.2 [a]	7.37 ± 0.4 [b]

Table 6. Cultivation methods' main effects. Data are shown as the least-squares means ± least-squares sigma estimated from a multifactorial ANOVA of the original data. Different letters indicate statistically significant differences according to a Tukey multiple comparisons test ($p \leq 0.05$).

Cultivation Method	**Total Phenolic Content (mg g^{-1} GAEq)**	**Total Flavonoid Content (mg g^{-1} CAEq)**	**Apigenin (mg g^{-1})**	**DPPH (mg g^{-1} TEq DW)**	**FRAP (mg g^{-1} TEq DW)**
T0	48.7 ± 1.2 [a]	9.08 ± 0.2 [a]	1.63 ± 0.01 [a]	68.39 ± 1.3 [a]	73.93 ± 2.3 [a]
IAA	38.2 ± 1.2 [b]	4.99 ± 0.2 [b]	1.26 ± 0.01 [b]	64.99 ± 1.3 [a]	73.19 ± 2.3 [a]
CC	15.5 ± 1.2 [c]	4.56 ± 0.2 [b]	0.05 ± 0.01 [c]	52.06 ± 1.3 [b]	61.12 ± 2.3 [b]

It should be noted that for both species, their values in apigenin concentrations (Figure 2) were closer at the beginning of the experiment, this not being the case for their contents of phenols and total flavonoids. The results show significant differences in the concentration of apigenin between treatments. At the beginning of the experiment, the apigenin content in *C. hyssopifolia* was 1.06 mg g^{-1}, whilst at the end of the trial period, the content in leaves from IAAS-H increased more than 60% (1.63 mg g^{-1}), and its CCH concentration decreased by about a 93% (0.10 mg g^{-1}). Regarding *C cyanea*, the apigenin concentration started at 2.2 mg g^{-1}, and by the end of the trial, it had decreased by around 40% (0.89 mg g^{-1}). CCC decreased in concentration by 97% (0.0067 mg g^{-1}).

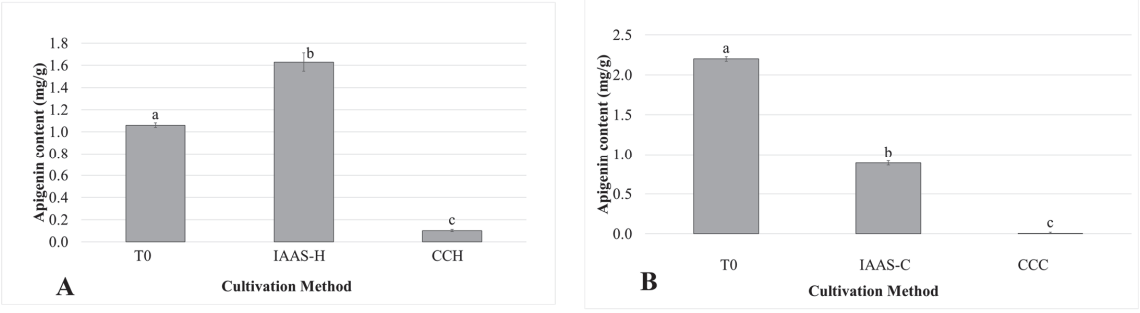

Figure 2. Simple main effects on the apigenin content of (**A**) *Cuphea hyssopifolia* and (**B**) *Cuphea cyanea* due to cultivation method at time zero (T0) and in Integrated Agri-aquaculture Systems with *C. hyssopifolia* (IAAS-H) and in Integrated Agri-aquaculture Systems with *C. cyanea* (IAAS-C), with their controls, Conventional *C. hyssopifolia* cultivation (CCH) and Conventional *C. cyanea* cultivation (CCC), respectively, at the end of the trial time. Bars represent the mean ± SD for three replicates for each system. Different letters indicate significant statistical differences according to Tukey's test ($p < 0.05$).

4. Discussion

This short study aimed to analyze the effect of an Integrated Agri-aquaculture Systems on the content of bioactive compounds in the medicinal plants *Cuphea hyssopifolia* and *Cuphea cyanea*. The content of water in *C. hyssopifolia* changed around 16% from time zero to the end, and the water content of *C. cyanea* decreased by nearly 30% from time zero to the last day. According to Graham (1994) [27], *C. hyssopifolia* has a root system of a short primary root and many lateral roots of equal thickness, and the tertiary root is fibrous; while *C. cyanea* has not been fully described in terms of its root system, we observed that it is less fibrous and abundant. *C. cyanea* needed manipulation during the first week of the experiment, because its long and creeping leaves moved the roots out of the NFT tube; after this period, and together with the sediment that accumulated in the roots, it could be kept in place. At the end of the experiment, we observed the death of flowers, then of leaves, and at the end, of complete branches; however, we did not observe the death of the complete plant. Some similar problems were also reported by Abdel-Rahim (2019) [28], where of the four medicinal plants that were produced, only mint and rosemary survived until the end of the trial period. This effect was associated with a gel-like rot of the roots in thyme and marjoram due to the sedimentation of fish faeces that ended up covering the roots. In this study, we observed this effect only in certain parts of the roots of *C. hyssopifolia*. Whereas the other roots looked healthy, those of *C. cyanea,* which had more parts with this gel, were not. This is probably because the method used was NFT, which allowed the roots to form new shoots in the air. At the end of the trial test, only *C. hyssopifolia* showed "full bloom" (floral and leaf growth), as described by Berti et al. (2008) [29], while for the use of *C. cyanea,* a design within IAAS should be reconsidered, either in its use with an inert substrate or with another aquaculture species. Because of the limitations in studies growing this native species with different media, methods, and nutrient solutions, it is not possible to directly compare the effect of IAAS on *Cuphea* spp. Yang et al. (2019) [30] reported a positive effect of integrated systems on basil growth, but the overall growth of basil is different from these species. The species utilized in this study are medicinal and recognised by local herbalists for use as medications, as an insecticide, and for treating sore throats [15]. These species also possess specific activities, such as an antitumor effect on human promyelocytic leukemia (HL-60 cells) [31], and on the ability to decrease effects on lipid peroxidation due to paracetamol-induced hepatoxicity [32]. In their study, Flanigan and Niemeyer (2014) [33] describe that variety affects the composition of the bioactive compounds, and Oladimeji et al. (2020) [6] reported that using an inert substrate as a culture medium

modifies water quality (and therefore nutrient accumulation and secondary metabolite contents). While this aspect needs further investigation, it suggests that *C. cyanea* can grow in aquaponic culture if some variables are modified, such as the aquatic species, the use of some inert substrate as a support medium for the roots, water temperature, etc. It is possible that the decreased growth and death of *C. cyanea* occurred due to a distressing effect; it may be that the roots cannot tolerate being in constant contact with OEW, or this variety cannot accept nitrogen in the same way that *C. hyssopifolia* can.

Polyphenols in plants and their antioxidant activity are beneficial for human health. Nevertheless, in plants, these compounds are a part of the defensive response to stress. This observation may result from the fact that phenylpropanoids are secondary metabolites related to the activation of plant stress and defence [34] and have been shown to have protective functions against oxidative stress [35]. The cultivation method, that is, the integration into IAAS, showed differences in the concentration of the phytochemical profile (phenols and flavonoids) of *Cuphea* spp. [36]. It is well documented that plants that grow with optimum levels of environmental stimuli are less likely to stimulate defense mechanisms, such as secondary metabolite synthesis, which ultimately leads to a loss in adaptability [37]. The batch of plants in the initial cultivation site was only irrigated with tap water. This is an irrigation custom in local greenhouses because these species grow "anywhere", without the need to add special nutrients. This probably generated nutritional stress in the plants, and thus raised their secondary metabolism. So, once the soil in which they came was removed, transplanted to the NFT, and irrigated with OEW, we observed a decrease in the immune activity of the plants that was detected in the analysis even when 7-day acclimatization was carried out. Then, during the days of integration, the levels of secondary metabolites decreased in each IAAS, but to some extent, the immune system remained in the plants; meanwhile, in the control, the production of phenols and flavonoids reached a minimum (Table 5). It is important to note that despite belonging to the same genus, the morphology of the two species is different, even contrasting, a remark that can be observed in the main effects analysis for the species **(**Table 5). Phenol and flavonoids compounds activate plant defense mechanisms against biotic and abiotic stressors through the shikimate and acetate pathways [38], so it can be confirmed that IAAS promotes sufficient stress (eustress), causing plants to activate and maintain a "waiting" state for a long time, in anticipation of a future stress situation [39].

It was also observed that the leaves of the plants In the IAAS appeared larger and greener, while the control showed small leaves with an opaque green color, a clear sign of biostimulation/elicitation [40]. However, the analyses showed no significant differences. A significant main effect of the species was observed for both growth variables, leaf area and plant height, and no main or simple main effects for of cultivation method were significant. These results imply that the cultivation method does not provoke significant changes in leaf area and plant height. Instead, the differences between species respond to the genetic identity of the plants, which can be inferred because the values for both groups are consistently different from each other for all types of cultivation (see the Supplementary Materials for interaction plots). No significant differences were found in the species' growth in both IAAS-H and IAAS-C and each of its conventional cultivations; this is probably due to the measurement methods used. For future studies, it is necessary to scale the system and the number of plants to conserve the aquaponic feeding rate ratio [41]. In this way, an approach for commercial-scale production and more precise methodologies such as image analysis would have to be used.

Another observation was that the C. *hyssopifolia* species showed its roots, and this promoted the retention of some solids from the water. According to Olness et al. 2005 [42] when this genus is grown hydroponically, deficiency in root growth is manifested due to a lack of nutrients (e.g., vanadium) or the ionic ratio. In this study, *C. cyanea* showed the same result of low root growth, although further studies on the nutrient dynamics in aquaponics with medicinal plants are needed to clarify these findings. Basil (*Ocimum basilicum*) presents morphological characteristics like those of *C. hyssopifolia* (e.g., shrub type, pivoting roots,

antioxidant properties correlated with disease prevention in humans); parsley (*Petroselinum crispum*), on the other hand, has been identified as a condiment or herb with beneficial effects on health due to its contents of phenolic acids and flavonoids [42,43]. In a study conducted by [9] with ornamental fish and both the aforementioned species, the authors found that the growing method has a significant effect on plant performance. Concerning the flavonoid content in parsley, aquaponics caused a significant increase in quercetin. Additionally, a remarkable increase was reported in other compounds such as myricetin and rosmarinic acid (+1861% and 633%, respectively).

Biostimulants are compounds of biotic origin that can induce a pre-stress conditioning effect, which promotes various physiological responses. Responses stimulated in this way can reach values between 30–60% higher than the values reported for the control [43,44]. Elgindi et al. 2011 [15] described 35 flavonoids found in 16 *Cuphea* spp; however, the presence of catechin, caffeic acid, and p-coumaric acid has not been described. Thus, it was proposed in this study that they could be detected using the UPC^2 method. Although it is reported that kaempferol, catechin, and quercetin have been isolated in *C. hyssopifolia*, in this study, we did not find any of the above; however, we did find apigenin (Figure 2), which has only been described in a limited number of *Cuphea* spp. This represents an apparent contradiction, since Braglia et al. 2022. [9] also describe that aquaponic culture promotes the biosynthesis of resveratrol and therefore the production of p-coumaric acid; however, this standard was not found.

Apigenin is a natural flavonoid found in medicinal plants and other fruits and vegetables. It is recognised for being found in large quantities in garlic, chamomile, orange, and propolis [9,45]. Its importance lies in its biological functions, which are beneficial to human health (i.e., its antitumor effect, beneficial for the cardiovascular system, and its effects on the liver, respiratory, endocrine, and central nervous systems). Apigenin acts specifically as an anti-inflammatory, antibacterial, antiviral, antiallergic, cytotoxic, antitumor treatment, and as a treatment for neurodegenerative diseases [44,46]. In this short study, an increase of more than 60% in the apigenin content in IAAS-H was found by the end of the trial, so a eustress effect related to cultivation using IAAS can be confirmed, as can a consequent increase in the production of bioactive compounds. Moreover, from the multifactorial ANOVA, a significant interaction between cultivation method and plant species was observed for apigenin, total phenolic content, and total flavonoids, meaning that the plant metabolic response to the cultivation method varies differently depending on the species tested. The analysis of the main effects of the cultivation methods shows that aquaponic cultivation has a global significant effect, yielding higher means for the total phenolic and apigenin contents, and for antioxidant activity in comparison to conventional cultivation (Table 6). In addition, during the analysis of the samples using the UPC^2 method, peaks for other different flavonoids were detected without identification, because standards were not available. More specific studies are needed to identify the other compounds that are present in these two medicinal species, possibly using gas-chromatography–mass spectrometry (GC-MS) analysis.

5. Conclusions

The results found in this short study show that *C. hyssopifolia* in IAAS-H has appropriate synergy, and that cultivation under aquaponic conditions has biostimulant effects. This allowed phenotypically better development than that of *C. cyanea* in IAAS-C. The results obtained in the present study show that the aquaponic system design is suitable for keeping *Cuphea* spp. in a greenhouse. IAAS-H was better for maintaining growth, high conversion of ammonia to nitrates in the water, and a high polyphenolic compound concentration in the plants; it also increased the content of a specific flavonoid, apigenin, compared with the conventional culture. This indicates that aquaponic cultivation can promote the biostimulation of medicinal plants, causing plants to activate second metabolism pathways, and thereby improving phenotypic variables (i.e., growth and development) and/or activating immunity by sacrificing previous ones. Future studies may involve comparing different

scales of hydroponic units, testing various inert substrates, and analyzing the resulting waters of tilapia in different growth stages. It is important to conduct additional studies on the elicitor and biostimulant effects of *organically enriched waters* in aquaponics. Analyzing the activities of enzymes related to stress responses, such as superoxide dismutase, catalase, and phenylalanine ammonia-lyase, is necessary to confirm whether the use of aquaponics causes eustress or distress. In this case, the aquaponic integration of *Cuphea* spp. with *C. carpio* increases the production of polyphenolic compounds, with each variety in a specific concentration. It was observed that *C. cyanea* is not an excellent candidate for introduction into aquaponic systems because it needs support for its roots. We must also consider the limited data concerning medicinal plants such as *Cuphea* spp. grown using different methods. The findings reported here contribute to the use of aquaponics as a sustainable system to stimulate the immune system of plants, raise the antioxidant content in leaves and fruit, and thus impact the zero hunger, good health and well-being goals of the 2030 agenda at the local level.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13102018/s1, File S1 includes real images, species interaction graphs, water dynamics data and species identification certificates.

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Institutional Review Board Statement: The protocol entitled "Cultivo acuapónico vertical Carpa Koi (*Cyprinus carpio* L. 1758--*Cuphea* spp. con potencial nutracéuticos" was approved by the Applied Research Ethics Committee, Faculty of Engineering, CEAIFI of the Autonomous University of Querétaro (registration 7164-08-12-2014) for studies with animals, in adherence with the ethical guidelines of beneficence, non-maleficence, justice, and autonomy.

Data Availability Statement: All data generated and analyzed during this study are included in this published article in the Supplementary Materials.

Conflicts of Interest: All authors have reviewed the manuscript and report that they have no competing interests to declare. The sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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The Biosurfactants Mannosylerythritol Lipids (MELs) as Stimulant on the Germination of *Lactuca sativa* L.

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Abstract: The application of pesticides in agriculture leads to improved crop quality and promotes high productivity. However, the uninterrupted use of these chemicals is directly related to environmental impacts, affecting biodiversity and the health of ecosystems and humans. In this sense, mannosylerythritol lipids (MELs) are a promising alternative, as they are biosurfactants with antimicrobial, amphiphilic characteristics, and low toxicity. Thus, in search of a partial reduction in the use of chemical pesticides in agriculture, this work aimed to evaluate the biostimulant effect of one of the homologs of MELs–MEL-B on the germination of Monica lettuce seeds (*Lactuca sativa* L.) and the influence on plant growth and root development. The seeds germinated in different concentrations of MEL-B. The incidence of germinated seeds, the germination index, and the average germination time were evaluated. MEL-B at 158 mg/L stimulated seed germination, growth, and seedling development parameters by 65%, while concentrations of 316 and 632 mg/L did not exceed 45% for these parameters. It was observed that MEL-B at 158 mg/L biostimulated the appearance of lateral roots and promoted only 7% of root stress, a difference of 47% for roots grown with MEL-B at 632 mg/L. Furthermore, MEL-B at 158 mg/L was the highest concentration at which there was no phytotoxic effect of MEL-B on seeds. The increase in enzymatic activity corroborates the phytotoxic effect and seed stress at concentrations of 316 and 632 mg/L, showing results of 47% and 54% of stressed roots. In an unprecedented way, this study proved that MEL-B has a biostimulant and phytotoxic effect related to its concentration.

Keywords: agriculture; enzyme activity; stressed roots; glycolipid; lettuce

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1. Introduction

The application of pesticides in agriculture leads to higher productivity. However, pesticides are easily diffused in soil, air, and water—resulting in large environmental impact [1,2]. In addition, they are vectors for simple and chronic human health problems, such as nausea and headaches, diabetes, and cancer [3]. The commercialization of active ingredients used in the production of pesticides exceeds 4 million tons annually [4]. In Brazil, since 1990, the use of pesticides has been increasing over time. In 2019, approximately 13.300 chemicals were registered [5]. Pesticides are essential to high crop yields and a high level of quality. On the other side, modern agriculture should be efficient and also environmentally friendly.

Biostimulants are eco-friendly compounds that can stimulate plant metabolism and improve the absorption of nutrients in the soil [6]. Biostimulants can be classified into

four main groups based on amino acids and protein hydrolysates; humic substances; microorganisms; and inoculum and algae extracts [7].

To date, the biostimulant potential of biosurfactants has been subtly investigated. For example, the inhibitory effects (in vitro) of *Pseudozyma aphidis* metabolites on phytopathogenic fungi were studied. The results indicate that *P. aphidis* has a potential application as a biocontrol agent for fungal pathogens [8]. In another study, the authors applied *Rhodotorula glutinis* and rhamnolipids on cherry tomatoes infected with *Alternaria alternata*. They concluded that, even at low concentrations, the mixture of *R. glutinis* and rhamnolipids is a safe alternative for controlling *A. alternata* infection [9]. The screening of cultivation conditions with sophorolipids and the application of them at different stages of plant growth was investigated. In response, it was observed that sophorolipids present efficient biocontrol activity for biotic and abiotic stress in the primary stage of plant germination [10]. The production of the biosurfactant with an anionic characteristic from *Candida sphaerica* UCP 0995 was investigated, and then, the germination index was used to evaluate the toxicity of the biosurfactant in the germination of *Lactuca sativa* L., indicating that the solutions of 0.125, 0.25, and 0.5 g/L did not inhibit the germination of seeds or the elongation of roots [11]. The biosurfactant production from *Candida lipolytica* UCP 0988 at 0.15, 0.3, and 0.6 g/L did not inhibit the germination of the seeds of *Lactuca sativa* L. [12].

MELs are glycolipid biosurfactants [13,14]. The acetylation-based classification of MELs includes MEL-A, MEL-B, MEL-C, and MEL-D. In this sense, MEL-B has an acetyl group in its chemical structure [15–17]. The application of biostimulants has been reported in agricultural practices; however, there is no practical research on the biostimulant activity of MELs in seed cultivation.

However, the surface-active properties of biosurfactants exhibit relevant pesticidal and antimicrobial properties [18]. Thus, biosurfactants are a promising alternative that may lead to the sustainable management of pathogens and agricultural pests, partially reducing chemical pesticides and contributing to a more sustainable agricultural practice [19,20]. The hydrophilic and hydrophobic portions of biosurfactants contribute to promoting interactions between immiscible liquids in agricultural pesticide formulations. Therefore, understanding biosurfactants, biostimulants, and biopesticide behavior expands opportunities in the surfactant market [19,21].

The continued use of chemical pesticides in agricultural practices has had a negative impact on ecosystem health and also on plant development. This major environmental problem can be tackled through environmentally correct solutions using the properties of biosurfactants to totally or partially reduce plant pathogenicity and increase the concentration of chemical pesticides in the environment [19,22].

Thus, among biosurfactants, MELs are well-reported in the literature for presenting promising results on their antimicrobial activity against pathogens associated with food and crop management [23].

Therefore, from the correlation between the properties of MELs and other glycolipids with potential application in agriculture, a screening of the concentration of MEL-B was carried out to evaluate the biostimulant activity of MEL-B in seeds of Monica lettuce (*Lactuca sativa* L.), taking into account the morphological behavior, physiological characteristics, and physical-chemical and biochemical analyses performed after the germination phase.

2. Material and Methods

2.1. Material

Monica lettuce (*Lactuca sativa* L.) seeds were purchased from the company Feltrin Sementes Ltd.a.(Farroupilha/Brazil); Sgima and Merck (Florianópolis/Brazil), registered in the National Registry of Seeds and Seedlings (RENASEM) of the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA). MEL-B at 95% purity was kindly provided by TOYOBO CO., LTD., Osaka, Japan. Guaiacol, sodium phosphate, phosphoric acid, hydrogen peroxide, PBS, and BSA reagents of analytical grades were obtained from Sigma and Merck.

2.1.1. Growing Medium Containing MEL-B for Lettuce Seed Germination

The germination tests were carried out in a Petri dish containing purified agar (% agar) at different concentrations of MEL-B (0, 3.16, 31.6, 158, 316, and 632 mg/L). Simultaneously, the MEL-B was weighed on an analytical balance (AD-500, Marte, São Paulo/Brazil) and subsequently solubilized into agar using a vortex mixer (K45-2820, Kasvi, São Paulo/Brazil). After homogenizing, the media were transferred to Petri dishes inside a flow chamber. The plates were sealed and stored in the refrigerator until use [7].

2.1.2. Contact Angle and Surface Tension

The influence of different concentrations of MEL-B (0, 3.16, 31.6, 158, 316, and 632 mg/L) on the measurement of contact angle and surface tension was performed in a goniometer where drops of soybean oil or diiodomethane were placed on the surface of agar containing MEL-B by using a micropipette of 100 µL. The procedure was performed in triplicate at 25 °C. The drops were photographed by a digital camera (Ramè-Hart, 250-F1, São Paulo/Brazil). The photo was subjected to digital processing to obtain the width and height [24]. The Drop Image provided the contact angle and surface tension values.

2.2. Germination Test

Lettuce seeds were sterilized with an aqueous alcohol solution (95% alcohol) for 5 min, and then the seeds were subjected to a hypochlorite solution (2%) for 1 min and posteriorly abundantly washed with distilled water. Then, the 100 seeds were distributed in 10 plates and incubated in a BOD chamber (New Lab, NL-41-02, São Paulo/Brazil) with controlled relative humidity (60%) for seven days, with day and night simulation at 25 and 20 °C, respectively. The number of lettuce germinated seeds was monitored for each concentration of treatment with MEL-B (0, 3.16, 31.6, 158, 316, and 632 mg/L). The germination speed index (GSI) and mean germination time (MGT) were calculated using Equations (1) and (2) [25].

2.2.1. Germination Speed Index (GSI)

The germination speed index of emerged seedlings was carried out on a daily basis and calculated according to Maguire [26]:

$$GSI = {N1}/{D1} + {N2}/{D2} + \cdots + {Nn}/{Dn} \quad (1)$$

in which *GSI* = germination speed index; *N*1, *N*2, *N*i = number of seeds germinated in the first count, second count, i-th count, respectively; *D*1, *D*2, *D*i = number of days in the first count, second count, i-th count, respectively. Unit: dimensionless.

2.2.2. Mean Germination Time (MGT)

The plates containing lettuce seeds were monitored daily, and the average germination time was calculated as proposed by Labouriau [27]:

$$MGT = {\sum ni \times ti}/{\sum ni} \quad (2)$$

in which *MGT* = mean germination time; *ni* = number of seeds germinated in time *ti* (not the accumulated number but the one referred to the *i*-th observation); *ti* = time between the beginning of the experiment and the *i*-th observation. Unit: days.

2.3. Morphological Parameters in Lettuce Cultivation

The seeds were cultivated with different concentrations of MEL-B. The behavior of lateral roots, stressed roots, length, and mass were evaluated. Regarding the appearance of lateral roots, the emergence of lateral roots was evaluated from the 3rd day of cultivation. One hundred Petri dishes containing 10 seeds per dish were monitored. All samples were observed visually and under a magnifying microscope (Technical, stereoscopic) [28].

Stressed roots were counted, as they did not germinate and/or showed low performance. In addition, the seeds that showed delay in the germination process during the observation of the 7 days of the experiment were taken into account. The accumulated records were represented in percentage at the end of the experiment.

The length of the roots (cm) was measured from the third day of germination to the seventh day. The procedure was performed in triplicate [29].

The mass of the samples used to prepare the crude enzymatic extract was determined using an analytical balance (Marte, AD-500). Seedlings (leaves and roots) were collected from Petri dishes daily and weighed. For each concentration, three runs were performed.

Morphological Analysis by Scanning Electron Microscopy (SEM)

The lettuce root samples were fixed with glutaraldehyde (2.5%) for 30 min. Then, they were dehydrated with an alcohol series (10, 30, 50, 70, 80, 90, and 100%) and dried at room temperature. For the analysis, the lettuce was distributed on carbon tapes on the surface of stubs and then coated with a layer of gold. After recovering, the samples were analyzed in SEM (JEOL JSM (6390LV)), with a tungsten electron source secondary electron detector at 10 kv [30].

2.4. Physicochemical Characterizations of Total Proteins and Activity of Peroxidase and Polyphenol Oxidase Enzymes

The study of the influence of MEL-B on germination and the induction of stress conditions in cultivation was carried out by quantifying total proteins and analyzing the activity of peroxidase and polyphenol oxidase enzymes.

Crude Enzymatic Extraction

Crude enzyme extraction was performed daily from the 3rd day of cultivation. After this period, the seeds that visibly started the germination process were selected. To extract the enzymes, the selected roots were weighed and macerated in a crucible under an ice bath. The addition of 1 mL of 50 mM sodium phosphate buffer was added until a homogeneous mass was obtained. Then, the plant material was transferred to microcentrifuge tubes and then centrifuged at 15,952× *g*-force for 10 min. The supernatant was used to determine the enzymes activity and protein content [31].

2.5. Protein Content

For protein quantification, the Lowry method was used [32]. A total of 100 µL of crude extract and 2 mL of solution C (Na_2CO_3 (2%)) in 1M NaOH and $CuSO_4$ (0.5%) were added to the test tubes. The mixtures stand for 10 min. Subsequently, 200 µL of Folin reagent was added, homogenized, and left to rest for another 30 min. The reading was performed under absorbance at a wavelength of 750 nm and calculated concerning the mass (g) of the sample used to prepare the crude extract. Mean of the absorbances obtained was obtained to determine the protein concentration in each analyzed sample. The calibration factor of the calibration curve was determined and, finally, the protein concentration for each sample was estimated according to the equation:

$$C = \frac{Abs \times F}{m} \quad (3)$$

in which C = concentration of protein in each sample, F = calibration curve factor, Abs = Absorbance of sample, and m = mass of the sample (g).

This determination was performed in triplicate.

2.5.1. Peroxidase Activity

A 140 µL aliquot of sodium phosphate buffer (50 mM, pH 6.4) containing 0.3% (v/v) guaiacol was used. An aliquot of 100 µL of crude enzyme extract and 60 µL of H_2O_2 (0.3%) was added. Enzyme activity was determined by spectrophotometer by observing the variation in absorbance at 470 nm and 25 °C for 5 min [33].

2.5.2. Polyphenoloxidase Activity

Polyphenoloxidase (PPO) activity was performed according to the methodology presented by Matsuno and Uritani [34]. This analysis was determined using catechol (0.02 mol/L) as a substrate for the enzyme. The reading was determined in proportions of 0.30 mL of sample and 1.85 mL of 0.10 M solution of phosphate buffer pH 6.0 with catechol. The absorbance was read in a UV-Vis spectrophotometer (Spectra Max, 384 plus) at 395 nm. The reading was performed every 1 min for 10 min, and water was used as a blank.

2.6. Statistical Analysis

The experimental plots consisted of 100 seeds at each concentration of treatment with MEL-B for daily monitoring of germination and 1.600 destructive samples to evaluate the behavior of germinated seeds and physical-chemical analyses, totaling 2.200 analyzed seeds in the period of 7 days of cultivation. Data were submitted to a one-way analysis of variance (ANOVA) significance test, and the difference was compared using Tukey's test ($p \leq 0.05$).

3. Results

3.1. Contact Angle and Surface Tension

Figure 1 shows the influence of different concentrations of MEL-B on the interaction of drops of diiodomethane (DIIM) and soybean oil with the treated surfaces.

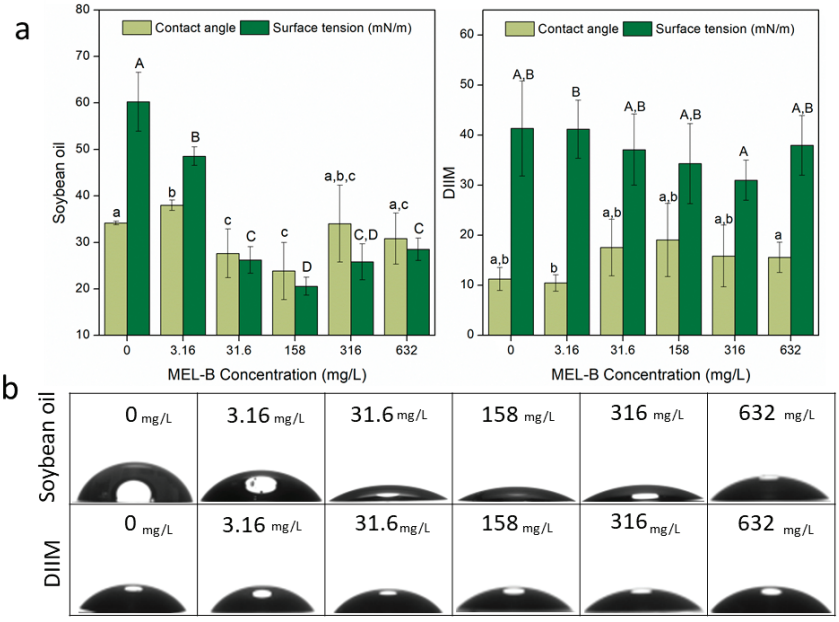

Figure 1. Effect of the drops of DIIM and soybean oil in the treated surfaces. (**a**) Contact angle and surface tension using oil and diiodomethane, (**b**) oil and diiodomethane drops in contact with the surface of the medium containing different concentrations of MEL-B. Means followed by the same letter do not differ from each other by the Tukey test ($p \leq 0.05$).

Figure 1a shows that this molecule did not undergo a significant variation in contact angle and surface tension after contact with agar surfaces treated with different concentrations of MEL-B. Therefore, despite having a contact angle lower than 90° and showing a wetting aspect, MEL-B did not promote the interaction of DIIM at the surface. Furthermore, the variation of MEL-B concentrations did not imply the reduction of the contact angle, as shown in Figure 1b.

On the other hand, a different response was observed after adding soybean oil. The surface tension reduced as the MEL-B concentration increased up to 31.6 mg/L, followed by a surface tension (quasi)plateau for higher MEL-B concentrations. MEL-B at 158 mg/L showed a significant difference in surface tension reduction about the control (Figure 1a). The behavior of the contact angle corroborates with this speculation since from the treatment carried out with 31.6 mg/L of MEL-B, wettability tended to increase. Due to the glycolipidic characteristics of MEL-B, the culture media supplemented with the biosurfactant suffered a weakening of the binding of water molecules.

3.2. Germination Properties

Figure 2 shows the influence of the MEL-B concentration added to the media on the number of germinated lettuce seeds (Figure 2a), germination speed index—GSI and mean germination time—MGT (Figure 2b), and the appreciation of secondary roots appearance (Figure 2d).

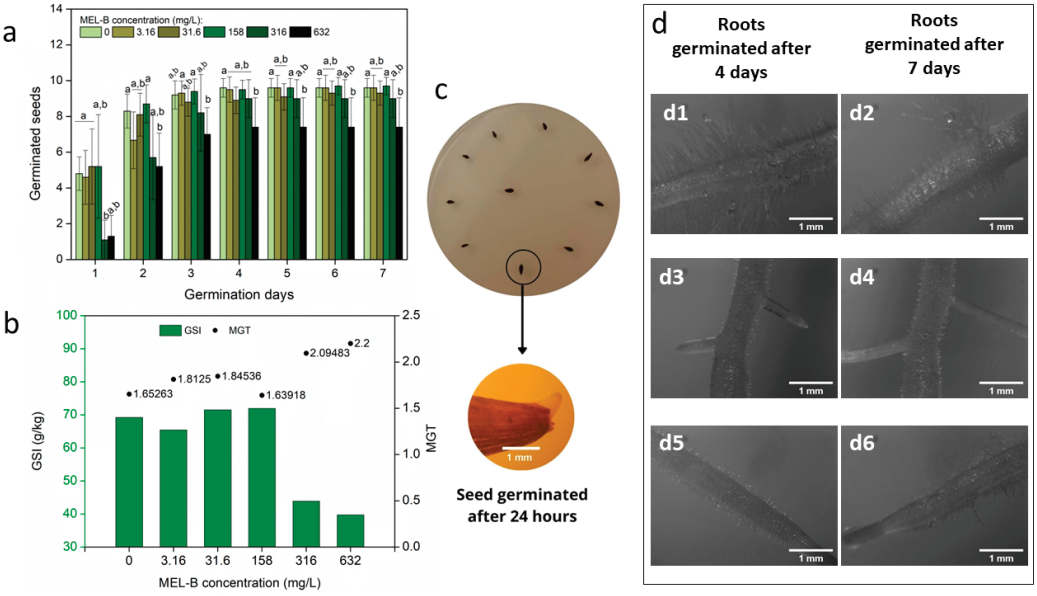

Figure 2. Effect of the different concentrations with MEL-B on the germination of *Lactuca sativa* L. (**a**) Cumulative germination of seeds, (**b**) GSI represented by vertical bars and MGT represented by points, and (**c**) representative image of lettuce seeds germinated after 24 h of cultivation. (**d**) Optical microscopical image of lettuce seeds germinated after 4 and 7 days of cultivation. Means followed by the same letter do not differ from each other by the Tukey test ($p \leq 0.05$).

Figure 2c reports the characteristic behavior of seeds that germinated after 24 h of cultivation. The observation was realized using an optical microscopical at 10× enlargement. The concentrations of MEL-B added to the culture medium influenced the incidence of germination (Figure 2a); however, all the seeds germinated on the 1st day showed the same morphological characteristics, independent of the MEL-B content present.

The incidence of germination was observed cumulatively during the germination of lettuce seeds (Figure 2a). On the 1st day of the experiment, it was observed that MEL-B at 316 and 632 mg/L affected seed germination, where only about 15% of the seeds germinated out of 100%. In contrast, at lower concentrations (0, 3.16, 31.6, and 158 mg/L) of MEL-B, more seeds (greater than 40%) germinated under each treatment condition. The differences in relation to the control were significant ($p \leq 0.05$) only in the concentration of 316 mg/L of MEL-B; it was noticed that the seeds cultivated with 316 and 632 mg/L of MEL-B

germinated less in comparison with the other conditions. The germination was higher than 80% in all growing conditions, except for the concentration of 632 mg/L of MEL-B, which was the highest used for seed germination and had lower levels since the first day of germination (Figure 2a).

One of the indicators of seed vigor is the GSI, which is directly proportional to each other. That is, the higher the GSI, the more vigorous the seed [34]. Regarding GSI, MEL-B promoted similar responses for control and intermediate conditions (3.16, 31.6, and 158 mg/L), indicating values above 65% for GSI. Differing significantly from concentrations of 316 and 632 mg/L, GSI showed an inhibitory effect by MEL-B with values of 43.9 and 39.7%, respectively (Figure 2b).

The results obtained for the GSI were corroborated by the MGT, where the time required for germination was greater for concentrations of 316 and 632 mg/L of MEL-B. For the culture containing 158 mg/L of MEL-B, there was a decrease in MGT compared to subsequent concentrations, returning to an increase in following treatments. This observation demonstrates that the average germination time of lettuce seeds is progressively increased under biotic stress. Treatment with MEL-B reduced GSI, increasing MGT at 316 and 632 mg/L. At the concentration of 158 mg/L, the results for the same parameters were the opposite, confirming a less pronounced inhibitory effect than the other cultivation conditions (Figure 2b).

Figure 2c shows the behavior of all roots in the first 24 h of cultivation. In all conditions, the same behavior of the germinated seeds was observed. Figure 2d shows morphological observations of the germinated roots after 4 and 7 days of cultivation. Figure 2 panels d1 and d2 demonstrates the predominant behavior of seeds grown in the medium without MEL-B treatment (control) in the medium containing MEL-B at 3.16 and 31.6 mg/L. In the records represented on Figure 2 panels d3 and d4, the evolutionary behavior of the germinated seeds in the culture medium containing MEL-B at 158 mg/L was compiled. The treatment performed with MEL-B at 316 and 632 mg/L is represented in Figure 2 panels d5 and d6. Thus, observations recorded with the aid of a microscope indicate that the different treatments with MEL-B caused morphological changes in the roots.

3.3. Morphology of the Roots

Root growth was monitored from the 4th day of seed germination (Figure 3). It was noted that on this 1st day of observation, all cultivation conditions showed similar behavior. All verified roots showed similar sizes in the measurements (~1.8 cm).

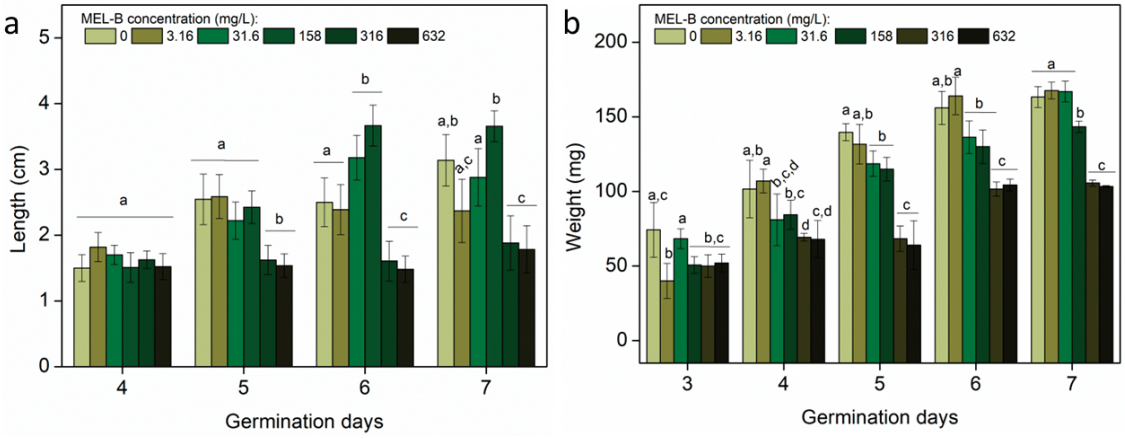

Figure 3. Monitoring of different concentrations of MEL-B on the morphology of *Lactuca sativa* L. roots. (**a**) Length and (**b**) weight of roots. Means followed by the same letter do not differ from each other by Tukey's test ($p \leq 0.05$).

However, from the 5th day of monitoring, the concentrations of 316 and 632 mg/L of MEL-B showed a significant difference in relation to the size of roots grown under control conditions. On the 6th day of cultivation, the roots cultivated at a concentration of 158 mg/L of MEL-B reached the largest size (less than 3.5 cm in length) compared to the other treatment conditions. In addition to presenting the largest size among the concentrations, it was the largest size observed among all the experiment days. In general, the seeds grown without MEL-B and with low concentrations (3.16, 31.6, and 158 mg/L) of MEL-B tended to grow with the days of cultivation. However, seeds germinated with 316 and 632 mg/L of MEL-B were less than 2 cm in length and did not show significant development since the 1st day of monitoring.

Another parameter monitored was the weight of the germinated roots (Figure 3b). On the 3rd day of the experiment, the roots germinated in the control conditions showed an average difference of 34.33 mg in relation to the roots germinated with medium containing 3.16 mg/L of MEL-B. On the 4th day of germination, the weights of the roots grown in the control and 3.16 mg/L of MEL-B were >100 mg and showed a significant difference in treatments made with 316 and 632 mg/L of MEL-B, which were lower than only 70 mg. From the 5th day, the behavior was similar for all concentrations. The tendency to increase in weight as the roots developed was observed. However, at MEL-B concentrations of 316 and 632 mg/L, the same pattern observed in Figure 3a was observed for the same treatment conditions. The roots developed less when compared to the control and other concentrations. On the last day of monitoring, the roots that developed the most (in this parameter) were without treatment and with 3.16 and 31.6 mg/L of MEL-B, obtaining an average weight >160 mg. The opposite was observed for roots treated with 632 mg/L of MEL-B. The lowest average weight (<105 mg) was observed in this condition compared to the control and the other concentrations on the 7th day.

Factors that cause adverse reactions in the development of lettuce seeds were noted in the experiment (Figure 4). The treatment performed with MEL-B on seed germination promoted the development of lateral roots (Figure 4a) at intermediate concentrations and created a stress (Figure 4b) medium at higher concentrations in addition to the morphological observations, as illustrated in Figure 4c.

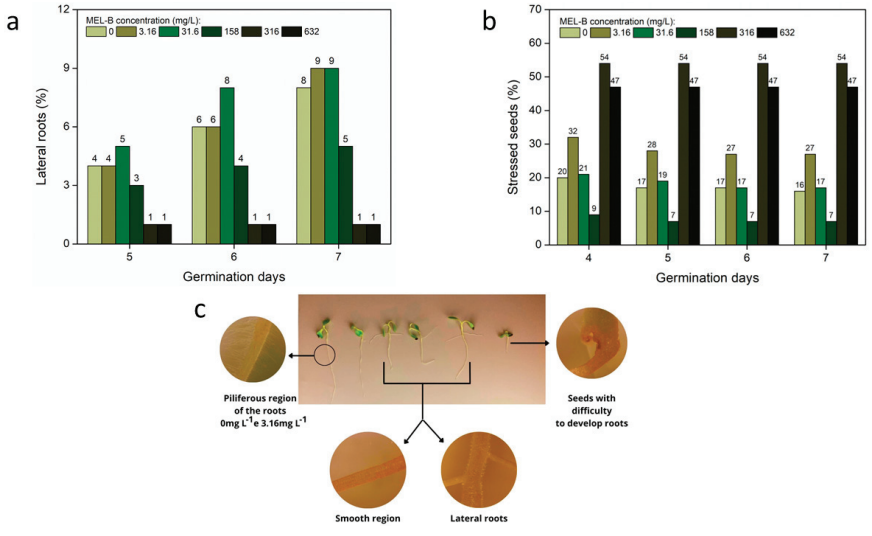

Figure 4. Monitoring (**a**) lateral roots, (**b**) stressed roots, and (**c**) illustrative image of root regions on the seventh day of cultivation under different concentrations of MEL-B.

As observed on the 4th day of germination (Figure 4b), there were increases of 34 and 27% for treatments made with 316 and 632 mg/L of MEL-B compared to the control. For

the treatment with MEL-B at 158 mg/L, the stressed roots were reduced by 11% when compared to the control. From the 5th day of cultivation, intermediate concentrations (3.16, 31.6, and 158 mg/L) and the control showed reduced stressed seeds. This behavior may reflect seed dormancy, a phenomenon that causes an intrinsic temporal block that provides additional time for germination. On the other hand, seeds grown under treatments of 316 and 632 mg/L of MEL-B remained with 54 and 47% of the stressed samples (Figure 4b). At the end of the experiment, it was noticed that the treatment performed with 158 mg/L of MEL-B presented only 7% of the roots stressed. That was the only concentration that showed a reduction of stressed roots among the treatments made with MEL-B compared to the control.

However, the treatment performed with MEL-B at 31.6 mg/L stimulated the same number of lateral roots in seven days of cultivation (Figure 4a). This behavior was also observed when seeds were germinated with MEL-B at 316 and 632 mg/L (Figure 4b).

The influence of different treatments with MEL-B is also recorded in Figure 4c. The behavior of the germinated roots after 7 days of cultivation shows that the intermediate treatments (3.16, 31.6, and 158 mg/L of MEL-B) did not inhibit the development of the roots. The opposite was registered when the seeds were germinated in the culture medium treated with MEL-B at 316 and 632 mg/L. Under these conditions, the roots showed adverse behavior in relation to the control and other treatments.

The microstructural analysis of the root surface was performed after 7th days (Figure 5). SEM is another alternative that makes it possible to interpret the surface of the roots, evaluate the microstructure, and correlate the potential influences of treatments with MEL-B in the cultivation. The evaluated roots presented plant tissue with an irregular shape and contracted cellular aspect in all treatments performed. In this way, the cell walls characterized the appearance of withered plant cells.

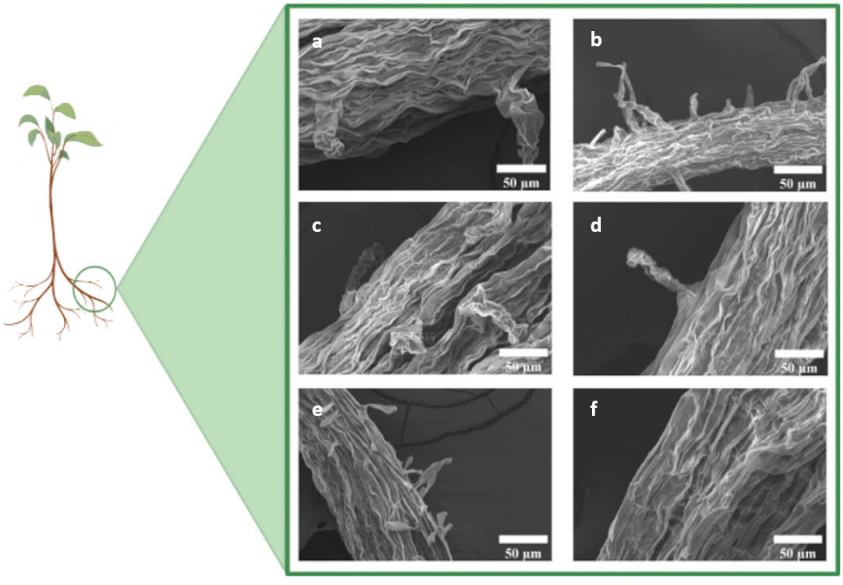

Figure 5. Scanning electron microscopy (SEM) of radicle and primary root of Monica lettuce after seven days of cultivation. (**a**) Root grown without treatment, and Radicle treated with MEL-B (**b**) 3.16 mg/L, (**c**) 31.6 mg/L, (**d**) 158 mg/L, (**e**) 316 mg/L and (**f**) 632 mg/L.

Under control conditions and treatment with 3.16 mg/L of MEL-B, the plant tissue of the primary roots showed a well-developed hairy region (Figure 5a,b). Although apparently in smaller amounts, the development of the piliferous region was observed in seeds cultivated under treatment of 31.6 and 158 mg/L of MEL-B (Figure 5c,d). For 7 days

of cultivation, the seeds treated with up to 158 mg/L of MEL-B presented root growth superior to those treated with 316 and 632 mg/L of MEL-B. The observations made with SEM corroborate the behavior illustrated in Figure 4c, where a visual comparison of root development under different treatment concentrations was performed.

Furthermore, it was noted that the plant tissue of the primary roots of seeds treated with 316 mg/L of MEL-B had a shape and structure similar to those observed in the root tissue of seeds germinated with 632 mg/L (Figure 5f). However, a considerable reduction in the development of the hairy region with these treatments was seen. In addition, the integrity of the roots was compromised. However, with a low rate of seeds germinating under these conditions, the root growth and morphological development were significantly lower than the control and other treatment concentrations.

3.4. Quantification of Protein and Enzyme Activity

The protein quantified in each treatment condition was evaluated from the crude extract of the sprouted roots. The crude extract obtained by the roots on the 3rd day of germination was produced with roots of lower size and mass than the roots that germinated for 4 days and successively until the 7th day of germination. In this sense, it was observed that even though the root mass decreased with increasing concentrations of MEL-B in the culture medium (Figure 3b), the protein quantification remained at similar values and without a statistical difference ($p \leq 0.05$)—for the roots germinated until the 4th day of the experiment (Figure 6a). This physiological alteration can corroborate with the interpretation of the behavior of the germinated roots under treatment of 316 and 632 mg/L of MEL-B and validate the stress that these concentrations cause in the germination of the seeds. From the 4th day of the experiment, it was noted that all concentrations presented a higher or equal amount of protein than the control. In addition, the highest amount of protein obtained in this study was 11.33 mg/g on the 5th day of germination, with the extract of germinated roots under treatment performed with 632 mg/L of MEL-B.

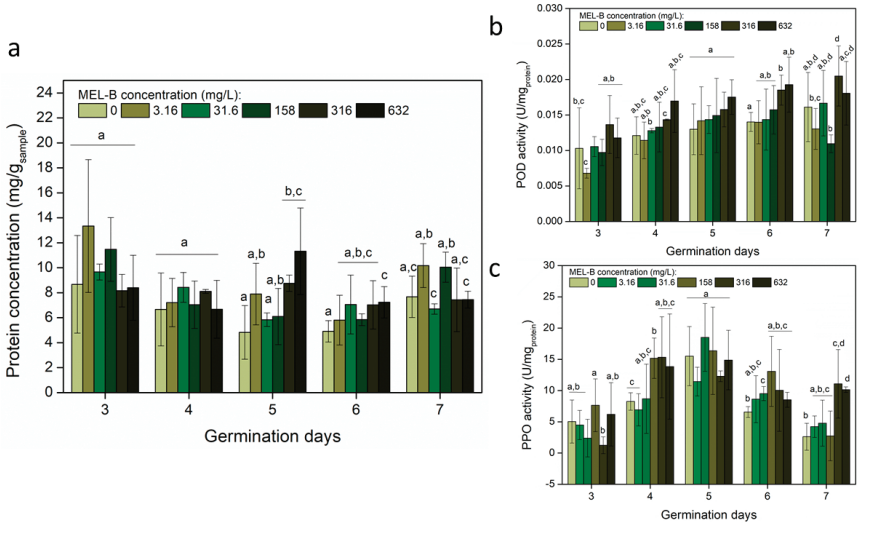

Figure 6. Effect of different concentrations of MEL-B on the physiology of *Lactuca sativa* L. roots. (**a**) Total protein in cultivated lettuce seeds, (**b**) Peroxidase enzymatic activity, and (**c**) Polyphenoloxidase. Means followed by the same letter do not differ from each other by Tukey's test ($p \leq 0.05$).

Peroxidase and polyphenol oxidase enzymes are pathogenesis-related enzymes involved in the cell wall lignification process and plant defense development processes in response to biotic and abiotic stresses.

When analyzing the peroxidase activity (Figure 6b), MEL-B treatments caused changes in the peroxidase enzyme activity. On the 3rd day of germination, only concentrations of 316 and 632 mg/L of MEL-B showed higher enzyme activity than the control. In the evaluations carried out from the fourth day onwards, it was noted that there was no difference in enzyme activity between the treatments and the control. On the 7th day of the experiment, the highest enzyme activity was 0.0205 $U/mg_{protein}$ in the treatment with 316 mg/L of MEL-B. On the other hand, the concentration of 158 mg/L showed the lowest enzyme activity for the same day of culture. In general, regarding enzymatic activities, the results showed that the levels of the peroxidase enzyme were low. However, plants may have suffered oxidative stress after treatment with MEL-B, which resulted in higher levels of peroxidase enzyme activity at higher treatment concentrations.

The polyphenol oxidase enzyme (Figure 6c) had its activity increased until the 5th day of germination. On the 6th and 7th days, lower levels of activity were observed. The treatment performed with 31.6 mg/L of MEL-B showed 18.5 $U/mg_{protein}$ on the 5th day of germination, which was the highest level of enzyme activity among all the days of cultivation. On the 7th day of germination, treatments made with 316 and 632 mg/L of MEL-B showed the greatest differences in enzyme activity from the control at 8.48 and 7.54 $U/mg_{protein}$, respectively. In addition, treatments performed with 316 and 632 mg/L of MEL-B influenced lower root development when compared to control and intermediate concentrations. However, it was noted that the enzyme activity levels remained above 10 $U/mg_{protein}$. The increase in polyphenol oxidase activity occurred without increasing the protein concentration, indicating that the roots were subjected to water stress.

4. Discussion

4.1. Interpretation of the Behavior of the Contact Angle and Surface Tension

The hydration of the plant cell is essential for biochemical reactions and seed metabolism [35]. In this sense, MEL-B is an surface active agent—it reduces the surface tension [17]. DIIM is a highly non-polar molecule that is not very water-soluble [36]. Therefore, this behavior was expected since naturally, the DIIM molecule has a low surface tension [37]. Thus, the surface tension reduced as the concentration of MEL-B increased. In the case of the soybean oil drop, the observed behavior may be related to the lipophilic character of the biosurfactant [38].

In general, the behavior observed with soybean oil under different cultivation media can predict the behavior of the lettuce seeds in the present study. In this sense, the lower surface tension—due to the increase in MEL-B concentration in the culture medium—may reflect higher levels of seed wettability in contact with the treated surfaces. Therefore, greater seed hydration is expected along with, consequently, a more significant influence of the bioactive properties of MEL-B on seed germination as biosurfactant concentrations increase.

4.2. Assessing Germination Properties

In observations of the germination incidence, the same incidence level in soybean seed germination (less than 80%) after treatment with rhamnolipids was observed. However, the concentrations of rhamnolipids used for seed treatment were higher than the concentrations used in this study [39]. Another study evaluated the effect of rhamnolipid on lettuce germination and growth. They noted that the concentration of 750 mg/L stimulated lettuce seed germination but impaired radicle development compared to control [29]. According to Karthika et al. [40], the biosurfactant produced by the *Bacillus* sp. also indicated an improvement in the germination percentage of tomato seeds after treatment.

This work observed that MEL-B showed more significant interaction with the external tissue surrounding the seed due to its chemical structure. MEL-B increased the permeability of the seeds and contributed to a better performance in the germination of the seeds in some concentrations [41].

4.3. Morphological Changes of Roots

The seeds subjected to rhamnolipid treatment in another study showed decreased root length as the concentration increased. This observation may suggest a phytotoxic effect of this by-product against seedlings at high concentrations. In relation to root mass, the highest treatment concentration with rhamnolipid (1 g/L) showed a lower mass than control and other treatments [29]. This behavior was also observed in the treatments with MEL-B at 316 and 632 mg/L in this work. In another study that used the biostimulant Coveron in lettuce germination, the observed effects were positive about the control, where the length of the lettuce roots grew up to 2.1 cm at the end of the experiment [42]. Compared to this study, MEL-B showed superior results, with roots up to 3 cm in length under treatment at a concentration of 158 mg/L.

Sophorolipids were applied to the germination of barley seeds. In 10 days of germination, 195 mg/L of this biosurfactant stimulated the development of nine lateral roots. In comparison to the control, the application of a sophorolipid was superior by 2% in the stimulation of the lateral roots [43]. Regarding stressed roots, researchers noted that the development of germinated roots after treatment with rhamnolipid at 1 g/L was lower compared to control and intermediate concentrations [29]. In this sense, these bioproducts have an inhibitory effect on seed germination at high concentrations. However, the biostimulant effect of MEL-B can be noticed in lower amounts when compared to rhamnolipids and sophorolipids.

Khare and Arora applied biosurfactants to *Lycopersicon esculentum* and showed antiphytopathogenic and biocontrol activities. Similar to MEL-B, the authors noted that the biosurfactant promoted increased root growth and improved plant evolution and potential antimicrobial activity in several spectrums and was also environmentally better than chemical pesticides [44].

On the 10th day of tomato seed germination, the authors reported that the vermicompost treatment promoted the growth of 3.57 cm of roots [45]. However, MEL-B obtained the same result with only 6 days of germination.

Thus, it can be said that the mechanisms of action of MEL-B in lettuce seeds contribute to the oxidative stress of roots when treated with MEL-B at 316 and 632 mg/L. Furthermore, this imbalance in plant tissue and root development indicates a lack of control in primary plant metabolism in which ATP synthesis may be compromised.

4.4. Biochemical Analyzes after Treatment with MEL-B

The reduction in germination and root length can be attributed to the decrease in cell divisions due to morphological and physiological changes caused by the treatment used in seed germination. Figure 6 illustrates the biochemical responses in relation to enzymatic activities and the quantification of total proteins after seed cultivation with MEL-B. Studies indicate that proteins are covalently linked to the lignin molecule and, therefore, associated with cellulose in the cell wall and can confer rigidity, impermeability, and resistance against biological attacks to plant tissues. In addition, the lignification of root tissues can promote anatomical changes and influence water absorption, affecting root cell elongation [46].

Regarding the evaluated enzymes, the size and mass of the roots decreased as MEL-B concentrations were increased. This fact may be related to the fact that the enzymatic activity was evaluated using the crude extract of these roots. The responses obtained in this step corroborate the observations made in the previous steps in this work, indicating that the highest treatment concentrations (MEL-B at 316 and 632 mg/L) caused stress in seed cultivation.

As the concentration of vermicompost increased sharply in seed germination, the authors noticed an increase in protein content, POD, and PPO activity [45]. The same behavior was observed for treatments performed with MEL-B at 316 and 632 mg/L.

5. Conclusions

Unprecedentedly, this is the first report on the influence of MEL-B on seed germination. MEL-B at 158 mg/L showed promising results in the biostimulation of cultivated seeds. On the other hand, the responses observed in the physiological and biochemical behavior indicate that MEL-B at 316 and 632 mg/L influenced oxidative stress and inhibited the germination and development of the seeds. However, it is fundamental to identify the mechanisms of biosurfactant-plant interaction. These biomolecules have great potential to replace chemical pesticides based on new formulations with biosurfactants, and the analysis of obtained results indicated that MEL-B has great potential to replace, even if partially, the chemical components present in conventional pesticides, aiming to combat phytopathogens and promote the application of MELs to improve the solubility and/or degradation of chemical pesticides, the biostimulation of plants, and the use of MELs to promote soil quality by removing heavy metals and crude oil.

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Physiological and Molecular Analysis Revealed the Role of Silicon in Modulating Salinity Stress in Mung Bean

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Abstract: Salinity stress acts as a significant deterrent in the course of optimal plant growth and productivity, and mung bean, being a relay crop in the cereal cropping system, is severely affected by salinity. Silicon (Si), on the other hand, has exhibited promising outcomes with regards to alleviating salinity stress. In order to understand the critical mechanisms underlying mung bean (*Vigna radiata* L.) tolerance towards salt stress, this study examined the effects of different salinity concentrations on antioxidant capacity, proteome level alterations, and influence on Si-transporter and salt-responsive genes. Salinity stress was seen to effect the gaseous exchange machinery, decrease the soluble protein and phenolic content and NR activity, and increase the accumulation of reactive oxygen species. An efficient regulation of stomatal opening upon Si application hints towards proficient stomatal conductance and CO_2 fixation, resulting in efficient photosynthesis leading to proficient plant growth. The soluble protein and phenolic content showed improved levels upon Si supplementation, which indicates an optimal solute transport system from source to sink. The content of superoxide radicals showed a surge under salinity stress treatment, but efficient scavenging of superoxide radicles was noted under Si supplementation. Salinity stress exhibited more damaging effects on root NR activity, which was notably enhanced upon Si supplementation. Moreover, the beneficial role of Si was further substantiated as there was notable Si accumulation in the leaves and roots of salinity-stressed mung bean plants. Furthermore, Si stimulated competent ROS scavenging by reinforcing the antioxidant enzyme activity, as well coordinating with their isozyme activity, as expressed by the varying band intensities. Similarly, the Si-mediated increase in peroxidase activity may reveal changes in the mechanical characteristics of the cell wall, which are in turn associated with salinity stress adaptation. Proteomic investigations revealed the upregulation or downregulation of several proteins, which were thereafter identified by LC−MS/MS. About 45 proteins were identified and were functionally classified into photosynthesis (24%), metabolic process (19%), redox homeostasis (12%), transmembrane transport (10%), stress response (7%), and transcription regulation (4%). The gene expression analysis of the silicon transporter genes (*Lsi1*, *Lsi2*, and *Lsi3*) and SOS pathway genes (*SOS1*, *SOS2*, and *SOS3*) indicated the role of silicon in mitigating salinity stress. Hence, the findings of this study can facilitate a profound understanding of the potential mechanisms adopted by mung bean due to exogenous Si application during salinity stress.

Keywords: antioxidant activity; mung bean; mass spectrometer; proteomics; silicon; salinity stress

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1. Introduction

Severe and persistent droughts in various regions of the world have compelled farmers to resort to low-quality irrigation water sources and to exploit unsustainable irrigation and fertilization approaches, which have escalated the problem of soil salinization [1]. Salinity stress negatively affects plant growth, development, and productivity as it reduces the osmotic potential of the rhizosphere, damages cell membranes, causes ionic imbalances

and photosynthesis impedance, and creates a significant increase in light-dependent respiration [2]. The interplay of ionic and osmotic stress coupled with nutrient deficiencies paves the way for oxidative stress development [3]. Because of their chemical makeup, ROS are extremely unstable and reactive and can start radical chain reactions that can deactivate proteins, oxidize membrane lipids, and harm nucleic acids [4]. Thus, unraveling the responses of plants towards salinity stress, in order to augment plant production, has been a pressing objective among plant breeders. In this regard, a comprehensive study on the mechanism of the molecular and biochemical responses of plants towards salinity stress tolerance is obligatory.

Mung bean (*Vigna radiata* L.), a leguminous crop, serves as a significant source of protein, carbohydrates, isoflavones, vitamins, fiber, and minerals [5]. However, salinity stress deleteriously disturbs plant germination, growth, the reproductive stage, and the capacity to biologically fix nitrogen in legumes. In mung bean, salinity has been observed to affect seedling germination and development [6], photosynthesis, nodulation [7], the accumulation of ROS, water status, membrane stability, and the content of pigments [7,8]. Of the various salinity management strategies, we intended to take advantage of the application of exogenous Si to combat salinity stress in mung bean because Si research so far has largely neglected legumes.

The position of silicon (Si) in terms of its "essentiality" for plant growth and development has been a reasonably debated topic among researchers. However, the plethora of research findings that have established Si as a proficient player in the alleviation of various abiotic stresses, such as salinity stress, drought stress, and metal toxicity, cannot be undermined either [7,9–11]. The protective role of Si is usually seen in plants due to the polymerization of silicates in the endodermis and exodermis. This leads to the obstruction of the Na^+ bypass route, resulting in lignification and suberization, as well as the formation of casparian bands, which disrupt the flow of solutes from roots to shoots by altering the properties of the membrane transport system [12]. Owing to the existence of precise transporters found in the cellular membranes of plant roots, silicon can be rapidly transported [13]. The soil-to-root influx of Si is mediated by the influx transporter *Lsi1* and its homolog *Lsi6*, whereas the efflux transporter *Lsi2* dictates the apoplastic release of Si, which is followed by Si-translocation to shoots mediated by a transpiration stream. Moreover, Si transporters *Lsi6* and *Lsi3* are involved in xylem unloading and re-loading, respectively [14].

Previously, efforts have been made to study the alterations in the protein expression of plants under salinity stress using proteomic approaches in alfalfa [15], maize [16], Halophytes *Suaeda maritima* (L.), and *Salicornia brachiate* [17]. However, the fact that very limited research has attempted to elucidate the effect of Si on the protein expression profiles of plants under salinity stress is concerning. Nevertheless, the proteomics analysis of tomato [18], capsicum [19], and rose [20] under salinity stress revealed a downregulation of the functional proteins, which were upregulated under Si supplementation. However, to the best of our knowledge, a proteomic analysis to elucidate Si's role in providing salinity stress resistance to mung bean is not available.

The orchestration of the multifaceted molecular events regulating the initiation or suppression of various salt-stress responsive genes, such as the salt overly sensitive (*SOS*) gene, culminate in conferring salt tolerance to plants [21]. Genes encoding the SOS proteins, which play a key role in maintaining a well-adjusted ion level inside the cell and providing salt tolerance, have been found in wheat [22], barley [23], and mustard [24]. Furthermore, plants overexpressing *SOS* genes have shown an increase in salt tolerance [25]. However, the role of Si in the regulation of *SOS* genes has not been widely studied as far as legumes are concerned.

It is of paramount importance to scrutinize and comprehend the underlying molecular mechanisms that are involved in the course of salt-stress tolerance in mung bean with Si supplementation, such that the improvement of this important legume crop is conceivable. To this point, proteome and transcriptome level analysis of mung bean under salinity

stress and Si supplementation has not been reported, which makes our study all the more indispensable and worth exploring. In this study, we aim to understand the mechanism behind protection against excessive ROS, connect the various dots related to changes in protein expression under salinity stress and Si supplementation using LC−MS/MS, and examine the role of mRNA level regulation of Si-transporter genes and salt-responsive genes in mung bean under salinity stress and Si supplementation. Investigation of mung bean protein expression patterns in response to salt stress will open up new avenues for understanding the regulatory networks of mung bean salt-stress acclimation and aid in the selection of candidate proteins for modification to increase salt-stress tolerance.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

This research was carried out at the polyhouse of School of Agricultural Innovations and Advanced Learning (VAIAL), Vellore Institute of Technology, Vellore, India. The polyhouse's growing conditions were as follows: temperature regime of 30 °C and 25 °C day and night, lighting period of 16:8 h, and relative humidity of approximately 65 ± 5%. Mung bean (*Vigna radiata* L.) seeds were subjected to surface sterilization with 5% (*v/v*) sodium hypochlorite solution for 30 min before being rinsed in distilled water. Three seeds were sown in plastic pots (with a diameter of 13 cm and height of 17 cm). The pots were filled at a 1:1 ratio with sterilized red soil and vermicompost, and the soil salinity and pH were 0.35 dS m^{-1} and 7.86, respectively.

2.2. Experimental Design

The experiment utilized a completely randomized design (CRD) with four replicates for each treatment. The plants were allowed to grow until they reached the vegetative stage (30 days after germination) and were then divided into eight groups for a combination of silicon (Si) (5 mM) and salinity treatments (10 mM NaCl, 20 mM NaCl, and 50 mM NaCl) for 10 days. The various treatments included the following: (a) control (T1) (b) –NaCl + Si (T2), (c) 10 mM NaCl/-Si (T3), (d) 10 mM NaCl/+Si (T4), (e) 20 mM NaCl/-Si (T5), (f) 20 mM NaCl/+Si (T6), (g) 50 mM NaCl/-Si (T7), and (h) 50 mM NaCl/+Si (T8). For silicon treatment, the plants were irrigated with a sodium silicate (5 mM) solution and intermittently irrigated with water for 10 days. The leaf and root samples were collected after 10 days of treatment and stored at −80 °C until further use.

2.3. Scanning Electron Microscope (SEM Analysis)

Fresh leaves were cut into small pieces, treated in 2.5% glutaraldehyde solution (pH7.4), stored at 4 °C, and then dehydrated using a wide range of ethanol concentrations (95–50%) for the SEM analysis. The leaves were further oven dried at 60 °C for 48 h [26]. A scanning electron microscope (model: EVO-18 Research, Carl Zeiss, Birmingham, UK) was used to examine the structure of the stomata.

2.4. Determination of Total Soluble Protein, Total Soluble Sugars and Total Phenolic Content

To determine the total soluble protein content, leaf samples (0.5 g) were homogenized in a mortar and pestle with 200 mM phosphate buffer (pH7), followed by centrifugation at 8000 rpm for 10 min. To 0.5 mL of supernatant, 10% TCA was added, followed by centrifugation at 3300 rpm for 30 min. The supernatant obtained was discarded, the pellets were then washed with water and dissolved in 1 mL of 0.1 N NaOH. Furthermore, 0.2 mL of the supernatant was mixed with 5 mL of Bradford reagent, then incubated for 5 min and the absorbance was read at 595 nm [27]. To determine the total soluble sugar content, the leaf samples (0.5 g) were homogenized in 5 mL of 80% ethanol, followed by centrifugation at 6000 rpm for 15 min. To the supernatant, 12.5 mL of 80% ethanol and 1 mL of 0.2% anthrone solution was added. The reaction was placed in a water bath at 100 °C for 10 min. The absorbance was read at 620 nm [28]. To determine the total phenolics content, 0.1 g of leaf samples were suspended in a test tube containing 1.5 mL of 50% (*v*/*v*) methanol and

1% (*v*/*v*) HCL. The reaction was placed in a water bath at 80 °C for 15 min. Furthermore, 0.02 mL of leaf extract (diluted in 0.08 mL extraction solution) was mixed with 0.7 mL of Folin−Ciocalteu solution (diluted in 1:10 ratio) and 0.7 mL of 6% (*w*/*v*) Na_2CO_3. The samples were placed in the dark for 1 h and the absorbance was measured at 765 nm [29]. Gallic acid was used as a standard.

2.5. Determination of Nitrate Reductase (NR) Activity

The NR activity was measured according to Lopez-serrano et al. [30]. Briefly, 0.2 g of leaf and root samples were immersed in 100 mM potassium phosphate buffer (pH 7.5), 1% (*v*/*v*) propanol, and 100 mM KNO_3, and then incubated in a hot water bath for 60 min at 30 °C. The reaction was stopped by placing the test tubes in a boiling water bath for 5 min. From this, 1 mL of supernatant was taken, to which 1 mL of 0.02% N-naphthyl ethylenediamine and 1 mL of 1% sulfanilamide were added and the absorbance was measured at 540 nm. To determine the quantity of NO_2 in the samples, a standard curve using KNO_2 was prepared.

2.6. Estimation of Silicon Concentration

From each pot, approximately 10 expanded leaves and masses of roots were selected at random. Each treatment group had five replications. The leaves were dried in a hot air oven at 80 °C for 48 h and then ground into a fine powder. Approximately 100 mg of leaf and root samples were acidified with HNO_3 for 12 h and further digested using the microwave-digestion method. Silicon concentrations were measured with a Perkin Elmer Optimum 5300 DV inductively coupled plasma optical emission spectrometer (ICP-OES) [9].

2.7. Determination of Hydrogen Peroxide (H_2O_2) and Superoxide (O_2^-) Content

The content of H_2O_2 was measured according to Velikova et al. [31] with minor modifications. Fresh leaves (0.25 g) were homogenized in 2 mL of 0.1% (*w*/*v*) trichloroacetic acid (TCA), followed by centrifugation at 10,000 rpm for 8 min at 4 °C. To the supernatant, 0.6 mL of 0.1% (*w*/*v*) TCA was added and it was then incubated for 1 h at room temperature in a dark place. Absorbance was read at 390 nm. The H_2O_2 content was calculated from a H_2O_2 standard curve. The O_2^- content was measured according to Muneer et al. [9], with slight modifications.

2.8. Estimation of Antioxidants Enzyme Activity and Their Relative Staining

To measure the antioxidant enzyme activity, the leaf samples (0.1 g) were homogenized in an extraction buffer comprising 50 mM potassium phosphate buffer (pH7.0) with 1 mM EDTA, 0.05% triton X, and 1 mM polyvinylpyrrolidone (PVP); centrifugation was then carried out at 10,000 rpm for 20 min at 4 °C. The supernatant was then utilized to determine the antioxidant enzyme activity. The superoxide dismutase (SOD) activity was analyzed using the nitro blue tetrazolium (NBT) inhibition method of Giannopolitis and Ries et al. [32]. One unit (U) of SOD activity was defined as the quantity of the enzyme that inhibited the photochemical reduction of NBT by 50%. Catalase (CAT) activity was performed according to Manivannan et al. [19]. One unit of catalase decomposed 1.0 μmole of H_2O_2 per minute, while the H_2O_2 concentration declined from 10.3 mM to 9.2 mM. The APX activity was determined according to Nakano and Asada et al. [33]. One unit (U) of APX activity corresponded to the amount of enzyme required to oxidize 1 μmole of ascorbic acid per minute per mg of protein.

For native staining, the antioxidant enzymes (30 μg) were electrophoresed in 10% resolving and 4% stacking gel, respectively, for APX and CAT isozymes, whereas, 15% resolving gel and 5% stacking gel were used for separating the SOD isozymes at 4 °C for 4 h at 80 V in a Tris-Glycine (pH8.3) running buffer. The active staining of isozymes of SOD, CAT, and APX were performed according to Pham et al. [34].

2.9. Native PAGE Profiling of Isozymes of Peroxidases' Enzyme(s)

The leaf samples (0.5 g) were homogenized in an extraction buffer composed of 100 mM K-PO_4 buffer (pH7.0) and 2 mM phenylmethylsulphonyl fluoride, and centrifugation was done at 14,000 rpm for 20 min at 4 °C. Active staining of GPOX, SPOX, and BPOX was carried out according to Lee et al. [35].

2.10. Protein Extraction and One-Dimensional Gel Electrophoresis (SDS-PAGE)

Protein extraction and SDS-PAGE were performed according to Muneer et al. [36]. The protein content was measured using the Bradford test and a standard curve of bovine serum albumin (BSA). After electrophoresis, the gel was stained with Comassie brilliant blue stain (CBBS), which is commercially available (Bio-Rad).

2.11. In-Gel Digestion of Protein Bands and Mass Spectrometer Analysis

The method of Muneer et al. [37] was used for the protein in gel digestion (for a detailed methodology of the in-gel digestion, please refer to Muneer et al.) [37].

The MS and MS/MS spectra data were analyzed with a mass tolerance of 50 ppm using the NCBI and Protein Pilot V.3.0 database software (with the MASCOT V.2.3.02 database search engine). Oxidation of methionines and carbamidomethylation of cysteines were permitted for database searches of the MS/MS spectra. A statistically significant threshold value of $p = 0.05$ was used to search for individual peptide ion scores. According to the gene ontology analysis (http://www.geneontology.org, accessed on 1 August 2022), the identified proteins were further categorized on the basis of the biological processes in which they contribute. The identified proteins were also analyzed to observe possible protein−protein interactions using the STRING database.

2.12. RNA Isolation, cDNA Preparation, and RT-PCR

RNA was isolated from the leaves using an RNA isolation Kkit according to the manufacturer's instructions (Hi-Media). Real-time PCR was carried out in Applied Biosystems using SYBR Green Chemistry (Sensifast HiRoxkit Bioline, Memphis, TN, USA) for 5 min at 95 °C, followed by 35 cycles of 20 s at 95 °C, 30 s at 57 °C, and 30 s at 72 °C, followed by 10 min at 72 °C. Actin was utilized to normalize all quantifications. For the RT-PCR reactions and qPCR, three distinct RNA preparations from independently grown plants were utilized. The results were analyzed using qBase plus 13 software. Table 1 lists the gene-specific primers utilized in our investigation.

Table 1. Primer sequences used for the RT-PCR analysis.

Gene	Forward Sequence (5′---------3′)	Reverse Sequence (5′---------3′)
Lsi-1	ATGGAGAGTGAAGGAGGGAA	TTAGAGGGTAACACATTGTT
Lsi-2	CGATGACTTTGCCCATCGTG	GCAATATGAACCTCGTCCGC
Lsi-3	TATTTYTTCCTGGCCAACCT	TTAAGCTATAGATGAGGGGG
SOS1	GCCAGCTATAAGCTAAGCAC	GCAATCCCTAAAGCAAGACC
SOS2	GCATTCATCGTGCAGCATC	GTATAGTCTCGCCATCACCTC
SOS3	ACGAAGAATTTCAGCTCGC	TCACCTAACTCGATGACTCC
Actin	ATCCTCCGTCTTGACCTTG	TGTCCGTCAGGCAACTCAT

2.13. Statistical Analysis

For the physiological parameters, a complete randomized design was employed with four replicates. The percentage change was calculated using: [(Treatment−Control)/(Treatment) × 100]. To compare the means of distinct replicates, Tukey's studentized range test was applied. Unless otherwise noted, the results are based on differences between means, with a level of significance of $p < 0.05$.

3. Results

3.1. Effect of Salinity Stress on Structure and Opening/Closing of Stomatal Pore of Mung Bean Supplemented with Si

Salinity stress impairs photosynthetic machinery and thus adversely affects the gaseous exchange in plants subjected to abiotic stress conditions by intervening with the opening and closing of the stomata. Hence, in our study, after 10 days of salinity stress treatment, the stomatal structure was found to be affected (Figure 1). It was evident that the stomatal pore was found to be closed when different concentrations (T3, T5, and T7) of salinity stress were provided when compared with the control. On the contrary, the stomatal opening was observed when Si was supplemented to the salinity stress-treated plants in T4, T6, and T8, respectively.

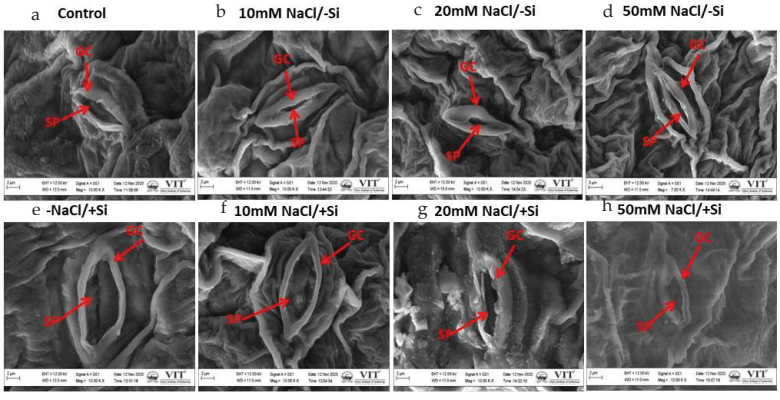

Figure 1. Representative image of stomatal opening/closing of mung bean (*Vigna radiata*) under Si supply (5 mM) and salinity stress (**a**) Control (T1) (**b**) –NaCl + Si (T2) (**c**) 10 mM NaCl/-Si (T3) (**d**) 10 mM NaCl/+Si (T4) (**e**) 20 mM NaCl/-Si (T5) (**f**) 20 mM NaCl/+Si (T6) (**g**) 50 mM NaCl/-Si (T7) (**h**) 50 mM NaCl/+Si (T8) for a duration of 10 days.

3.2. Effect of Salinity Stress on the Soluble Protein, Sugar, and Phenolic Content of Mung Bean Supplemented with Si

The content of soluble protein was seen to be reduced by 25% in the highest concentration (T7) of salinity stress provided when compared with the control (T1) (Figure 2A). However, Si supplementation increased the levels of soluble protein by 93% in T4 when compared with T3, but reduced the soluble protein content in T6 and did not have many significant changes in T8. The total soluble sugar content in salinity-treated plants was seen to be increased upon Si supplementation in T4, but there was no significant difference in T6 and T8 upon the supplementation of Si (Figure 2B). For the total phenolic content, the phenolics levels were found to be increased through the supplementation of Si to e-salinity-stressed plants (Figure 2C). The phenolic content was increased by 53% and 50% in T6 and T8, respectively, when compared with the salinity treatments. However, no significant change was observed upon Si supplementation in T4.

3.3. Effect of Salinity Stress on the Nitrate Reductase Activity of Mung Bean Supplemented with Si

Salinity stress was seen to affect the NR activity in the leaves in T3 when compared with the control, but it did not show significant changes in the root NR activity (Figure 3A,B). The NR activity in the leaves was seen to be reduced by 38% in T3 when compared with the control (T1). After supplementation with Si, the NR activity in leaves did not show significant changes. However, after Si supplementation, the root NR activity was found to be increased, and the most significant increase was that of 92% in T4 and 59% in T8. However, no significant change was observed in T6 upon Si supplementation.

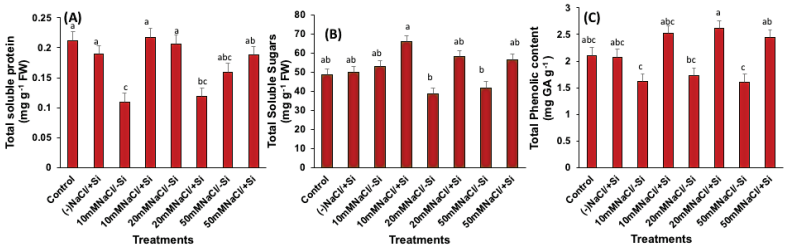

Figure 2. Changes in content of the (**A**) total soluble protein, (**B**) total soluble sugars, and (**C**) total phenolic contents of mung bean (*Vigna radiata*) under Si supply (5 mM) and salinity stress: (a) Control (T1), (b) –NaCl + Si (T2), (c) 10 mM NaCl/-Si (T3), (d) 10 mM NaCl/+Si (T4), (e) 20 mM NaCl/-Si (T5), (f) 20 mM NaCl/+Si (T6), (g) 50 mM NaCl/-Si (T7), and (h) 50 mM NaCl/+Si (T8) for a duration of 10 days. Vertical bars indicate mean ± SE of the mean for $n = 4$. Means denoted by different letter are significantly different at $p \leq 0.05$ according to Tukey's studentized range test.

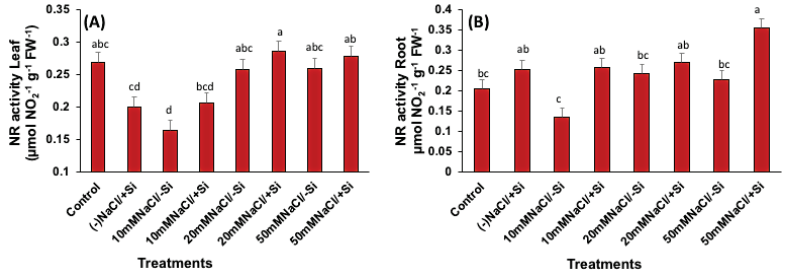

Figure 3. Changes in NR activity for the (**A**) leaves and (**B**) roots of mung bean (*Vigna radiata*) under Si supply (5 mM) and salinity stress: (a) control (T1), (b) –NaCl + Si (T2), (c) 10 mM NaCl/-Si (T3), (d) 10 mM NaCl/+Si (T4), (e) 20 mM NaCl/-Si (T5), (f) 20 mM NaCl/+Si (T6), (g) 50 mM NaCl/-Si (T7), and (h) 50 mM NaCl/+Si (T8) for a duration of 10 days. Vertical bars indicate mean ± SE of the mean for $n = 4$. Means denoted by a different letter are significantly different at $p \leq 0.05$ according to the Tukey's studentized range test.

3.4. Silicon Concentration in Leaves and Roots

The Si content in the leaves was slightly more than the Si content in the roots. In the leaves, the highest Si content was observed in T2 and T8 (Figure 4A). The Si content increased in T4, T6, and T8 by 21%, 49%, and 81%, respectively. The highest change in Si content was observed in T8 compared with T7. In the roots, the Si content was the highest in T4 followed by T6 and T8 (Figure 4B). The root Si content in T6 and T8 did not show a significant difference when compared with the Si alone treatment (T2). The Si content in T3 and T5 did not show significant changes either. However, the highest significant percentage change of 51% was observed in T4 when compared with T3.

3.5. Effects of Salinity Stress on Hydrogen Peroxide (H_2O_2) and Superoxide (O_2^-) Content of Mung Bean Supplemented with Si

Following salinity stress treatments for 10 days, the H_2O_2 content was found to have increased by 73.2% in T3 compared with the control (Figure 5A). However, the H_20_2 content was found to be decreased in T5 and T7, but only the decrease in T7 was significant. Moreover, after the supplementation of Si, the content of H_2O_2 did not significantly reduce in T4 and T8, but a significant reduction of 49% was seen in T6 when compared with T5. For the O_2^- content, the levels increased in salinity treatments T3, T5, and T7 by 238%, 270%, and 217%, respectively, when compared with the control (T1) (Figure 5B). After Si supplementation in T4 and T6, the levels of O_2^- were found to be reduced significantly by

32% and 31%, respectively, but the levels of O_2^- increased in T8 when compared with T7 after Si supplementation.

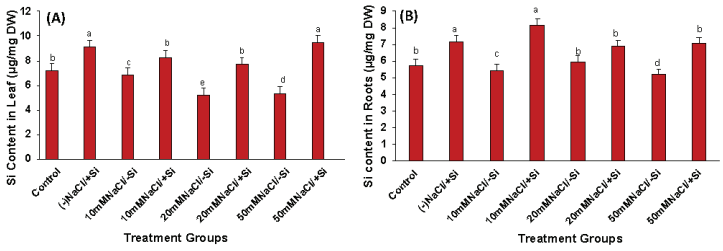

Figure 4. Changes in Si content of (**A**) leaves and (**B**) roots of mung bean (*Vigna radiata*) under Si supply (5 mM) and salinity stress: (a) control (T1), (b) –NaCl+Si (T2), (c) 10 mM NaCl/-Si (T3), (d) 10 mM NaCl/+Si (T4), (e) 20 mM NaCl/-Si (T5), (f) 20 mM NaCl/+Si (T6), (g) 50 mM NaCl/-Si (T7), and (h) 50 mM NaCl/+Si (T8) for a duration of 10 days. Vertical bars indicate mean ± SE of the mean for $n = 4$. Means denoted by s different letter are significantly different at $p \leq 0.05$ according to the Tukey's studentized range test.

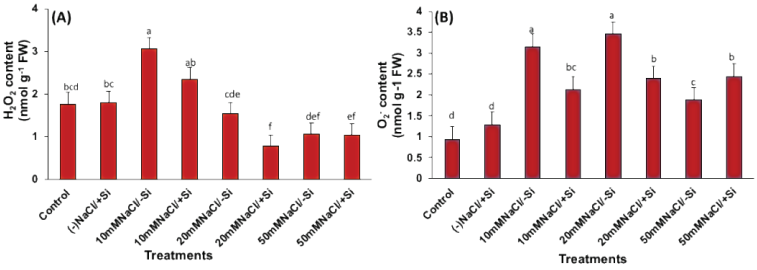

Figure 5. Changes in the content of (**A**) H_2O_2 and (**B**) O_2^- of mung bean (*Vigna radiata*) under Si supply (5 mM) and salinity stress: (a) control (T1), (b) –NaCl + Si (T2), (c) 10 mM NaCl/-Si (T3), (d) 10 mM NaCl/+Si (T4), (e) 20 mM NaCl/-Si (T5), (f) 20 mM NaCl/+Si (T6), (g) 50 mM NaCl/-Si (T7), and (h) 50 mM NaCl/+Si (T8) for a duration of 10 days. Vertical bars indicate mean ± SE of the mean for $n = 4$. Means denoted by a different letter are significantly different at $p \leq 0.05$ according to the Tukey's studentized range test.

3.6. Effect of Salinity Stress on Antioxidant Activity and Their Isozyme Patterns in Mung Bean Supplemented with Si

The SOD activity was seen to be affected in all of the salinity treatment groups, with the exception of T3 (Figure 6A). Si supplementation was, however, seen to enhance the activity of SOD significantly by 185% and 101% in T6 and T8, respectively, when compared with T5 and T7, respectively. However, a significant difference between SOD activity in the Si-treatment alone and control was missing. Moreover, Si supplementation in T4 was found to have decreased the SOD activity when compared with T3. The isozyme bands of SOD were found to be more intense in the treatment groups where Si was supplemented along with salinity stress (Figure 6D). For the isozymes of SOD, SOD-3 displayed a reduced band intensity in T3, T5, and T6; however, after Si supplementation, SOD-3 had greater band intensities in T4, T6, and T8, respectively. The SOD-2 isozyme bands were also expressed more in T4 compared with T3. The APX activity increased by 120% and 48% in T6 and T8, respectively, when compared with T5 and T7, respectively, upon Si supplementation (Figure 6B). However, there was no significant difference between the APX activity in the Si-treatment alone and the control. Moreover, Si supplementation in T4 was found to have decreased the APX activity when compared with T3. The band intensity of isozyme APX-2 diminished under salinity treatments T3 and T5, whereas Si supplementation in T4 and T6

showed increased band intensities of APX-2 (Figure 6D). The CAT activity followed the same trend as observed in the SOD and APX activity. After Si supplementation, the activity of CAT increased by 241% and 26% in T6 and T8, respectively, when compared with T5 and T7, respectively (Figure 6C). However, the CAT for T3 and T4 were not significant when compared with T2 and T1, respectively. Moreover, Si supplementation in T4 was found to have decreased the CAT activity when compared with T3. Of the two CAT isozymes stained, CAT-2 showed expression changes across salinity stress treatments and Si supplemented groups (Figure 6D). CAT-2 isozyme bands showed lesser band intensity in T3 and were highly expressed in T3 and T5, whereas the expression of CAT-2 was increased in T4 and T6, respectively.

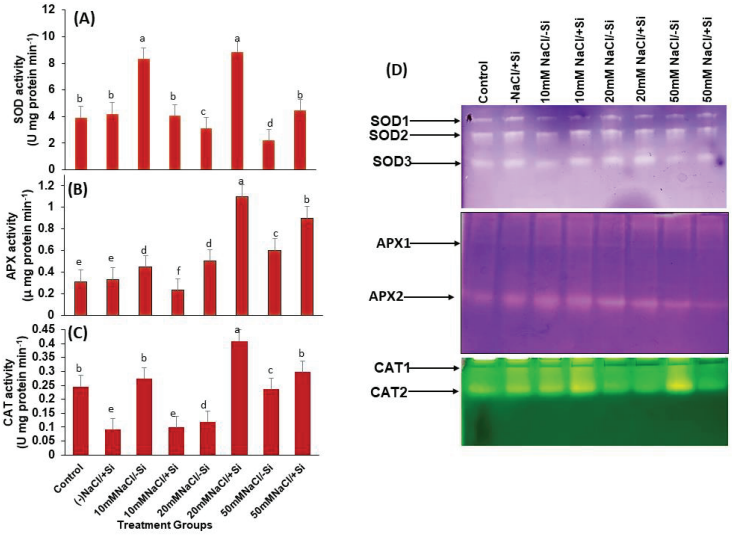

Figure 6. Changes in the antioxidant enzyme activity and isozyme profiles of (**A**,**D**) superoxide dismutase, (**B**,**D**) ascorbate peroxidase, and (**C**,**D**) catalase of mung bean (*Vigna radiata*) under Si supply (5 mM) and salinity stress: (a) control (T1), (b) –NaCl+Si (T2), (c) 10 mM NaCl/-Si (T3), (d) 10 mM NaCl/+Si (T4), (e) 20 mM NaCl/-Si (T5), (f) 20 mM NaCl/+Si (T6), (g) 50 mM NaCl/-Si (T7), and (h) 50 mM NaCl/+Si (T8) for a duration of 10 days. Vertical bars indicate mean ± SE of the mean for $n = 4$. Means denoted by a different letter are significantly different at $p \leq 0.05$ according to Tukey's studentized range test.

3.7. Effect of Salinity Stress on the Isozymes of Peroxidase Enzymes' in Mung Bean Supplemented with Si

The active staining of BPOX revealed four isozymes, BPOX-1, BPOX-2, BPOX-3, and BPOX-4, among which the expression pattern on BPOX-4 was seen to be more prominent (Figure 7A). BPOX-4 had a lower band intensity in T5 and T7; however, after Si supplementation in T6 and T8, the band intensities were seen to have increased. Similarly, the GPOX isozyme GPOX-2 was seen to have a higher expression profile under Si supplementation in T6 and T8 when compared with T5 and T7, respectively (Figure 7B). SPOX isozymes did not display many changes in expression among the salinity-treated groups and Si-supplemented groups (Figure 7C). However, under Si supplementation, the band intensity of SPOX-2 was found to be higher in T8 compared with that of T7.

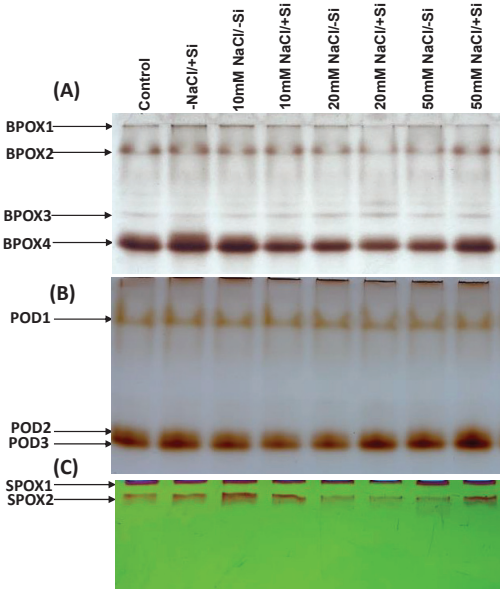

Figure 7. Profiles of peroxidase isozymes: (**A**) BPOX, (**B**) POD, and (**C**) SPOX of mung bean (*Vigna radiata*) under Si supply (5 mM) and salinity stress: (a) control (T1), (b) –NaCl+Si (T2), (c) 10 mM NaCl/-Si (T3), (d) 10 mM NaCl/+Si (T4), (e) 20 mM NaCl/-Si (T5), (f) 20 mM NaCl/+Si (T6), (g) 50 mM NaCl/-Si (T7), and (h) 50 mM NaCl/+Si (T8) for a duration of 10 days.

3.8. Changes in the Expression of Proteins

The protein profile was analyzed by one-dimensional gel electrophoresis (SDS-PAGE), and the proteins were observed to be either upregulated or downregulated (Figure 8, graphical representation shown in Figure 9B). Following this, these up or downregulated proteins were identified using a mass spectrometer (LC−MS/MS) (Table 2). Direct gene ontology consortium (http://www.geneontology.org/, accessed on 1 August 2022) was used to determine the percent variation of identified proteins for functional categorization. All of the differentially expressed proteins were grouped into photosynthesis (24%), metabolic process (19%), redox homeostasis (12%), transmembrane transport (10%), stress response (7%), and transcription regulation (4%) (Figure 9A). Additionally, the STRING database was used to examine the identified proteins for any protein−protein interactions (Figure 10). The proteins mostly interacted with cell division, ATP synthase, photosynthesis, transport, metabolism, and other signaling-pathway-related proteins.

3.8.1. Proteins Related to Photosynthesis

Salinity stress was found to have decreased the expression of vital proteins that are involved in photosynthesis, such as ribulose bisphosphate carboxylase large chain (band 3A, 3C, 7A, 7B, and 7C). However, Si supplementation positively influenced the expression of ribulose bisphosphate carboxylase large chain (band 6A and 6D), thus restoring the normal functioning of the photosynthetic process.

3.8.2. Proteins Related to Metabolic Processes

Salinity stress was seen to downregulate the enzyme fumarylacetoacetase (band 3B) involved in tyrosine and phenylalanine catabolism, fructose-bisphosphatase (band 3G) and putative phosphoketolase (band 3G) involved in carbohydrate metabolism, and putative phospho-2-dehydro-3-deoxyheptonate aldolase (band 3H) involved in amino acid synthesis. After Si supplementation to the salinity-stressed plants, enzyme 1-phosphatidylinositol 4-kinase (band 7D) involved in lipid metabolism, fructose-bisphosphate aldolase (band

7F) involved in carbohydrate metabolism, cyclase family protein (7F) involved in amino acid metabolism, tRNA(Ile)-lysidine synthetase (band 7F) involved in tRNA metabolic process, and precorrin-2 dehydrogenase (band 7H) involved in porphyrin biosynthesis were observed to be upregulated. Moreover, Si supplementation alone could upregulate various key proteins such as cellulose (band 2C), peptide hydrolase (band 2F), 3-deoxy-7-phosphoheptulonate synthase (band 2G), and UDP-N-acetylglucosamine transferase subunit ALG13 (band 2I) involved in key metabolic processes.

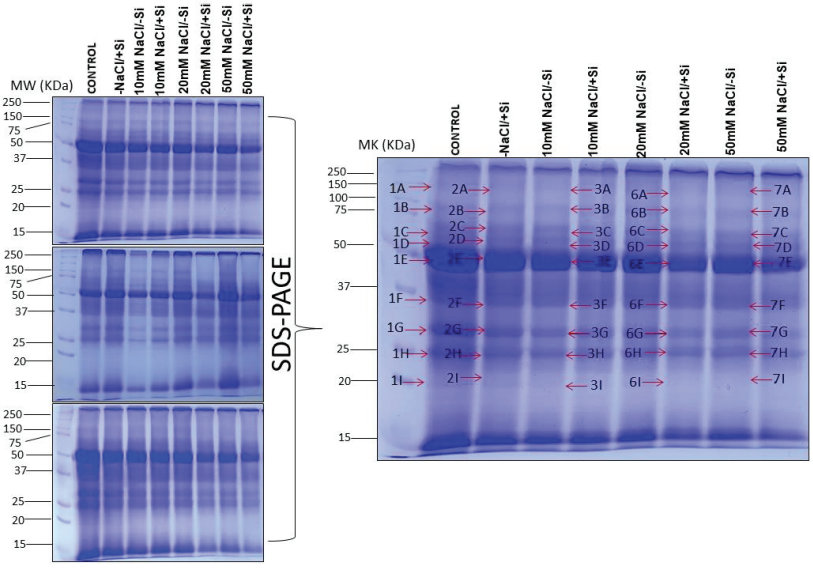

Figure 8. Representative image of protein profiles (SDS-PAGE) of mung bean (*Vigna radiata*) under Si supply (5 mM) and salinity stress: (a) control (T1), (b) –NaCl+Si (T2), (c) 10 mM NaCl/-Si (T3), (d) 10 mM NaCl/+Si (T4), (e) 20 mM NaCl/-Si (T5), (f) 20 mM NaCl/+Si (T6), (g) 50 mM NaCl/-Si (T7), and (h) 50 mM NaCl/+Si (T8) for a duration of 10 days. Differentially expressed bands excised for protein identification by LC−MS/MS are marked by arrows.

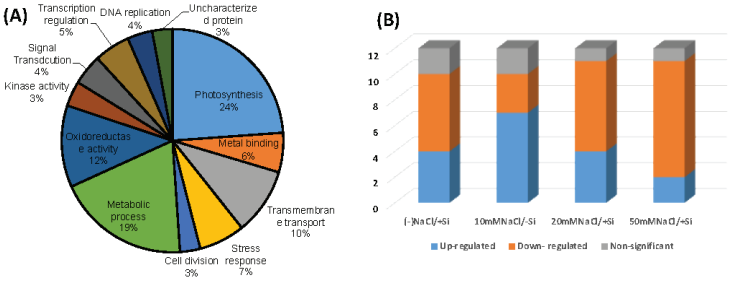

Figure 9. Comparative analysis of the proteome profiles between the treatments. (**A**) Functional classification of the proteins identified by Gene ontology analysis and (**B**) Venn diagram illustration of the up, down, or non-significantly regulated proteins.

Table 2. Identification of differentially-expressed proteins by LC/MS-MS in mung bean (*Vigna radiata*).

Band no.	Protein Name	Plant Species	Accession Number	Protein Score	Biological Function
1A	Fumarylacetoacetase	*Cephalotus follicularis*	A0A1Q3BBE1	28	Metal ion binding, chaperone binding
	Uncharacterized protein	*Apolygus lucorum*	A0A6A4KDN9	26	Integral component of membrane
	Protein kinase domain-containing protein	*Rhizophagus irregularis*	U9V622	26	ATP binding, protein kinase activity
1B	Ribulose bisphosphate carboxylase large chain	*Alfaroa guanacastensis*	A0A068L6A4	43	Photorespiration, photosynthesis
	5B protein like protein	*Arabidopsis thaliana*	Q9SUZ2	30	Stress response
	DHA1 family multidrug resistance protein-like MFS transporter	*Paenibacillus prosopidis*	A0A368W0U8	29	Transmembrane transport
1C	Ribulose bisphosphate carboxylase large chain	*Soleirolia soleirolii*	A0A0F7C9I4	101	Photosynthesis
	Uracil permease	*Paludibacterium purpuratum*	A0A4R7BCH9	39	Transmembrane transport
	Putative metallothionein expression activator	*Diaporthe ampelina*	A0A0G2HMQ0	35	Metal binding
1D	Ribulose bisphosphate carboxylase large chain	*Trifolium repens*	A0A023HPA0	186	Photosynthesis
	Peptidyl-tRNA hydrolase	*Glycomyces artemisiae*	A0A2T0UIK1	45	Translation
	Flavodoxin	*Eggerthella lenta*	A0A369MMS0	42	Metal binding
1E	Ribulose bisphosphate carboxylase large chain	*Berchemia lineata*	A0A7L8XJV8	223	Photosynthesis
	Ribulose bisphosphate carboxylase large chain	*Pycnarrhena cauliflora*	B3FWZ0	223	Photosynthesis
	Ribulose bisphosphate carboxylase large chain	*Soleirolia soleirolii*	A0A0F7C9I4	193	Photosynthesis
1F	Biosynthetic peptidoglycan transglycosylase	*Xanthomonas arboricola*	A0A7W9QLL5	30	Peptidoglycan synthesis
	LRRNT_2 domain-containing protein	*Quercus lobata*	A0A7N2MTJ2	30	Transmembrane transport
	Cell division protein FtsQ	*Aeromonas veronii*	A0A6S4V1U8	29	Cell division
1G	Ribulose bisphosphate carboxylase large chain	*Agathis borneensis*	Q9MVV3	61	Photosynthesis
	Lysine-specific demethylase 3A	*Capsicum chinense*	A0A2G3CFW6	33	Methylation
	Glucose-1-phosphate adenylyl transferase	*Rhodospirillaceae bacterium*	A0A2E5LIM1	31	Glycogen biosynthetic process
1H	Lysozyme	*Enterobacter phage vB_EkoM5VN*	A0A7I8HQY3	32	Defense response, catabolic process
	Putative N-glycosyltransferase	*Frankia alni*	Q0RGF9	32	Transferase activity
	probable transcription factor KAN4	*Juglans regia*	A0A2I4EQ27	31	Transcription regulation
1I	Ribulose bisphosphate carboxylase small subunit	*Arachis duranensis*	A0A6P4D9J6	94	Photosynthesis
	Uncharacterized protein	*Marchantia polymorpha*	A0A2R6WJ30	31	NIL
	Uncharacterized protein	*Pseudocercospora fijiensis*	M2ZM51	31	NIL
2A	Ribulose bisphosphate carboxylase large chain	*Trifolium repens*	A0A023HPA0	39	Photosynthesis
	Uncharacterized protein	*Setaria italica*	K4A228	32	NIL
	Endonuclease/exonuclease/phosphatase family metal-dependent hydrolase	*Rhizobium pisi*	A0A7W5BJJ3	27	Endonuclease activity

Table 2. *Cont.*

Band no.	Protein Name	Plant Species	Accession Number	Protein Score	Biological Function
2B	tRNA wybutosine-synthesizing protein 4	*Trichoderma arundinaceum*	A0A395NCK0	38	Endonuclease activity
	ABC-type branched-subunit amino acid transport system substrate-binding protein	*Streptomyces* sp. *BK022*	A0A4Q7Z6F6	34	Transmembrane transport
	TPX2 domain-containing protein	*Marchantia polymorpha* subsp. *ruderalis*	A0A176W329	33	Kinase activity, cell cycle/division
2C	Uncharacterized protein	*Kribbella* sp. *VKM Ac-2527*	A0A4R6KBE5	35	NIL
	Cellulase	*Bosea* sp. *AK1*	A0A542B873	30	Metabolic process
	Multiple sugar transport system permease protein	*Kribbella* sp. *VKM Ac-2569*	A0A4Q7QE40	29	Transmembrane transport
2D	Ribulose bisphosphate carboxylase large chain	*Kayea stylosa*	Q8MCX9	213	Photosynthesis
	Precorrin-2 dehydrogenase	*Winogradskyella arenosi*	A0A368ZJY7	53	Oxidoreductase
	Uncharacterized protein	*Klebsormidium nitens*	A0A1Y1HR37	47	Transmembrane transport
	Haemolysin activation/secretion protein	*Cupriavidus plantarum*	A0A316F4A6	43	Protein transport
2E	Ribulose bisphosphate carboxylase large chain	*Cornus eydeana*	Q2TV61	143	Photosynthesis
	Ribulose bisphosphate carboxylase large chain	*Crossostylis grandiflora*	A0ZQX2	120	Photosynthesis
	Acyltransferase	*Tardiphaga robiniae*	A0A7G6TUN4	41	Transmembrane transport, transferase activity
2F	Uncharacterized protein	*Salix brachista*	A0A5N5L7N0	33	Electron transport
	Peptide hydrolase	*Trichoderma arundinaceum*	A0A395NEC7	33	Catabolic process
	Shugoshin_C domain-containing protein	*Kalanchoe fedtschenkoi*	A0A7N0U758	33	Cell cycle/division
2G	Ribulose bisphosphate carboxylase large chain	*Aloe vera (Aloe)*	Q6VW13	82	Photosynthesis/ photorespiration
	Nitronate monooxygenase	*Paraburkholderia unamae*	A0A328WWJ8	33	Nitronate monooxygenase activity
	3-deoxy-7-phosphoheptulonate synthase	*Paenibacillus peoriae*	A0A0K2F5Q8	30	Metabolic process
2H	Superoxide dismutase	*Phaseolus lunatus*	Q3S614	46	Stress response
	Histidine kinase	*Massilia aurea*	A0A7W9U5Y5	34	Signaling, protein modification process
	Positive regulator of purine utilization	*Pyrenophora seminiperda CCB06*	A0A3M7MHM2	34	Transcription, metal binding
2I	Ribulose bisphosphate carboxylase large chain	*Kalanchoe fedtschenkoi*	A0A7N0TKL3	141	Photosynthesis/ photorespiration
	UDP-N-acetylglucosamine transferase subunit ALG13	*Botryotinia fuckeliana*	A0A384JFM8	30	Glycosylation, metabolic process
	2-keto-3-deoxy-L-fuconate dehydrogenase	*Rhizobium* sp. *PP-F2F-G48*	A0A4R1X109	28	Oxidoreductase
3A	Ribulose bisphosphate carboxylase large chain	*Trifolium repens*	A0A023HPA0	88	Photosynthesis/ photorespiration
	DUF4328 domain-containing protein	*Streptomyces violarus*	A0A7W4ZSR6	41	Transmembrane transport
	PAS domain S-box-containing protein	*Mucilaginibacter* sp. *E4BP6*	A0A7Y9HYI9	38	Signaling, kinase activity

Table 2. *Cont.*

Band no.	Protein Name	Plant Species	Accession Number	Protein Score	Biological Function
3B	Fumarylacetoacetase	*Cephalotus follicularis*	A0A1Q3BBE1	26	Catabolic process
	Protein kinase domain-containing protein	*Phaeosphaeria nodorum*	Q0UFQ8	26	Transcription, phosphorylation
	Uncharacterized protein	*Prunus persica*	A0A251QEC7	25	Defense response
3C	Ribulose bisphosphate carboxylase large chain	*Trifolium repens*	A0A023HPA0	96	Photosynthesis/ photorespiration
	Ribulose bisphosphate carboxylase large chain	*Spirodela polyrhiza*	A0A0F7EWB6	96	Photosynthesis/ photorespiration
	Ribulose bisphosphate carboxylase large chain	*Cladopus austro-osumiensis*	O03046	93	Photosynthesis/ photorespiration
3D	Ribulose bisphosphate carboxylase large chain	*Vanilla planifolia*	A0A0D3M9U3	151	Photosynthesis/ photorespiration
	Uncharacterized protein	*Lactuca sativa*	A0A2J6KPN6	33	Protein auto phosphorylation
	ATPase subunit of ABC transporter with duplicated ATPase domains	*Rhizobium* sp. *BK049*	A0A7W5KML0	31	ATP binding
3E	Uncharacterized protein	*Chara braunii*	A0A388KU15	31	Polymerase activity, DNA integration
	ATP-binding protein	*Raoultella ornithinolytica*	A0A225U1S1	29	ATP binding
	Methyltransferase family protein	*Cellulomonas* sp. *PhB143*	A0A3N2JFI2	29	Methylation
3F	Chromosome partition protein Smc	*Cohnella lupini*	A0A3D9IW82	35	DNA replication, chromosome condensation
	Long-chain acyl-CoA synthetase	*Streptomyces* sp. *BK239*	A0A4Q7XQB8	34	Aspartic-type endopeptidase activity
	SOS response UmuD protein	*Arthrobacter* sp. *SLBN-122*	A0A542G4S1	32	SOS response, DNA repair, transcription
	Diacylglycerol kinase iota	*Aegilops tauschii*	N1QUQ4	32	Defense response
3G	Fructose-bisphosphatase	*Brassica napus (Rape)*	A0A078FJK5	45	Metabolic process
	Putative phosphoketolase	*Fusarium culmorum*	A0A2T4H188	38	Carbohydrate metabolic process
	Ferredoxin-NADP reductase	*Xanthomonas campestris*	A0A7W6KYF2	34	Oxidoreductase
3H	Superoxide dismutase	*Glycine max*	Q71UA1	60	Stress response
	GntR family transcriptional regulator	*Rathayibacter* sp. *PhB93*	A0A3N1NHP1	37	Transcription
	Putative phospho-2-dehydro-3-deoxyheptonate aldolase	*Phaeomoniella chlamydospora*	A0A0G2EP81	36	Amino acid biosynthesis, metal binding
3I	Ribulose bisphosphate carboxylase small subunit	*Arachis duranensis*	A0A6P4D9J6	80	Photosynthesis/ photorespiration
	Uncharacterized protein	*Punica granatum*	A0A218W2T9	31	Transcription
	UDP-N-acetylglucosamine transferase subunit ALG13	*Botryotinia fuckeliana*	A0A384JFM8	31	Protein glycosylation, lipid metabolic process
6A	Ribulose bisphosphate carboxylase large chain	*Psychotria* sp. *PSN 1*	D6C638	118	Photosynthesis/ photorespiration
	Putative oxidoreductase, NAD(P)-binding domain	*Frankia alni*	Q0RJX8	33	Oxidoreductase
	Phosphomethylpyrimidine synthase	*Halomonas songnenensis*	A0A2T0V5C0	31	Metabolic process

Table 2. *Cont.*

Band no.	Protein Name	Plant Species	Accession Number	Protein Score	Biological Function
6B	Signal recognition particle subunit SRP68	*Klebsormidium nitens*	A0A1Y1IMJ0	34	Transport
	Pseudouridine synthase	*Azospirillum brasilense*	A0A560AZY8	32	Ribosome biogenesis
	Histidine kinase	*Pseudomonas putida*	A0A7D6A9J0	31	Kinase activity
6C	Alpha-mannosidase	*Penicillium expansum*	A0A0A2JQF2	32	Metabolic process
	Cytochrome c domain-containing protein	*Nitrospirillum amazonense*	A0A560G155	31	Metal binding
	Ubiquitin-like domain-containing protein	*Lupinus angustifolius*	A0A1J7GL54	31	Cell cycle
	Peroxidase	*Hibiscus syriacus*	A0A6A3AV07	28	Stress response
	PNP_UDP_1 domain-containing protein	*Fusarium poae*	A0A1B8A859	27	Metabolic process
6D	Ribulose bisphosphate carboxylase large chain	*Trichocladus crinitus*	O98531	226	Photosynthesis/ photorespiration
	Ribulose bisphosphate carboxylase large chain	*Ceriops tagal*	O20035	201	Photosynthesis/ photorespiration
	Ribulose bisphosphate carboxylase large chain	*Trifolium aureum*	A0A023HQ08	196	Photosynthesis/ photorespiration
6E	Fructose-bisphosphatase	*Brassica napus*	A0A078FJK5	45	Sucrose biosynthesis
	Ferredoxin-NADP reductase	*Xanthomonas campestris*	A0A7W6KYF2	34	Oxidoreductase, metal binding
	LacI family transcriptional regulator	*Cohnella phaseoli*	A0A3D9INY3	33	Transcription
	GDSL esterase/lipase	*Noccaea caerulescens*	A0A1J3D4B7	32	Lipid metabolic process
6F	Fructose-bisphosphate aldolase	*Spinacia oleracea*	A0A0K9QFF9	86	Glycolytic process
	GH43 family beta-xylosidase	*Novosphingobium* sp. *PhB57*	A0A4R3T5L4	32	Carbohydrate metabolic process
	Thioesterase domain-containing protein	*Microbispora* sp. *GKU 823*	A0A1V4EJK0	32	Biosynthetic process
	Protein kinase domain-containing protein	*Jatropha curcas*	A0A067L8H1	32	Protein kinase activity, ATP binding
6G	Phosphoinositide 5-phosphatase	*Penicillium italicum*	A0A0A2KL89	39	Lipid metabolic process
	Amidase domain-containing protein	*Fusarium poae*	A0A1B8AW33	39	Oxidoreductase
	SNF2 domain-containing protein	*Bradyrhizobium huanghuaihaiense*	A0A562QI78	36	ATP binding, helicase activity
	Acyl-CoA reductase-like NAD-dependent aldehyde dehydrogenase	*Halomonas stenophila*	A0A7W5EU67	36	Oxidoreductase
6H	Superoxide dismutase	*Phaseolus lunatus*	Q3S614	95	Stress response, metal ion binding
	Putative phospho-2-dehydro-3-deoxyheptonate aldolase	*Phaeomoniella chlamydospora*	A0A0G2EP81	32	Amino acid biosynthesis
	Formate dehydrogenase subunit alpha	*Citrobacter freundii*	A0A2S4Q6X5	31	Formate metabolic process
	Two-component system alkaline phosphatase synthesis response regulator PhoP	*Staphylococcus* sp. *AtHG25*	A0A318R5E0	31	Transcription
6I	Ribulose bisphosphate carboxylase small subunit	*Arachis duranensis*	A0A6P4D9J6	89	Photosynthesis/ photorespiration
	Epidermal patterning factor-like protein	*Nicotiana tabacum*	A0A1S3YWQ1	38	Cell differentiation, developmental protein
	Predicted protein	*Hordeum vulgare*	F2DSS8	32	RNA catabolic process

Table 2. *Cont.*

Band no.	Protein Name	Plant Species	Accession Number	Protein Score	Biological Function
7A	Ribulose bisphosphate carboxylase large chain	*Phalaenopsis* sp. *SH-2010*	E0D9N8	146	Photosynthesis/ photorespiration
	CopA family copper-resistance protein	*Sphingomonas* sp. *BK481*	A0A7W5SGK6	49	Oxidoreductase
	Protein TonB	*Bacteroidales bacterium*	A0A7Y5A3N0	34	Protein transport
	Replicative DNA helicase	*Candidatus Xiphinematobacter*	A0A0P0FJI7	34	DNA replication
7B	Ribulose bisphosphate carboxylase large chain	*Kayea stylosa*	Q8MCX9	216	Photosynthesis/ photorespiration
	Ribulose bisphosphate carboxylase large chain	*Trifolium aureum*	A0A023HQ08	204	Photosynthesis/ photorespiration
	Ribulose bisphosphate carboxylase large chain	*Crossostylis grandiflora*	A0ZQX2	204	Photosynthesis/ photorespiration
7C	Ribulose bisphosphate carboxylase large chain	*Mucuna* sp. *SH-2010*	E0D986	263	Photosynthesis/ photorespiration
	Ribulose bisphosphate carboxylase large chain	*Kayea stylosa*	Q8MCX9	262	Photosynthesis/ photorespiration
	Ribulose bisphosphate carboxylase large chain	*Adenophora liliifolioides*	H6VPA5	258	Photosynthesis/ photorespiration
7D	Cytochrome bo(3) ubiquinol oxidase subunit 1	*Pseudomonas putida*	A0A059URU4	45	Transmembrane Transport, respiration
	1-phosphatidylinositol 4-kinase	*Cucurbita maxima*	A0A6J1I6A0	43	Lipid metabolic process, signal transduction
	Protoporphyrinogen oxidase	*Dothistroma septosporum*	N1PK36	32	Oxidoreductase
	Rho-GAP domain-containing protein	*Botryotinia fuckeliana*	A0A384JQ00	30	Signal transduction
7E	Uncharacterized protein	*Sorghum bicolor*	A0A1B6QGR0	37	Transmembrane transport
	Histidine kinase	*Cellulomonas cellasea*	A0A7W4UJ46	37	Signaling
	Putative oxidoreductase	*Clavibacter michiganensis*	B0RF25	37	Oxidoreductase
7F	Fructose-bisphosphate aldolase	*Spinacia oleracea*	A0A0K9QFF9	48	Carbohydrate metabolic process
	Ferredoxin-NADP reductase	*Xanthomonas campestris*	A0A7W6KYF2	33	Metal binding, oxidoreductase activity
	tRNA(Ile)-lysidine synthetase	*Setosphaeria turcica*	R0JM12	31	tRNA metabolic process
	Cyclase family protein	*Streptomyces* sp. *CAI-21*	A0A7Y6LZ28	29	Amino acid metabolic process
7G	Peptidylprolyl isomerase	*Micractinium conductrix*	A0A2P6V266	33	Isomerase activity
	Quinone oxidoreductase, putative	*Colletotrichum orbiculare*	N4V6N5	33	Oxidoreductase
	AAHS family benzoate transporter-like MFS transporter	*Arthrobacter* sp. *SLBN-122*	A0A542G607	30	Transmembrane transport
	Protein translocase subunit SecE	*Thermobifida cellulosilytica TB100*	A0A147KLB0	29	Protein transport, translocation
7H	Precorrin-2 dehydrogenase	*Winogradskyella arenosi*	A0A368ZJY7	38	Porphyrin biosynthesis, oxidoreductase
	Putative K(+)-stimulated pyrophosphate-energized sodium pump	*Gemmatimonadales bacterium*	A0A7Y4VZE7	37	Sodium ion transport, metal binding
	BHLH domain-containing protein	*Physcomitrium patens*	A0A2K1KTX7	36	Transcription
	2,5-diketo-D-gluconate reductase A	*Microbacterium* sp. *SLBN-154*	A0A542N566	33	Oxidoreductase

Table 2. *Cont.*

Band no.	Protein Name	Plant Species	Accession Number	Protein Score	Biological Function
7I	Uncharacterized protein	*Algoriphagus boseongensis*	A0A4R6T7V4	43	Transmembrane transport
	GntR family transcriptional regulator	*Klebsiella quasipneumoniae*	A0A2N4VV92	37	Transcription
	Cytochrome P450	*Mycobacterium* sp. *BK558*	A0A4Q7PXL0	37	Oxidoreductase
	TonB-dependent receptor plug domain-containing protein	*Nitrospiraceae bacterium*	A0A7Y4SCD5	33	Transmembrane transport

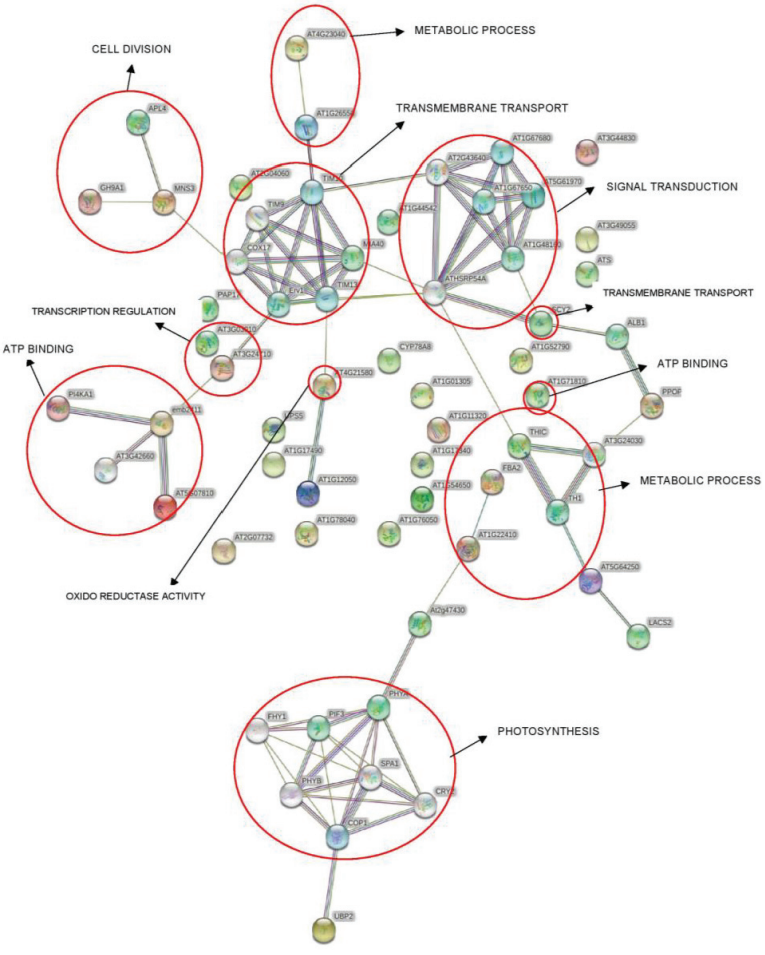

Figure 10. Analysis of the protein identified for protein−protein interaction by STRING 9.1 of mung bean (*Vigna radiata*) under Si supply (5 mM) and salinity stress.

3.8.3. Proteins Having Oxidoreductase Activity

It was observed that proteins such as ferredoxin-NADP reductase (band 3G, 7F), involved in oxidation/reduction reactions; copA family copper-resistance protein (band 7A), which mediates copper resistance via the sequestration of copper in the periplasm along

with the copper-binding protein CopC; protoporphyrinogen oxidase (band 7D), which is a precursor to heme and chlorophyll; putative oxidoreductase (band 7E); quinone oxidoreductase putative (band 7G); cytochrome P450 (band 7I), which functions as monooxygenase; and 2,5-diketo-D-gluconate reductase A (band 7H) are all downregulated under salinity stress treatments. However, Si supplementation restored the activities of the proteins involved in oxidoreductase activity, such as the putative oxidoreductase NAD(P)-binding domain (band 6A), ferredoxin-NADP reductase (band 6E), amidase domain-containing protein (band 6G), acyl-CoA reductase-like NAD-dependent aldehyde dehydrogenase (band 6G), 2-keto-3-deoxy-L-fuconate dehydrogenase (band 2I), and precorrin-2 dehydrogenase (band 2D).

3.8.4. Proteins Involved in Stress Response

Proteins involved in defense responses, such as SOS response UmuD protein (band 3F), which is involved in SOS mutagenesis; diacylglycerol kinase iota (band 3F); and superoxide dismutase (band 3H), were shown to be affected by salinity stress, where the upregulation of stress responsive proteins such as superoxide dismutase (bands 6H and 2H) and peroxidase (band 6C) was witnessed when Si was supplemented.

3.8.5. Proteins Responsible for Transmembrane Transport

Transmembrane transport proteins such as DUF4328 domain-containing protein (band 3A); cytochrome b (3); ubiquinol oxidase subunit 1 (band 7D) involved in electron transport; AAHS family benzoate transporter-like MFS transporter (band 7G), which transport a variety of aromatic acids; and cis, cis-muconate, and tonB-dependent receptor plug domain-containing protein (band 7I), which engage in high-affinity binding and energy-dependent uptake with the outer membrane receptor proteins, are downregulated by salinity stress. Meanwhile, Si supplementation upregulated the transmembrane proteins ABC-type branched-subunit amino acid transport system substrate-binding protein (band 2B) involved in amino acid transport, multiple sugar transport system permease protein (band 2C) involved in sugar transport, and acyltransferase (band 2E).

3.8.6. Proteins Involved in Signal Transduction

Salinity stress was seen to downregulate proteins such as PAS domain S-box-containing protein (band 3A), which acts as a molecular sensor for sensing redox changes in the electron transport system; 1-phosphatidylinositol 4-kinase (band 7D), which acts as an early signaling system during abiotic stress in plants; Rho-GAP domain-containing protein (band 7D); and histidine kinase (band 7E). However, treatment with Si alone only showed upregulation of the signal transduction protein histidine kinase (band 2H), whose periplasmic domains act as receptor to transduce a signal through its transmembrane domain to the cytoplasmic enzymatic domains.

3.8.7. Proteins Involved in Metal Binding

Proteins such as ferredoxin-NADP reductase (band 7F), which acts as the electron acceptor associated with photosystem I, and putative K (+)-stimulated pyrophosphate-energized sodium pump (band 7H), which uses the energy of pyrophosphate hydrolysis as the driving force for Na (+), transport across the membrane. Salinity stress also downregulated the ATPase subunit of the ABC transporter with duplicated ATPase domains (band 3D) and ATP-binding protein (band 3E), and Putative phospho-2-dehydro-3-deoxyheptonate aldolase (band 3H), which were upregulated upon Si supplementation.

3.8.8. Proteins Involved in Cell Division

Among the identified proteins, salinity stress was seen to downregulate the protein chromosome partition protein Smc (band 3F). However, silicon treatment alone was seen to upregulate proteins such as TPX2 domain-containing protein (band 2B), which has microtubule binding activity, and shugoshin_C domain-containing protein (band 2F), which

is involved in kinetochore attachment. When Si was supplemented to salinity-stressed plants, upregulation of proteins such as ubiquitin-like domain-containing protein (band 6C) and epidermal patterning factor-like protein (band 6I), involved in cell division and differentiation, were seen to be upregulated.

3.8.9. Proteins Responsible for Transcription and DNA Replication

Salinity stress was seen to downregulate protein kinase domain-containing protein (band 3C) methyltransferase family protein (band 3E), chromosome partition protein Smc (band 3F), SOS response UmuD protein (band 3F), GntR family transcriptional regulator (bands 3H and 7I), and replicative DNA helicase (band 7A), all of which are involved in essential process such as transcription, translation, phosphorylation, DNA replication, etc. Furthermore, Si supplementation enhanced the activities of proteins such as pseudouridine synthase (band 6B) involved in Ribosome biogenesis, LacI family transcriptional regulator (band 6E), and two-component system alkaline phosphatase synthesis response regulator PhoP (band 6H) involved in transcription.

3.9. Expression of Si-Transporter and Salt-Responsive Genes

The expression of *Lsi2* and *Lsi3* genes was increased in the salinity- and Si-supplemented groups (Figure 11A–C). The Si-alone treatment showed the highest expression levels of Si transporters, indicating the efficient uptake and transport of Si in mung bean plants under salinity stress. The *Lsi1* gene showed a somewhat increased expression in the salinity plus Si treatment groups. Overall, the expression of Si-transporter genes indicated proficient efflux and influx of Si in plants. Furthermore, salt responsive genes *SOS1* and *SOS3* showed reduced expression levels in the salinity plus Si treatments, whereas the expression of *SOS2* was increased in the salinity plus Si treatment groups (Figure 11C–E).

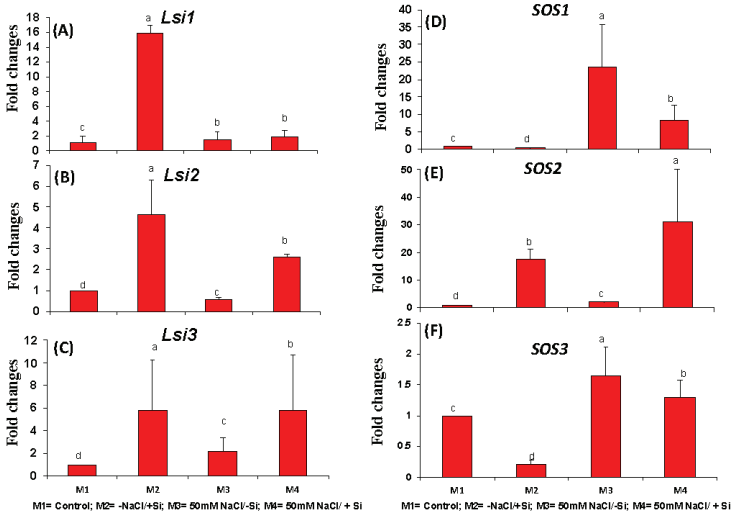

Figure 11. The relative expression level of the *Lsi* and *SOS*-related genes: (**A**) *Lsi1*, (**B**) *Lsi2*, (**C**) *Lsi3*, (**D**) *SOS1*, (**E**) *SOS2*, and (**F**) *SOS3* of mung bean (*Vigna radiata*) under Si supply (5 mM) and salinity stress: (a) control (M1), (b) –NaCl + Si (M2), (c) 50 mM NaCl/-Si (M3), (d) 50 mM NaCl/+Si (M4) for a duration of 10 days. Vertical bars indicate mean ± SE of the mean for $n = 4$. Means denoted by a different letter are significantly different at $p \leq 0.05$ according to Tukey's studentized range test.

4. Discussion

Numerous external and internal cues, frequently functioning concurrently, govern the stomata aperture. This comprises of lower soil water potential, water stress-induced

abscisic acid (ABA) generation, and hydrogen peroxide (H_2O_2) accumulation in the leaves of plants that are grown in saline soils [38]. Consequently, growth reduction as a result of impairment in photosynthesis may be linked to stomatal closure and the limited CO_2 uptake by plants under salinity stress [1]. In our study, we found a similar occurrence of stomatal closure in mung bean exposed to salinity stress, which was also observed in wheat and sweet pepper (Figure 1) [39,40]. However, an efficient regulation of stomatal opening upon Si application hints towards proficient stomatal conductance and CO_2 fixation, resulting in superlative photosynthesis leading to proficient plant growth. As far as we are aware, no investigations have been reported on the stomatal opening and closing of plants under salinity stress and Si application; therefore a clear mechanism of Si mediated regulation of stomatal aperture is missing. With the information that ion concentrations of K^+, Cl^-, and Ca^{2+} mediate the guard cell turgor pressure [41], we may hypothesize that Si application reduced the activity of outward rectifying K^+ channels relative to inwardly rectifying K^+ channels, and/or reduced Cl^- release from guard cells and lowered the Ca^{2+} concentration inside them, resulting in stomatal opening.

Plants rapidly build up osmotic regulatory substances (such as soluble sugars and soluble protein) in response to abiotic stress to increase the cell-fluid concentration. The primary function of these substances involves the maintenance of cell turgor, balancing protoplasm infiltration and the outside environment, and permitting cells to execute routine physiological processes [42]. In our study, we found that salinity stress inflicted a decline in the levels of soluble protein in a low salinity concentration (10 mM NaCl), which was improved upon Si supplementation. However, a significant impact of Si on increasing the soluble sugar content under salinity was not seen (Figure 2A,B). In wheat plants subjected to salinity stress, Si application improved the soluble protein content significantly, which is assumed to be due to enhance protein kinase synthesis and better cell signaling, which resulted in improved soluble protein levels [43]. Si augmentation increased the soluble protein levels in barley plant leaves and roots [44]. This increase in soluble protein accumulation indicates that the endogenous defense system of plants was strengthened in response to salt stress. Si-mediated regulation of osmolytes has also been reported in alfalfa and basil [45,46], where it has been documented that the movement of osmolytes may progressively provide energy for root development and contribute to the correction of the root's osmotic potential. Thus, Si-mediated accumulation of osmotic regulatory substances in mung bean may be correlated to its enhanced salinity tolerance. Furthermore, secondary metabolites, such as phenolic compounds, are generated in response to adverse environmental circumstances, and are essential for plant growth and reproduction [47]. The buildup of phenolic compounds has been associated with increased ROS scavenging via several mechanisms, including the inhibition of the enzymes involved in ROS generation and quenching [48]. In our study, we observed an increase in the content of phenolic compounds under Si supplementation to intermediate concentrations (20 mM and 50 mM NaCl) of salinity-stressed mung bean plants (Figure 2C). However, the addition of Si to low concentrations of salinity-stressed (10 mM NaCl) plants did not have any considerable impact on the total phenolic content. Comparable observations were also made in alfalfa [45] and tomato [47], where Si supplementation under salinity stress enhanced the plants' phenolic content. The Si-mediated adjustments to the plant secondary metabolism under oxidative stress could increase the phenolic content.

Nitrogen (N) is an indispensable nutrient for plants as it is a building block for many different biomolecules (including proteins, nucleic acids, amino acids, pigments, and hormones) [49]. NR is a substrate-inducing enzyme that is largely active at the transcriptional level and is induced, among other things, by NO3-N, carbohydrates, and light. [50]. However, salinity stress has been shown to deleteriously disturb these progressions, particularly the inorganic uptake of N and the enzymes required in its assimilation into organic compounds, which is not surprising considering the wide range of roles that N plays in plants and the years of study that have gone into understanding the effect of salinity on N metabolism [51]. In cucumber seedlings, growth inhibition instigated by salinity

stress has been linked to changes in nitrogen absorption and enzyme activity involved in nitrogen assimilation [52]. Additionally, salinity stress also inflicts metabolic disorders in compounds of a nitrogenous nature in *Arabidopsis thaliana* [53]. Our study displayed a disruption of NR activity in the roots of mung bean exposed to salinity stress, when compared with the NR activity in leaves. The NR activity in roots was, however, counteracted by Si application to salinity-stressed (10 mM NaCl and 50 mM NaCl) plants, resulting in an improved NR activity in the roots, hinting towards competent N metabolism and nitrogen fixation by mung bean plants (Figure 3A,B). This is in agreement with the findings reported in licorice [54], sunflower [55], and cucumber [56].

Most of the Si research revolves around drawing a relation between Si accumulation and the defensive role it displays in providing abiotic stress tolerance to plants. However, variations in Si accumulation levels among species have been reported and the status of mung bean with regards to efficient Si translocation from roots to shoots remain unexplored. In our study, we aimed to map out the distribution of Si by tracking its accumulation in different parts of the plants. We observed that Si accumulation was affected under salinity stress in both the roots and leaves of mung bean (Figure 4A,B). Furthermore, the accumulation of Si in leaves was slightly greater than the Si accumulation in the roots. These findings suggest a dissimilarity in Si accumulation between the leaves and roots, which is in agreement with the findings in *Glycyrrhiza uralensis* where the accumulation of Si was greater in the shoots than the roots [54]. The deposition of Si on root cell walls, which can inhibit salt transfer to the shoots, may explain why mung bean plants exposed to high salinity experience increased silicon buildup. Additionally, biomass formation aided by Si supplementation may also be a reason for the slightly higher Si accumulation in the shoots [19,20]. The favorable effects of Si were highly associated with the level of Si accumulation in mung bean plants, which could serve as an adaptation strategy for mung bean to decrease salt stress by absorbing and transporting more Si, hence increasing plant growth under salt stress.

A higher concentration of NaCl promotes malfunctioning of the cell membranes, which results in the excessive permeability of ions and electrolytes, which tends to exacerbate oxidative burst in the cells [57]. This was evident by the increase in content of ROS, such as H_2O_2 and O_2^-, in our study. The H_2O_2 content was found to have increased in a low concentration (10 mM NaCl) for the salinity-stressed plants; however Si supplementation could only efficiently scavenge H_2O_2 in intermediate concentration (20 mM NaCl) of salinity-stressed mung bean plants. The O_2^- content was also increased upon salinity stress induction. But a competent scavenging of O_2^- was seen under Si supplementation to two concentrations (10 mM and 20 mM NaCl) of salinity-stressed mung bean plants (Figure 5A,B). Our results are in agreement with studies conducted in cucumber [57], rose [20], and wheat [58], where Si conveyed protection against oxidative damage by bolstering the structural integrity of the cell membranes, especially under salt stress.

Several studies have found that stress causes increased reactive oxygen species (ROS) generation, and that supplementing the plant with silicon increases the quantitative changes in the activity of antioxidant enzymes to scavenge these ROS. However, the quantitative shifts on their own are not enough to confirm or depict the intricate changes happening at the cellular level. Protein profiles may change as a result of altered enzyme activity, which may be caused by the downregulation or de novo production of stress-specific antioxidant enzyme proteins [59]. In this regard, not many attempts have been made to demonstrate the changes in isozyme expression profiles, as well as the quantitative changes of the antioxidant enzymes in plants under salinity stress and Si supplementation. However, in our study, a tight regulation of Si in ROS metabolism is demonstrated by Si's constitutive participation in the expression of isozymes of antioxidant enzymes such as SOD, CAT, and APX, which was examined by native-PAGE assay (Figure 6A–D). We observed elevated levels of antioxidant enzyme activity for SOD, CAT, and APX under a low salinity concentration, which was in accordance with the results obtained by Yousif et al. [60] in sorghum, Singh et al. [61] in wheat, and Abdelaal et al. [62] in sweet pepper.

Nonetheless, Shekari et al. [63] reported that Si treatment for herbal *Anethum graveolens* plants under salinity stress significantly increased the activities of CAT, APX, and SOD. Similarly, in rapeseed, Si's participation in increasing the antioxidant enzyme activities was reported by Alam et al. [64]. It is likely that the comprehensive coordination of antioxidant enzymes is indispensable for the redox homeostasis mechanism in mung bean when it is exposed to oxidative stress.

Lignification is a recurrent response of many plant species to several environmental circumstances and mechanical injury, as it reinforces the cell wall for long-distance water transport and gives conducting tissues a structural stiffness and tenacity [65]. The enzymes most directly engaged in lignin production are peroxidases [35]. Previously, it has been demonstrated that salt stress affects secondary cell wall production, as indicated by altered lignification, and that salinity stress is connected with changed anatomical advantageous modifications, such as an increase in lignin deposition in vascular tissues of salt-treated tomato plants and xylem root components in maize [66,67]. However, there are no reports on the role of Si in the lignification of salinity-stressed plants with regards to the expression of peroxidase enzymes. Therefore, in our study, we observed that there was a decreased expression of BPOX, POD, and SPOX in salinity-stressed mung bean, but upon Si supplementation, the expression of these peroxidase enzymes was significantly improved (Figure 7A–C). An increase in the observed peroxidase activity observed may reflect changes in cell wall mechanical properties related to salinity-stress adaption. However, it would be very interesting to find out if there is cross talk between lignin synthesis and Si deposition in salinity-stressed plants, both of which function by providing mechanical protection to the plant cell.

Salinity stress has either direct impacts on photosynthesis, such as stomatal and mesophyll diffusion restrictions and changes in photosynthetic metabolism, or oxidative damage caused by the superimposition of several stressors [68]. Simultaneous stomatal growth redemption and photosynthesis-related protein stimulation indicated Si participation in important carbon fixation processes. Nwugo and Huerta [69] previously demonstrated the augmentation of photosynthesis-related proteins in rice plants exposed to cadmium stress. The primary enzyme required for CO_2 fixation during photosynthesis is ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO). The RuBisCO small subunit (band 6I), in particular, is required for carboxylation catalytic efficiency and CO_2/O_2 selectivity. Salinity stress reduced the abundance of RuBisCO large subunit (bands 3C and 3D), whereas Si supplementation increased the abundance of the RuBisCO large subunit (band 6D). Our results are similar to those observed in rose and capsicum under salinity stress and Si supply, where an increased abundance of the RuBisCO small and large subunits was reported [19,20]. Enhanced RuBisCO protein accumulation in Si treatments allowed for photoprotection as well as an improvement in the light-harvesting mechanism for plant physiological growth.

In reaction to abiotic stresses, amino acid, carbohydrate, and amine metabolic pathways undergo various modifications. The activation of early metabolic reactions is essential for cellular adaptation and survival, as it helps correct the chemical and energy imbalances caused by stress. Phosphomethylpyrimidine synthase (band 6A), an important enzyme in pyrimidine biosynthesis, was seen to be upregulated after the addition of Si. A similar protein involved in nucleotide metabolism, adenylosuccinate synthase, was also upregulated when Si was added in capsicum under salinity stress [19]. We have observed that salinity stress downregulated the protein fructose-bisphosphatase (band 3G). However, Si addition upregulated the protein fructose-bisphophatase (band 6E), which is involved in sucrose biosynthesis, indicating that the synthesis and transport of solutes from source to sink have been improved under salinity stress. Under salinity stress, the protein 1-phosphatidylinositol 4-kinase (band 7D), an essential enzyme in the salicylic acid mediated signal transduction pathway is downregulated, but the addition of Si was found to have upregulated the protein lipase (band 6E). The large majority of lipid-associated plant defense responses are facilitated by lipase activation that cleave or modify lipid

substrates in several subcellular compartments (Lee et al., 2019). Thus, Si supplementation promotes effective regulation of the metabolic processes in plants, allowing them to cope with stress [70]. Thus, Si supplementation mediates the efficient regulation of metabolic processes in the plants, such that the plants can cope up with stress.

During oxidative stress, ROS production is often significantly elevated. The excessive ROS production under conditions of salt stress primarily depletes vital metabolic pathways and protein synthesis [18]. The superoxide dismutase (SODs) is the first line of defense against ROS within a cell [71]. We found the upregulation of SOD (band 2H) after Si supplementation alone as well as when Si was supplemented to salinity-stressed plants (band 6H). Similarly, Si supplementation to salinity-stressed plants also upregulated peroxidases (band 6C), whose functions in ROS scavenging, lignin biosynthesis, and the consequent defense response activity in plants have been well documented. Plant peroxygenase is generally engaged in the H_2O_2-dependent hydroxyl catalysis of aromatics, sulfoxidation of xenobiotics, and oxidation of unsaturated fatty acids [72]. Our results are in agreement with studies conducted in rice under Cd stress [73], alfalfa under salt stress [15], and rose under salinity stress and Si supply [20], where stress-induced activation of peroxidases and its protective role have been documented. Thus, the presence of a competent balance between ROS formation and its scavenging helps mung bean in ameliorating the negative effects of salinity stress.

Under abiotic stress conditions, plant tissues endure substantial oxygen variations, resulting in a highly hypoxic environment [74]. Furthermore, ROS exposure produces an oxidative environment that alters the redox equilibrium of the cell. As numerous intracellular signaling pathways governing cell division and stress response systems are sensitive to redox conditions, intracellular alterations in redox status also have a significant impact on cell functioning [75]. Several physiological activities, including redox activity, are mediated by electron-transporting oxidoreductases in biological membranes. In our study, we found that salinity stress downregulated the protein Ferredoxin-NADP reductase (band 3G) whereas Si application lead to an upregulation of Ferredoxin-NADP reductase (bands 6A and 6E). During the linear electron transport process of photosynthesis, ferredoxin, NADP (H) oxidoreductase (FNR) transports electrons from ferredoxin (Fd) to $NADP^+$. For reductive assimilation and light/dark activation/inactivation of enzymes, both NADPH and reduced Fd (Fd^{red}) are essential [76]. As a result, FNR acts as a hub that connects electron transport in photosynthesis to redox metabolism in chloroplasts, and Si supplementation positively affects the redox balance in the plants. Reactive aldehydes formed as a by-product of lipid peroxidation are detoxified by reducing their carbonyl group to alcohol or oxidizing it to the corresponding carboxylic acid [77]. This oxidative reaction is known to be catalyzed by $NAD(P)^+$-dependent aldehyde dehydrogenases (ALDHs), whose accumulation was improved under Si supplementation (band 6G); this was similar to the findings on alfalfa under salt stress, where alcohol dehydrogenase activity involved during oxygen deprivation was increased [15]. Our results are also consistent with the findings on redox homeostasis of rose and tomato under salinity stress and Si supplementation [18,20].

The degree to which plants profit from Si depends on its deposition in the tissues, which normally ranges from 0.1% to 10% (by dry weight) and exhibits significant cultivar, species, and wider evolutionary variations [10]. This is made conceivable by the various Si-transporter genes that mediate the coordinated uptake and distribution of Si along the plant parts. The plasma-membrane transporters encoded by these genes are hypothesized to coordinate the symplastic migration of Si to circumvent the apoplastic (casparian band) barriers [78]. The identification of *OsLsi1* and *OsLsi2* in rice [14], *ZmLsi1* and *ZmLsi2* in maize [79], *CsLsi1* and *CsLsi2* in cucumber [80], and *HvLsi1* and *HvLsi2* in barley [81], has enlightened the research community about the uptake and distribution of Si in plants and its relation with the suppressed or enhanced expression of *Lsi* (influx and efflux) genes in providing ameliorative benefits to plants under various biotic and abiotic stresses. To the best of our knowledge, the role of Si transporters in mung bean under salinity stress has not been explored before. Consequently, we observed that even under salt stress, *Lsi1*, *Lsi2*, and

Lsi3 were considerably expressed in Si-treated plants; however under salt stress without Si treatment, the expression levels were lower (Figure 11A–C). Our results are in line with Muneer and Jeong [18], who illustrated an increased expression of *LeLsi1*, *LeLsi2*, and *LeLsi3* genes in tomato under salinity stress and Si supplementation. This synergistic activation of silicon transporter genes under salt stress implies a role for Si in salt-stress mitigation. The expression of Si transporters is regulated differently in different plant species. The mechanisms that modulate Si transporter gene expression, however, remain unclear.

The SOS pathway is seen as crucial for managing both Na^+ efflux out of the root cortex, as well as long-distance transport into the plant tissue via the xylem [82]. SOS machinery, consisting of *SOS1*, *SOS2*, and *SOS3* proteins, were extensively studied in an effort to comprehend the process of ion homeostasis and salinity tolerance in a vast array of plant species [83]. In *Arabidopsis*, sos1 mutants subjected to moderate salinity displayed decreased Na accumulation in the leaves, indicating *SOS1* involvement in Na^+ xylem loading [84]. In salinity-stressed maize plants supplemented with Si, an increased expression of *SOS1* and *SOS2* genes was observed along with an increase in the Na^+ in the xylem and leaf tissues [85]. Transgenic rice overexpressing *SOS1* have a significantly higher salt tolerance than wild-type rice when supplemented with Si. This improvement in tolerance is coupled with enhanced Na^+ efflux in transgenic roots and greater K^+ absorption, resulting in less Na^+ buildup in cells and a higher K^+/Na^+ ratio [86]. This is in agreement with our results where we observed an increased expression of *SOS1* and *SOS2* in mung bean plants under 50 mM of salinity stress supplemented with Si, indicating competent Na^+ efflux from the cell (Figure 11D–F). *SOS3* is known to be a Ca^{2+} regulated SOS pathway upstream regulatory protein that plays critical roles in pathways related to salt-stress response [87]. Kim et al. [88] demonstrated that NaCl treatment induces *At*SOS3 expression significantly. Moreover, the overexpression of *Le*SOS3-1 improves salt-stress tolerance in tobacco through the regulation of stress-related physiological changes. Similarly, we observed an increased expression of *SOS3* under Si supplementation in mung bean under salinity stress, indicating the efficient phosphorylation of *SOS1*, thus leading to competent efflux of Na^+ ions from the cells. This is in accordance with another study on sugarcane, where, in response to salt stress, most *VviSOS3* genes were regulated similarly in all three organs. These findings imply that *VviSOS3* genes may play a role in ionic and osmotic homeostasis establishment and maintenance [89]. Thus, it can be concluded that the Si-mediated expression of SOS genes leads to the sequential efflux, compartmentalization, and blockage of Na^+ ions in mung bean plants, thus retaining optimum levels of beneficial ions, which allow the cells to remain viable and hence assist towards tissue growth even under salt stress. The precise methods through which Si assists SOS gene transcription remain unknown; one can only hypothesize that Si indirectly impacts transcription factors, but additional study is required.

5. Conclusions

In conclusion, Si-mediated regulation of stomatal aperture, osmoregulatory substances, N metabolism, and ROS homeostasis, provided the physiochemical basis of salinity stress tolerance in mung bean. Although soluble protein and phenolic content were seen to be enhanced under Si supplementation, a convincing improvement in soluble sugar content upon Si supplementation was missing. Similarly, the increase in root NR activity after Si supplementation also shed light on the active N uptake and its metabolism. Antioxidant defense against oxidative-stress-induced damage was maintained by antioxidant enzymes and their isozyme activity. We observed that isozymes and antioxidant molecules appeared to play a significant role in providing competent defense to plants against salinity. Furthermore, the dynamic role of Si in the regulation of proteins engaged in diverse cellular functions and metabolic pathways may benefit from a better understanding of the potential mechanism(s) evolved in plants to ameliorate the adverse effects of salinity stress. Gene expression studies of Si transporter and salt responsive genes revealed an optimum expression of these genes in response to salinity stress and Si supplementation. An optimal expression of Si transporters in plants with inefficient Si absorption systems may result in

an increase in Si accumulation, hence boosting the plants' resilience to numerous stressors. However, investigating probable interaction partners and expression patterns of Si transporter genes will lend evidence to their likely roles in plant stress responses. Our research brings up the possibility of using Si transporters for breeding objectives. However, the roles of this transporter in Si fluxes and plant physiology in general must be clarified if we are to successfully use Si as a preventive measure against environmental stress.

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Use of Bioinoculants Affects Variation in Snap Bean Yield Grown under Deficit Irrigation

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Abstract: The use of beneficial microorganisms, such as plant growth promoting rhizobacteria (PGPR) and mycorrhizal fungi, for organic farming could improve the productivity and the resilience of vegetable crops. Both PGPR and PGPF are allowed for organic farming, and they represent new important tools for regenerating poor and marginal soils in transition to environmentally friendly farming. In the experiment, the effects of PGPM-based products were evaluated on snap bean in combination with two irrigation regimes. The experimental design adopted was split-plot, with the main plot represented by the irrigation regime (reintegration of 100 and 60% of the ETc), the sub-plot by the microbial consortia, and finally the sub-sub-plot by genotype ('Domino' and 'Maxi'). Seeds were sown in a cold greenhouse and the growing cycle finished after 86 days from sowing. The results showed a significant increase of the yield due to the application of PGPM compared to the control. The deficit irrigation applied (ETc 60%) affected plants growth in the two genotypes and their related production differently (in average 2.20 kg m^{-2} for Domino and 3.63 kg m^{-2} for Maxi), showing a positive effect of PGPM on yield (in average 2.47 kg m^{-2} without PGPM and 3.36 kg m^{-2} with PGPM) and product quality. Furthermore, an interesting negative correlation between the number of nodules and the yield was also observed, as a consequence of their early outcome which increased plant productivity in relation to the experimental factors.

Keywords: PGPM; drought stress; nodules; organic farming; sustainability

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1. Introduction

Nowadays, sustainable agricultural methodologies based on ecological principles and natural rules is of primary importance in order to respond to the intensification of agriculture based worldwide on the efficient use of available resources [1]. The key challenge is to increase the production of foods and feeds with minimal environmental impacts in terms of nutrient leaching, biodiversity loss, greenhouse gas emissions, and resource exhaustion [2]. This frame-low input in farming practices represents a primary goal of enhancing the sustainability of cropping schemes in order to cope with climate change and achieve high yields in more environmentally friendly conditions [3–6]. To meet what was mentioned—besides a number of approaches which can be adopted, such as low nitrogen supply [7], cropping in soilless conditions [8,9], and overall breeding for resistance [10–14]—deficit irrigation, where possible, represents a sustainable way to save water [15–21]. Although deficit irrigation represents a limiting factor in horticulture, researchers' interest in assessing protocols to save water in agriculture has increased [17,22]. To this aim, the use of helpful microorganisms, such as plant-growth-promoting rhizobacteria (PGPR), added in the rhizosphere, has been shown to increase plants' potential resistance to abiotic stresses such as water shortage in a number of crops [11], including tomato wheat, rice [23], and common bean [24]. The naturally occurring soil-dwelling microbiota, in fact, represents a useful way to establish long-term resilient farming systems [25]. The rhizosphere is the soil region that is adhered to plant roots and represents

the area with the highest microbial activity where chemical, physical and microbiological interactions take place with intensive feedbacks on plant growth [26]. The rhizosphere includes plant-growth-promoting rhizobacteria (PGPR) which exert their enhancing roles through both direct and indirect pathways [27]. Direct mechanisms involve phytohormone production, atmospheric nitrogen fixation, iron sequestration, and inorganic phosphate solubilization [28]. On the other hand, PGPR indirectly promote plant growth by inducing anti-phytopathogen compound production and, as a consequence, they develop abiotic stress tolerance abilities such as drought and salinity resistance [29–32].

According to scientific literature, [23,24,33], common bean, *Phaseolus vulgaris* L., has a high agronomic interest worldwide [34]. It belongs to the Fabaceae family, and similar to other legume crops, it has a key role in improving soil fertility by boosting nitrogen input by symbiotic N fixation [35]. Common bean represents 50% of grain legumes used for direct human consumption and remains the most important grain legume and vegetable crop in the twenty-first century [36]. Originating from two different gene pools, Mesoamerican and Andean, its broad adaptation, consumer preference, and easiness of production for both dry seeds and green pods allows it to keep an edge over the other legumes [37]. Common bean presently offers a distinctive opportunity to understand how both the host and the environment contribute to rhizosphere microbiome assembly and vice versa, due to the pre-existing genetic differences in each gene pool followed by divergent breeding history [25,38].

Within the framework of the H2020 BRESOV project, of which the overall target is to increase plants' tolerance to biotic and abiotic stresses and to adapt varieties to the specific requirements of organic and low-input production processes, we evaluated the effects of a commercial product based on PGPM (*Trichoderma* spp., *Bacillus* spp., *Pseudomonas* spp., etc.) on two different green bean cultivars under a deficit irrigation regime.

2. Materials and Methods

2.1. Plant Material

The experiment was carried out during spring 2021. The seeds of snap beans were sown directly into the soil in a cold greenhouse at the experimental farm of ITAKA s.r.l. company located in Comiso — South East of Sicily— (37°00′09.7″ N, 14°.34′45.4″ E) on 11 March 2021. Two commercial varieties, 'Maxi' (*Phaseolus vulgaris* L. var *nanus*) from Hild company and 'Domino' (*Phaseolus vulgaris* L. var *vulgaris*) from De Bolster, were adopted in the experiment. The two varieties are both present on the market for green pod consumption and were part of a much larger seed set used in the contest of the BRESOV project by different partners.

Seeds were sown in rows with a space of 50 cm between rows and 40 cm along the row. For each spot, a total of 5 seeds were sown with a density of 20 plants m^{-2}.

2.2. Irrigation Regimes

Before experiment onset, the field was prepared with a tiller and abundantly watered during the first week of March [39]. The irrigation system was arranged by using driplines with drippers at a distance of 0.20 m from each other, and served by a reservoir located near the greenhouse. Water counters were installed upstream the dripline, one for each of the two main plots. A weather station (Watchdog 2500 series) was installed in the field provided by Ecosearch s.r.l. (Montone, Italy) From the weather station, 6 probes were installed—2 for each repetition, at 0.05 and at 0.25 m of depth, respectively—in order to monitor the percentage of humidity in the soil. For the determination of water stress, crop reference evapotranspiration (ET_0) provided by the weather station was taken into account daily, and it was assumed that ET_c 100 was the gradient corresponding to soil saturation.

For the first 3 weeks from experiment onset, plants were irrigated with the same water volumes until the unfolding of the 4th leaf; after this period, the irrigation was differentiated considering 100% of water requirement (ET_c 100) and a deficit irrigation corresponding to 60% of ET_c (ET_c 60).

2.3. Microorganisms Treatments

Exactly one week before sowing the snap bean seeds, the first treatment with PGPM (MO) was carried out according to the protocol provided by ITAKA s.r.l.; to this purpose, the commercial product Maxi Soil® was used. This formulate consists of a microbial consortium containing three species of *Trichoderma*(*T. harzianum*, *T. asperelum*, *T. atroviride*) and *Bacillus amylofiquefaciens*, *B. azotoformans*, *B. megaterium*, *B. pumilus*, *B. subtilis*, *Pseudomonas lurida*, *P. fluorescence*, *Streptomyces griseus*, and *S. lydicus*.

One week after sowing, the second treatment with MO was carried out. Maxi Soil was diluted in water at a rate of 0.5 g L^{-1} (0.5 g m^{-2}) and applied to the soil by fertigation.

2.4. Morpho-Physiological Parameters

Pods were harvested at commercial maturity after 65, 72, 79, and 86 days from sowing. At every harvest, the total yield and the number of pods for each plant were recorded; moreover, starting from the second harvest, three pods per plant were randomly collected in order to analyze the pod's weight and diameter. At the end of the cropping cycle, ten entire plants per plot were removed from the field in order to register the fresh and dry weight of both epigeal and hypogeal portions. To calculate the percentage of dry matter, the epigeal and hypogeal portions of the plants were dried in a heater for 72 h at 68 °C until constant weight, then the dry weight was weighted and the percentage of dry matter was calculated. Before the destructive assay, the number of the ramification of the first order and the number of root nodules were recorded.

During the cropping cycle, 55 days after sowing, the SPAD (Soil Plant Analysis Development) index was registered using a "SPAD 502 Plus Chlorophyll Meter" (Spectrum technologies, Inc., Aurora, IL, USA).

2.5. Experimental Design

The experimental design was a "Split-plot" with 3 factors (Figure 1). The main factor was represented by the two different irrigation regimes based on crop evapotranspiration (ET_c). ET_c was calculated according to the Penman–Monteith formula [39]. The sub-plot was represented by application or no application of PGPMs (MO or NMO); the sub-sub-plot was represented by the two adopted genotypes (GE) of snap bean. Each repetition was divided into 2 equal plots corresponding to the two different water regimes. Each plot was divided into 4 sub-plots, 1.00 m equidistant between each other, and representing the combination of the 3 experimental factors. Each elemental plot was 4.60 m long and consisted of 3 rows with 0.50 m equidistant between them. Each row was divided in half in such a way to obtain 6 sub-sub-plots within, in which a randomization of the 2 varieties of snap bean with 3 repetitions for each was arranged.

2.6. Statistical Analysis

The data obtained were subjected to statistical analysis with the Student-Newman-Keuls ANOVA 1 test performed with the software CoStat version 6.451(CoHort Software, Birmingham, England). Correlation and PCA were performed by using IBM SPSS Statistics for Windows, Version 28.0 (IBM Corp: Armonk, NY, USA).

2.7. Climatic and Soil Conditions

During the experiment, climatic conditions were stable over the whole cultivation season, with the relative humidity of the air ranging from a minimum of 30% to a maximum of 80% (Figure 2). The night and day shift in air temperature during the growing period varied from 3 °C to 18 °C at night and from 22 °C to 46 °C during the daytime (Figure 2). Concerning the soil temperatures, at 25 cm of depth, the temperatures ranged from a minimum of 7.8 °C (Tmin) to a maximum of 29.4 °C (Tmax), while on the surface, at 5 cm of depth, it ranged from a minimum of 18.8 °C (Tmin) to a maximum of 46.1 °C (Tmax), as shown on Figure 1. Concerning the relative humidity (R.U.) of the soil, it varied from 11 to 54% and from 63 to 93% for the minimum and the maximum R.U., respectively (Figure 2).

The greenhouse temperature was maintained on a range between 10 °C Tmin and 35 °C Tmax (Figure 2) by opening or closing both the windows and doors.

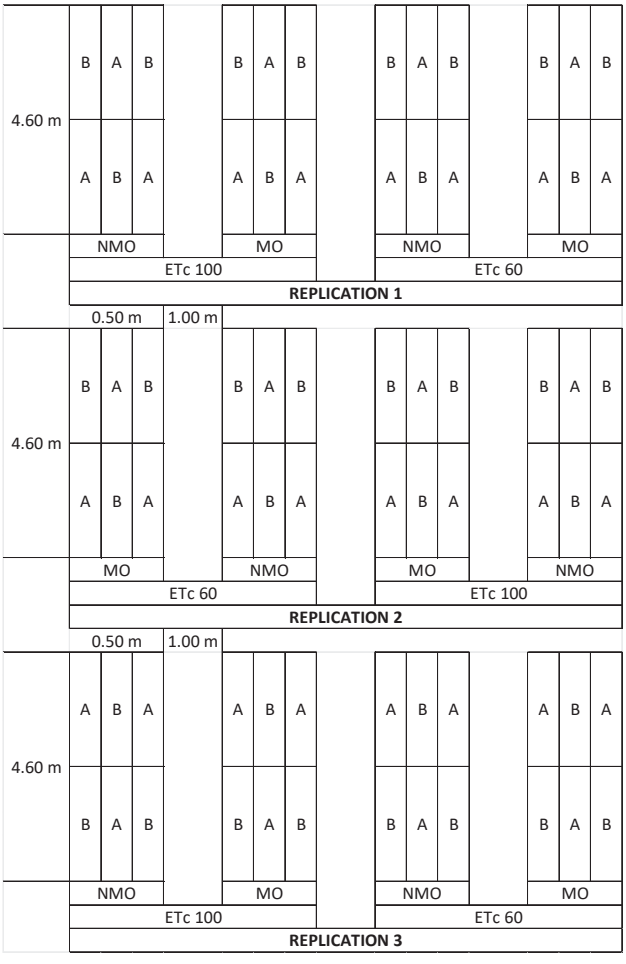

Figure 1. Experimental design. The experimental field was arranged as "split plot" with three experimental factors and three replications. The two different irrigation regimes were based on ETc (ETc 100 and ETc 60). The sub-plot was represented by application or not of PGPMs (MO or NMO); the sub-sub-plot was represented by the two genotypes of snap bean adopted: Domino (A) and Maxi (B).

In order to evaluate the soil characteristics for the snap bean cultivation, soil samples were collected at 30 cm depth and uniformed in bulk. The soil characteristics were uniform among the field and belonged to the sandy-loamy typology. These kinds of soil characteristics are optimal for snap bean cultivation [40–42], and are shown in Table 1.

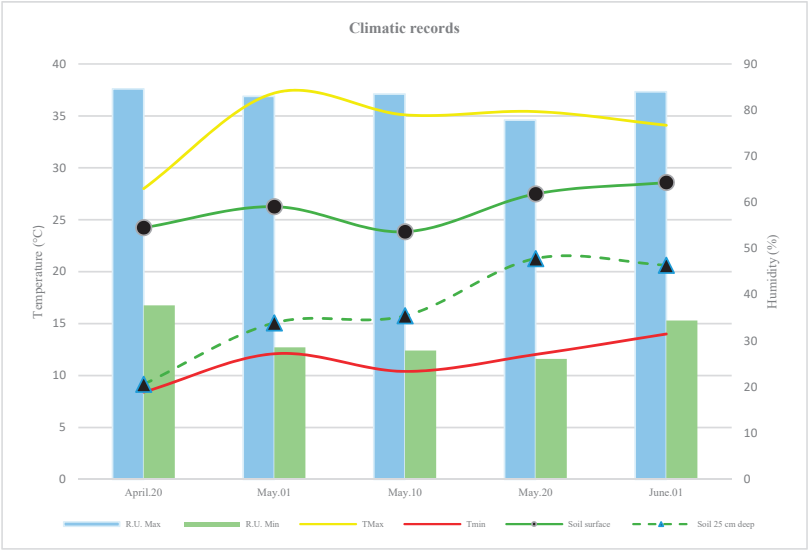

Figure 2. Records of air and soil temperatures (°C) and relative humidity (%) in greenhouse during the growing period.

Table 1. Soil analysis of the experimental field.

Soil Analysis		
Fine ground (<2 mm)	989	g/kg
Sand (0.02–2 mm)	856	g/kg
Silt (0.002–0.02 mm)	53	g/kg
Clay (<0.002 mm)	91	g/kg
Total Limestone	5	g/kg
Total Nitrogen (N)	1	g/kg
Organic carbon	6.7	g/kg
C/N Ratio	6.7	
Assimilable phosphorus (P_2O_5)	144	mg/kg
Exchangeable potassium (K_2O)	706	mg/kg
pH	7.6	
specific conductivity (25 °C)	3.63	dS/m
Cation exchange capacity (CSC)	11.5	meq/100 g
Degree of saturation in bases (GDB)	100	%
Exchangeable Calcium	7.9	meq/100 g
Exchangeable Magnesium	1.7	meq/100 g
Exchangeable sodium	0.4	meq/100 g
Exchangeable potassium (saturated extract)	1.5	meq/100 g
Calcium	68.89	%
Magnesium	14.45	%
Sodium (ESP)	3.63	%
Potassium	13.03	%
K/Mg Ratio	0.9	
Mg/K Ratio	1.11	

3. Results

3.1. Production and Plants Characteristics

The yield in pods (kg m^{-2}) was significantly affected by ET_c, MO, and GE.

Considering the effect of ET_c, the yield ranged from 2.92 to 4.05 kg m^{-2} for ET_c 60 and ET_c 100, respectively. Regarding the influence of MO, the yield varied from 3.08 to 3.89 kg m^{-2} for NMO and MO, respectively. Different yield was also observed due to GE, as B was observed to have a higher yield (4.04 kg m^{-2}) compared to A (2.92 kg m^{-2}) (Table 2 and Figure 3).

Table 2. Table with all characters analyzed with statistical analysis (ANOVA—Student-Newman-Keuls).

	ET_c 100						ET_c 60						MEAN						
	NMO			MO			NMO			MO									
	A	B	$\bar{x}$	A	B	$\bar{x}$	A	B	$\bar{x}$	A	B	$\bar{x}$	ET_c 100	ET_c 60	NMO	MO	A	B	TOT
Yield kg m^{-2}	3.45	3.91	3.68	3.84	5.00	4.42	2.00	2.95	2.47	2.40	4.32	3.36	4.05	2.92	3.08	3.89	2.92	4.04	3.48
Pod N° m^{-2}	820.0	335.9	578.0	803.6	504.6	654.1	651.2	341.4	496.3	747.1	548.9	648.0	616.0	572.1	537.1	651.0	755.5	432.7	594.1
Pod Ø (mm)	5.88	6.88	6.38	6.71	9.00	7.86	6.09	5.78	5.93	6.49	8.76	7.63	7.12	6.78	6.16	7.74	6.29	7.61	6.95
Pod length (cm)	11.7	11.3	11.5	12.0	14.3	13.1	10.8	9.0	9.9	12.4	13.1	12.8	12.3	11.3	10.7	12.9	11.7	11.9	11.8
Pod weight (g)	4.3	12.4	8.4	5.0	10.8	7.9	3.1	7.9	5.5	3.2	7.8	5.5	8.1	5.5	6.9	6.7	3.9	9.8	6.8
N° Branch	4.3	4.0	4.2	5.3	4.7	5.0	6.3	5.7	6.0	6.0	5.0	5.5	4.6	5.8	5.1	5.3	5.5	4.8	5.2
E.F.W. (g)	252.0	170.7	211.3	278.7	282.0	280.3	125.0	146.7	135.8	163.3	210.0	186.7	245.8	161.3	173.6	233.5	204.8	202.3	203.5
I.F.W. (g)	27.3	14.0	20.7	20.7	16.0	18.3	15.0	10.0	12.5	21.7	16.7	19.2	19.5	15.8	16.6	18.8	21.2	14.2	17.7
E.D.M. (%)	16.9	17.3	17.1	16.0	15.9	16.0	31.7	40.1	35.9	34.2	33.0	33.6	16.5	34.7	26.5	24.8	24.7	26.6	25.6
I.D.M. (%)	19.6	24.1	21.8	53.3	46.8	50.0	51.4	52.3	51.9	60.3	70.5	65.4	35.9	58.6	36.9	57.7	46.2	48.4	47.3
N° nodules	85.0	40.7	62.8	80.0	26.3	53.2	114.7	56.0	85.3	70.3	17.7	44.0	58.0	64.7	74.1	48.6	87.5	35.2	61.3
SPAD	43.5	44.4	44.0	45.2	45.5	45.3	43.6	45.2	44.4	46.3	48.2	47.3	44.6	45.9	44.2	46.3	44.6	45.8	45.2

Analysis of variance—Student-Newman-Keuls

	ET_c	MO	GE	ETc × MO	ETc × GE	MO × GE	ETc × MO × GE
Yield kg m^{-2}	***	***	***	n.s.	n.s.	n.s.	n.s.
Pod N° m^2	n.s.	*	***	n.s.	n.s.	n.s.	n.s.
Pod Ø (mm)	n.s.	**	*	n.s.	n.s.	*	n.s.
Pod length (cm)	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.
Pod weight (g)	***	n.s.	**	n.s.	**	n.s.	n.s.
N° Branch	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
E.F.W. (g)	**	*	n.s.	n.s.	n.s.	n.s.	n.s.
I.F.W. (g)	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.
E.D.M. (%)	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
I.D.M. (%)	***	***	n.s.	*	n.s.	n.s.	n.s.
Nod N°	n.s.	**	***	n.s.	n.s.	n.s.	n.s.
SPAD	*	***	*	n.s.	n.s.	n.s.	n.s.

n.s.: not significant; *: *p* value = 0.05%; **: *p* value = 0.01%; ***: *p* value = 0.001%.

Furthermore, the number of pods per m^{-2} was significantly affected by MO and GE. Concerning the effect of MO, the values ranged from 537.1 to 651.0 for NMO and MO, respectively, whereas GE ranged from 432.7 to 755.5 for B and A, respectively (Table 2). The pod diameter was affected by the interaction of MO × GE. Among A, the values varied in average from 5.99 to 6.60 mm for NMO and MO, respectively, and among B from 6.33 to 8.88 for NMO and MO, respectively (Table 2). The pod length was significantly affected by MO; longer pods were observed for MO (12.9 cm) than NMO (10.7 cm) (Table 2). The pod weight was statistically influenced by the interaction of ET_c × GE. Among A, the values fluctuated from 3.2 to 4.6 g for ETc 60 and Etc 100, respectively, and values for B ranged from 7.9 to 11.6 g for ETc 60 and ETc 100, respectively (Table 2).

Significant variations were noted in the snap bean development between the different treatments among the cultivars. Plant epigeous fresh weight (E.F.W.) was significantly affected by ET_c and MO, ranging from 161.3 g to 245.8 g for ETc 60 and ETc 100, respectively, and from 173.6 to 233.5 g for NMO and MO, respectively (Table 2). Otherwise, plants' hypogeous fresh weight (I.F.W.) was significantly affected by GE, with values ranging from 14.2 to 21.2 g for B and A, respectively. The plant epigeous dry matter (E.D.M. %) was significantly influenced by ETc, ranging from 16.5 to 34.7% for ETc 100 and Etc 60, respectively, whereas the ipogeous dry matter (I.D.W.) significantly differed according to the interaction between ET_c × MO. Among ET_c 100, the values ranged from 21.84 to 50.0% for ET_c 100 NMO and MO, respectively, whereas among ET_c 60, values ranged from 51.9

to 65.4% for NMO and MO, respectively (Table 1). The number of first-order branches was significantly influenced only by ETc, with values ranging from 4.6 to 5.8 for ETc 100 and ETc 60, respectively. Regarding the nodulation expressed by the number of nodules, it was significantly affected by MO and GE. Concerning the effect of MO, the number of nodules varied from 48.6 to 74.1 for MO and NMO, respectively, and regarding GE, the values fluctuated from 35.2 to 87.5 for B and A, respectively (Table 2 and Figure 4).

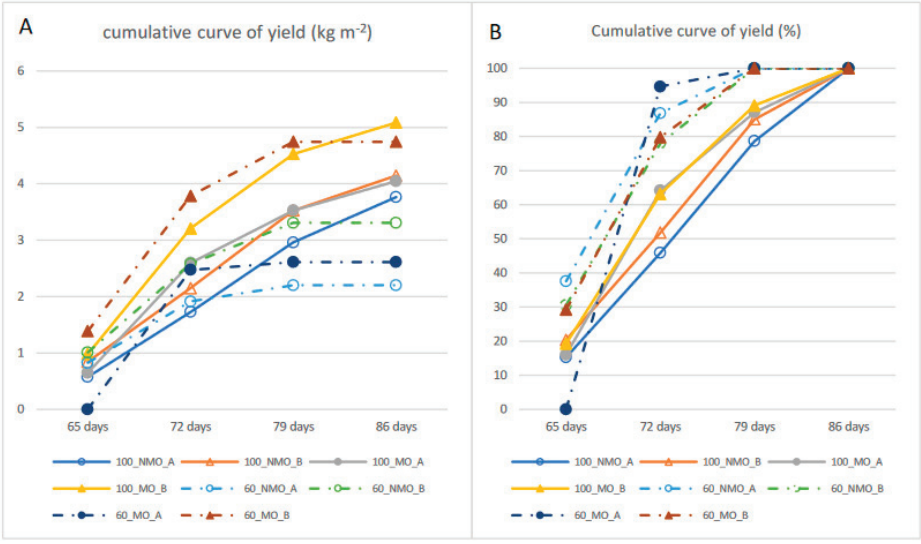

Figure 3. Yield cumulative curves. (**A**) yield of all harvest expressed in kg m^{-2}; (**B**) percentage of yield collected per harvest.

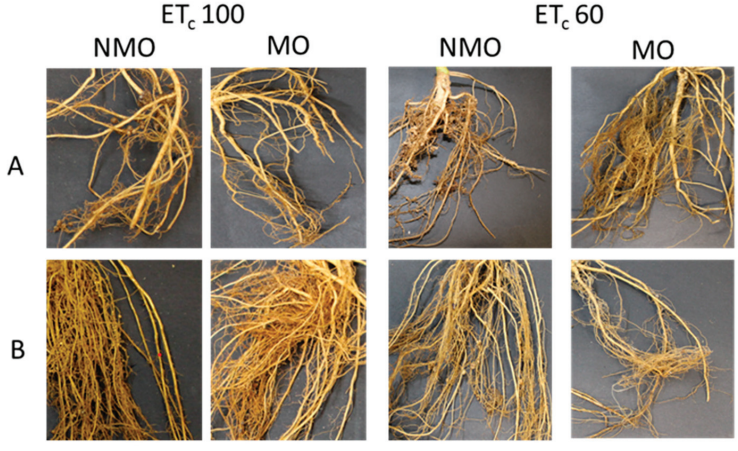

Figure 4. Details of roots and nodules among the thesis. Differences in nodulation can be observed between the two different irrigation regimes (ETc 100 and ETc 60), by application of PGPMs (MO or NMO) and between the two genotypes of snap bean adopted: Domino (**A**) and Maxi (**B**).

The SPAD was significantly influenced by ET_c, MO, and GE. Regarding ETc, the values ranged from 44.6 to 45.9 for ETc 60 and ETc 100, respectively. Concerning the MO application, the values varied from 44.2 to 46.3 for NMO and MO, respectively. Regarding GE values, the range fluctuated from 44.6 to 45.8 for A and B, respectively (Table 2).

3.2. Correlations

The Pearson's correlations determined among the experimental factors highlighted some parameters for better understanding and distinguishing the effect of the microbial treatment in deficit regime conditions between the two genotypes studied (Table 3). The yield was positively correlated with pod diameter, pod length, and the pod weight, which indicates how the pod's characteristics influenced the yield. It is interesting that the yield was also positively correlated to the E.F.W. and negatively correlated to the N° of branches and the N° of nodules. Regarding the pod number, it was positively correlated with the I.F.W and the N° of nodules, and negatively correlated with the pod weight. The pod diameter was positively correlated with the pod length and the pod weight, and was otherwise negatively correlated with the N° of nodules. The pod length was positively correlated with E.F.W. instead, and the pod weight was negatively correlated with the N° of branches and the N° of nodules. The N° of branches was positively correlated with E.D.M., I.D.M., and the N° of nodules. Concerning E.F.W., it was positively correlated with I.F.W. and negatively correlated with E.D.M; otherwise, E.D.M. was positively correlated with I.D.M.

Table 3. Pearson's correlations of the characteristics analyzed.

	Yield kg m^{-2}	**Pod N° m^2**	**Pod Ø (mm)**	**Pod Length (cm)**	**Pod Weight (g)**	**N° Branch**	**E.F.W. (g)**	**I.F.W. (g)**	**E.D.M. (%)**	**I.D.M. (%)**	**N° Nodules**	**SPAD**
Yield kg m^{-2}	1											
Pod N° m^2	−0.092	1										
Pod Ø (mm)	0.582 **	−0.177	1									
Pod length (cm)	0.506 *	0.257	0.730 **	1								
Pod weight (g)	0.670 **	−0.745 **	0.475 *	0.169	1							
N° Branch	−0.520 **	0.229	−0.163	−0.150	−0.523 **	1						
E.F.W. (g)	0.413 *	0.221	0.355	0.415 *	0.102	−0.035	1					
I.F.W. (g)	−0.050	0.608 **	−0.137	0.317	−0.391	0.182	0.502 *	1				
E.D.M. (%)	−0.472 *	−0.189	−0.142	−0.260	−0.269	0.423 *	−0.598 **	−0.353	1			
I.D.M. (%)	−0.050	0.076	0.359	0.168	−0.233	0.432 *	−0.116	−0.318	0.589 **	1		
N° nodules	−0.622 **	0.518 **	−0.604 **	−0.261	−0.691 **	0.464 *	−0.161	0.398	0.045	−0.226	1	
SPAD	0.444 *	−0.068	0.432 *	0.230	0.207	−0.039	−0.172	−0.301	0.299	0.583 **	−0.446 *	1

*: Correlation significative at 0.05; **: correlation significative at 0.01.

Interesting correlations were also found regarding the SPAD, which was positively correlated with yield, pod diameter, and I.D.M, whereas it was negatively correlated with the number of nodules.

3.3. Principal Component Analysis (PCA)

From the analysis of the data by principal component analysis, a total of 12 principal components (PC) were observed, and among them, the first two were responsible for 70.39% of the total variance registered. The first two PC were used to describe the distribution in a two-dimensional space limited by the principal detected components (Figure 5). The PCA analysis showed that the PC1 is positively correlated with yield, pod Ø, pod length, pod weight, and E.F.W., and was negatively correlated with N° of branches, E.D.M., and nodule N°, representing 44.70% of the total variance (Table 4, Figure 5). Concerning the PC2, it was positively correlated to Pod N° and I.F.W., and it represented 29.09% of the total variance (Table 4). The distribution of the studied parameters can be subdivided into two main blocks, one represented (in the space at the bottom) by genotype B while genotype A is distributed in the space at the top (Figure 5). The PCA clearly shows different responses to MO under the different ETcs; the distance between the MO and NMO sample is higher in B than A in both ETc 60 and 100. Interestingly, there is poor distance between 60_MO_B and 100_MO_B.

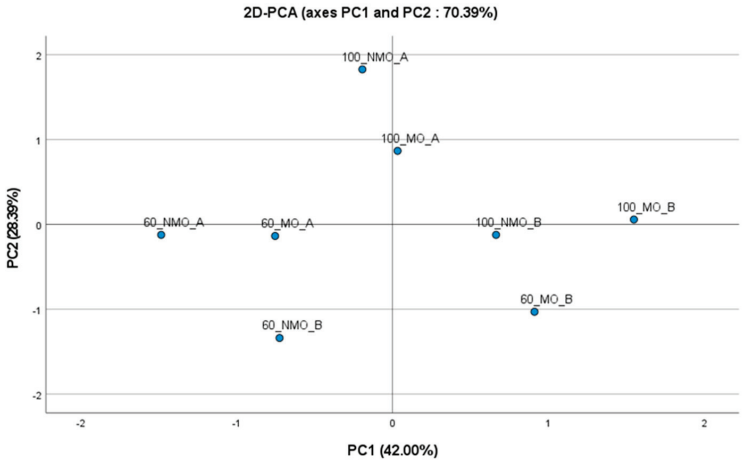

Figure 5. Two-dimensional principal component analysis (2D-PCA) that showed the characteristics analyzed.

Table 4. PCs matrix related to the characteristics analyzed.

	Component Scores	
	PC1	**PC2**
Yield kg m^{-2}	0.976	0.068
Pod N° m^{-2}	−0.299	0.722
Pod Ø (mm)	0.849	−0.246
Pod length (cm)	0.705	0.229
Pod weight (g)	0.762	−0.335
N° Branch	−0.745	−0.353
E.F.W. (g)	0.668	0.576
I.F.W. (g)	−0.032	0.844
E.D.M. (%)	−0.547	−0.750
I.D.M. (%)	−0.076	−0.624
N° nodules	−0.850	0.458
SPAD	0.417	−0.570
% of Variance	42.00	28.39

Extraction Method: Principal Component Analysis with two components extracted.

Extraction Method: Principal Component Analysis with two components extracted.

4. Discussion

Abiotic stresses are hostile to plant growth and development. In particular, water deficiency is a severe constraint that affects growth and limits agricultural productivity on a global scale, a reason why several authors focused their attention on the optimization of strategies to ameliorate water deficit [43–46].

Plant-growth-promoting microbe (PGPM) treatment may be advantageous in the contest of water deficit regimes; it is demonstrated, in fact, that both PGPR and PGPF guarantees the survival of the plant during a drought through a variety of processes including osmotic adjustments, improved phytohormone synthesis, and antioxidant activity, among others, and these mechanisms also promote the plant's development while improving crop yield [27,47–50].

Farmers and companies now recognize the usefulness of PGPM in promoting plant growth and yield. In fact, several PGPM-based formulates are commercialized and widespread [27,51]. On the basis of the recent literature [52–54], the hypothesis to verify

is that this type of formulation could be useful in overcoming drought stress by improving plants' growth and final yield. The results obtained from the present experiment are compatible with what has already been reported in literature [54–57], and confirm the usefulness of the formulation in improving yields both in optimal water supply and in case of drought stress. In literature, it is reported that the factors influencing the efficacy of microbial treatments are complex and genotype dependent [27]. Consequently, a different effectiveness of the consortium was found between the two cultivars used in the experiment (Domino and Maxy). Despite this, for both genotypes, an increase in terms of yield, pod diameter, and fresh weight were observed for the plants where the PGPM-based product was applied in both irrigation regimes. Between the two genotypes, Maxy benefited more from the treatment than Domino (Table 2), confirming the hypothesis that applying soil microorganisms to cropping schemes is genotype dependent. In particular, comparing the data obtained in ET_c 100 in the untreated control theses with the ET_c 60 theses treated with MO, it's clear that the values are comparable (Figure 5), thus observing compensation of the stress by the treatment. This result is of high importance when concerning water shortages in many environments and/or the need to save water for a better approach to the sustainability of farming practices.

Another interesting point of discussion concerns the nodulation. The nodules of legumes are of particular interest for the scientific community, as the site of nitrogen fixation by means of symbiotic nitrogen-fixing bacteria. Root nodules of legumes are the product of a highly specific interaction between the bacteria involved (rhizobia) and plants' roots or stems [58]. The inverse correlations observed between yield, pod size, and number of nodules are interesting. The number of nodules observed on the roots was lower in plants with higher yield and pod diameter, both in relation to ETc, GE, and to the Maxy Soil application. However, as reported in literature and as confirmed by the cumulative yield curve (Figure 2), this can be explained by assuming a greater metabolic activity of the plant, which close to the end of the cropping cycle, has reinvested its resources by subtracting nutrients from the nodules to reinvest them in the growth of the pods. In fact, the literature reports how the plant can regulate nodulation according to its specific needs [59]. Obviously, in order to better clarify the effect of this type of commercial microbial formulations on nodulation, more specific and in-depth studies are needed, focusing on the interaction between applied PGPMs, symbiotic rhizobia, and the plant response.

5. Conclusions

The PGPM based products mentioned in the present paper has brought an increase in yield both in optimal irrigation conditions and in deficit water conditions. The increase of yield was observed in both genotypes, but between these, the cultivar "Maxy" took the greatest advantage of the treatment, observing an almost complete compensation of the water stress. Furthermore, the inverse correlation between nodulation and yield suggests a reinvestment of the plant's nutritional resources in the last phases of the cropping cycle, which leads to the detriment of the nodules, and benefits the pods' growth.

According to what is reported in literature, the study confirms the effectiveness of PGPM applications in improving the growth and yield of crops both under optimal conditions and under stress, while taking into account the variability found between genotypes.

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

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

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