horticulturae

Optimising Soilless Culture Systems and Alternative Growing Media to Current Used Materials

Edited by

Nazim S. Gruda and Juan A. Fernández

Printed Edition of the Special Issue Published in *Horticulturae*

www.mdpi.com/journal/horticulturae

MDPI

Optimising Soilless Culture Systems and Alternative Growing Media to Current Used Materials

Optimising Soilless Culture Systems and Alternative Growing Media to Current Used Materials

Editors

Nazim S. Gruda
Juan A. Fernández

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

Editors
Nazim S. Gruda
University of Bonn
Germany

Juan A. Fernández
Technical University of
Cartagena
Spain

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

This is a reprint of articles from the Special Issue published online in the open access journal *Horticulturae* (ISSN 2311-7524) (available at: https://www.mdpi.com/journal/horticulturae/special_issues/Soilless_Culture_Systems).

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

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

ISBN 978-3-0365-3901-0 (Hbk)
ISBN 978-3-0365-3902-7 (PDF)

Cover image courtesy of Juan A. Fernández

© 2022 by the authors. Articles in this book are Open Access and distributed under the Creative Commons Attribution (CC BY) license, which allows users to download, copy and build upon published articles, as long as the author and publisher are properly credited, which ensures maximum dissemination and a wider impact of our publications.
The book as a whole is distributed by MDPI under the terms and conditions of the Creative Commons license CC BY-NC-ND.

Contents

About the Editors

Nazim S. Gruda, Professor of Horticulture at the University of Bonn, Germany. Professor Gruda is an internationally recognized and a leading authority in soilless culture, growing media, and controlled environment cultivation. He has been working as a successful researcher, research advisor, project expert and evaluator, reviewer, member of editorial boards and scholar for more than three decades. He obtained his Doctorate at the Technical University of Munich and his Habilitation at the Humboldt University of Berlin, both in Germany. His research focused on the scientific understanding and application of innovative and sustainable horticultural food production. He has achieved an impressive track record of publications with nearly 300 articles. He is Chair of an International Society for Horticultural Science (ISHS) Working Group and has edited three Acta Horticulturae for the ISHS. In recognition of his excellent research, Professor Gruda was awarded the "Dr Heinrich-Baur-Prize" 2003 by the Technical University of Munich, Germany, the "National Scientific Prize" 2018 by the Academy of Science of Albania, and has been elected as "Distinguished Scientist" 2020 from the Academy of Science of China. In addition, professor Gruda is a foreign correspondent member of the worldwide oldest agricultural institution, "Accademia dei Georgofili" in Italy and an honorary member of the Academy of Agricultural and Forestry Sciences in Romania.

Juan A. Fernández, Professor of Horticulture at the Technical University of Cartagena (UPCT), Spain. Professor Fernández is an Agricultural Engineering from the Technical University of Valencia, and a doctorate in Agricultural Engineering at the University of Murcia. He has been working on the introduction and development of techniques for the production of new horticultural and ornamental species, and recently on the hydroponic cultivation of baby leaf vegetables, the use of compost from agri-food industry in vegetable production, the use of LED lighting for indoor cultivation of leafy vegetables and the molecular characterization of neglected crops. He has a large participation in research projects and contracts and is currently an Editorial Board member of several prestigious journals. His research work is notable for his numerous publications (more than 140 articles in journals indexed in the SCOPUS database). He was Chair of two International Society for Horticultural Science (ISHS) Working Groups and has edited four Acta Horticulturae for the ISHS. He has supervised several doctoral thesis and numerous final projects and master's degree projects. He has been characterized by its continuous transfer of knowledge to the productive sector, co-authoring the various books and publications. Finally, he has been President of the Ornamentals Group of the Spanish Society of Horticultural Sciences and is currently 2nd Vice President of the above-mentioned Society and the Researcher in charge of the R & D Group of the UPCT, Hortofloricultura Mediterránea".

horticulturae

MDPI

Editorial

Optimising Soilless Culture Systems and Alternative Growing Media to Current Used Materials

Nazim S. Gruda [1,*] and Juan A. Fernández [2]

[1] Division of Horticultural Sciences, Institute of Crop Science and Resource Conservation, University of Bonn, Auf dem Hügel 6, 53121 Bonn, Germany
[2] Department of Agricultural Engineering, Technical University of Cartagena, Paseo Alfonso XIII, 48, 30203 Cartagena, Spain; juan.fernandez@upct.es
* Correspondence: ngruda@uni-bonn.de

1. Introduction

In the last few decades, soilless culture systems (SCSs) have been gaining worldwide popularity, making them one of the fastest-growing sectors in agriculture [1,2]. As a result, there is increased interest in the production of seedlings and transplants and the growth of pot ornamentals, small/soft fruit crops, greens, herbs, and medicinal and aromatic plants in soilless container systems [3].

Growing media, i.e., crop cultivation in solid, inorganic, or organic materials, are relevant to efficient and intensive horticultural plant production within soilless systems. Therefore, today, horticultural science focuses on searching for alternative materials to peat, mineral wool, and other non-renewable raw materials. Thus, problems related to the despoiling of ecologically important peat bog areas, pervasive waste, and the sustainability of materials production, including transportation, have been moved to the forefront.

Currently, interest in organic production is continually increasing. However, the regulations that concern hydroponic production are different in different countries. For instance, EU rules do not allow plants grown hydroponically to be marketed as organic except when they grow naturally in water. This regulation applies to plants grown in aquaponics systems [4]. In contrast, the USDA organic regulations do not currently prohibit hydroponic production. Certification to the USDA organic standards is currently allowed if it is certifying to comply with the NOSB recommendations. However, which hydroponic practices align or do not align with the Organic Foods Production Act and USDA organic regulations is the subject of intense debate [5–7].

New strategies and technologies, including new sustainable raw materials, should be continually developed to solve specific cultivation limitations, optimise existing systems, reduce related environmental impacts, and address the impacts of climate change.

2. Special Issue Overview and a Short Discussion

Moving horticultural production from open fields to greenhouses means that all environmental conditions can be controlled better. The application of SCS means that conditions in the rootzone can also be controlled. After the reviewers' evaluation, nine original papers and one review from 41 authors from different countries were published in this Special Issue.

The focuses of the review papers included in this Special Issue were recent scientific evidence regarding the effects of several environmental and cultivation factors on the morphology, architecture, and performance of the root systems of plants grown in SCS. In this review, different issues were comprehensively discussed: the effect of root restriction, nutrient solution, irrigation frequency, rootzone temperature and pH, oxygenation, vapour pressure deficit, lighting, root exudates, CO_2, and beneficiary microorganisms [8].

Citation: Gruda, N.S.; Fernández, J.A. Optimising Soilless Culture Systems and Alternative Growing Media to Current Used Materials. *Horticulturae* **2022**, *8*, 292. https://doi.org/10.3390/horticulturae8040292

Received: 29 March 2022
Accepted: 29 March 2022
Published: 30 March 2022

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

Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

One of the topics addressed in the current Special Issue was the optimisation of soilless culture systems. For instance, as ammonium is preferentially taken up, the rhizosphere of blueberry plants tends to become acidified over time [9]. The authors found that substrate amendment with low rates of $CaCO_3$ and fertigation with a low-pH nutrient solution (pH 4.5) are viable tools with which the pH buffering capacity can be increased in coconut coir-based substrates used for blueberry cultivation. $CaCO_3$ neutralised H^+ and contributed to Ca and Mg for plant uptake.

The application of organic fertilisation is complex because organic compounds first need to be mineralised. Cannavo et al. [10] reported that the release of mineral N is strongly dependent on the growing media, temperature, humidity, and fertiliser used. However, as the results described in this study were only derived from incubation trials, they should be additionally verified using plant experiments. Rhizosphere conditions and growing media properties influence the uptake of mineral elements.

Moreover, Loera-Muro et al. [7] recommended using vermicompost leachate fertiliser as a feasible replacement for inorganic fertiliser in hydroponic systems to achieve sustainable and eco-friendly agricultural production. The use of vermicompost leachate allows the maintenance of rosemary (*Rosmarinus officinalis* L.) or the increase in the production of mint (*Mentha spicata* L.) and with neither the modification of the bacterial communities for both plants nor changes to their ability to form biofilms. The product quality of both plants remained unaltered.

Avdouli et al. [11] investigated the performance of basil in a soilless culture cascade system. In such as system, the used nutrient solution drained from a primary crop is directed to a secondary crop, enhancing resource-use efficiency while minimising waste. The authors found that the performance of basil in the cascade system was subject to a compromise between a reduction in fresh produce and an increase in total amino acids and ascorbate content with an electric conductivity (EC) of 5 dS m^{-1} as the upper limit/threshold of tolerance to stress. They concluded that basil might be a good candidate for use as a secondary crop in a soilless culture cascade system.

The impacts of environmental issues and climate change required alternative peat materials in growing media. Peat is a limited resource in high demand, and the extraction of peat bogs has negative impacts on the environment. Covering only about 3% of Earth's land area, they may store nearly one-third of the entire world's terrestrial organic carbon [2,12]. In the long-term, peatlands are the largest stores of organic carbon out of all of the terrestrial ecosystems [13], i.e., they store more organic carbon than forests.

In the current Special Issue, different raw materials such as composts of spent mushrooms, composted heather, different coir types, alder, cattail, and reed were analysed as alternatives for the partial replacement of peat in growing media [14–16]. Hernández et al. [14] showed 3 to 7-times higher yields of red baby leaf lettuce compared to peat when composts from *Agaricus bisporus* and *Pleurotus ostreatus* were used, even under the pressure of the plant pathogen *Pythium irregulare*. The combinations of two compost types affected the higher suppressiveness of 50% against *Pythium*. Machado et al. [15] reported a high fresh yield and total flavonoids by cultivating spinach in coir pith. In contrast, the levels of other phytochemicals and antioxidant activity were not affected and remained within normal ranges for spinach. Moreover, Leiber-Sauheitl et al. [16] developed a preliminary test procedure for the identification of new raw materials as peat substitutes in growing media.

With sustainability in mind, the performance of greenhouse beit-alpha-cucumber in pine bark and perlite fertigated with biofloc aquaculture effluent was analysed [17]. In another study, hemp fibres were used to cultivate tomato plants as an organic alternative to mineral wool [18]. Hemp fibres led to similar yields to those achieved using conventionally used mineral wool. Likewise, no adverse effects on plant growth parameters and the quality of fruits were observed. Nevertheless, the authors reported low air volume and easily available water and very rapid microbial decomposition associated with high nitrogen immobilisation in hemp [18]. However, a question arises: what is the contribution of these changes and transformations of hemp during the cultivation on the total greenhouse gas

emissions? The need to evaluate biological decomposition throughout the cultivation cycle should be considered for further research. At the same time, the greenhouse gas emissions should be calculated from the production of the material until its disposal. For instance, mineral wool has a high energy demand associated with the expansion of the minerals during the manufacturing process with disposal problems. At the same time, hemp is a renewable material that can be composted at the end of cultivation.

3. Conclusions

This Special Issue provides insight into the optimisation of the existing SCS. Furthermore, it contributes to an extension of the research that concerns finding and utilising novel alternative raw materials to those currently preparing sustainable growing media.

It is clear that while much has been achieved in this Special Issue, many challenges remain. The use of new, practical, and effective tools and technologies in SCS and the continuous pursuit and validation of novel and renewable soilless substrate materials may assist in solving some of the challenges in a climate-smart agriculture approach and dealing with the environmental problems in soilless cropping. We expect these publications to promote further discussion about these two exciting topics.

Author Contributions: N.S.G. prepared the outline of the manuscript and analysed the Special Issue topics. J.A.F. wrote the concluding remarks and future perspectives. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

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

References

1. Savvas, D.; Gruda, N. Application of soilless culture technologies in the modern greenhouse industry—A review. *Eur. J. Hortic. Sci.* **2018**, *83*, 280–293. [CrossRef]
2. Gruda, N.S. Increasing Sustainability of Growing Media Constituents and Stand-Alone Substrates in Soilless Culture Systems. *Agronomy* **2019**, *9*, 298. [CrossRef]
3. Gruda, N. (Ed.) *Advances in Horticultural Soilless Culture*; Burleigh Dodds Science Publishing Limited: Cambridge, UK, 2021; p. 442, ISBN 13-9781786764355. Available online: https://www.taylorfrancis.com/books/edit/10.1201/9781003048206/advances-horticultural-soilless-culture-nazim-gruda (accessed on 18 March 2022).
4. EU-Organic-Farming. Available online: https://ec.europa.eu/info/food-farming-fisheries/farming/organic-farming/organic-production-and-products_en (accessed on 18 March 2022).
5. USDA. Available online: https://www.ams.usda.gov/sites/default/files/media/Hydroponics%20package.pdf (accessed on 18 March 2022).
6. NOSB. *Hydroponic and Aquaponic Task Force Report*; National Organic Standards Board (NOSB): Washington, DC, USA, 2016; p. 75.
7. Loera-Muro, A.; Troyo-Diéguez, E.; Murillo-Amador, B.; Barraza, A.; Caamal-Chan, G.; Lucero-Vega, G.; Nieto-Garibay, A. Effects of Vermicompost Leachate versus Inorganic Fertilizer on Morphology and Microbial Traits in the Early Development Growth Stage in Mint (*Mentha spicata* L.) Additionally, Rosemary (*Rosmarinus officinalis* L.) Plants under Closed Hydroponic System. *Horticulturae* **2021**, *7*, 100. [CrossRef]
8. Balliu, A.; Zheng, Y.; Sallaku, G.; Fernández, J.; Gruda, N.; Tuzel, Y. Environmental and Cultivation Factors Affect the Morphology, Architecture and Performance of Root Systems in Soilless Grown Plants. *Horticulturae* **2021**, *7*, 243. [CrossRef]
9. Schreiber, M.; Nunez, G. Calcium Carbonate Can Be Used to Manage Soilless Substrate pH for Blueberry Production. *Horticulturae* **2021**, *7*, 74. [CrossRef]
10. Cannavo, P.; Recous, S.; Valé, M.; Bresch, S.; Paillat, L.; Benbrahim, M.; Guénon, R. Organic Fertilization of Growing Media: Response of N-Mineralization to Temperature and Moisture. *Horticulturae* **2022**, *8*, 152. [CrossRef]
11. Avdouli, D.; Max, J.; Katsoulas, N.; Levizou, E. Basil as Secondary Crop in Cascade Hydroponics: Exploring Salinity Tolerance Limits in Terms of Growth, Amino Acid Profile, and Nutrient Composition. *Horticulturae* **2021**, *7*, 203. [CrossRef]
12. Weissert, L.F.; Disney, M. Carbon storage in peatlands: A case study on the Isle of Man. *Geoderma* **2013**, *204*, 111–119. [CrossRef]
13. UNEP. *Frontiers 2018/19 Emerging Issues of Environmental Concern*; United Nations Environment Programme: Nairobi, Kenya, 2019; Available online: https://www.unep.org/resources/frontiers-201819-emerging-issues-environmental-concern (accessed on 28 March 2022).
14. Hernández, D.; Ros, M.; Carmona, F.; Saez-Tovar, J.; Pascual, J. Composting Spent Mushroom Substrate from Agaricus bisporus and Pleurotus ostreatus Production as a Growing Media Component for Baby Leaf Lettuce Cultivation under Pythium irregulare Biotic Stress. *Horticulturae* **2021**, *7*, 13. [CrossRef]

15. Machado, R.; Alves-Pereira, I.; Ferreira, R.; Gruda, N. Coir, an Alternative to Peat—Effects on Plant Growth, Phytochemical Accumulation, and Antioxidant Power of Spinach. *Horticulturae* **2021**, *7*, 127. [CrossRef]
16. Leiber-Sauheitl, K.; Bohne, H.; Böttcher, J. First Steps toward a Test Procedure to Identify Peat Substitutes for Growing Media by Means of Chemical, Physical, and Biological Material Characteristics. *Horticulturae* **2021**, *7*, 164. [CrossRef]
17. Ayipio, E.; Wells, D.; Smith, M.; Blanchard, C. Performance of Greenhouse-Grown Beit Alpha Cucumber in Pine Bark and Perlite Substrates Fertigated with Biofloc Aquaculture Effluent. *Horticulturae* **2021**, *7*, 144. [CrossRef]
18. Nerlich, A.; Karlowsky, S.; Schwarz, D.; Förster, N.; Dannehl, D. Soilless Tomato Production: Effects of Hemp Fiber and Rock Wool Growing Media on Yield, Secondary Metabolites, Substrate Characteristics and Greenhouse Gas Emissions. *Horticulturae* **2022**, *8*, 272. [CrossRef]

horticulturae

MDPI

Review

Environmental and Cultivation Factors Affect the Morphology, Architecture and Performance of Root Systems in Soilless Grown Plants

Astrit Balliu [1], Youbin Zheng [2], Glenda Sallaku [1], Juan A. Fernández [3], Nazim S. Gruda [4] and Yuksel Tuzel [5,*]

[1] Faculty of Agriculture and Environment, Agricultural University of Tirana, 1029 Tirana, Albania; aballiu@ubt.edu.al (A.B.); gsallaku@ubt.edu.al (G.S.)
[2] School of Environmental Sciences, University of Guelph, Guelph, ON N1G 2W1, Canada; yzheng@uoguelph.ca
[3] Department of Agronomical Engineering, Universidad Politécnica de Cartagena, 30203 Cartagena, Spain; juan.fernandez@upct.es
[4] INRES—Institute of Crop Science and Resource Conservation, Division of Horticultural Sciences, University of Bonn, 53121 Bonn, Germany; ngruda@unibonn.de
[5] Department of Horticulture, Faculty of Agriculture, Ege University, Bornova, Izmir 35040, Turkey
* Correspondence: yuksel.tuzel@ege.edu.tr

Abstract: Soilless culture systems are currently one of the fastest-growing sectors in horticulture. The plant roots are confined into a specific rootzone and are exposed to environmental changes and cultivation factors. The recent scientific evidence regarding the effects of several environmental and cultivation factors on the morphology, architecture, and performance of the root system of plants grown in SCS are the objectives of this study. The effect of root restriction, nutrient solution, irrigation frequency, rootzone temperature, oxygenation, vapour pressure deficit, lighting, rootzone pH, root exudates, CO_2, and beneficiary microorganisms on the functionality and performance of the root system are discussed. Overall, the main results of this review demonstrate that researchers have carried out great efforts in innovation to optimize SCS water and nutrients supply, proper temperature, and oxygen levels at the rootzone and effective plant–beneficiary microorganisms, while contributing to plant yields. Finally, this review analyses the new trends based on emerging technologies and various tools that might be exploited in a smart agriculture approach to improve root management in soilless cropping while procuring a deeper understanding of plant root–shoot communication.

Keywords: soilless culture systems; root restriction; nutrient solution; irrigation frequency; rootzone temperature; oxygenation; vapour pressure deficit; lighting; rootzone pH; root exudates; CO_2; plant-microorganism relationships

Citation: Balliu, A.; Zheng, Y.; Sallaku, G.; Fernández, J.A.; Gruda, N.S.; Tuzel, Y. Environmental and Cultivation Factors Affect the Morphology, Architecture and Performance of Root Systems in Soilless Grown Plants. *Horticulturae* **2021**, *7*, 243. https://doi.org/10.3390/horticulturae7080243

Academic Editor: Pietro Santamaria

Received: 9 July 2021
Accepted: 7 August 2021
Published: 12 August 2021

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

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Roots are an essential organ that provides physical anchorage, water, nutrient uptake, stress avoidance mechanisms, and specific signals to the aerial part biome [1]. Root architecture considers the root elongation and hairiness, and lateral and adventitious roots (ARs) developed during plant evolution. It enables plants to respond to changing environmental conditions and adapt to different growing media [2]. Understanding how plant root system architecture enables plants to adapt to their environment and enhance this potential is essential for effective crop management [3].

While taking up water and nutrients, roots compete with other plants, fungi, and microorganisms in the rootzone, where positive or negative interactions occur due to complex processes [3]. Root architecture under abiotic stress conditions is regulated by phytohormones, inducing or repressing the process depending on the adverse condition [4–6]. However, environmental factors, such as temperature, nutrient elements, and water and salt stress [2,7] play significant roles.

Due to increasing problems in soil-based crop production, such as the loss of arable land, soil degradation, and, mainly, the impacts of climate change and soil-borne pathogens, soilless culture systems (SCS) are currently one of the fastest-growing sectors in horticulture [8–10]. They are used both in simple greenhouses and in advanced controlled-environment conditions. Recently, alongside typically grown plants, such as fruit and leafy vegetables and ornamental plants, there is an increasing interest in growing and producing other plants in soilless culture systems. For instance, figs (*Ficus carica* L.), table grape (*Vitis vinifera* L.), and other traditionally soil-grown fruit and vegetable crops, greens and herbs, wild vegetables, and recently cannabis have been cultivated in these systems [11].

In SCS, the roots are confined into a specific rootzone, one of the main distinctions between plants grown in soil and SCS [12]. In response to small rooting volume, plants increase root density, which involves greater water, nutrient, and oxygen consumption per unit volume of the rootzone [8]. Moreover, there are differences in root growth based on the type of SCS. For instance, root morphology is visually distinct among hydroponic types, such as deep water culture, ebb-flood, and aeroponic sub-mist systems, compared to a solid medium [13].

Growing plants in a limited rooting volume, root restriction, is a powerful technique to improve the utilization efficiency of agricultural resources such as space, water, and nutrition [14]. However, in container plants, the root system is more exposed to every environmental change and human-imposed mistake. According to [15], the following issues can be caused by mistakes in container-grown plants: root death due to oxygen shortage as a result of over-irrigation (particularly during hot growing periods), salt accumulation in the rootzone when it is not sufficiently leached by irrigation water, ammonium toxicity as a result of the application of high concentrations of fertilizer throughout extreme high-temperatures periods, or exposure of the plant container to direct solar radiation that may provoke over-heating and subsequently, root death.

The physical and chemical characteristics of the growing medium, changes in the nutrient solution, rootzone volume and depth, water availability, and microbial organisms inhabiting the rhizosphere can all affect root growth [16]. Bláha [17] reported a general acceptation that a 1% change in root system size corresponds to a 2% change in the yield. Hence, appropriate conditions should be provided in the rootzone for healthy root development, although extensive root growth may not be the best for most SCSs [16].

Considering the essential role of the root system in plant growth, yield, and product quality, in this review, we summarize the recent scientific evidence regarding the effects of several environmental and cultivation factors on the morphology, architecture, and performance of the root system of plants grown in soilless culture systems. Root restriction, nutrient solution, irrigation frequency, rootzone temperature, oxygenation, vapour pressure deficit, lighting, rootzone pH, root exudates, CO_2, and beneficiary microorganisms are discussed.

2. Root Restriction

Apart from porosity that is much higher in growing media, the difference between soil and SCS is the limited volume of plant roots [9]. Root restriction affects the root system (by reducing root dry matter and inducing ARs formation and a dense mat of roots) and yield. There are reported cases of reduced yield, but there is always a significant increase in plants' harvest index. A summary of several recently published papers regarding the influences of root restriction on root morphology and plant yield are presented in Table 1.

Table 1. Plant responses to root restriction.

Plant Response	Crop	Production System	Additional Information	Reference
Reduced dry matter of roots	Chili pepper	Polyvinyl-chloride (PVC) columns, filled with a mixture of coconut coir dust and empty fruit bunch compost (70:30, *v:v*)	9570 mL (control) vs. 2392 mL (root-restricted) columns	[18]
	Pepper	Plastic pots (three seeds per pot) containing Fafard 2B mix (Sun Gro Horticulture, Agawam, MA)	500 mL (control) vs. 60 mL (restricted) containers	[19]
	Cucumber	Floating system (F.S.)	Control vs. 40 mL (restricted) vessels	[20]
AR formation	Cucumber	Floating system (F.S.)	Control vs. 40 mL (restricted) vessels	[20]
	Tomato	Flow-through hydroponic culture system (FTS)	1500 mL (control) vs. 25 mL (restricted) containers	[21]
Dense mat of roots	Cucumber	Floating system (F.S.)	Control vs. 40 mL (restricted) vessels	[20]
	Tomato	Flow-through hydroponic culture system (FTS)	1500 mL (control) vs. 25 mL (restricted) containers	[21]
Yield reduction	Sweet potato	A mixed system of solid media and nutrient solution	4.5 L, 3.0 L, and 1.6 L pots	[22]
	Tomato	Different alternatives of solid growing media (perlite, pumice, volcanic ash, perlite + peat, pumice + peat, volcanic ash + peat)	8 L and 4 L pots	[23]
	Processing tomato	Solid growing media (Metro-Mix 350, Sun Gro Horticulture)	26 L, 16, 6, and 1 L pots	[24]
Non-significant yield reduction	Pepper	Growth media (Fafard 2B mix; Sun Gro Horticulture, and Turface clay) mixed in a 3:1 ratio	1500 mL, 500 mL, and 250 mL plastic pots	[19]
	Tomato	Coconut fiber substrate	10, 7.5 and 5 L pots	[25]
Increased harvest index	Pepper	Growth media (Fafard 2B mix; Sun Gro Horticulture, and Turface clay) mixed in a 3:1 ratio	1500 mL, 500 mL, and 250 mL plastic pots	[19]
	Chili pepper	Polyvinyl-chloride (PVC) columns, filled with a mixture of coconut coir dust and empty fruit bunch compost (70:30, *v:v*)	9570 mL (control) vs. 2392 mL (root-restricted) columns	[18]

Commonly, roots in container-grown plants are very dense to compensate for limited rootzone volume. On the other hand, the increased root density means more oxygen and an increased nutrient consumption per unit volume of the rootzone. While in general, no changes in root anatomy have been seen in unrestricted plants [20,21], small volume causes significant changes in the morphology of the root system. These changes are mainly manifested by forming ARs, a rapid elongation of apical meristematic tissues, barriers to radial oxygen loss, and air films in the upper cuticle [26]. The replacement of primary root by ARs [21] is a typical adaptive change in root morphologyin response to stress conditions [27]. ARs can promote the exchange of gases by alleviating the adverse effects of oxygen deficiency [26,28] and enhancing the absorption of nutrients [26,27,29]. Experiments conducted with cucumbers grown in a floating system confirmed that the primary roots of root-restricted plants, grown in a container with a 40 mL volume, proliferate towards the bottom of the container producing numerous shorter lateral roots (LR) that filled the entire volume with a dense mat of roots [20]. The mat of ARs accelerating the loss of primary roots was also observed in root-restricted tomato plants [21]. Due to the volume restriction, the LRs impede their growth, and the root system displays an apparent water-logging performance indicated by the browning of roots [20]. The 'root turnover' progresses with the loss of older roots and the subsequent gain of new roots.

Root restriction significantly depresses root and shoot growth [14,19,20]. However, the effects of root restriction on reduced shoot growth are not implemented through nutrient deficiency or water stress [30]. Root-restricted plants develop more densely branched root systems than root-unrestricted plants [31]. Since the distal root orders play a key role in the

uptake and translocation of minerals [32], new, fine roots might be a reason for a higher nutrient uptake rate in root-restricted plants.

Plant photosynthetic capacity can also be depressed by root restriction [18,33]. The reduction in the photosynthesis rate in root-restricted plants is often explained by a feedback inhibition mechanism of the excessive carbohydrate accumulation in leaves [34,35]. This was related to decreased sink activity due to removing active sinks [36] or reducing phloem transport to the available sinks [37]. However, some recent evidence does not support that claim. No carbohydrate built-up was found in root-restricted chili pepper plants [18]. Similarly, Shi et al. [33] found that decreased photosynthesis rate due to carbohydrate-induced feedback inhibition did not occur because carbohydrate concentration was lower in root-restricted tomato plants. The decreased plant ability to capture photosynthetically active radiation due to reduced leaf area is the main factor for the decreased photosynthetic activity of root-restricted plants [33]. Further, [14] have found a significant decrease in root respiration, cytochrome pathway capacity, hydrolytic ATP-ase activities, and root cell viability. In addition, they reported a significant decrease in leaf water potential, stomatal conductance, intercellular CO_2 concentration, and increases in the stomatal limitation and the xylem sap ABA concentration [33].

Usually, a larger container size provides higher yields. Thus, the total and first quality yields of pepper plants grown in a closed irrigation system were highest in the variant with 16.6 L plant^{-1} perlite, followed by 6.7 and 3.3 L plant^{-1} [38]. Similarly, Sakamoto and Suzuki [22] reported that sweet potato plants grown in small-sized pots (1.6 L) decreased the fresh weight of tuberous roots compared with plants grown in 3.0 L and 4.5 L pots. By analyzing the effects of pot volume on tomato growth and yield, Tüzel et al. [23] found that 8 L rooting volume per plant resulted in a higher total yield (7.4 kg plant^{-1}) than 4 L plant^{-1}(6.2 kg plant^{-1}). Saito et al. [24] found that, in 1 and 6 L root volume, fruit number per plant, fruit fresh weight, and yield of processing tomatoes were significantly smaller than 16 L and 26 L treatment. However, differently from the above, Pires et al. [25] found that the medium volume did not affect the number of fruits and the total yield of tomatoes grown in pots (5, 7.5, and 10 L plant^{-1}) filled with coconut fiber substrate. However, the number of non-marketable fruits was higher in the lowest volume irrigated once a day, due to calcium deficiency.

Despite partly contradictory results, there are fine pieces of evidence that root restriction increases the harvest index—the ratio of edible to total biomass [18,19,30]. Any loss in edible biomass production is offset by including more plants in a given volume [19]. As such, root restriction can save up to 50% of medium volume and would be beneficial in reducing production costs [18]. In addition, if properly managed, root restriction can be a tool for increasing volume use efficiency in both terrestrial and space-flight plant production systems and reducing inedible biomass burdens in bio regenerative life-support systems [19]. However, to maximize the benefits of root restriction, further studies should be conducted focusing on manipulating the limited root system by ensuring adequate nutrition, optimum irrigation frequency, and maintaining proper rootzone temperature and oxygenation level.

3. Nutrient Solution

Plants in soil typically exhibit good root growth to gain water and nutrients from less-explored regions. Contrary to that, in frequently flushed soilless rootzones, the near-absence of clear depletion zones somewhat diminishes the need by the plant for such active 'foraging' [12]. A considerable number of research publications have shown that variation in root system architecture plays a key role in crop nutrient efficiency [39,40]. A summary of plant responses to nutrient solutions in SCSs is presented in Table 2. Correspondingly, root architecture can also be significantly influenced by nutrient availability, the heterogeneity of the nutrient supply, and symbiotic microorganisms [41]. Forde and Lorenzo [42] reported two ways to monitor the nutrient supply: directly through localized changes in the nutrient solution or indirectly through changes in the internal nutrient status of the plant itself. The

direct pathway allows plants to respond to short-term changes of nutrients and provides roots with spatial information about the nutrient distribution within the medium profile. Thus, the developmental responses are concentrated to that region of the medium to benefit from the nutrient acquisition. The indirect pathway has the advantage of enabling the plant to integrate its nutritional signals with those coming from the range of other physiological processes, such as photosynthesis [42].

The phenotypic consequence of a change in nutrient supply in a given genotype depends on exact nutrient concentration, nutrient distribution and gradients, concentrations of other nutrients, developmental stage of the plant, and environmental factors [43]. Awika et al. [44] tested baby spinach accessions in small pots to determine phenotypic and genetic correlations between root traits and the shoot fresh weights under low and high nitrogen concentrations. They also found that, in a restricted soilless medium, the architecture of roots is a function of genetics defined by the soilless matrix and exogenously supplied nutrients. When plants face nutrient starvation, root morphology is affected, and its root surface area (RSA) usually increases. However, the specific effects depend on the element supplied in lower quantities, as the root response is focused on the assimilation of a specific nutrient [45]. Thus, although the response to low P is species-dependent, the general plant response includes primary root growth inhibition, increase in LR and root hairs, and cluster root formation [46,47].

The general response to low N includes an increase in vertical, deep roots [47]. Gruda and Schnitzler [48] reported differences in root length (RL) and root mass of tomato transplants within and outside of containers, depending on N supply. The root mass inside the container was higher with higher N-application rates. In contrast, outside the containers, the root mass was significantly higher at low N-application rates. Thus, the RL increased to search for more nutrients outside the containers.

On the other hand, the effect of nitrate on LR initiation is controversial. Several studies report a positive effect of nitrate on LR density, while others have found no effects of nitrate on LR number or density [43]. In general, at the morphological level, the inhibition of primary root (PR) growth is a typical response to most nutrient deficiencies, except for sulphur and zinc. In contrast, deficiency-induced LR responses vary considerably between nutrients producing nutrient-specific patterns of LR length, density, and branching [43]. However, how different root architectures affect the nutrient status of aboveground tissues and vice versa is a question that cannot be fully understood if nutrients are investigated in isolation [49]. The crosstalk between different nutrient signals and the benefits of RSA responses in a particular condition are yet to be characterized [43].

Table 2. Plant responses to nutrient solution.

Plant Response	Crop	SCS	Additional Information	Reference
No increase in plant yield by increasing N fertilization rates	Lettuce	Washed sand; 2.5 L (no confinement, the control); 1.0 L (moderate) and 0.4 L (severe root restriction)	Total nitrogen concentrations in mM L1, 5.55, 8.05, 10.55, 13.05 and 15.55.	[50]
	Spinach	Styrofoam trays floated into 80 cm × 44 cm·× 19 cm (52 L) plastic basins	"Full dose" nutrient solution (mg L^{-1}: N 150, P 50, K 150, Ca 150, Mg 50, Fe 5.0, Mn 0.50, Zn 0.05, B 0.50, Cu 0.03, Mo 0.02), "half dose" (with macro elements reduced by 50%)	[51]
	Baby leaf lettuce	Styrofoam trays floated into 135 cm × 125 cm × 20 cm a flotation bed	Nutrient solutions with 12 and 4 mM L^{-1} N	[52]
Primary root growth inhibition, increase in lateral roots and root hairs	Various crops	Various production system	Limited P supply	[39,41,46]
Increase in vertical, deep roots	Various crops	Various production systems	Limited N supply	[40,43,47]
Increased root dry weight, specific root length, root tissue density, and root length density due to increased irrigation intervals	Tomato, Zinnia	450 mL plastic pots containing either Metromix 360 (MM360) or Ball Professional Growing Mix (BPGM)	24-h, 48-h, and 96-h irrigation intervals	[53]
	Chili pepper	31 cm × 15 cm × 60 cm container filled with sandy-loamy soil	1-, 3- and 5-day irrigation intervals	[54]

When the growing system enables maintaining a constant concentration of each nutrient at the root surface, as is the case of NFT, DFT, and aeroponics, the ability of the restricted root system to meet plant requirements is not a limiting factor [12]. This is because frequent fertigation might improve the uptake of nutrients through the continuous replenishment of nutrients in the depletion zone at the vicinity of the root interface and enhance the transport of dissolved nutrients by mass flow [55].

In commercial production, soilless grown crops are commonly provided with high levels of inorganic nutrients. While this practice prevents growth from being limited by nutrient supply, it can exacerbate the release of nutrients into the environment. According to Grewal et al. [56], the drainage water contained 59% of applied N, 25% of applied P, and 55% of applied K. Similarly, Yang and Kim [57] reported in a recent study that only 30–40% and 46–62% of total N and P inputs, were assimilated into aquaponic crops. Lower proportions of 14–25% and 11–21% of total N and P inputs were assimilated into hydroponic crops. Therefore, it is recommended to decrease nutrient concentrations, especially N, in feeding recipes [58].

According to Cardoso et al. [50], root confinement reduces plant growth. However, the increase of N concentrations in the nutrient solution does not compensate the entire reduction in plant growth; the increase of N concentration in the nutrient solution enhanced shoot growth at the expense of decreased root growth. By studying the effect of different nutrient solution concentrations in a floating system, Öztekinet et al. [51] found no differences between full and half dose applications in many measured parameters of spinach. The authors concluded that half-dose application might be preferred in terms of yield and water consumption. Meantime, a reduction in leaf nitrate content due to reduced N concentration in the nutrient solution (4 mM vs. 12 mM) was reported in baby leaf lettuce grown in a floating system [52]. The limitation of nutrient element supply (3-0.5-1.25mM of N-P-K), particularly combined with the restriction of root volume (9 L), tended to induce early flowering, fruit set and maturation, and enhanced the allocation of assimilates to pepper fruits [30]. Savvas and Gruda [10] and Gruda et al. [59] also reported some methods to reduce the nitrate contents in SCS-leafy vegetables by lowering or eliminating the NO_3-N supply a few days before harvesting. However, nitrate-lowering strategies require appropriate calibration based on species-/genotype-specific responses interacting with climate and growing conditions [60].

4. Water Supply and Irrigation

The exact time to initiate an irrigation event and the respective amount of water are the most critical factors for efficient irrigation management and saving water [61,62]. Irrigation frequency affects plant growth and productivity (Table 3), either directly by affecting the wetting patterns and water distribution in the medium volume, modulating root distribution and growth, or indirectly on nutrient availability [63].

Table 3. Plant responses to irrigation frequency.

Plant Response	Crop	SCS	Additional Information	Reference
Increased irrigation frequency increases plant yield	Chrysanthemum	Seedling tray contained coconut peat	Irrigation frequencies of 4, 6, and 8 times/day	[64]
	Tomato	40-L (15 cm × 18 cm × 120 cm) bags containing expanded perlite	Irrigation applied when the plants had consumed 0.4-, 0.8-, or 1.2-L of water	[65]
Vertical root-density distribution mimics container moisture content. Denser at the lower part of the container.	Tomato	Wood fiber substrate		[66]
	Chili pepper	31 × 15 × 60 cm container filled with sandy-loamy soil	1-, 3-, and 5-day irrigation intervals	[54]

The irrigation method, rate, timing, and interval affect root initiation, elongation, branching, development, and dry-matter partitioning between roots and shoots [67]. Roberts et al. [53] reported that plug-cell transplants irrigated at intervals of 48 h for

zinnia (*Zinnia elegans* Jacq.) or 96 h for tomatoes (*Lycopersicon esculentum* Mill.) showed significantly higher root parameters than similar transplants watered daily [53]. Similarly, Ismail and Ozawa [54] found that a 3-day irrigation interval showed a remarkably higher root development for chili pepper than 1 or 5 d treatments.

According to Savvas and Gruda [10], the particle size of the growing media and container geometry affects water availability and aeration in the rootzone. Generally, root development is better in well-aerated growing media with high air volume and high saturated hydraulic conductivity. For instance, root development of plants grown in wood fibers and coir is better than in a peat-based substrate [68]. The growing medium's interaction with water supply influences wetting patterns in the rootzone, easily available water, leaching fraction, water availability [68–70], and consequently, root formation [66]. Variation in water supply led to different heights of substrate moisture in containers. Usually, the wetted layer of the substrate is larger in optimum water supply treatments and reduced in drought conditions. Gruda and Schnitzler [66] reported that the substrate moisture of the whole container could be achieved only for the treatments with a high matrix potential. This is reflected in a reduced development of rooting mass in drought treatments [66]. The vertical distribution of moisture content in containers affects the vertical root-density distribution [71]. Typically, the root distribution pattern mimics moisture distribution [54]; the geotropic and hydrotropic nature of roots favor the formation of a root layer at the bottom of the container.

The main mechanisms by which irrigation frequency enhances nutrient acquisition by the plant are the frequent replenishment of the nutrient solution in the depletion zone adjacent to the root surface and the enhancement of mass flow transport [55,63]. Thus, the increase of irrigation in SCS fertigation frequency could serve as an efficient tool to enhance crop yield by improving the availability of less mobile nutrients, such as P and K and water [55,72]. In addition, altering irrigation frequency increases N's availability in the growing medium or the ability of roots to absorb it with a generally increased N use efficiency [73]. However, at high irrigation frequencies, as the time interval between consecutive fertigations is reduced, the NH_4 concentration increases. Therefore, an adjustment of the NH_4/NO_3 ratio to diminish the risks of NH_4 toxicity in sensitive crops is recommended [55,63].

Irrigation frequency affects the target nutrient concentration, which Bar Yosef [74] defines as a concentration providing an uptake rate equalling the target nutrient consumption rate by the crop at the specific growth stage. For example, pepper fertigated 18 times per day gave a similar total yield, large fruit yield, and unmarketable yield under target N concentrations of 70 and 140 mg L. In contrast, tomato response to N target concentration was even more assertive than pepper, showing an evident decline in total, marketable, and large fruit yields as N concentration increased from 50 to 150 and 250 mg L^{-1} N [74]. As a general rule, at a higher frequency, the nutrient depletion zones around roots are more often replenished by a fresh solution, increasing the time-averaged concentration in the rootzone. Therefore, under similar weather and substrate conditions, a higher target nutrient concentration is required under low irrigation frequency [38].

Irrigation frequency, directly or indirectly, influences plant yield and several physiological aspects [63]. However, the results regarding the effect of irrigation frequency on plant production are sometimes contradictory. For instance, Nikolaou et al. [74] reported that irrigation frequency did not influence cucumber crop's growth and production. According to the authors, plants at low irrigation frequency induce water stress conditions, whereas high irrigation frequency increases the plants' transpiration rate, resulting in less water and nutrient losses. On the contrary, Taweesak et al. [64] reported that increased irrigation frequency improves plant growth and the number of flowers of chrysanthemum plants grown under restricted root conditions. Similarly, Rodriguez-Ortega et al. [65] concluded that for the optimal fertigation management of tomato plants grown in growing bags filled with perlite, moderate- or high-frequency irrigation is required. According to them, low-frequency irrigation is not recommended because it causes water deficit in plants due to

salts accumulation in the medium. The effects of irrigation frequency and water availability in the rootzone in SCSs could be related to the heterogeneity of root distribution in the rootzone [61] and photoassimilate partitioning between shoot and root [25]. However, container geometry, the temperature in the rootzone [75], and the hydraulic conductivity of the medium affect the water status characteristics [76].

Lastly, high irrigation frequency can positively affect the radicle length of different species by washing phytotoxic compounds when forestry products, such as bark, sawdust, and woodchips, were used. These results were not only found in bioassays [77,78] but also in container plants [77].

5. Rootzone Temperature

The environment temperature is a key factor in seed germination and subsequent root system development [79]. A summary of recent publications studying the effects of rootzone temperature is presented in Table 4. Optimum root temperatures will stimulate constant growth and the formation of new roots and improve nutrients and water uptake, crucially essential for the rapid growth of SCS plants [80]. The mechanisms regulating root growth under a specific temperature remain unclear [81]. However, in addition to changes in assimilate partitioning between roots and shoots [82], cold temperatures affect the growth rate of single root tips and the total root system architecture, especially the formation and orientation of LRs [83].

The root system comprises embryonic roots (radicles) and post-embryonic roots formed from the existing roots LRs or ARs. LRs affect the root system architecture [84]. Lateral root primordia development (LRP), LR emergence, organ growth, and the periodic branching of higher-order LRs are the main processes that increase the size of the root system [85]. Although the times and places of LRP morphogenesis are genetically controlled [86], plants can have very different root system architectures when grown in varying environmental conditions [2]. The exposure of plant roots to temperatures below or above their optimum temperature generally decreases (i) primary root length, (ii) LR density, and (iii) the angle under which LRs emerge from the primary root, whereas the average LR length is unaffected [79].

Roots growing in containers are more exposed to extreme ambient temperatures than soil-grown roots [12]. As a rule, the smaller the medium/nutrient solution volume is, the larger the temperature fluctuations are expected. In a study by Xu et al. [30], rootzone temperature (RZT) in a small container (9 L) varied between 14.1 and 26.9 °C. It was close to the variation of air temperature in small containers. In contrast, in large- and middle-sized containers, a narrower temperature variation was maintained during the daily cycle, and a higher temperature was recorded at night. Usually, an increased root:shoot ratio was recorded under unfavourable, low RZTs. This adaptation may overcome water and nutrient uptake restrictions due to increased water viscosity or decreased root hydraulic conductance [79].

Table 4. Plant responses to rootzone temperature.

Plant Response	Crop	Production System	Additional Information	Reference
Increased root length	Cucumber	Plastic pots filled with sand	12 °C vs. 20 °C	[87]
	Garden pea	Foam trays filled with peat	12 °C vs. 20 °C	[88]
Increased root branching	Oilseed rape	Petri dishes filled with agar	10, 15 and 20 °C	[83]
	Several species	Transparent cylinders, filled with a growth medium made from half-strength Hoagland solution and 0.2% Phytagel	18–34 °C	[88]
Increased root density	Oilseed rape	Petri dishes filled with agar	10, 15 and 20 °C	[83]
	Garden pea	Foam trays filled with peat	12 °C vs. 20 °C	[89]
Reduced yield	Lettuce	DFT hydroponic system with	25 °C and 30 °C vs. 10 °C	[90]
	Tomato	Rockwool, cubs and slabs	16–27 °C vs. 10 °C	[91]
	Tomato	NFT hydroponic system	20.3 vs. 16.6 °C and14.2 vs. 5.8 °C	[92]
	Baby leaves of lettuce and rocket	Floating system	30 vs. 21.9 °C	[93]

The increase of RZT to an optimum level significantly increases RL [87], primarily due to the increased density of LR [89]. At temperatures of 10–15 °C, the emerged LRs were densest near the basal part of the tap root and declined acropetally along with it in garden pea grown in a soilless culture system. Root branching is also affected by temperature. According to Nagel et al. [83], lateral root formation in oilseed rape at 10 °C started later, and the branching rate was reduced by 60% compared with the treatment of 20 °C. The same effect was reported by Luo et al. [88] in the seedlings of different subtropical species. On the other side, increased average root diameter [94] and the initiation of second and third-order laterals [95] were reported in roots suffering from supra optimal temperature stress. Changes in root system morphology are adaptive plant responses to temperature stress, providing greater surface area for absorption per unit root weight or length [96].

In addition to root system architecture, especially the formation, density, and orientation of LRs [83,89], rootzone temperature affects the functionality of the root system [92,97]. Root water uptake decreases drastically when the temperature goes down because of the decrease in the vapour pressure deficit (VPD) and the increase in the viscosity of water [98]. Also, root hydraulic conductance decreases faster than stomatal conductance when only the roots were subjected to low-temperature stress [98,99]. Hence, although the transpiration rate decreases under low-temperature conditions because of a decrease in VPD between the leaf surface and the atmosphere, the stomata of the sensitive plants remain open. In contrast, those of the tolerant plants close more rapidly. Under such conditions, sensitive plants, such as cucumber and melon, start losing water from their leaves at dawn, while the roots are still cold [99]. The decreased root-sourced water supply negatively affects leaf growth [100] and stomatal conductance [101], and consequently, the overall assimilation capability of a plant [97].

The rate of nutrient uptake in a plant might also be disturbed by low rootzone temperature. The magnitude of these effects depends on the crop's physiological stage [99] and growing season and cultivar [91]. According to Xu et al. [30], increasing root medium temperatures can increase N, P, and K uptake in pepper plants and enhance branch growth and total fruit yield, despite delayed flowering and fruit set. Similar results were reported by Tachibana [102] and Kawasaki et al. [103]. Increased rootzone temperature advanced the internal xylem's structure near the root tip [92,103]. This increases both xylem exudation and root respiration, which improves nutrient transport to the shoot and increases shoot growth [92,103]. The enhancement of nutrient uptake and the improvement in nutrient transportation from roots to the aerial part of plants in optimum root temperature have different reasons. Apart from changes in root structure, a higher transpiration rate of the root system was recorded [104].

High rootzone temperature can also affect the functionality of the root system. The adverse effects of high root temperature result from a significant increase in root respiration rate [99], reduced oxygen solubility in the nutrient solution, and decreased oxygen consumption and cell viability [105]. The increased enzymatic oxidization of phenolic compounds in root epidermal and cortex tissues could be a reason as well [106]. The effects of high temperature have to be counted in both the short and long term. In the short term, a high solution temperature activates water and nutrient uptake through decreased water viscosity and affects membranes transport. In contrast, in the long-term, high temperature cause growth depression and browning in roots, accompanied by depressed water and nutrient uptake rates [106]. Often, heat affects roots' incomplete recovery even after several days of post-heat recapture [107].

The adverse effects of high rootzone temperature on the root system and whole plant growth and development might further worsen when ammonium is applied as the source of N [12]. At rootzone temperatures as high as 25 °C, plant tolerance to high NH_4 concentrations is often reduced due to low carbohydrate concentration in the cytoplasm available to detoxify cytoplasmic ammonia (NH_3) [99].

The growth and yield of many plants are influenced by rootzone temperature [92,97]. A 7-day low temperature (10 °C) exposure reduced leaf area, stem size, fresh weight, and

the water content of lettuce, compared with ambient rootzone temperature (20 °C) exposure [90]. Similarly, a reduction of marketable yield per plant was observed in two different cocktail tomato cultivars in response to root cooling in winter, but not in summer [91]. On the other side, the increment of rootzone temperature from 13–19 °C had a significant positive effect on the growth of cucumber seedlings [97]. This preserved the photosynthetic capability of the already existing leaves and promoted the expansion rate of the newly developed leaves [97].

In the same way, in tomatoes grown in a NFT system, the fruit yield was higher in the heating treatment than the control; the increased individual fruit dry weight was responsible for this difference [92]. During extreme weather conditions, the yield of baby lettuce and rocket was 31.4% and 18.9% higher with controlled RZT than the control, respectively, whilst quality parameters and chemical composition were not affected significantly [93]. Contrary to that, high rootzone temperatures have reduced shoot and root growth and water content in carrots grown in a DFT hydroponic system. In contrast, total phenolic compounds and soluble-solid content were increased [108].

In most cases, root cooling had a positive effect on the functional quality of tomatoes [91]. Sakamoto and Suzuki [90] have also reported that lettuce leaves under low rootzone temperature contained higher anthocyanin, phenols, sugar, and nitrate concentrations than leaves under optimum temperatures. Similarly, according to Kawasaki et al. [92], the soluble solid content of tomato fruits decreased in a rootzone heating treatment. Slightly different from above, the contents of ascorbic acid and sugar in strawberry fruits were not significantly influenced by the rootzone cooling [105].

6. Oxygenation

Plants adapt to low soil oxygen availability through root morphology, anatomy, and architecture to maintain root system functioning [109]. A summary of plant reactions under O_2 deficiency is presented in Table 5. Total root length, surface area, and the volume and number of forks are significantly reduced under O_2 deficiency conditions [110]. In addition, the formation, elongation, and growth angle of roots change under flooding conditions, resulting in an overall altered root architecture [109]. Therefore, the flooding-induced inhibition of root growth ultimately would lead to nutrient limitation and negatively impact the survival of the whole plant [79]. The phenomenon of hypoxiais particularly acute in hot periods when water temperatures increase, because the quantity of dissolved oxygen in water decreases and the rate of root respiration increases [111].

There are differences in sensitivity to oxygen deficiency in the rooting medium among plant species [112]. Under O_2 deficiency stress, tolerant plants develop several belowground adaptations, including adventitious root, aerenchyma, radial oxygen-loss barrier development, and a change in root hydraulic conductance [109,113]. The formation of aerenchyma in the root is one of the best-studied adaptations of plants to oxygen depletion, providing an alternative pathway for oxygen supply to the root tissue [114]. This requires that new, well-adapted, adventitious roots be formed. Thus, axial oxygen loss can be kept to a minimum so that the root tip becomes a well-oxygenated micro-climate [79]. In addition, a greater cortex-to-stele ratio and a smaller surface area to the volume also encourage the diffusion of O_2 along roots. In contrast, barriers within the outer cell layers to prevent radial O_2 loss from the cortex to the rhizosphere further improves O_2 movement to the growing apex of roots in waterlogged growing media [109].

Lack of oxygen in the rootzone induces developmental responses in the shoot, such as epinastic leaf curvature, stomatal closure, and the slowing of leaf expansion—all reactions to compensate for the diminished input of resources from the roots [115]. Leaf yellowing, wilting, roots rotting, and root blackening are also common symptoms of waterlogged plants [113]. The appearance of wilting in waterlogged plants has generally been attributed to the effects of ethylene production by roots rather than to a shortage of water to maintain leaf turgor. In addition, the observation of altered aquaporin activity and lower hydraulic

conductance in response to hypoxia stress suggests that the leaves of waterlogged plants are water deficit stressed. However, this hypothesis needs testing [113].

Table 5. Plant responses to rootzone oxygen.

Plant Response	Crop	Production System	Additional Information	Reference
Alterations in formation, elongation, and growth angle of roots.	Various crops	Various systems	O_2 deficiency	[109]
Adventitious root formation, aerenchyma, and radial oxygen-loss barrier development	Cucumber	Floating system	O_2 deficiency	[20]
	Tomato	Flow-through hydroponic culture system (FTS)	O_2 deficiency	[21]
	Various crops	Various systems	O_2 deficiency	[113]
Increased yield	Melon	Rockwool, cubs, and slabs	Oxygen enrichment	[116]
	Melon	Rockwool, cubs, and slabs	Oxygen enrichment	[117]
	Lettuce	Nutrient solution	Oxygen enrichment	[118]

Available oxygen is mainly determined by the layout of the hydroponic system and the substrate's physical properties. In contrast, oxygen diffusion rates into the water depend directly on volumetric air content, partial oxygen pressure, and temperature [119]. Morard and Silvestre [111] reported that the rate of root respiration depends on plant species and can differ from 1.44 to 7.8 µmol O_2 h^{-1} g^{-1} root FW. Considering that oxygen concentration in the rootzone of plants in soilless systems is quite variable and rapidly changes [12], attention should be paid not to let it fall below a plant-specific critical value [120].

High root respiration rate, high medium temperature, and high crop water demand are factors that may provoke oxygen deficiency, even in well-aerated substrate crops [116]. In container-grown plants, an accumulation of roots at the bottom of the container is usually observed. This results in intense root-to-root competition for oxygen and nutrients, leading to more rapid decreases in the concentration of dissolved oxygen due to the respiration of an extensive mass of dense roots and as a consequence of the existence of a perched water layer on the bottom of the container [12]. This situation may be aggravated by the consumption of oxygen from microorganisms under warm condition, which can complete O_2 depletion in less than 24 h. Consequently, roots will quickly be exposed to a transition from a fully aerobic to an anaerobic environment [121].

Hydroponically grown plants may also suffer from oxygen deficiency. Especially in NFT, the oxygen concentration can heavily deplete during the daytime [120]. Furthermore, when roots start to intertwine and shield each other, the flow rate of the nearby root nutrient solution is reduced. This means the transport rate of oxygen to dense root layers, even in deep flow systems, can be limited, despite large flows in the adjacent nutrient solution [122].

Under root asphyxia conditions, plants might use the oxygen from the reduction reactions of nitrates to nitrites to ensure water and nitrate uptake processes, relying on the metabolism of the "nitrate respiration" type [123]. In these conditions, switching from aerobic respiration to the glycolytic generation of ATP results in a severe decrease in energy available for maintenance, growth, and ion uptake [79]. Since less ATP is produced, this implies that adaptation has a cost that will probably result in reduced growth and yield [113]. According to Morard et al. [123], oxygen deprivation of the nutrient solution has an immediate effect on the water and nutrient uptake of the whole plant. Thus, root asphyxia of a tomato plant causes a 20 to 30% decrease in water uptake after 48 h, and the active uptake of nutrients, namely nitrate, potassium, and phosphate, is rapidly reduced. In addition, oxygen deficiency inhibits plant gas exchange parameters and net photosynthetic rate [35]. On the contrary, aeration promotes plant growth, leaf K, P, Mg, and water uptake [124], and plant net photosynthetic rate [35].

Oxygenation is a common practice in soilless commercial production, and several oxygenation methods are practised [118,125]. A higher yield of marketable and first category fruits was reported in melon plants grown in rockwool slabs for the oxygen-enriched treatment [116]. Also, increased head size and leaf number were reported by Öztekin and Tüzel [118] in lettuce plants grown with an aerated nutrient solution. Furthermore, Bonachela et al. [117] showed an increase in total and marketable yield for the oxygen-enriched melon grown on rockwool slabs. No significant differences were found for the melons grown on perlite grow bags. Therefore, they concluded that oxygen enrichment should be restricted to rockwool and to crop periods when a high oxygen demand concurs with low oxygen availability. In addition, no effects of oxygen enrichment on yieldwere found in pepper and cucumber plants grown in porous mediasuch as cedar sawdust and perlite [126]. Lee et al. [127] warned that excessive aeration inhibits root respiration, nutrients, bioactivity, and water uptake, resulting in reduced plant growth and fruit yield. Some modern oxygenation technologies can increase the nutrient solution dissolved oxygen (DO) level to a few times higher than the saturation level at ambient conditions. To find out whether too high of a rootzone DO can negatively affect the plant in SCS, Zheng et al. [128] grew young tomato plants in a deep water culture system with DO at 8, 20, 30, or 40 mg L^{-1}. They found that two weeks from the start of the experiment, the roots in the 40 mg L^{-1} treatment were stunted and thicker, with fewer side and fine roots compared to roots in the other three treatments, and the root respiration rate increased linearly with the increasing DO. Therefore, they recommend, for soilless cultivation, rootzone DO should not go higher than 30 mg L^{-1}.

7. Water Pressure Deficit

Vapour pressure deficit (VPD) is the difference between the saturated vapour pressure and the actual vapour pressure at a given temperature. VPD can affect plant root morphology, architecture, and performance in soilless grown plants. For example, Zhang et al. [129] grew *Lycopersicon esculentum* seedlings in perlite and vermiculite mix under either high VPD (4–5 kPa at noon) or low VPD (<1.5 kPa) conditions. They found that the seedlings grown under low VPD had longer total root length, larger root diameter, higher total root volume, total root surface area, number of root tips, number of root forks, and biomass. They further divided the roots into three diameter ranges (0–0.5, 0.5–1.0, and >1.0 mm) and found that root lengths for root diameters in the 0–0.5 and 0.5–1.0 mm ranges were greater under the low VPD condition than those under the high VPD condition. VPD did not have any effect on root length of the roots with diameter >1.0 mm. By growing *Lycopersicon esculentum* plants under either low VPD (0.23 kPa) or moderate VPD (0.7 kPa), Arve and Torre [130] found that plants grown under the low VPD developed adventitious roots at the base of the stem. However, those under the moderate VPD did not. Low VPD increases root biomass for plants in the soilless system reported in other studies [131]. The response of root growth to VPD is species specific, and the VPD is range dependent. Zheng and Shimizu [132] grew four species of conifer tree seedlings in vermiculite under four different VPDs (2.40/1.32, 2.00/1.06, 1.60/0.79, or 1.20/0.53 kPa during light/dark), they found the root biomass of *Pinus massoniana* increased linearly with the increase of VPD; however, there was no VPD effect on the root biomass of *Pinus tabulaeformis*; *Platycladus orientalis* and *Cunninghamia lanceolata*.

Roots with different diameters can have different abilities in water and nutrient uptake; it is generally believed that finer roots are better in water and nutrient uptake. VPD not only can affect plant morphology and architecture and eventually, affect plant root water and nutrient uptake, but VPD is also a major driving force for plant water and nutrient uptake. When VPD is high, water is readily transpired from the leaf to the air, resulting in high water and nutrient uptake. However, when VPD is too high, water cannot flow up quickly enough, resulting in stomatal closure and reduced water and nutrient uptake. When VPD is low, root uptake of nutrients can also be limited. The leaf Ca content dramatically decreased by growing *Lycopersicon esculentum* in a low VPD environment [133].

Most of the existing studies were focusing on how rootzone moisture levels affect root morphology and architecture. More research is needed to investigate how different species respond to the realistic VPD ranges in soilless plant production facilities.

8. Lighting

Lighting has three aspects: light intensity, spectral quality, and photoperiod. Lighting can affect root initiation, growth, and ultimately, affect root water and nutrient uptake. The generally accepted view for root initiation and growth is that there is an optimal light intensity for different species, and different species may have different optimal light spectra [134]. Gil et al. [135] rooted *Dendranthema* × *grandiflorum* cuttings in the soilless medium under either a blue (peaked at 460 nm) or red (peaked at 625 nm) light-emitting diode (LED) or fluorescent lights, all with photosynthetic photon flux density (PPFD) at 5, 35, or 65 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Their results showed that the number of adventitious roots and root dry weights were the highest for the cuttings under the 65 $\mu mol \cdot m^{-2} \cdot s^{-1}$, regardless of the light spectrum used; under the same PPFD of 35 $\mu mol \cdot m^{-2} \cdot s^{-1}$, the blue LED treatment had the highest number of adventitious roots, root length, and root dry weight among the three light spectrum treatments, in general. Their finding indicates that blue light can stimulate root initiation and growth. This is also supported by a few other recent studies. For example, Rasool et al. [136] exposed cuttings (inserted in plugs containing soilless medium) of *Kalanchoe blossfeldiana* under LEDs with different red and blue ratios,90:10, 70:30, and 15:85; results showed that the root-covered plug surfaces were highest under the two higher blue ratios. Navidad et al. [137] grew *Abies laciocarpa* and *Piceaabies* seedlings under either highpressure sodium light (HPS) with 5% blue (low blue) or under the same HPS but increased the blue portion to 30% (high blue) using LED. They found that the high blue treatment resulted in a 3.8 times increased root dry weight and a reduced total root length in *P. abies* but had no effect on the root growth of *A. laciocarpa.* Olschowski et al. [138], also showed that *Calibrachoa* cuttings rooted in soilless plugs generally had higher root dry weight and total root length under higher vs. lower light intensity (i.e., PPFD of 80 vs. 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$), regardless of the light spectrum. They also showed that plants had higher root dry weight under HPS light and a combination of white, blue, and red LEDs than those under the red-, blue-, or white-only LEDs.

Light can also affect root water and nutrient uptake performance. For example, by growing *Brassica oleracea* var. Alboglabra under either fluorescent or LED lights, with different blue and red light ratios of 1:9, 2:8, and 4:6, Barickman et al. [139] found that the shoot tissue concentrations of P, S, K, Ca, and Mg increased under the LEDs, compared to those under the fluorescent light. However, the root tissue concentrations increased for K and decreased for Mg under LEDs vs. fluorescent, and no light-treatment effects were observed on Ca or P uptake. By growing *Larix principis-rupprechtii* seedlings under different fertilisation levels and two LED spectral combinations, Zhao et al. [140] found light×nutrient interactions on root dry weight, uptake of N and P, and the nutrient utilisation efficiencies. There is no clear cut whether light affects root morphology and architecture, leading it to affect water and nutrient uptake. It mainly affects plant aboveground (e.g., leaf morphology and, size and stomatal conductance) and ultimately leads to affecting on water and nutrient uptake.

Based on the limited available literature, we are not able to generalize which spectrum or spectra combination are the best in promoting root initiation, root growth, and root water and nutrient uptake; however, both light quality and intensity can affect water and nutrient uptake, and the effects are species, lighting, and environment-dependent [141]. Future research needs to investigate how light spectrum, intensity, and photoperiod affect root growth, morphology, architecture, and nutrient and water uptake during the entire plant growth and development period, rather than only during the early propagation stage.

9. CO_2

Since roots are one of the major organs for the storage of photosynthates, the growth, architecture, and nutrient contents of roots will be considerably impacted by elevated CO_2 [eCO_2]. eCO_2 increases root to shoot ratio in nutrient-limited conditions because the increased biomass by eCO_2 will be preferentially allocated to roots to exploit and acquire more nutrients [142]. By using a meta-analysis, Dong et al. [143] found an 8% increase in the root-to-shoot ratio of vegetables and a 35% increase in yield of root vegetables under [eCO_2] conditions compared to ambient [CO_2] conditions. The improvement of vegetable root growth by [eCO_2] may be attributed to plants' higher nutrient requirement, leading to more allocation of photosynthates to roots [142]. The biomass and the morphology of vegetable roots could dramatically change under [eCO_2] conditions. Li et al. [144] found that the total root length, root surface area, root volume, average diameter, and the number of root tips of cucumber plants were also significantly increased by elevating [CO_2] from 400 to 1200 μmol mol^{-1} with sufficient nitrogen supply [144]. The authors also found that the concentrations of three soluble sugars (glucose, fructose, and sucrose) and three organic acids (citric acid, malic acid, and oxalic acid) were all increased with [eCO_2]. Those results indicated that [eCO_2] strongly promoted the robust root growth of vegetables and facilitated the transportation of photosynthates from aerial part to roots.

10. Rootzone pH

Rootzone pH can affect nutrient availability and the microorganism community and activities and cause effects on root initiation and growth and ultimately, influence root water and nutrient uptake [141]. For soilless production, it is recommended to keep rootzone pH between 5.5 and 6.5 for most plant species. Lower than pH 5.5, there is a potential for toxicity caused by an excess concentration of Mn levels; higher than 6.5, many elements, such as P, Fe and Mn, can become unavailable to plants. Dysko et al. [145] compared different pH levels (4.5, 5.0, 5.5, 6.0, and 6.5) of nutrient solution of tomato plants grown on mats made of shredded rye straw, peat, or rockwool slabs. The authors found that the concentration of available phosphorus in the root zone was strictly linked with the pH level of the nutrient solution, and the substrate used and available phosphorus was lower in organic substrates (straw, peat) than in rockwool, and, regardless of the substrate type, the best yield performance was obtained at pH 5.5 of the nutrient solution. However, higher nitrogen, calcium, and magnesium concentrations were obtained in organic substrates [146]. In gerbera plants grown in pumice, the high pH level of a nutrient solution (5.0 vs. 5.8) increased the pH of RZ, resulting in significant restrictions in Cu, Mn and Zn uptake [147].

Different plant species can have different sensitivities to rootzone pH, and different plant species can also influence their rootzone pH differently. By growing different species in a soilless cultivation system, Huang et al. [148] found that *Viola* × *wittrockiana*, *Petunia* × *hybrida*, and *Catharanthus roseus* seedlings raised rootzone pH. However, *Celosia cristata*, *Lycopersicon esculentum*, and *Zinnia elegans* seedlings lowered rootzone pH. Growing *Echinacea purpurea* and *E. angustifolia* in three different soilless cultivation systems with three different growing media and three NO_3/NH_4 ratios, Zheng et al. [149] found that rootzone pH remained stable in both *Echinacea* species, regardless of growing media or the ratio of NO_3/NH_4. Zhang et al. [150] studied the effects of Ca at different pH levels of RZ in jack pine (*Pinus banksiana*) seedlings and found that high pH and Ca concentrations decreased root dry weight and inhibited root cell elongation

More studies need to be designed to investigate how rootzone pH affects root growth, morphology, and architecture in soilless cultivation.

11. Root Exudates and Allelopathy

Plant root exudates include carbohydrates, organic acids (e.g., aminoacids), nucleosides, flavonoids, phenolics, glucosinolates, salicylic and jasmonic acid catabolites, enzymes, and vitamins [141]. More than 100 compounds were detected in the root exudates from *Arabidopsis thaliana* grown in a hydroponic system [151]. Plants allocate about 27%

of carbon to their roots, and roots release about11% of the net fixed carbon in to the rootzone [1]. The amount and type of exudates depend on plant species, ages, rootzone microorganisms, and the growing environment. Root exudates can improve plant root and shoot growth and improve plant resistance to unfavourable conditions by attracting beneficial microbiota, toxic chelating compounds in the rootzone, changing rootzone pH, and increasing certain nutrient elements [1]. More research is needed to investigate what compounds can be beneficial to root initiation, growth, morphology, and architecture in order to utilize them to promote plant growth in a soilless cultivation system.

Some root exudates can have inhibitory effects on themselves or other species. These chemicals are known as allelochemicals, which can cause a variety of stresses (e.g., oxidative stress) to plants. Allelochemicals can cause injury to roots, reduce root water and nutrient uptake, and ultimately, reduce photosynthesis and plant growth [141]. By growing *Dactylis glomerata* L., cv. Amba, *Lolium perenne* L. cv.'Belida', and *Rumex acetosa* L. cv. 'Belleville', in a soilless medium, Hussain and Reigosa [152], found that the root length of all the three species was reduced when there was a presence of either one of the allelochemicals, benzoxazolin-2(3H)-one or cinnamic acid, at a concentration of 0.1 mM or higher.

In soilless cultivation systems, nutrient solutions are often reused. This practice can lead to the accumulation of certain allelochemicals, which can negatively impact plant roots. For example, by growing *Lactuca sativa* cv. Southern in solution culture, Talukder et al. [153] demonstrated that the length of the longest root and total root dry weight of plants were reduced when the nutrient solution was continuously used without replacement for six weeks, compared with the control. When the solution was treated by different technologies to degrade the harmful allelochemicals, these root growth attributes were the same as the control. Future research needs to investigate what allelochemicals at what critical level can cause adverse effects on what species in order to provide recommendations for soilless cultivators to decide which species can be grown within the same nutrient solution and when and what technologies to use for extending nutrient solution life for reuse.

12. Root–Microbial Relationships

Plant roots release a vast range of low- and high-molecular weight compounds, including carbohydrates, amino acids, organic acids, fatty acids, proteins, enzymes, nucleotides, and vitamins [154–156]. The type and amount of root exudates are affected by plant species, growth stage, the physico-chemical properties of the growing medium, and other factors. The latter could be (i) physical, such as light, water status, salinity, and temperature; (ii) chemical, nutrient quantity, toxic ions, and pH; and (iii) biological, such as a microbial community [1]. Among other functions, root exudates have a crucial role in the communication between plants and rhizosphere-inhabiting microorganisms [1,157,158]. The chemical communication and interaction between plant roots and microorganisms may be positive or negative [159]. The ones having positive interactions are called plant-beneficial microorganisms. They include mycorrhizal fungi and plant growth-promoting bacteria (PGPR), which help plants by enhancing nutrient availability, inducing plant defence mechanisms, and improving the effectiveness and interaction of plants in SCS [59,160]. The ones having negative interactions, such as competition, parasitism, and pathogenesis, include pathogenic fungi, viruses, and bacteria [158].

The population of microorganisms is low before planting in a solid growing media or nutrient solution. However, high numbers of aerobic, heterotrophic bacteria are present within twenty hours after transplanting [161], derived from plant material, growing media, water, and insects [162]. The contamination of microorganisms starts immediately after planting and is affected by the growing system and media, e.g., organic vs. inorganic, moisture content; nutrient status (e.g., pH, concentration and sources of organic and inorganic nutrients); species and growth stage of the plant and environmental factors [163].

The number of aerobic bacteria is significantly lower in a deep water culture (DWC) compared with NFT, inorganic (rockwool) and organic (coconut fiber) substrate culture, whereas beneficial fungi are significantly higher in coconut-fiber culture than other

SCSs [164]. However, the composition and function of the microbial population on the root and nutrient solution changes during the growing season [165,166].

Arbuscular mycorrhizal fungi (AMF) could increase yield and improve the quality of vegetables [167,168] and other horticultural crops [169]. The improved performance of AM-inoculated plants has been attributed to the more efficient uptake of nutrients, increase in photosynthesis efficiency, the facilitation of water uptake, and the mitigation of ionic imbalances [170–172]. Root association with AMF enhances nutrient acquisition, particularly for diffusion-limited mineral nutrients, such as P, Zn, and Cu [173]. In the case of P acquisition, it may be attributed to integrative physiological/biochemical events, including the proliferation of mycorrhizal hyphae, improved root morphology, increased soil acid phosphatase activity, and the AMF-up-regulated expression of roots [172]. In accordance, Nurbaity et al. [174] recommended that phosphorus concentration in ebb-flow techniques could be reduced up to 50% when AMF is used.

In addition, AMF may lower the root infections of pathogens in SCSs [175], such as *Pythium aphanidermatum* in cucumber [176], *Fusarium oxysporum f. sp. Radicis lycopersici* in tomato [177], or *Phytophthora cryptogea* in gerbera [178]. Furthermore, Song et al. [179] reported that mycorrhizal inoculation with AMF *Funneliformis mosseae* significantly alleviated tomato early blight (*Alternaria solani*) in sand culture due to significantly increased activity of β-1,3-glucanase, chitinase, phenylalanine ammonia-lyase, and lipoxygenase in leaves.

PGPR typically promote plant growth in two ways: direct stimulation and bio control [180]. Growth promotion is implemented through nitrogen fixation, phosphate solubilization, iron sequestration, synthesis of phytohormones, modulation of plant ethylene levels, and the control of phytopathogenic microorganisms [180,181]. PGPR colonizing the surface of the root system (and sometimes inner tissues) have been used both in soil and soilless culture systems due to their positive effects on nutrient uptake (e.g., nitrogen fixation, solubilization of phosphorus), plant stress control, and competition or antagonism with pathogens, suppression, etc. [182]. Recently, they were successfully used in many crops grown hydroponically, such as tomato [183], cherry tomato [184], and lettuce [181]. In addition, PGPR is able to modify root architecture and root tissue structures through the production of phytohormones, secondary metabolites and enzymes. They reduce the growth rate of the primary root and increase the number and length of LR and root hairs [185]. A comprehensive list of reports regarding PGPR effects on root traits was recently published by Grover et al. [186].

Plant inoculation with AMF or rhizobial bacteria, separately or combined, significantly influences and alters root architecture [187,188]. Two different types of root architecture remodelling associated with AMF or rhizobial associations have been reported. In type I, AMF colonization promotes root growth, with a greater number and length of lateral roots and more fine roots. In contrast, in type II root-rhizobium symbiotic associations, in different crop species such as legume crops infected by AMF, often result in inhibited root growth, probably due to the carbon costs of developing nodules maintaining N_2 fixation [41]. Separately, significant increases in root dry weight due to mycorrhizal inoculation were reported in pepper [189] and tomato seedlings [170]. However, since a decrease in root-hair density was reported in specific crops [173,190], the mycorrhizal effects on root hairs seem to be related to plant species [191].

Both AM fungi and PGPB are negatively affected by adverse environmental conditions. Salt stress can affect AM fungi by slowing down root colonization, spore germination, and hyphal growth [192,193]. On the other hand, salinity leads to a failure in the establishment of rhizobia, either by decreasing the survival rate and proliferation of rhizobia or by inhibiting root hair colonization [194]. However, AM fungi have alleviated the salinity stress in transplanted cucumber plants by extending their RL and RSA [195]. In addition, combined applications of AMF and PGPB in garden pea were able to sustain RL, RSA, and root volume (RV) at the level of non-saline plants and provide a significantly higher yield than control plants [187]. Similarly, inoculation with both rhizobia and mycorrhiza fungi provided the best results regarding the length and the weight of faba bean primary

roots, suggesting that co-inoculation could be a potential solution to alleviate the harmful effects low rootzone temperatures [196]. More information regarding the response of the root-associated microbiome under different stress conditions can be found in a recent review by Pascale et al. [197].

13. Conclusions, Trends and Outlook

The recent scientific evidence about the effects of several environmental and cultivation factors on the morphology, architecture, and performance of the root system of plants grown in SCS, which have been presented in this review, point to the high degree of research carried out in recent years intending to achieve high efficiency in water and nutrient supply by using proper pH, temperature, and oxygen levels at the rootzone, proper lightening and CO_2 concentration, and an effective root–microbial relationship while helping a plant to achieve its target yields.

Using rootzone variables, specific models can be developed and used to efficiently manage the irrigation or fertigation needed for optimizing root behaviour in specific horticultural plants grown in SCS. In addition, by using tools, such as multi-element sensors and interpretation algorithms based on machine learning logic, it is possible to monitor the availability of nutrients in the hydroponic solution and modify its composition in real-time while reducing economic costs and minimizing the environmental impact of SCSs. In this context, computer-controlled nutrient management systems with an array of ion-selective electrodes represent a useful tool for the online and real-time monitoring of nutrient solutions, intending to satisfy the nutritional requirements of plants for optimal growth. However, several disadvantages of ion-selective electrodes, such as signal drift and distortions due to interfering ions, make application in SCS difficult. Therefore, it is essential to develop an effective data-processing method to compensate for signal drift and interference. Similarly, advanced Big Data analytics and simulation techniques might allow forecasting the quality and quantity of greenhouse vegetable and fruit production under various conditions and, in turn, to determine the optimal parameters, such as the composition and concentration of the hydroponic nutrient solution temperature, humidity, CO_2 levels, and lighting.

Further investigation of rootzone temperature regulation is required for a deeper understanding of plant root–shoot communication and developing proper environmental control strategies. In addition, the differential thermal regulation of shoots and roots would be an effective strategy to increase plant growth and improve the yield and quality of crops with minimum stress. Notably, the effects of rootzone temperature on crops to increase phytochemical compounds, which are beneficial components for human health, are another important research area with practical interest.

Even hydroponically grown plants, especially in NFT, may suffer from oxygen deficiency, affecting water and nutrient uptake. Despite several techniques already developed to facilitate the oxygen enrichment of growing media or nutrient solution, conflicting results are obtained regarding crop yield and quality. Therefore, comprehensive studies are required to identify the best oxygenation methods depending on different SCSs, growing media, crops, and cultivation cycles.

Lastly, but very importantly, more research is required to study the response of root-associated microorganisms under different stress conditions on root behaviour in different SCSs. Furthermore, further studies are needed to select and detect efficient microorganisms under different SCSs to obtain superior responses on crop productivity.

Author Contributions: A.B., Y.T. and G.S.; writing—original draft preparation (root restriction, nutrient solution, water supply and irrigation, root zone temperature, oxygenation, root–microbial relationships), Y.Z.; writing—original draft preparation (vapor pressure deficit, lighting, rootzone pH, root exudates, and allelopathy), N.S.G.; writing—original draft preparation (CO_2 and growing media), N.S.G. and J.A.F.; review and editing the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

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

References

1. Vives-Peris, V.; de Ollas, C.; Gómez-Cadenas, A.; Pérez-Clemente, R.M. Root exudates: From plant to rhizosphere and beyond. *Plant Cell Rep.* **2020**, *39*, 3–17. [CrossRef]
2. Nibau, C.; Gibbs, D.; Coates, J.C. Branching out in new directions: The control of root architecture by lateral root formation. *New Phytol.* **2008**, *179*, 595–614. [CrossRef]
3. Teixeira, J.S.; Tusscher, K.T. The Systems Biology of Lateral Root Formation: Connecting the Dots. *Mol. Plant* **2019**, *12*, 784–803. [CrossRef]
4. Atkinson, J.A.; Rasmussen, A.; Traini, R.; Voss, U.; Sturrock, C.; Mooney, S.J.; Wells, D.; Bennett, M.J. Branching Out in Roots: Uncovering Form, Function, and Regulation. *Plant Physiol.* **2014**, *166*, 538–550. [CrossRef]
5. Lavenus, J.; Goh, T.; Roberts, I.; Guyomarc'H, S.; Lucas, M.; De Smet, I.; Fukaki, H.; Beeckman, T.; Bennett, M.; Laplaze, L. Lateral root development in Arabidopsis: Fifty shades of auxin. *Trends Plant Sci.* **2013**, *18*, 450–458. [CrossRef]
6. Vives-Peris, V.; López-Climent, M.F.; Pérez-Clemente, R.M.; Gómez-Cadenas, A. Root Involvement in Plant Responses to Adverse Environmental Conditions. *Agronomy* **2020**, *10*, 942. [CrossRef]
7. Dubrovsky, J.G.; Forde, B. Quantitative Analysis of Lateral Root Development: Pitfalls and How to Avoid Them. *Plant Cell* **2012**, *24*, 4–14. [CrossRef] [PubMed]
8. Tüzel, Y.; Balliu, A. Advances in liquid -and solid-medium soilless culture systems. In *Advances in Horticultural Soilless Culture*; Gruda, N.S., Ed.; Burleigh Dodds Science: Cambridge, UK, 2021; ISBN 978 1 78676 292 4. Available online: www.bdspublishing.com (accessed on 10 July 2021). [CrossRef]
9. Sabatino, L. Increasing Sustainability of Growing Media Constituents and Stand-Alone Substrates in Soilless Culture Systems—An Editorial. *Agronomy* **2020**, *10*, 1384. [CrossRef]
10. Savvas, D.; Gruda, N. Application of soilless culture technologies in the modern greenhouse industry—A review. *Eur. J. Hortic. Sci.* **2018**, *83*, 280–293. [CrossRef]
11. Gruda, N. Soilless Culture Systems and Growing Media in Horticulture: An Overview. In *Advances in Horticultural Soilless Culture*; Gruda, N.S., Ed.; Burleigh Dodds Science: Cambridge, UK, 2021; pp. 1–20. ISBN 978 1 78676 292 4. Available online: https://shop.bdspublishing.com/store/bds/detail/workgroup/3-190-89123 (accessed on 10 July 2021). [CrossRef]
12. Raviv, M.; Lieth, J.H.; Bar-Tal, A.; Silber, A. Growing plants in soilless culture: Operational conclusions. In *Soilless Culture*; Elsevier BV: Alpharetta, GA, USA, 2008; pp. 545–571.
13. Maclellan, L. Effect of Hydroculture Methods on Tomato Root Morphology and Anatomy. *Honor. Coll.* **2019**, *567*. Available online: https://digitalcommons.library.umaine.edu/honors/567 (accessed on 11 April 2021).
14. Shi, K.; Hu, W.-H.; Dong, D.-K.; Zhou, Y.-H.; Yu, J.-Q. Low O_2 supply is involved in the poor growth in root-restricted plants of tomato (*Lycopersicon esculentum* Mill.). *Environ. Exp. Bot.* **2007**, *61*, 181–189. [CrossRef]
15. Kafkafi, U. Root Temperature, Concentration and the Ratio NO_3^- / NH_4^+ Effect on Plant Development. *J. Pl. Nutr.* **1990**, *13*, 1291–1306. [CrossRef]
16. Jones, J.B. *Growing Plants Hydroponically*; CRC Press: Boca Raton, FL, USA, 2014. [CrossRef]
17. Bláha, L. Crop Science Institute Importance of Root-Shoot Ratio for Crops Production. *Agron. Agric. Sci.* **2019**, *2*, 1–7. [CrossRef]
18. Zakaria, N.I.; Ismail, M.R.; Awang, Y.; Wahab, P.E.M.; Berahim, Z. Effect of Root Restriction on the Growth, Photosynthesis Rate, and Source and Sink Relationship of Chilli (*Capsicum annuum* L.) Grown in Soilless Culture. *BioMed Res. Int.* **2020**, *2020*, 1–14. [CrossRef] [PubMed]
19. Graham, T.; Wheeler, R. Root restriction: A tool for improving volume utilization efficiency in bioregenerative life-support systems. *Life Sci. Space Res.* **2016**, *9*, 62–68. [CrossRef]
20. Kharkina, T.G.; Ottosen, C.-O.; Rosenqvist, E. Effects of root restriction on the growth and physiology of cucumber plants. *Physiol. Plant.* **1999**, *105*, 434–441. [CrossRef]
21. Peterson, T.A.; Reinsel, M.D.; Krizek, D.T. Tomato (*Lycopersicon esculentum* Mill., cv. 'Better Bush') Plant Response to Root Restriction. I. Alteration of plant morphology. *J. Exp. Bot.* **1991**, *42*, 1233–1240. [CrossRef]
22. Sakamoto, M.; Suzuki, T. Effect of pot volume on the growth of sweetpotato cultivated in the new hydroponic system. *Sustain. Agric. Res.* **2018**, *77*, 137–145. [CrossRef]
23. Tuzel, I.; Tüzel, Y.; Gül, A.; Altunlu, H.; Eltez, R. Effect of different irrigation schedules, substrates and substrate volumes on fruit quality and yield of greenhouse tomato. *Acta Hortic.* **2001**, *548*, 285–292. [CrossRef]
24. Saito, T.; Fukuda, N.; Iikubo, T.; Inai, S.; Fujii, T.; Konishi, C.; Ezura, H. Effects of Root-volume Restriction and Salinity on the Fruit Yield and Quality of Processing Tomato. *J. Jpn. Soc. Hortic. Sci.* **2008**, *77*, 165–172. [CrossRef]
25. Pires, R.C.D.M.; Furlani, P.R.; Ribeiro, R.V.; Junior, D.B.; Sakai, E.; Lourenção, A.L.; Neto, A.T. Irrigation frequency and substrate volume effects in the growth and yield of tomato plants under greenhouse conditions. *Sci. Agricola* **2011**, *68*, 400–405. [CrossRef]

26. Pan, J.; Sharif, R.; Xu, X.; Chen, X. Mechanisms of Waterlogging Tolerance in Plants: Research Progress and Prospects. *Front. Plant Sci.* **2021**, *11*. [CrossRef]
27. Steffens, B.; Rasmussen, A. The Physiology of Adventitious Roots. *Plant Physiol.* **2016**, *170*, 603–617. [CrossRef]
28. Ayi, Q.; Zeng, B.; Liu, J.; Qiaoli, A.; van Bodegom, P.; Cornelissen, J.H.C. Oxygen absorption by adventitious roots promotes the survival of completely submerged terrestrial plants. *Ann. Bot.* **2016**, *118*, 675–683. [CrossRef] [PubMed]
29. Gonin, M.; Bergougnoux, V.; Nguyen, T.D.; Gantet, P.; Champion, A. What Makes Adventitious Roots? *Plants* **2019**, *8*, 240. [CrossRef] [PubMed]
30. Xu, G.; Wolf, S.; Kafkafi, U. Interactive effect of nutrient concentration and container volume on flowering, fruiting, and nutrient uptake of sweet pepper. *J. Plant Nutr.* **2001**, *24*, 479–501. [CrossRef]
31. Bar-Tal, A.; Feigin, A.; Sheinfeld, S.; Rosenberg, R.; Sternbaum, B.; Rylski, I.; Pressman, E. Root restriction and N-NO_3 solution concentration effects on nutrient uptake, transpiration and dry matter production of tomato. *Sci. Hortic.* **1995**, *63*, 195–208. [CrossRef]
32. Massa, D.; Magán, J.J.; Montesano, F.F.; Tzortzakis, N. Minimizing water and nutrient losses from soilless cropping in southern Europe. *Agric. Water Manag.* **2020**, *241*, 106395. [CrossRef]
33. Shi, K.; Ding, X.-T.; Dong, D.-K.; Zhou, Y.-H.; Yu, J.-Q. Root restriction-induced limitation to photosynthesis in tomato (*Lycopersicon esculentum* Mill.) leaves. *Sci. Hortic.* **2008**, *117*, 197–202. [CrossRef]
34. Nishizawa, T.; Saito, K. Effects of Rooting Volume Restriction on the Growth and Carbohydrate Concentration in Tomato Plants. *J. Am. Soc. Hortic. Sci.* **1998**, *123*, 581–585. [CrossRef]
35. Pezeshki, S.; Santos, M. Relationships among Rhizosphere Oxygen Deficiency, Root Restriction, Photosynthesis, and Growth in Baldcypress (*Taxodium distichum* L.) Seedlings. *Photosynthrtica* **1998**, *35*, 381–390. [CrossRef]
36. Osorio, S.; Ruan, Y.-L.; Fernie, A.R. An update on source-to-sink carbon partitioning in tomato. *Front. Plant Sci.* **2014**, *5*, 516. [CrossRef]
37. Lemoine, R.; La Camera, S.; Atanassova, R.; Dédaldéchamp, F.; Allario, T.; Pourtau, N.; Bonnemain, J.L.; Laloi, M.; Coutos-Thévenot, P.; Maurousset, L.; et al. Source-to-sink transport of sugar and regulation by environmental factors. *Front. Plant Sci.* **2013**, *4*, 272. [CrossRef]
38. Bar-Yosef, B. Fertigation management and crop response to solution recycling in semi-closed greenhouses. In *Soilless Culture: Theory and Practice*; Raviv, M., Lieth, J.H., Eds.; Elsevier: Alpharetta, GA, USA, 2008; pp. 545–573.
39. Lynch, J. Root Architecture and Plant Productivity. *Plant Physiol.* **1995**, *109*, 7–13. [CrossRef]
40. Lynch, J.P. Steep, cheap and deep: An ideotype to optimize water and N acquisition by maize root systems. *Ann. Bot.* **2013**, *112*, 347–357. [CrossRef]
41. Li, X.; Zeng, R.; Liao, H. Improving crop nutrient efficiency through root architecture modifications. *J. Integr. Plant Biol.* **2015**, *58*, 193–202. [CrossRef] [PubMed]
42. Forde, B.; Lorenzo, H. The nutritional control of root development. *Plant Soil* **2001**, *232*, 51–68. [CrossRef]
43. Shahzad, Z.; Amtmann, A. Food for thought: How nutrients regulate root system architecture. *Curr. Opin. Plant Biol.* **2017**, *39*, 80–87. [CrossRef] [PubMed]
44. Awika, H.O.; Mishra, A.K.; Gill, H.; DiPiazza, J.; Avila, C.A.; Joshi, V. Selection of nitrogen responsive root architectural traits in spinach using machine learning and genetic correlations. *Sci. Rep.* **2021**, *11*, 9536. [CrossRef]
45. Marzec, M.; Melzer, M. Regulation of Root Development and Architecture by Strigolactones under Optimal and Nutrient Deficiency Conditions. *Int. J. Mol. Sci.* **2018**, *19*, 1887. [CrossRef]
46. Niu, Y.F.; Chai, R.S.; Jin, G.L.; Wang, H.; Tang, C.X.; Zhang, Y.S. Responses of root architecture development to low phosphorus availability: A review. *Ann. Bot.* **2012**, *112*, 391–408. [CrossRef]
47. Rogers, E.D.; Benfey, P.N. Regulation of plant root system architecture: Implications for crop advancement. *Curr. Opin. Biotechnol.* **2015**, *32*, 93–98. [CrossRef] [PubMed]
48. Gruda, N.; Schnitzler, W.H. Transplants Influence of Wood Fiber Substrates and N Application Rates on the Growth of Tomato Transplants. *Adv. Hortic. Sci.* **1999**, *13*, 20–24.
49. Kellermeier, F.; Armengaud, P.; Seditas, T.J.; Danku, J.; Salt, D.E.; Amtmann, A. Analysis of the Root System Architecture of Arabidopsis Provides a Quantitative Readout of Crosstalk between Nutritional Signals. *Plant Cell* **2014**, *26*, 1480–1496. [CrossRef]
50. Cardoso, F.L.; Andriolo, J.L.; Picio, M.D.; Piccin, M.; Souza, J.M. Nitrogen on growth and yield of lettuce plants grown under root confinement. *Hortic. Bras.* **2015**, *33*, 422–427. [CrossRef]
51. Öztekin, G.B.; Uludağ, T.; Tüzel, Y. Growing spinach (*Spinacia oleracea* L.) in a floating system with different concentrations of nutrient solution. *Appl. Ecol. Environ. Res.* **2018**, *16*, 3333–3350. [CrossRef]
52. Balanza, V.; Martínez, J.; Conesa, E.; Egea-Gilabert, C.; Niñirola, D.; Marin, J.L.; González, A.; Fernández, J.A. Effect of pgpr application and nitrogen doses on baby leaf lettuce grown in a floating system. *Acta Hortic.* **2012**, *952*, 679–687. [CrossRef]
53. Roberts, B.R.; Wolverton, C.; Janowicz, L. The impact of substrate and irrigation interval on the post-transplant root growth of container-grown zinnia and tomato1. *J. Environ. Hortic.* **2017**, *35*, 1–5. [CrossRef]
54. Ismail, S.M.; Ozawa, K. Effect of Irrigation Interval on Growth Characteristics, Plant Water Stress Tolerance and Water Use Efficiency for Chile Pepper. In Proceedings of the Thirteenth International Water Technology Conference (IWTC 130), Hurghada, Egypt, 12–15 March 2009; pp. 545–556.

55. Silber, A.; Xu, G.; Wallach, R. High irrigation frequency: The effect on plant growth and on uptake of water and nutrients. *Acta Hortic.* **2003**, *627*, 89–96. [CrossRef]
56. Grewal, H.S.; Maheshwari, B.; Parks, S. Water and nutrient use efficiency of a low-cost hydroponic greenhouse for a cucumber crop: An Australian case study. *Agric. Water Manag.* **2011**, *98*, 841–846. [CrossRef]
57. Yang, T.; Kim, H.-J. Comparisons of nitrogen and phosphorus mass balance for tomato-, basil-, and lettuce-based aquaponic and hydroponic systems. *J. Clean. Prod.* **2020**, *274*, 122619. [CrossRef]
58. LE Bot, J.; Jeannequin, B.; Fabre, R. Growth and Nitrogen Status of Soilless Tomato Plants Following Nitrate Withdrawal from the Nutrient Solution. *Ann. Bot.* **2001**, *88*, 361–370. [CrossRef]
59. Gruda, N.; Savvas, D.; Colla, G.; Rouphael, Y. Impacts of genetic material and current technologies on product quality of selected greenhouse vegetables—A review. *Eur. J. Hortic. Sci.* **2018**, *83*, 319–328. [CrossRef]
60. Conversa, G.; Bonasia, A.; Lazzizera, C.; La Rotonda, P.; Elia, A. Reduction of Nitrate Content in Baby-Leaf Lettuce and Cichorium endivia Through the Soilless Cultivation System, Electrical Conductivity and Management of Nutrient Solution. *Front. Plant Sci.* **2021**, *12*, 645671. [CrossRef]
61. Lieth, J.H.; Oki, L.R. Irrigation in soilless production. In *Soilless Culture: Theory and Practice Theory and Practice*; Elsevier BV: Edinburgh, UK, 2019. [CrossRef]
62. Nikolaou, G.; Neocleous, D.; Katsoulas, N.; Kittas, C. Irrigation of Greenhouse Crops. *Horticulturae* **2019**, *5*, 7. [CrossRef]
63. Silber, A. Fertigation frequency and nutrient uptake by plants: Benefits and constraints. *Int. Fertil. Soc. Proc.* **2005**, *571*, 1–36.
64. Taweesak, V.; Abdullah, T.L.; Hassan, S.A.; Kamarulzaman, N.H.; Yusoff, W.A.W. Growth and Flowering Responses of Cut Chrysanthemum Grown under Restricted Root Volume to Irrigation Frequency. *Sci. World J.* **2014**, *2014*, 1–6. [CrossRef] [PubMed]
65. Rodriguez-Ortega, W.; Martinez, V.; Rivero, R.M.; Cámara-Zapata, J.-M.; Mestre, T.; Garcia-Sanchez, F. Use of a smart irrigation system to study the effects of irrigation management on the agronomic and physiological responses of tomato plants grown under different temperatures regimes. *Agric. Water Manag.* **2017**, *183*, 158–168. [CrossRef]
66. Gruda, N.; Schnitzler, W.H. The effect of water supply on bio-morphological and plant-physiological parameters of tomato transplants cultivated in wood fiber substrate. *J. Appl. Bot.* **2000**, *74*, 233–239. [CrossRef]
67. Leskovar, D.I. Root and Shoot Modification by Irrigation. *HortTechnology* **1998**, *8*, 510–514. [CrossRef]
68. Gruda, N.; Schnitzler, W. Suitability of wood fiber substrates for production of vegetable transplants II: The effect of wood fiber substrates and their volume weights on the growth of tomato transplants. *Sci. Hortic.* **2004**, *100*, 333–340. [CrossRef]
69. Gruda, N.; Schnitzler, W. Suitability of wood fiber substrate for production of vegetable transplants: I. Physical properties of wood fiber substrates. *Sci. Hortic.* **2004**, *100*, 309–322. [CrossRef]
70. Machado, R.; Alves-Pereira, I.; Ferreira, R.; Gruda, N. Coir, an Alternative to Peat—Effects on Plant Growth, Phytochemical Accumulation, and Antioxidant Power of Spinach. *Horticulturae* **2021**, *7*, 127. [CrossRef]
71. Wallach, R. Physical Characteristics of Soilless Media. In *Soilless Culture: Theory and Practice*; Raviv, M., Lieth, J.H., Eds.; Elsevier: Alpharetta, GA, USA, 2008; pp. 41–116.
72. Xu, G.; Levkovitch, I.; Soriano, S.; Wallach, R.; Silber, A. Integrated effect of irrigation frequency and phosphorus level on lettuce: P uptake, root growth and yield. *Plant Soil* **2004**, *263*, 297–309. [CrossRef]
73. Scagel, C.F.; Bi, G.; Fuchigami, L.H.; Regan, R.P. Effects of Irrigation Frequency and Nitrogen Fertilizer Rate on Water Stress, Nitrogen Uptake, and Plant Growth of Container-grown Rhododendron. *HortScience* **2011**, *46*, 1598–1603. [CrossRef]
74. Nikolaou, G.; Neocleous, D.; Katsoulas, N.; Kittas, C. Effect of irrigation frequency on growth and production of a cucumber crop under soilless culture. *Emir. J. Food Agric.* **2017**, *29*, 863. [CrossRef]
75. Heller, H.; Bar-Tal, A.; Assouline, S.; Narkis, K.; Suryano, S.; De La Forge, A.; Barak, M.; Alon, H.; Bruner, M.; Cohen, S.; et al. The effects of container geometry on water and heat regimes in soilless culture: Lettuce as a case study. *Irrig. Sci.* **2014**, *33*, 53–65. [CrossRef]
76. Fields, J.S.; Owen, J.; Scoggins, H.L. The Influence of Substrate Hydraulic Conductivity on Plant Water Status of an Ornamental Container Crop Grown in Suboptimal Substrate Water Potentials. *HortScience* **2017**, *52*, 1419–1428. [CrossRef]
77. Gruda, N.; Rau, B.J.; Wright, R.D. Laboratory bioassay and greenhouse evaluation of a pine tree substrate used as a container substrate. *Eur. J. Hortic. Sci.* **2009**, *74*, 73–78.
78. Ortega, M.; Moreno, M.T.; Ordovas, J.; Aguado, M. Behaviour of different horticultural species in phytotoxicity bioassays of bark substrates. *Sci. Hortic.* **1996**, *66*, 125–132. [CrossRef]
79. Koevoets, I.T.; Venema, J.H.; Elzenga, J.T.M.; Testerink, C. Roots Withstanding their Environment: Exploiting Root System Architecture Responses to Abiotic Stress to Improve Crop Tolerance. *Front. Plant Sci.* **2016**, *7*, 1335. [CrossRef]
80. Gruda, N. Impact of Environmental Factors on Product Quality of Greenhouse Vegetables for Fresh Consumption. *Crit. Rev. Plant Sci.* **2005**, *24*, 227–247. [CrossRef]
81. Zhu, J.; Zhang, K.-X.; Wang, W.-S.; Gong, W.; Liu, W.-C.; Chen, H.-G.; Xu, H.-H.; Lu, Y.-T. Low Temperature Inhibits Root Growth by Reducing Auxin Accumulation via ARR1/12. *Plant Cell Physiol.* **2014**, *56*, 727–736. [CrossRef] [PubMed]
82. Chen, X.; Song, F.; Liu, F.; Tian, C.; Liu, S.; Xu, H.; Zhu, X. Effect of Different Arbuscular Mycorrhizal Fungi on Growth and Physiology of Maize at Ambient and Low Temperature Regimes. *Sci. World J.* **2014**, *2014*, 1–7. [CrossRef]
83. Nagel, K.A.; Kastenholz, B.; Jahnke, S.; van Dusschoten, D.; Aach, T.; Mühlich, M.; Truhn, D.; Scharr, H.; Terjung, S.; Walter, A.; et al. Temperature responses of roots: Impact on growth, root system architecture and implications for phenotyping. *Funct. Plant Biol.* **2009**, *36*, 947–959. [CrossRef]

84. Du, Y.; Scheres, B. Lateral root formation and the multiple roles of auxin. *J. Exp. Bot.* **2017**, *69*, 155–167. [CrossRef]
85. Waidmann, S.; Sarkel, E.; Kleine-Vehn, J. Same same, but different: Growth responses of primary and lateral roots. *J. Exp. Bot.* **2020**, *71*, 2397–2411. [CrossRef]
86. Torres-Martínez, H.H.; Rodriguez-Alonso, G.; Shishkova, S.; Dubrovsky, J.G. Lateral Root Primordium Morphogenesis in Angiosperms. *Front. Plant Sci.* **2019**, *10*, 206. [CrossRef] [PubMed]
87. Yan, Q.-Y.; Duan, Z.-Q.; Mao, J.-D.; Li, X.; Dong, F. Low Root Zone Temperature Limits Nutrient Effects on Cucumber Seedling Growth and Induces Adversity Physiological Response. *J. Integr. Agric.* **2013**, *12*, 1450–1460. [CrossRef]
88. Luo, H.; Xu, H.; Chu, C.; He, F.; Fang, S. High Temperature can Change Root System Architecture and Intensify Root Interactions of Plant Seedlings. *Front. Plant Sci.* **2020**, *11*, 160. [CrossRef] [PubMed]
89. Balliu, A.; Sallaku, G. The environment temperature affects post-germination growth and root system architecture of pea (*Pisum sativum* L.) plants. *Sci. Hortic.* **2021**, *278*, 109858. [CrossRef]
90. Sakamoto, M.; Suzuki, T. Effect of Root-Zone Temperature on Growth and Quality of Hydroponically Grown Red Leaf Lettuce (*Lactuca sativa* L. cv. Red Wave). *Am. J. Plant Sci.* **2015**, *6*, 2350–2360. [CrossRef]
91. He, F.; Thiele, B.; Watt, M.; Kraska, T.; Ulbrich, A.; Kuhn, A.J. Effects of Root Cooling on Plant Growth and Fruit Quality of Cocktail Tomato during Two Consecutive Seasons. *J. Food Qual.* **2019**, *2019*, 1–15. [CrossRef]
92. Kawasaki, Y.; Matsuo, S.; Kanayama, Y.; Kanahama, K. Effect of Root-zone Heating on Root Growth and Activity, Nutrient Uptake, and Fruit Yield of Tomato at Low Air Temperatures. *J. Jpn. Soc. Hortic. Sci.* **2014**, *83*, 295–301. [CrossRef]
93. Karnoutsos, P.; Karagiovanidis, M.; Bantis, F.; Chatzistathis, T.; Koukounaras, A.; Ntinas, G.K. Controlled root-zone temperature effect on baby leaf vegetables yield and quality in a floating system under mild and extreme weather conditions. *J. Sci. Food Agric.* **2020**, *101*, 3933–3941. [CrossRef]
94. Qin, L.; He, J.; Lee, S.K.; Dodd, I.C. An assessment of the role of ethylene in mediating lettuce (*Lactuca sativa*) root growth at high temperatures. *J. Exp. Bot.* **2007**, *58*, 3017–3024. [CrossRef]
95. Pardales, J.R.J.; Banoc, D.M.; Yamauchi, A.; Iijima, M.; Kono, Y. Root System Development of Cassava and Sweetpotato during Early Growth Stage as Affected by High Root Zone Temperature. *Plant Prod. Sci.* **1999**, *2*, 247–251. [CrossRef]
96. MacDuff, J.H.; Wild, A.; Hopper, M.J.; Dhanoa, M.S. Effects of temperature on parameters of root growth relevant to nutrient uptake: Measurements on oilseed rape and barley grown in flowing nutrient solution. *Plant Soil* **1986**, *94*, 321–332. [CrossRef]
97. Wang, X.; Zhang, W.; Miao, Y.; Gao, L. Root-Zone Warming Differently Benefits Mature and Newly Unfolded Leaves of Cucumis sativus L. Seedlings under Sub-Optimal Temperature Stress. *PLoS ONE* **2016**, *11*, e0155298. [CrossRef]
98. Aroca, R.; Porcel, R.; Lozano, J.M.R. Regulation of root water uptake under abiotic stress conditions. *J. Exp. Bot.* **2011**, *63*, 43–57. [CrossRef]
99. Kafkafi, U. Functions of the Root System. In *Soilless Culture: Theory and Practice*; Raviv, M., Lieth, J.H., Eds.; Elsevier: Alpharetta, GA, USA, 2008; pp. 13–40.
100. Poiré, R.; Schneider, H.; Thorpe, M.R.; Kuhn, A.J.; Schurr, U.; Walter, A. Root cooling strongly affects diel leaf growth dynamics, water and carbohydrate relations inRicinus communis. *Plant Cell Environ.* **2010**, *33*, 408–417. [CrossRef] [PubMed]
101. Dodd, I.; He, J.; Turnbull, C.; Lee, S.; Critchley, C. The influence of supra-optimal root-zone temperatures on growth and stomatal conductance in *Capsicum annuum* L. *J. Exp. Bot.* **2000**, *51*, 239–248. [CrossRef]
102. Tachibana, S. Effect of Root Temperature on the Rate of Water and Nutrient Absorption in Cucumber Cultivars and Figleaf Gourd. *J. Jpn. Soc. Hortic. Sci.* **1987**, *55*, 461–467. [CrossRef]
103. Kawasaki, Y.; Matsuo, S.; Suzuki, K.; Kanayama, Y.; Kanahama, K. Root-zone Cooling at High Air Temperatures Enhances Physiological Activities and Internal Structures of Roots in Young Tomato Plants. *J. Jpn. Soc. Hortic. Sci.* **2013**, *82*, 322–327. [CrossRef]
104. Li, X.; Gruda, N.; Dong, J.; Duan, Z. Greenhouse soil warmed by capillary network and its effect on the growth of cucumber. *Acta Hortic.* **2020**, *1296*, 149–158. [CrossRef]
105. Masaru, S.; Uenishi, M.; Miyamoto, K.; Suzuki, T. Effect of Root-Zone Temperature on the Growth and Fruit Quality of Hydroponically Grown Strawberry Plants. *J. Agric. Sci.* **2016**, *8*, 122. [CrossRef]
106. Falah, M.A.F.; Wajima, T.; Yasutake, D.; Sago, Y.; Kitano, M. Responses of root uptake to high temperature of tomato plants (*Lycopersicon esculentum* Mill.) in soil-less culture. *J. Agric. Technol.* **2010**, *6*, 543–558.
107. Giri, A.; Heckathorn, S.; Mishra, S.; Krause, C. Heat Stress Decreases Levels of Nutrient-Uptake and -Assimilation Proteins in Tomato Roots. *Plants* **2017**, *6*, 6. [CrossRef] [PubMed]
108. Sakamoto, M.; Suzuki, T. Elevated Root-Zone Temperature Modulates Growth and Quality of Hydroponically Grown Carrots. *Agric. Sci.* **2015**, *06*, 749–757. [CrossRef]
109. Pedersen, O.; Sauter, M.; Colmer, T.D.; Nakazono, M. Regulation of root adaptive anatomical and morphological traits during low soil oxygen. *New Phytol.* **2021**, *229*, 42–49. [CrossRef] [PubMed]
110. Li, Y.; Niu, W.; Cao, X.; Wang, J.; Zhang, M.; Duan, X.; Zhang, Z. Effect of soil aeration on root morphology and photosynthetic characteristics of potted tomato plants (*Solanum lycopersicum*) at different NaCl salinity levels. *BMC Plant Biol.* **2019**, *19*, 1–15. [CrossRef]
111. Morard, P.; Silvestre, J. Plant injury due to oxygen deficiency in the root environment of soilless culture: A review. *Plant Soil* **1996**, *184*, 243–254. [CrossRef]

112. Nicola, S.; Egea-Gilabert, C.; Niñirola, D.; Conesa, E.; Pignata, G.; Fontana, E.; Fernández, J.A. Nitrogen and aeration levels of the nutrient solution in soilless cultivation systems as important growing conditions affecting inherent quality of baby leaf vegetables: A review. *Acta Hortic.* **2015**, *1099*, 167–177. [CrossRef]
113. Shaw, R.E.; Meyer, W.S.; McNeill, A.; Tyerman, S.D. Waterlogging in Australian agricultural landscapes: A review of plant responses and crop models Waterlogging in Australian agricultural landscapes: A review of plant responses and crop models. *Crop. Pasture Sci.* **2013**, *64*, 549–562. [CrossRef]
114. Lara, L.J.; Egea-Gilabert, C.; Niñirola, D.; Conesa, E.; Fernández, J.A. Effect of aeration of the nutrient solution on the growth and quality of purslane (*Portulaca oleracea*). *J. Hortic. Sci. Biotechnol.* **2011**, *86*, 603–610. [CrossRef]
115. Jackson, M.B. Long-distance signalling from roots to shoots assessed: The flooding story. *J. Exp.Bot.* **2002**, *53*, 175–181. [CrossRef]
116. Acuña, R.; Gil, I.; Bonachela, S.; Magán, J. Oxyfertigation of a greenhouse melon crop grown in rockwool slabs in a mediterranean area. *Acta Hortic.* **2008**, *779*, 447–454. [CrossRef]
117. Bonachela, S.; Acuña, R.A.; Magan, J.J.; Marfa, O. Oxygen enrichment of nutrient solution of substrate-grown vegetable crops under Mediterranean greenhouse conditions: Oxygen content dynamics and crop response. *Span. J. Agric. Res.* **2010**, *8*, 1231. [CrossRef]
118. Öztekin, G.B.; Tüzel, Y. Effects of oxyfertigation and plant growth promoting rhizobacteria on greenhouse lettuce grown in perlite. *Acta Sci. Pol. Hortorum. Cultus* **2020**, *19*, 97–105. [CrossRef]
119. Schroeder, F.-G.; Lieth, J. Gas composition and oxygen supply in the root environment of substrates in closed hydroponic systems. *Acta Hortic.* **2004**, *644*, 299–305. [CrossRef]
120. Suhl, J.; Oppedijk, B.; Baganz, D.; Kloas, W.; Schmidt, U.; van Duijn, B. Oxygen consumption in recirculating nutrient film technique in aquaponics. *Sci. Hortic.* **2019**, *255*, 281–291. [CrossRef]
121. Drew, M.C. Oxygen deficiency and root metabolism: Injury and acclimation under hypoxia and anoxia. *Annu. Rev. Plant Physiol.Plant Mol. Biol.* **1997**, *48*, 223–250. [CrossRef] [PubMed]
122. Blok, C.; Jackson, B.E.; Guo, X.; de Visser, P.H.B.; Marcelis, L.F.M. Maximum Plant Uptakes for Water, Nutrients, and Oxygen Are Not Always Met by Irrigation Rate and Distribution in Water-based Cultivation Systems. *Front. Plant Sci.* **2017**, *8*, 562. [CrossRef] [PubMed]
123. Morard, P.; Lacoste, L.; Silvestre, J. Effect of oxygen deficiency on uptake of water and mineral nutrients by tomato plants in soilless culture. *J. Plant Nutr.* **2000**, *23*, 1063–1078. [CrossRef]
124. Alvarado-Camarillo, D.; Valdez-Aguilar, L.A.; González-Fuentes, J.A.; Rascón-Alvarado, E.; Peña-Ramos, F.M. Response of hydroponic lettuce to aeration, nitrate and potassium in the nutrient solution. *Acta Agric. Scand. Sect. B—Plant Soil Sci.* **2020**, *70*, 341–348. [CrossRef]
125. Marfà, O.; Cáceres, R.; Guri, S. Oxyfertigation: A new technique for soilless culture under mediterranean conditions. *Acta Hortic.* **2005**, *697*, 65–72. [CrossRef]
126. Ehret, D.L.; Edwards, D.; Helmer, T.; Lin, W.; Jones, G.; Dorais, M.; Papadopoulos, A.P. Effects of oxygen-enriched nutrient solution on greenhouse cucumber and pepper production. *Sci. Hortic.* **2010**, *125*, 602–607. [CrossRef]
127. Lee, J.W.; Lee, B.S.; Kang, J.G.; Bae, J.H.; Ku, Y.G.; Gorinstein, S.; Lee, J.H. Effect of root zone aeration on the growth and bioactivity of cucumber plants cultured in perlite substrate. *Biologia* **2014**, *69*, 610–617. [CrossRef]
128. Zheng, Y.; Wang, L.; Dixon, M. An upper limit for elevated root zone dissolved oxygen concentration for tomato. *Sci. Hortic.* **2007**, *113*, 162–165. [CrossRef]
129. Zhang, J.; Jiao, X.; Du, Q.; Song, X.; Ding, J.; Li, J. Effects of Vapor Pressure Deficit and Potassium Supply on Root Morphology, Potassium Uptake, and Biomass Allocation of Tomato Seedlings. *J. Plant Growth Regul.* **2021**, *40*, 509–518. [CrossRef]
130. Arve, L.E.; Torre, S. Ethylene is involved in high air humidity promoted stomatal opening of tomato (*Lycopersicon esculentum*) leaves. *Funct. Plant Biol.* **2015**, *42*, 376–386. [CrossRef]
131. Lihavainen, J.; Ahonen, V.; Keski-Saari, S.; Kontunen-Soppela, S.; Oksanen, E.; Keinänen, M. Low vapour pressure deficit affects nitrogen nutrition and foliar metabolites in silver birch. *J. Exp. Bot.* **2016**, *67*, 4353–4365. [CrossRef]
132. Zheng, Y.; Shimizu, H. Plant growth and water use efficiency of four Chinese conifer tree species under different air humidity. *Phyton-Horn* **2005**, *45*, 575–582.
133. Adams, P. Crop nutrition in hydroponics. *Acta Hortic.* **1993**, *323*, 289–306. [CrossRef]
134. Christiaens, A.; Gobin, B.; Van Labeke, M. Light quality and adventitious rooting: A mini-review. *Acta Hortic.* **2016**, *1134*, 385–394. [CrossRef]
135. Gil, C.S.; Jung, H.Y.; Lee, C.; Eom, S.H. Blue light and NAA treatment significantly improve rooting on single leaf-bud cutting of Chrysanthemum via upregulated rooting-related genes. *Sci. Hortic.* **2020**, *274*, 109650. [CrossRef]
136. Rasool, A.; Graham, T.; Dayboll, C.; Stoochnoff, J.; Zheng, Y.; Dixon, M. Improving adventitious rooting in Kalanchoe blossfeldiana cuttings using sole source targeted LED lighting spectra. *Acta Hortic.* **2020**, *1296*, 629–636. [CrossRef]
137. Navidad, H.; Fløistad, I.S.; Olsen, J.E.; Torre, S. Subalpine Fir (*Abies laciocarpa*) and Norway Spruce (*Piceaabies*) Seedlings Show Different Growth Responses to Blue Light. *Agronomy* **2020**, *10*, 712. [CrossRef]
138. Olschowski, S.; Geiger, E.-M.; Herrmann, J.; Sander, G.; Grüneberg, H. Effects of red, blue, and white LED irradiation on root and shoot development of Calibrachoa cuttings in comparison to high pressure sodium lamps. *Acta Hortic.* **2016**, *1134*, 245–250. [CrossRef]

139. Barickman, T.; Kopsell, D.; Sams, C.; Morrow, R. Sole-Source LED Lighting and Fertility Impact Shoot and Root Tissue Mineral Elements in Chinese Kale (*Brassica oleracea* var. *alboglabra*). *Horticulturae* **2020**, *6*, 40. [CrossRef]
140. Zhao, J.; Chen, X.; Wei, H.-X.; Lv, J.; Chen, C.; Liu, X.-Y.; Wen, Q.; Jia, L.-M. Nutrient uptake and utilization in Prince Rupprecht's larch (*Larix principis-rupprechtii* Mayr.) seedlings exposed to a combination of light-emitting diode spectra and exponential fertilization. *Soil Sci. Plant Nutr.* **2019**, *65*, 358–368. [CrossRef]
141. Zheng, Y. Advances in understanding plant root behaviour and rootzone management in soilless culture system. In *Advances in Horticultural Soilless Culture*; Gruda, N.S., Ed.; Burleigh Dodds Science: Cambridge, UK, 2021; pp. 23–44.
142. Li, X.; Dong, J.; Gruda, N.; Chu, W.; Duan, Z. Does the short-term fluctuation of mineral element concentrations in the closed hydroponic experimental facilities affect the mineral concentrations in cucumber plants exposed to elevated CO_2? *Plant Soil* **2021**, 1–17. [CrossRef]
143. Dong, J.; Gruda, N.; Li, X.; Tang, Y.; Zhang, P.; Duan, Z. Sustainable vegetable production under changing climate: The impact of elevated CO_2 on yield of vegetables and the interactions with environments-A review. *J. Clean. Prod.* **2020**, *253*, 119920. [CrossRef]
144. Li, X.; Dong, J.; Chu, W.; Chen, Y.; Duan, Z. The relationship between root exudation properties and root morphological traits of cucumber grown under different nitrogen supplies and atmospheric CO_2 concentrations. *Plant Soil* **2018**, *425*, 415–432. [CrossRef]
145. Dyśko, J.; Kaniszewski, S.; Kowalczyk, W. The effect of nutrient solution on phosphoros availability in soilless culture of tomato. *J. Elementol.* **2008**, *13*, 189–198.
146. Dyśko, J.; Kowalczyk, W.; Kaniszewski, S. The Influence of pH of Nutrient Solution on Yield and Nutritional Status of Tomato Plants Grown in Soilless Culture System. *Veg. Crop. Res. Bull.* **2009**, *70*, 59–69. [CrossRef]
147. Savvas, D.; Karagianni, V.; Kotsiras, A.; Demopoulos, V.; Karkamisi, I.; Pakou, P. Interactions between ammonium and pH of the nutrient solution supplied to gerbera (*Gerbera jamesonii*) grown in pumice. *Plant Soil* **2003**, *254*, 393–402. [CrossRef]
148. Huang, J.-S.; Nelson, P.V.; Lee, J.-W. Seedling effect on root substrate pH. *J. Plant Nutr.* **2001**, *24*, 1133–1147. [CrossRef]
149. Zheng, Y.; Dixon, M.; Saxena, P. Greenhouse production of *Echinacea purpurea* (L.) and *E. angustifolia* using different growing media, NO_3^-/NH_4^+ ratios and watering regimes. *Can. J. Plant Sci.* **2006**, *86*, 809–815. [CrossRef]
150. Zhang, W.; Xu, F.; Zwiazek, J.J. Responses of jack pine (*Pinus banksiana*) seedlings to root zone pH and calcium. *Environ. Exp. Bot.* **2015**, *111*, 32–41. [CrossRef]
151. Strehmel, N.; Böttcher, C.; Schmidt, S.; Scheel, D. Profiling of secondary metabolites in root exudates of Arabidopsis thaliana. *Phytochemistry* **2014**, *108*, 35–46. [CrossRef]
152. Hussain, M.I.; Reigosa, M.J. Allelochemical stress inhibits growth, leaf water relations, PSII photochemistry, non-photochemical fluorescence quenching, and heat energy dissipation in three C_3 perennial species. *J. Exp. Bot.* **2011**, *62*, 4533–4545. [CrossRef]
153. Talukder, R.; Asaduzzaman, M.; Ueno, M.; Tanaka, H.; Asao, T. Alleviation of allelochemical stress-induced growth inhibition and oxidative damage in lettuce under closed hydroponics through electro-degradation. *Hortic. Sci.* **2020**, *47*, 53–68. [CrossRef]
154. Badri, D.V.; Vivanco, J.M. Regulation and function of root exudates. *Plant Cell Environ.* **2009**, *32*, 666–681. [CrossRef] [PubMed]
155. Mohanram, S.; Kumar, P. Rhizosphere microbiome: Revisiting the synergy of plant-microbe interactions. *Ann. Microbiol.* **2019**, *69*, 307–320. [CrossRef]
156. Nazir, N.; Kamili, A.N.; Zargar, M.Y.; Khan, I.; Shah, D.; Parray, J.A.; Tyub, S. Effect of Root Exudates on Rhizosphere Soil Microbial Communities. *J. Res. Dev.* **2016**, *16*, 88–95.
157. Canarini, A.; Kaiser, C.; Merchant, A.; Richter, A.; Wanek, W. Root Exudation of Primary Metabolites: Mechanisms and Their Roles in Plant Responses to Environmental Stimuli. *Front. Plant Sci.* **2019**, *10*, 157. [CrossRef] [PubMed]
158. Olanrewaju, O.S.; Ayangbenro, A.S.; Glick, B.R.; Babalola, O.O.; Ayangbenro, A. Plant health: Feedback effect of root exudates-rhizobiome interactions. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 1155–1166. [CrossRef]
159. Bais, H.P.; Weir, T.L.; Perry, L.; Gilroy, S.; Vivanco, J.M. The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol.* **2006**, *57*, 233–266. [CrossRef]
160. Baum, C.; El-Tohamy, W.; Gruda, N. Increasing the productivity and product quality of vegetable crops using arbuscular mycorrhizal fungi: A review. *Sci. Hortic.* **2015**, *187*, 131–141. [CrossRef]
161. Berkelmann, B.; Wohanka, W.; Wolf, G. Characterization of the bacterial flor in circulating nutrient solutions of a hydroponic system with rockwool. *Acta Hortic.* **1994**, 372–381. [CrossRef]
162. Stanghellini, M.E. Hydroponics: A Solution for Zoosporic Pathogens. *Plant Dis.* **1994**, *78*, 1129–1138. [CrossRef]
163. Chave, M.; Dabert, P.; Brun, R.; Godon, J.-J.; Poncet, C. Dynamics of rhizoplane bacterial communities subjected to physicochemical treatments in hydroponic crops. *Crop. Prot.* **2008**, *27*, 418–426. [CrossRef]
164. Koohakan, P.; Ikeda, H.; Jeanaksorn, T.; Tojo, M.; Kusakari, S.-I.; Okada, K.; Sato, S. Evaluation of the indigenous microorganisms in soilless culture: Occurrence and quantitative characteristics in the different growing systems. *Sci. Hortic.* **2004**, *101*, 179–188. [CrossRef]
165. Khalil, S.; Alsanius, B.W. Dynamics of the indigenous microflora inhabiting the root zone and the nutrient solution of tomato in a commercial closed greenhouse system. *Gartenbauwissenschaft* **2001**, *66*, 188–198.
166. Waechter-Kristensen, B.; Sundin, P.; Gertsson, U.; Hultberg, M.; Khalil, S.; Jensén, P.; Berkelmann-Loehnertz, B.; Wohanka, W. Management of microbial factors in the rhizosphere and nutrient solution of hydroponically grown tomato. *Acta Hortic.* **1997**, 335–342. [CrossRef]
167. Babaj, I.; Sallaku, G.; Balliu, A. The effects of endogenous mycorrhiza (*Glomus* spp.) on plant growth and yield of grafted cucumber (*Cucumis sativum* L.) under common commercial greenhouse conditions. *Albanian J. Agric. Sci.* **2014**, *13*, 24–28.

168. Vuksani, A.; Sallaku, G.; Balliu, A. The Effects of Endogenous Mycorrhiza (*Glomus* spp.) on Stand Establishment Rate and Yield of Open Field Tomato Crop. *Albanian J. Agric. Sci.* **2015**, *14*, 25–30.
169. Unal, N.; Tuzel, Y.; Tuzel, I.; Oztekin, G. Effects of mycorrhiza and irrigation programs on strawberry production in substrate culture. *Acta Hortic.* **2020**, *1273*, 177–182. [CrossRef]
170. Balliu, A.; Sallaku, G.; Rewald, B. AMF Inoculation Enhances Growth and Improves the Nutrient Uptake Rates of Transplanted, Salt-Stressed Tomato Seedlings. *Sustainability* **2015**, *7*, 15967–15981. [CrossRef]
171. Meça, E.; Sallaku, G.; Balliu, A. Artificial Inoculation of AM Fungi Improves Nutrient Uptake Efficiency in Salt Stressed Pea (*Pissum Sativum* L.) Plants. *J. Agric. Stud.* **2016**, *4*, 37. [CrossRef]
172. Mishra, V.; Ellouze, W.; Howard, R.J. Utility of Arbuscular Mycorrhizal Fungi for Improved Production and Disease Mitigation in Organic and Hydroponic Greenhouse Crops. *J. Hortic.* **2018**, *5*, 1–10. [CrossRef]
173. Shao, Y.-D.; Hu, X.-C.; Wu, Q.-S.; Yang, T.-Y.; Srivastava, A.; Zhang, D.-J.; Gao, X.-B.; Kuča, K. Mycorrhizas promote P acquisition of tea plants through changes in root morphology and P transporter gene expression. *S. Afr. J. Bot.* **2021**, *137*, 455–462. [CrossRef]
174. Nurbaity, A.; Istifadah, N.; A Haryantini, B.; Ilhami, M.F.; I Habibullah, M.; Arifin, M. Optimization of hydroponic technology for production of mycorrhiza biofertilizer. *IOP Conf. Series: Earth Environ. Sci.* **2019**, *347*, 012017. [CrossRef]
175. Utkhede, R. Increased Growth and Yield of Hydroponically Grown Greenhouse Tomato Plants Inoculated with Arbuscular Mycorrhizal Fungi and *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *BioControl* **2006**, *51*, 393–400. [CrossRef]
176. Postma, J.; Klein, M.J.E.I.M.W.-D.; Van Elsas, J.D. Effect of the Indigenous Microflora on the Development of Root and Crown Rot Caused by Pythium aphanidermatum in Cucumber Grown on Rockwool. *Phytopathology* **2000**, *90*, 125–133. [CrossRef]
177. Minuto, A.; Clematis, F.; Gullino, M.L.; Garibaldi, A. Induced suppressiveness to *Fusarium oxysporumf*.sp. *radicis lycopersici* in rockwool substrate used in closed soilless systems. *Phytoparasitica* **2007**, *35*, 77–85. [CrossRef]
178. Garibaldi, A.; Minuto, A.; Grasso, V.; Gullino, M. Application of selected antagonistic strains against Phytophthora cryptogea on gerbera in closed soilless systems with disinfection by slow sand filtration. *Crop. Prot.* **2003**, *22*, 1053–1061. [CrossRef]
179. Song, Y.; Chen, D.; Lu, K.; Sun, Z.; Zeng, R. Enhanced tomato disease resistance primed by arbuscular mycorrhizal fungus. *Front. Plant Sci.* **2015**, *6*, 786. [CrossRef]
180. Gamalero, E.; Glick, B.R. Mechanisms used by plant Growth-Promoting Bacteria. In *Bacteria in Agrobiology: Plant Nutrient Management*; Maheshwari, D.K., Ed.; Springer: Heidelberg, Germany, 2011. [CrossRef]
181. Moncada, A.; Vetrano, F.; Miceli, A. Alleviation of Salt Stress by Plant Growth-Promoting Bacteria in Hydroponic Leaf Lettuce. *Agronomy* **2020**, *10*, 1523. [CrossRef]
182. Lee, S.; Lee, J. Beneficial bacteria and fungi in hydroponic systems: Types and characteristics of hydroponic food production methods. *Sci. Hortic.* **2015**, *195*, 206–215. [CrossRef]
183. Kalozoumis, P.; Savvas, D.; Aliferis, K.; Ntatsi, G.; Marakis, G.; Simou, E.; Tampakaki, A.; Karapanos, I. Impact of Plant Growth-Promoting Rhizobacteria Inoculation and Grafting on Tolerance of Tomato to Combined Water and Nutrient Stress Assessed via Metabolomics Analysis. *Front. Plant Sci.* **2021**, *12*, 1–15. [CrossRef] [PubMed]
184. Aini, N.; Yamika, W.S.D.; Pahlevi, R.W. The effect of nutrient concentration and inoculation of PGPR and AMF on the yield and fruit quality of hydroponic cherry tomatoes (*Lycopersicum esculentum* Mill. var. cerasiforme). *J. Appl. Hortic.* **2019**, *21*, 116–122. [CrossRef]
185. Vacheron, J.; Desbrosses, G.; Bouffaud, M.-L.; Touraine, B.; Moënne-Loccoz, Y.; Muller, D.; Legendre, L.; Wisniewski-Dyé, F.; Prigent-Combaret, C. Plant growth-promoting rhizobacteria and root system functioning. *Front. Plant Sci.* **2013**, *4*, 356. [CrossRef]
186. Grover, M.; Bodhankar, S.; Sharma, A.; Sharma, P.; Singh, J.; Nain, L. PGPR Mediated Alterations in Root Traits: Way toward Sustainable Crop Production. *Front. Sustain. Food Syst.* **2021**, *4*, 1–28. [CrossRef]
187. Veselaj, E.; Sallaku, G.; Balliu, A. Combined application of arbuscular mycorrhizae fungi and plant growth promoting bacteria improves growth and nutrient uptake efficiency of pea (*Pisum sativum* L.) plants. *Acta Sci. Pol. Hortorum. Cultus* **2018**, *17*, 73–86. [CrossRef]
188. Veselaj, E.; Sallaku, G.; Balliu, A. Tripartite Relationships in Legume Crops Are Plant-Microorganism-Specific and Strongly Influenced by Salinity. *Agriculture* **2018**, *8*, 117. [CrossRef]
189. Ikiz, O.; Abak, K.; Dasgan, H.; Ortas, I. Effects of mycorrhizal inoculation in soilless culture on pepper plant growth. *Acta Hortic.* **2009**, *807*, 533–540. [CrossRef]
190. Shao, Y.; Zhang, D.; Hu, X.; Wu, Q.; Jiang, K.; Xia, T.; Gao, X.; KUČA, K. Mycorrhiza-induced changes in root growth and nutrient absorption of tea plants. *Plant Soil Environ.* **2018**, *64*, 283–289. [CrossRef]
191. Zou, Y.-N.; Zhang, D.-J.; Liu, C.-Y.; Wu, Q.-S. Relationships between mycorrhizas and root hairs. *Pak. J. Bot.* **2019**, *51*, 727–733. [CrossRef]
192. Abeer, H.; Ef, A.; Alqarawi, A.A.; Mona, A. Arbuscular mycorrhizal fungi mitigates NaCl inducedects on *Solanum lycopersicum* L. *Pak. J. Bot.* **2015**, *47*, 327–340.
193. Jahromi, F.; Aroca, R.; Porcel, R.; Ruiz-Lozano, J.M. Influence of Salinity on the In Vitro Development of Glomus intraradices and on the In Vivo Physiological and Molecular Responses of Mycorrhizal Lettuce Plants. *Microb. Ecol.* **2007**, *55*, 45–53. [CrossRef]
194. Egamberdieva, D.; Jabborova, D.; Wirth, S. Alleviation of Salt Stress in Legumes by Co-inoculation with Pseudomonas and Rhizobium. In *Plant Microbe Symbiosis: Fundamentals and Advances*; Springer Science and Business Media LLC: New Delhi, India, 2013; pp. 291–303.

195. Sallaku, G.; Sandén, H.; Babaj, I.; Kaciu, S.; Balliu, A.; Rewald, B. Specific nutrient absorption rates of transplanted cucumber seedlings are highly related to RGR and influenced by grafting method, AMF inoculation and salinity. *Sci. Hortic.* **2019**, *243*, 177–188. [CrossRef]
196. Senberga, A.; Dubova, L.; Alsina, I. Germination and growth of primary roots of inoculated bean (Vicia faba) seeds under different temperatures. *Agron. Res.* **2018**, *16*, 243–253. [CrossRef]
197. Pascale, A.; Proietti, S.; Pantelides, I.S.; Stringlis, I.A. Modulation of the Root Microbiome by Plant Molecules: The Basis for Targeted Disease Suppression and Plant Growth Promotion. *Front. Plant Sci.* **2020**, *10*, 1741. [CrossRef]

horticulturae

MDPI

Article

Effects of Vermicompost Leachate versus Inorganic Fertilizer on Morphology and Microbial Traits in the Early Development Growth Stage in Mint (*Mentha spicata* L.) And Rosemary (*Rosmarinus officinalis* L.) Plants under Closed Hydroponic System

Abraham Loera-Muro [1], Enrique Troyo-Diéguez [2], Bernardo Murillo-Amador [2], Aarón Barraza [1], Goretty Caamal-Chan [1], Gregorio Lucero-Vega [3] and Alejandra Nieto-Garibay [2,*]

[1] CONACYT-Centro de Investigaciones Biológicas del Noroeste, S.C., Instituto Politécnico Nacional 195, Playa Palo de Santa Rita Sur, La Paz, BCS C.P. 23096, Mexico; aloera@cibnor.mx (A.L.-M.); abarraza@cibnor.mx (A.B.); mcaamal@cibnor.mx (G.C.-C.)

[2] Centro de Investigaciones Biológicas del Noroeste S.C., Instituto Politécnico Nacional 195, Playa Palo de Santa Rita Sur, La Paz, BCS C.P. 23096, Mexico; etroyo04@cibnor.mx (E.T.-D.); bmurillo04@cibnor.mx (B.M.-A.)

[3] Academic Department of Agronomy, Universidad Autónoma de Baja California Sur (UABCS), La Paz, BCS C.P. 23085, Mexico; g.lucero@uabcs.mx

* Correspondence: anieto04@cibnor.mx; Tel.: +52-6121317264

Citation: Loera-Muro, A.; Troyo-Diéguez, E.; Murillo-Amador, B.; Barraza, A.; Caamal-Chan, G.; Lucero-Vega, G.; Nieto-Garibay, A. Effects of Vermicompost Leachate versus Inorganic Fertilizer on Morphology and Microbial Traits in the Early Development Growth Stage in Mint (*Mentha spicata* L.) And Rosemary (*Rosmarinus officinalis* L.) Plants under Closed Hydroponic System. *Horticulturae* **2021**, *7*, 100. https://doi.org/10.3390/horticulturae7050100

Academic Editors: Nazim Gruda and Juan A. Fernández

Received: 16 April 2021
Accepted: 28 April 2021
Published: 6 May 2021

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

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

Abstract: The objective of this study was to compare the morphology of *M. spicata* and *R. officinalis* plants, and the relative abundance quantification, colony-forming units, ribotypes, and biofilm former bacteria under an inorganic fertilizer and the use of vermicompost leachate in the rhizosphere under a closed hydroponic system. In mint (*Mentha spicata*) plants treated with the vermicompost leachate, growth increase was determined mainly in root length from an average of 38 cm in plants under inorganic fertilizer to 74 cm under vermicompost leachate. In rosemary (*Rosmarinus officinalis*), no changes were determined between the two treatments. There were differences in the compositions of microbial communities: For *R. officinalis*, eight ribotypes were identified, seven for inorganic fertilizer and four for vermicompost leachate. For *M. spicata*, eight ribotypes were identified, three of them exclusive to vermicompost leachate. However, no changes were observed in microbial communities between the two treatments. Otherwise, some changes were observed in the compositions of these communities over time. In both cases, the main found phylum was Firmicutes, with 60% for *R. officinalis* and 80% for *M. spicata* represented by the *Bacillus* genus. In conclusion, the use of vermicompost leachate under the hydroponic system is a viable alternative to achieve an increase in the production of *M. spicata*, and for both plants (mint and rosemary), the quality of the product and the microbial communities that inhabited them remained unaltered.

Keywords: organic fertilizer; hydroponic; ribotypes; vermicompost leachate

1. Introduction

At present, the growing global population has put pressure on agriculture in different ways: the increase in demand for food and the need to meet this demand in an environmentally friendly manner. Although the use of chemical fertilizers has led to an enhancement in crop production, several major health- and environment-related concerns are associated with their use [1,2]. Pollution and the increase in global temperature are predicted to have negative consequences for agriculture in the coming decades [3]. Likewise, future climate-change scenarios predict a more frequent occurrence of extreme conditions [4]. In this sense, hydroponic systems have emerged as an alternative to improve yield, product quality, water management, land saving, nutrient recycling, and environmental and pathogen control. Hydroponic systems are cultivation technologies that use nutrient solutions rather

than soil substrates, and can use natural or artificial media to provide physical support to plants [5–7]. However, there is an intense debate about which hydroponic practices align or do not align with the Organic Foods Production Act (OFPA) and USDA organic regulations [8]. Furthermore, hydroponic systems are a form of soilless food production, and one of the points of conflictive in points in organic agriculture is the use of inorganic nutrition in water solutions, which many people strongly believe should not be allowed [8]. Hydroponic production has increased in recent years due to its multiple benefits. Thus, it is convenient to understand the role of microorganisms and natural sources of nutrients to improve hydroponic systems for the production of healthy food beyond reaching certification in organic agriculture. At present, the use of vermicompost leachate coupled with hydroponic systems seems to be a viable alternative. Vermicompost is the resulting product from the processing of organic waste in the digestive tract of earthworms [9,10]. This process involves the bio-oxidation and stabilization of organic compounds by the joint action of earthworms and microorganisms [11]. Consequently, the obtained vermicompost is a fertilizer with available nutrients for plants and a strong charge of beneficial bacteria [12,13]. Likewise, vermicompost is an effective technique to reduce the toxicity of waste material [14]. Vermicompost leachate is a subproduct of the vermicompost process with nutrients, microorganisms, and biologically active substances, such as fulvic acids and humic acids, and the released water during the decomposition of the organic material [15,16]. One of the positive effects of the use of vermicompost leachate is an increase in the population of plant-growth-promoting bacteria (PGPB) [17]. PGPB can promote plant growth by both direct and indirect mechanisms. Direct mechanisms include the production of auxin, ACC deaminase activity, cytokinin, gibberellin, the nitrogen fixation process, phosphorus solubilization, and the sequestration of iron by bacterial siderophores. Indirect mechanisms refer to the bacterial capability to inhibit the proliferation of plant pathogenic organisms, such as fungi and bacteria [2,18]. Most studies on hydroponic systems reported the role of indigenous bacteria and the effects of bacterial addition, and indirect bacterial mechanisms for biological pathogen control, but scarce data are available about the existence of differences between the bacteria content and plant growth when applying vermicompost leachate to a hydroponic system [13,19]. The influence of agricultural management practices on plant microbial communities is not completely clear [20]. Opportune microorganism identification in hydroponic systems which uses vermicompost leachate as a low-cost organic fertilizer is essential to select the most adequate microorganisms for an efficient pathogen biocontrol program, also to define a fertilization protocol for this system environmentally friendly and accessible to any producer [21]. Mint (*Mentha spicata* L.) and rosemary (*Rosmarinus officinalis* L.) are two plants of agronomic importance belonging to the *Lamiaceae* family [19,22], a family with many wild and cultivated officinal species, rich in essential oils and antioxidant compounds that are useful to humans [23,24]. The leaves of *M. spicata* are dried and used for tea infusions, and cultivated for the production of essential oils that are widely used in the pharmaceutical and cosmetic industries [19]. *R. officinalis*, besides its culinary uses due to its characteristic aroma, is also widely employed by indigenous populations in areas where it spontaneously grows. Rosemary extracts are used as a natural antioxidant, improving the shelf life of perishable foods [22,25]. This study assessed the effect of two types of fertilizer (inorganic versus organic fertilizer) on the growth of mint (*M. spicata*) and rosemary (*R. officinalis*) plants under a hydroponic production system, as an alternative agronomic method contributing to a reduction in pollution, water use, and fertilizer consumption, and low-cost production.

2. Materials and Methods

2.1. Study Area

The experiment was conducted in a shade-enclosure environment that served as a greenhouse facility in La Paz, located in a Bw (h') hw (e) climate, which is considered to be semiarid and sustains the xerophytic vegetation of Baja California Sur, northwest Mexico, at 7 m above sea level. Mean, maximal, and minimal temperature in the shade-enclosure

facility were 21.4, 31.8, and 8.9 °C, respectively, with a mean of 70% relative humidity. Meteorological records were obtained during the study from an automated weather station located inside the shade-enclosure facility.

2.2. Plant Cultivation Conditions and Hydroponic System

The experiment was carried out from September to November. *M. spicata* and *R. officinalis* cuttings were obtained from mother plants within their regional cultivars and were placed in pots with vermiculite until they developed enough roots to be able to absorb nutrients from fertilizers after applying the treatments. The pots were placed in 30 propylene containers of 20 L (24.5 × 16 × 10 cm (length × width × height)) filled with water. Oxygen supplementation in containers was provided with a Blogger Sweetwater pump (model SST20, 50 Hz). The water volume was maintained constant to build a closed hydroponic system; there was no recirculating water because the study was on the early vegetative stage (September to November).

2.3. Treatments and Experimental Design

The experimental design consisted of two treatments: one applying vermicompost leachate (L) and the other applying inorganic fertilizer (SS; control group) [26]. Vermicompost leachate (L) was produced at the CIBNOR experimental field according to recommendations by Gunadi et al. [27]. The vermicomposting process was carried out in 200 L containers cut in half, to which 5 holes were made in its base. Subsequently, a 5 cm thick layer of gravel and an antiaphid mesh were placed to separate the gravel from the bed where the earthworms developed. Kitchen waste and manure were used as food for the earthworms in a ratio of 1:1 volume:volume. Both kitchen waste and manure were precomposted for 21 days before being used as food for the earthworms. The feeding process was carried out using 5 cm thick layers of precomposted food every week for 12 weeks. The vermicomposting process was considered to have ended when a homogeneous material was observed without the presence of remnants of the original material. The vermicompost was separated to be laid and sheltered in a dry place and away from light for 90 days for its mineralization. Vermicompost leachate was obtained according to the methodology described by García-Galindo et al. [28], where 5 kg of vermicompost was placed in a container. Three liters of distilled water was poured into the container, and the leachate was collected. Information of the nutrient content of both inorganic fertilizer and vermicompost leachate is shown in Table 1. The experiment was established under a completely randomized design with 15 replicates for each treatment (vermicompost leachate and inorganic fertilizer). Each replicate consisted in a container before descripted with 12 pots, each pot with one plan. Treatments were applied once at five days after sowing (DAS), for inorganic fertilizer a commercial fertilizer of 17% NPK was used to prepared 10 mL that contained 0.0079, 0.000087, 0.070 (parts per million of N, P K, respectively) diluted in 40 L of top water (the capacity of pot container). For the vermicompost-leachate treatment, 140 mL that contained 0.00709, 0.000259, and 0.074 (parts per million of N, P K, respectively) was diluted in 40 L of tap water. The nutrient doses of N–P–K corresponded to the minimum established for these crops in the region to examine if any differences could be detected in microbial and morphological traits in the use of an organic versus inorganic fertilizer. Plants were analyzed in early-stage growth at 35 days after fertilizer application.

2.4. Morphological Traits and Relative-Growth Analysis

Stem length (SL, cm), fresh stem weight (FSW), dry stem weight (DSW), foliar area (FA), fresh foliar weight (FFW), dry foliar weight (DFW), root length (RL), fresh root weight (FRW), and dry root weight (DRW) were evaluated in five *M. spicata* plants and five *R. officinalis* rosemary plants before treatment application and at the end of the experiment (35 DAS). Stem and root weights (g) were obtained using an analytical scale (Mettler Toledo, AG204); for dry weights, an oven was used with forced air circulation at 70 °C (Shel-Lab®, FX-5, series 1000203) until constant weight. Data of initial and final dry weights were used

to calculate total relative growth rate (TGR), foliar growth rate (FGR), root growth rate (RGR), and stem growth rate (SGR) in grams per day, according to Hunt [29], following Formula (1):

$$TGR = ((lnDW2) \times (lnDW1))/(t2 - t1), \quad (1)$$

where DW2 and DW1 are the total plant (TGR), foliar (FGR), root (RGR) and stem (SGR) dry weight (g), recorded at times t2 (time of sampling) and t1 (beginning of the experiment), respectively. The difference (t2 − t1) is expressed in days. TGR, FGR, RGR, and SGR are expressed in g^{-1} day^{-1}.

Table 1. Solution-component analyses of nutritional source for *M. spicata* and *R. officinalis* in hydroponic system.

SS		L	
pH	5.5–6.5	pH	5.5–6
Electric conductivity (dS/m)	1.84	Electric conductivity (dS/m)	1.36
	mg L^{-1}		mg L^{-1}
Potassium nitrate	53,330	Potassium nitrate	5490.6
Ammonium nitrate	10,200	Ammonium nitrate	0.021
Monoammonium phosphate	14,800	Nitrite	0.012
Calcium nitrate	60,200	Nitrate	1.500
Magnesium sulphate	42,200	Potassium total	0.074
Ferrous sulfate	2000	Nitrogen total	1.5
Manganese Sulfate	50	Manganese Sulfate	6.38

SS: inorganic fertilizer, L: vermicompost leachate.

2.5. Photosynthetic Pigments

For *M. spicata* and *R. officinalis* plants under organic and inorganic treatments, we determined chlorophyll with seven plants (one leaf per plant) per treatment. *M. spicata* SPAD values [30,31] were recorded for 20 consecutive days after the beginning of both organic and inorganic treatments application. In *R. officinalis* plants, chlorophyll was evaluated two times: before any treatment application, and 20 days after both treatment applications. For *R. officinalis*, the chlorophyll was extracted following the acetone extraction methodology from leaf tissue, and the absorbance measure was carried out with a UV/visible spectrophotometer (model HELIOS OMEGA, Thermo Scientific, Vantaa, Finland). Chlorophyll a and b concentrations were estimated by applying the following functions [32]:

$$\text{Chlorophyll a (mg mL}^{-1}) = 11.64\ (A663) - 2.16\ (A645) \quad (2)$$

$$\text{Chlorophyll b (mg mL}^{-1}) = 20.97\ (A645) - 3.94\ (A663), \quad (3)$$

where A663 and A645 correspond for the absorbance values at wavelengths (λ) of 663 and 645 nm, respectively.

2.6. Sampling for Bacterial-Community Characterization

To determine the influence of organic and inorganic fertilizers on rhizobial microbial communities from the plant rhizosphere, samples of the root rhizosphere were taken in the hydroponic system as follows: a water sample of 50 mL with the roots (0–0.5 cm) from three different reservoirs at three times (1, 7, and 35 DAS). The collected samples were processed immediately for: (i) total DNA isolation from water (rhizosphere) samples, and (ii) bacterial isolation from *R. officinalis* and *M. spicata* root samples with the methodology that follows below. Vermicompost was free of pathogens.

2.7. Colony-Forming Units (CFU) Quantification and Isolation of Bacteria from M. spicata and R. officinalis Cultivated by Hydroponic System

The water and root samples were vorticed for 30 s. Then, 25 mL of the sample was transferred to a new tube for DNA isolation. The remaining 25 mL was used to determine

the colony-forming units (CFU). One milliliter of the remaining sample was used to perform serial dilutions in saline solution 0.85% (w/v) (from 10-2 to 10-7). Lastly, 100 µL for each dilution (from 10-2 to 10-7) was plated on nutrient agar (NA) and incubated for 24 h at 30 °C. After 24 h, the CFU count was performed.

After the CFU count, bacterial colonies were isolated on the basis of their morphology. A representative colony of the five most abundant colonial morphologies was reseeded by streak dilution in a new plate of NA and incubated at 30 °C overnight. This step was repeated until a pure isolate in each case (a single bacterial morphology per isolate) was obtained. The obtained pure isolates were stored in glycerol 30% (v/v) at −80 °C until their use.

2.8. DNA Isolation

The total DNA isolation of the water samples and bacterial isolates was carried out according to the protocol with slight modifications [33]. For water samples, 25 mL was centrifuged at 5000× *g* for 10 min, and the supernatant was discarded. For bacterial isolates, 3 mL of liquid culture was placed in nutrient broth (NB) at 30 °C overnight and centrifuged at 5000× *g* for 5 min, and the supernatant was discarded. Both the pellet from water samples and the bacterial isolate pellets were processed in the same way. The resulting pellet was resuspended in 1 mL of a lysis buffer (15% sucrose, 0.3 mg/mL lysozyme, 0.05 M EDTA and 1 M Tris, pH 8) and incubated for 30 min at 37 °C. Then, 100 µL of 10% SDS (w/v), 100 µL of 5 M NaCl, and 5 µL of proteinase K (0.4 mg/mL) were added and incubated under agitation for 1 h at 50 °C. After incubation, 200 µL of phenol–chloroform–isoamyl alcohol (25:24:1) was added to 500 µL of the solution, briefly vorticed, and then centrifuged at 12,000× *g* for 5 min. The aqueous phase was recovered, and 200 µL of ammonium acetate (7.5 M) and 500 µL (1 volume) of absolute ethanol were added to be mixed by inversion and precipitate at 4 °C overnight to centrifuge at 4 °C at 12,000× *g* for 15 min. The supernatant was discarded, and the pellet was washed twice with 100 µL of ethanol 70% (v/v). The DNA was dried at room temperature, resuspended in molecular-biology-grade water, and stored at −20 °C until use.

2.9. Relative-Abundance Quantification by qPCR

The relative abundance of the bacterial population was assessed through qPCR to determine the effect of treatments. The qPCR was performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) according to the instructions of the iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA). The relative abundance of the total bacteria in the rhizosphere samples for each treatment was assessed according to the methodology described by López-Gutiérrez et al. [33] with slight modifications.

2.10. Characterization of Bacterial Communities by Ribotype Assay Analysis (16S rRNA Gene)

Ribotype assay analysis was conducted according to the Bogino et al. [34] methodology. A total DNA of 36 water samples (3 samples × 3 times × 2 treatments × 2 species of plants = 36 samples in total) and 60 bacterial isolate strains (30 isolate strains for each plant for both organic and inorganic fertilization treatments) were characterized by amplified ribosomal DNA restriction analysis (ARDRA). Bacterial genomic DNA was extracted from each isolate as mentioned previously. For 16S rRNA gene amplification, we used primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′- AAGGAGGTGATCCAGCC-3′). PCR amplification products (~1500 bp) were processed by a restriction endonuclease assay with HaeIII (Thermo Fisher Scientific), and the resulting fragments were electrophoretically separated on a 2% (w/v) agarose gel, stained with ethidium bromide to visualize them with UV radiation, and the corresponding image was photographed. Ribotype identification is directly associated with a specific restriction fragment fingerprint. The community structure dendrogram was constructed on the basis of ribotypes of the bacterial isolates with GelCompar II software. Bacterial isolate strains belonging to either unique majority ribotypes or common ribotypes were selected for further identification through 16S rRNA

gene nucleotide sequence analysis with primers COM 1 (5′-CAGCAGCCGCGGTAATAC-3′) and COM 2 (5′-CCGTCAATTCCTTTGAGTTT-3′) with the methodology described by Stach et al. [35]. The 16S rRNA gene sequences were analyzed using the BLAST (blastn) search program (National Center for Biotechnology Information (NCBI)).

2.11. Biofilm-Formation Assay

Biofilms are microbial communities that adhere to surfaces and are enclosed in a protective matrix; this is also the primary structure from which bacteria interact with plants and other eukaryotes. Thus, to characterize the bacterial capability of the rhizosphere (water samples) isolate strains from *M. spicata* and *R. officinalis* to form biofilms, we carried out the crystal violet (CV) staining quantitative assay of Labrie et al. [36] with slight modifications. CV staining absorbance was measured at 590 nm using a spectrophotometer (Multiskan Spectrum, Thermo Scientific, Wilmington, DE, USA).

2.12. Statistical Analysis

Data were analyzed using univariate and multivariate analysis of variance (ANOVA and MANOVA) for one-way classification, and the nutrition source was the study factor. For chlorophyll content, multiple analysis of variance (MANOVA) and significant differences between means for each recorded date were determined by two-way analysis of variance (ANOVA). Least significant differences (LSD) in Tukey's HSD test ($p = 0.05$) were estimated for one-way ANOVA. For all cases, significant differences between means were considered to be significant at $p < 0.05$. All statistical analyses were performed with Statistica software program v10.0 and GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Plant Morphology and Photosynthetic Pigments

3.1.1. *M. spicata*

Stem height (SL), dry foliar weight (DFW), fresh foliar weight (FFW), foliar area (FA), and root length (RL) showed a significant increase in the vermicompost leachate treatment compared with the inorganic treatment for *M. spicata* (Table 2). There was no difference between the vermicompost leachate treatment and the inorganic treatment for relative growth rates of leaves (FGR), stems (RGS), total growth rate (TGR), and roots (RGR), which was lower for vermicompost leachate than inorganic fertilizer was (Table 3). Chlorophyll a and b, and total content did not show any differences between plants with vermicompost leachate or inorganic treatment (Table 4 and Figure 1).

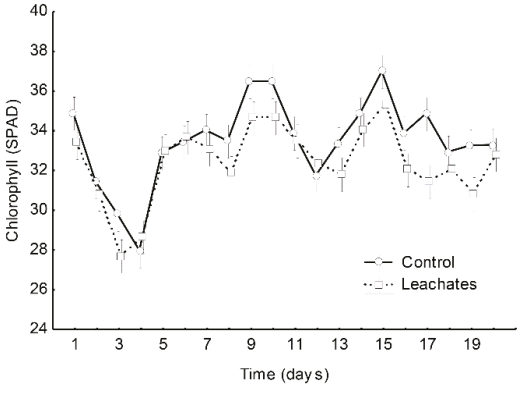

Figure 1. Chlorophyll SPAD readings in *M. spicata* plants under leachates of inorganic and vermicompost leachate fertilizers. Vertical bars represented mean ± standard error.

Table 2. Morphometric parameters in *M. spicata* and *R. officinalis* plants under fertilization treatments.

		SL (cm)	FSW (g)	DSW (g)	FA (cm^2)	FFW (g)	DFW (g)	RL (cm)	FRW (g)	DRW (g)
M. spicata	SS	11.8 ± 0.5 b	1.6 ± 0.5 a	0.4 ± 0.1 a	123 ± 51 b	4 ± 0.3 b	1 ± 0.1 b	38 ± 5 b	10 ± 2 a	1.5 ± 0.3 a
	L	14.7 ± 0.7 a	3 ± 0.5 a	0.6 ± 0.1 a	246 ± 21 a	7 ± 0.5 a	2 ± 0.2 a	54 ± 7 a	12 ± 2 a	1.8 ± 0.3 a
R. officinalis	SS	4.5 ± 0.5 a	4.5 ± 1 a	3.5 ± 0.6 a	32.7 ± 7 a	7.9 ± 1.4 a	4.6 ± 1 a	8.2 ± 0.6 a	10 ± 1 a	4 ± 0.6 a
	L	5.2 ± 0.4 a	4.9 ± 0.2 a	3.4 ± 0.1 a	33.5 ± 2 a	8 ± 0.8 a	4.5 ± 0.1 a	8.8 ± 0.5 a	11 ± 0.7 a	4.3 ± 0.2 a

SS: inorganic fertilizer, L: vermicompost leachate, SL: stem length, FSW: fresh stem weight, DSW: dry stem weight, FA: foliar area, FFW: fresh foliar weight, DFW: dry foliar weight, RL: root length, FRW: fresh root weight, DRW: dry root weight. Data represent means ± standard error (n = 3). *M. spicata* and *R. officinalis* data were treated as independent ANOVA analyses. Different letters for each column denote statistical difference.

Table 3. Total growth rate (TGR), foliar growth rate (FGR), root growth rate (RGR), and stem growth rate (SGR) expressed in grams per day of *M. spicata* and *R. officinalis* plants.

		TGR	FGR	RGR	RGS
M. spicata	SS	0.03 ± 0.00 a	0.023 ± 0.01 a	0.057 ± 0.02 a	0.013 ± 0.0 a
	L	0.02 ± 0.00 a	0.021 ± 0.01 a	0.048 ± 0.01 a	0.015 ± 0.0 a
R. officinalis	SS	0.0339 ± 0.008 a	0.0218 ± 0.01 a	0.0424 ± 0.001 a	0.034 ± 0.01 a
	L	0.0239 ± 0.007 a	0.0206 ± 0.01 a	0.0282 ± 0.001 b	0.022 ± 0.00 a

SS: inorganic fertilizer, L: organic fertilizer (vermicompost leachate). Data represent means ± standard deviation (n = 5). Different letters denote statistical differences.

Table 4. Chlorophyll (Chl) a and b, and total ($mg \cdot mL^{-1}$) content in *M. spicata* and *R. officinalis* plants under different nutrient sources in two times before (BT) and after (AT) application of vermicompost leachate and inorganic treatments.

	Treatment	Date *	Chl a	Chl b	Chl Tot
M. spicata	SS	BT b	60 ± 11 a	5 ± 0.4 a	86 ± 16 a
	L	AT a	67 ± 10 a	5.4 ± 0.6 a	97 ± 17 a
R. officinalis	SS	BT b	63 ± 9 a	4.9 ± 0.4 a	87 ± 13 a
	L	AT a	74 ± 10 a	5.5 ± 0.4 a	105 ± 15 a

SS: inorganic fertilizer, L: organic fertilizer (vermicompost leachate). Data represent means ± standard deviation (n = 5). Different letters denote statistical differences. * Denote statistical differences between sampling dates.

3.1.2. *R. officinalis*

For all morphological traits, there were no differences between the vermicompost leachate and inorganic treatments (Tables 2 and 3) except for rosemary under treatment with leachate in RGR, which showed lower growth (Table 3). Organic treatment did not affect chlorophyll a and b, and total content did not undergo alterations in either organic or inorganic treatment, and the only variable that exerted an effect was the time (date) of chlorophyll sampling (Table 4).

3.2. CFU Quantification and Relative Abundance of Bacterial Communities

The relative abundance of total bacterial communities due to the effect of treatments was assessed by CFU estimation and by a qPCR-based assay. For both *M. spicata* and *R. officinalis*, no differences were determined between the vermicompost leachate and inorganic treatments regarding the abundance of bacterial populations; however, an increase in relative abundance in time was more evident for the vermicompost leachate (Figure 2).

Bacterial community structure kinetics between both vermicompost leachate and inorganic treatments was analyzed. Thirty-six total DNA water samples were analyzed by amplified ribosomal DNA restriction analysis (ARDRA). As this test showed for *M. spicata* and *R. officinalis*, bacterial community structures underwent changes through time without a significant effect between treatments (Figure 3a,b). Thus, these results highlight the feasibility of replacing inorganic fertilizer with the vermicompost leachate without significant impact on the bacterial abundance or bacterial community structures of *M. spicata* and *R. officinalis* in hydroponic systems.

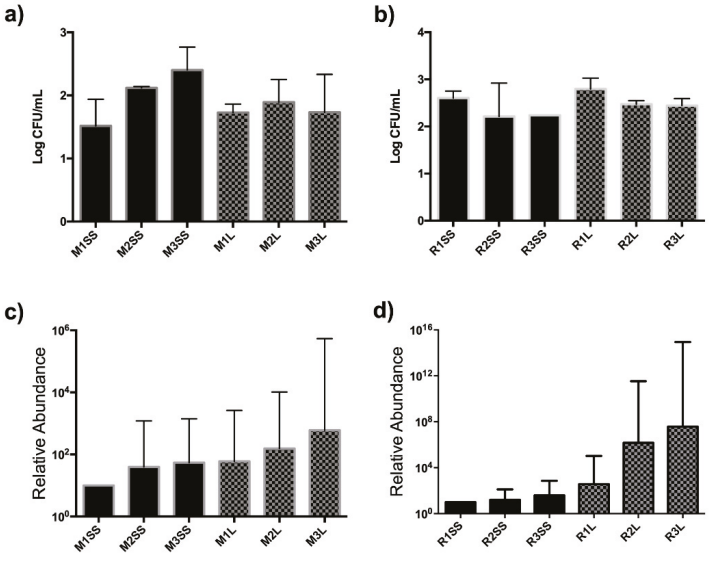

Figure 2. Colony-forming unit (CFU) quantification and relative abundance (qPCR) of bacterial communities in *M. spicata* and *R. officinalis*. CFU quantification in (**a**) *M. spicata* and (**b**) *R. officinalis*; relative abundance (qPCR) of bacterial communities in (**c**) *M. spicata* and (**d**) *R. officinalis* (M1SS: mint composed sample, time 1, inorganic fertilizer; M2SS: *M. spicata* composed sample, time 2, inorganic fertilizer; M3SS: *M. spicata* composed sample, time 3, inorganic fertilizer; M1L: *M. spicata* composed sample, time 1, vermicompost leachate; M2L: *M. spicata* composed sample, time 2, vermicompost leachate; M3L: *M. spicata* composed sample, time 3, vermicompost leachate; R1SS: *R. officinalis* composed sample, time 1, inorganic fertilizer; R2SS: *R. officinalis* composed sample, time 2, inorganic fertilizer; R3SS: *R. officinalis* composed sample, time 3, inorganic fertilizer; R1L: *R. officinalis* composed sample, time 1, vermicompost leachate; R2L: *R. officinalis* composed sample, time 2, vermicompost leachate; R3L: *R. officinalis* composed sample, time 3, vermicompost leachate.

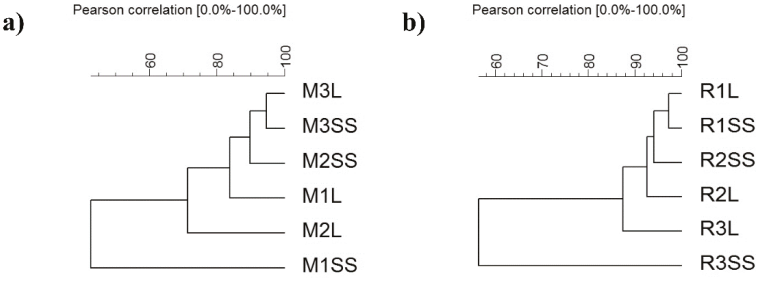

Figure 3. Dendrogram of general distribution of bacterial composition of communities between treatments in (**a**) *M. spicata* and (**b**) *R. officinalis* (M1SS: *M. spicata* composed sample, time 1, inorganic fertilizer; M2SS: *M. spicata* composed sample, time 2, inorganic fertilizer; M3SS: *M. spicata* composed sample, time 3, inorganic fertilizer; M1L: *M. spicata* composed sample, time 1, vermicompost leachate; M2L: *M. spicata* composed sample, time 2, vermicompost leachate; M3L: *M. spicata* composed sample, time 3, vermicompost leachate; R1SS: *R. officinalis* composed sample, time 1, inorganic fertilizer; R2SS: *R. officinalis* composed sample, time 2, inorganic fertilizer; R3SS: *R. officinalis* composed sample, time 3, inorganic fertilizer; R1L: *R. officinalis* composed sample, time 1, vermicompost leachate; R2L: *R. officinalis* composed sample, time 2, vermicompost leachate; R3L: *R. officinalis* composed sample, time 3, vermicompost leachate).

3.3. Composition and Diversity of Bacterial Communities

A total of 60 bacterial isolate strains (30 isolate strains for each plant for both vermicompost leachate and inorganic fertilization treatments) were characterized by ARDRA. From ARDRA, 15 ribotypes were identified in *M. spicata* and *R. officinalis* according to the yielded fingerprint after the restriction assay with the HaeIII restriction enzyme (Table 5). In the case of *R. officinalis*, eight different ribotypes were identified (Figure 4). Of these eight ribotypes, seven were present in inorganic treatment, and four in the vermicompost leachate. Of the ribotypes present in the inorganic treatment, four were exclusively present in this treatment, while only one ribotype was exclusive of the vermicompost leachate. In the case of *M. spicata*, there were also eight different ribotypes for both the vermicompost leachate and the inorganic treatment. For the inorganic treatment, there were five ribotypes, and none was exclusive to this treatment. For the vermicompost leachate treatment, eight ribotypes were present, and three ribotypes were exclusive of this treatment. However, it was not possible to characterize the ribotype to which three bacterial isolates from *M. spicata* belonged (two from inorganic treatment and one from organic treatment).

Representative bacterial strains were identified by 16S rRNA gene sequencing. Bacterial isolate strains were selected according to ribotype ARDRA profiles (Table 6). Most bacterial isolate strains belonged to the Firmicutes phylum, which was mainly composed of the Bacilli class, the Bacillaceae family, and the *Bacillus* genus. Bacterial isolate strains belonging to Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria classes from the Proteobacteria phylum were found (Table 6).

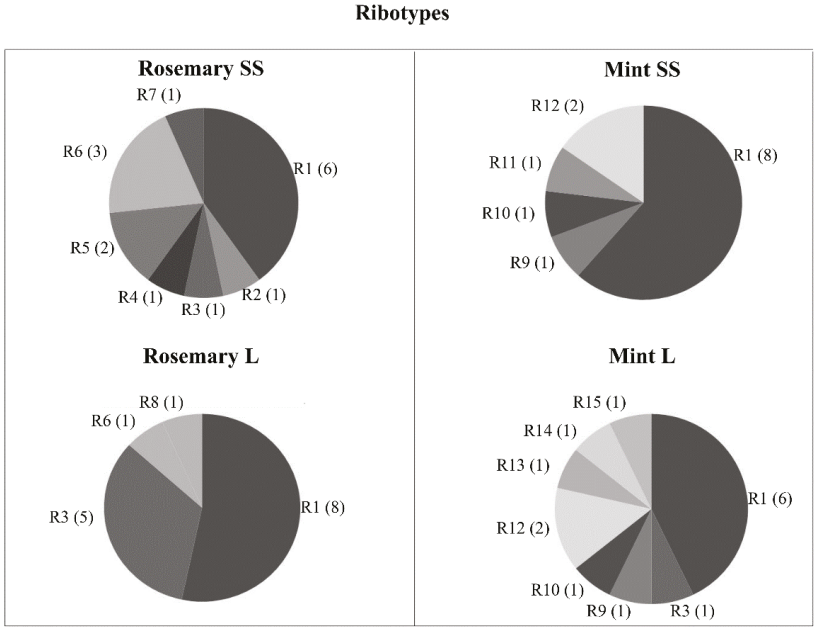

Figure 4. Ribotypes present in *M. spicata* and *R. officinalis* obtained by amplified ribosomal DNA restriction analysis (ARDRA; R: ribotype, number: number of ribotypes, and number in parentheses: number of isolates corresponding to each ribotype).

Table 5. Ribotypes of bacteria isolated from hydroponic system in *M. spicata* and *R. officinalis* plants.

Ribotype	Isolates from *M. spicata*		Isolates from *R. officinalis*	
	SS	L	SS	L
1	MSS1, MSS5, MSS10, MSSR1, MSSR4, MSS2, MSS6, MSSR5	ML6, ML7, ML8, ML10, MLR3, MLR5	RSS1, RSS5, RSS7, RSS8, RSS9, RSSR1	RL4, RL5, RL6, RL7, RL8, RL9, RL10, RLR2
2			RSS2	
3		MLR4	RSS3	RL2, RLR1, RLR3, RLR4, RLR5
4			RSS4	
5			RSS6, RSS10	
6			RSSR2, RSSR3, RSSR4	RL3
7			RSSR5	
8				RL1
9	MSS3	ML1		
10	MSS4	MLR2		
11	MSS7			
12	MSS8, MSS9	ML2, ML4		
13		ML5		
14		ML9		
15		MLR1		

SS: inorganic fertilizer, L: vermicompost leachate, MSS-number or MSSR-number: isolates from *M. spicata* inorganic fertilizer, ML-number or MLR-number: isolates from *M. spicata* vermicompost leachate, RSS-number or RSSR-number: isolates from *R. officinalis* inorganic fertilizer, and RL-number or RLR-number: isolates from *R. officinalis* vermicompost leachate. Note: MSSR2, MSSR3, and ML3 are missing from the table because they were unclassified.

Table 6. Identities of bacterial strains isolated from hydroponic system in *M. spicata* and *R. officinalis* plants.

Isolated	Rt	Most Closely Related Sequence (Accession Number) (Id %)	Phylogenetic Affiliation
RSS-1	1	*Bacillus koreensis* (NR_043084.1) (98)	*Firmicutes*
RSS-5	1	*Bacillus aryabhattai* (NR_118442.1) (99)	*Firmicutes*
MSS-2	1	*Bacillus aryabhattai* (NR_118442.1) (99)	*Firmicutes*
ML-6	1	*Bacillus vietnamensis* (NR_113995.1) (98)	*Firmicutes*
RSS-2	2	*Enterobacter cloacae* (NR_118568.1) (99)	*Gammaproteobacteria*
RSS-3	3	*Herbaspirillum chlorophenolicum* (NR_114143.1) (99)	*Betaproteobacteria*
RSS-4	4	*Bacillus pseudomycoides* (NR_114422.1) (99)	*Firmicutes*
RSS-6	5	*Bacillus subtilis* (NR_102783.1) (99)	*Firmicutes*
RSSR-2	6	*Novosphingobium pokkalii* (NR_149820.1) (94)	*Alphaproteobacteria*
RSSR-5	7	*Lysinibacillus tabacifolii* (NR_132691.1) (99)	*Firmicutes*
RL-1	8	*Novosphingobium capsulatum* (NR_113.591.1) (99)	*Alphaproteobacteria*
ML-1	9	*Bacillus paralicheniformis* (NR_137421.1) (99)	*Firmicutes*
MSS-4	10	*Pseudomonas entomophila* (NR_102854.1) (99)	*Gammaproteobacteria*
MLR-2	10	*Pseudomonas entomophila* (NR_102854.1) (99)	*Gammaproteobacteria*
MSS-7	11	*Brevibacterium frigoritolerans* (NR_117474.1) (99)	*Firmicutes*
MSS-8	12	*Staphylococcus petrasii* (NR_136463.1) (99)	*Firmicutes*
MSS-9	12	*Staphylococcus petrasii* (NR_136463.1) (99)	*Firmicutes*
ML-5	13	*Bacillus oceanisediminis* (NR_118440.1) (98)	*Firmicutes*
ML-9	14	*Bacillus flexus* (NR_118382.1) (99)	*Firmicutes*
MLR-1	15	*Bacillus toyonensis* (NR_121761.1) (98)	*Firmicutes*

Rt: ribotype.

Ribotypes found in rosemary bacterial isolate strains belonged to Firmicutes (60%), mainly composed of the *Bacillus* genus. Comparing the vermicompost leachate and inorganic treatments, we determined that the Firmicutes phylum was the most abundant between treatments, and the Alphaproteobacteria and Betaproteobacteria classes, and Gammaproteobacteria showed greater abundance in inorganic treatment than in the vermicompost leachate treatment (Figure 4, Table 6). The ribotypes found in *M. spicata* bacterial isolate strains belonged to Firmicutes (80% and were mainly composed of the *Bacillus* genus. Interestingly, 10% of the bacterial isolate strains were unclassified. Comparing the vermicompost leachate and inorganic treatments, the most abundant phylum was Firmicutes, followed by the Gammaproteobacteria class (Tables 5 and 6). For the vermicompost leachate, the Betaproteobacteria class showed greater abundance in the vermicompost

leachate treatment than in inorganic treatment (Tables 5 and 6). Therefore, the Firmicutes phylum was the most abundant in both *R. officinalis* and *M. spicata* plants, and in both the vermicompost leachate and the inorganic treatment.

3.4. Biofilm-Forming Ability of Bacterial Communities

All bacterial isolate strains from *R. officinalis* (30 isolates) and *M. spicata* (30 isolates) were assessed for adhesion and biofilm-establishment capability with a CV assay. The CV assay showed that all bacterial isolates were able to adhere to the surface and establish biofilms (Figure 5). Differences were found in biofilm formation that were categorized according to the capability to retain CV measured by the OD at 595 nm (CV-OD595) [28], for all bacterial isolate strains as follows: weak (<0.6), moderate (0.6–1.2), and strong (>1.2). *R. officinalis* bacterial isolate strains with the vermicompost leachate treatment showed that 3 bacterial isolates formed a moderate biofilm, 2 a strong biofilm, and the remaining 10 a weak biofilm. For the bacterial isolate strains from the inorganic treatment, 4 bacterial isolates formed a moderate biofilm, 1 a strong biofilm, and the remaining 10 a weak biofilm. The *M. spicata* bacterial isolate strains with the vermicompost leachate treatment showed that 1 bacterial isolate formed a strong biofilm, 2 a moderate biofilm, and the remaining 12 formed a weak biofilm. For the inorganic treatment, 2 bacterial isolates were able to form a strong biofilm, 1 a moderate biofilm, and the remaining 12 a weak biofilm. Altogether, for the *R. officinalis* and *M. spicata* plants and both the vermicompost leachate and the inorganic treatment, most bacterial isolates were able to form weak biofilms in the conditions assessed in this study.

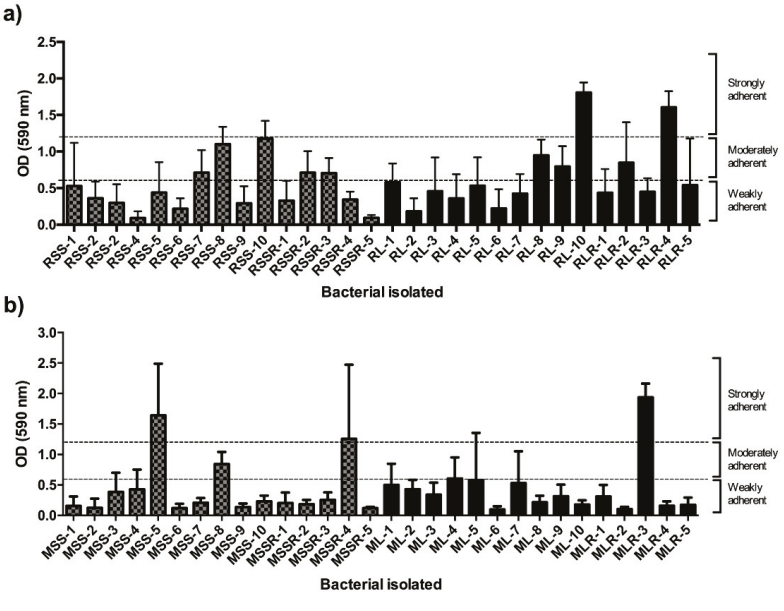

Figure 5. Biofilm formation quantified by staining with crystal violet of isolates from (**a**) *R. officinalis* and (**b**) *M. spicata* (RSS-number or RSSR-number: isolates from *R. officinalis* inorganic fertilizer, RL-number or RLR-number: isolates from *R. officinalis* vermicompost leachate, MSS-number or MSSR-number: isolates from *M. spicata* inorganic fertilizer, and ML-number or MLR-number: isolates from *M. spicata* vermicompost leachate.

4. Discussion

The vermicompost leachate treatment for both *M. spicata* (mint) and *R. officinalis* (rosemary) plants did not affect their growth; even for *M. spicata* plants, we were able

to determine a growth increase for several morphometric parameters. Moreover, for *R. officinalis* plant growth, for all morphometric parameters, there were only differences for root growth, which was lower for vermicompost than for inorganic leachate; similar results were found by Peng et al. [37]. This is important since the aim of healthy food production is avoiding the application of inorganic fertilizer [25,38–41]. Furthermore, vermicompost leachate contains a high amount of plant hormones, such as auxins, gibberellins, and cytokinins from microbial origin, giving rise to plant-growth enhancement, and acting as a liquid fertilizer [15,42–45]. Emperor and Kumar [45] determined that organic matter processed in the earthworm gut and then excreted as vermicast undergoes an increased level of microbial population, microbial respiration, microbial enzyme activity, and N, P, and K enrichment, bacterial exopolysaccharide production, lignocellulolytic activity establishment, nitrifying, and nitrogen-fixing microorganism proliferation. The above allow for us to conclude that the use of vermicompost to replace inorganic fertilizers is a viable option under the use of hydroponic systems [43,46–49].

The bacterial communities' relative abundance showed no differences between the vermicompost leachate and inorganic treatments for both *R. officinalis* and *M. spicata* plants, showing time-related differences, as expected, in accordance with previous works, where the analyzed bacterial communities underwent the same behavior [50,51]. The bacterial-community structure for the *R. officinalis* and *M. spicata* plants and for both treatment types were mainly composed by the Firmicutes phylum, followed by the Proteobacteria phylum, which was represented by the Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria classes; we were also able to determine the presence of beneficial bacteria from the *Bacillus* (Firmicutes phylum) and *Pseudomonas* (Proteobacteria phylum) genera. Those bacteria are designated as beneficial or plant-growth-promoting (PGPB), and the characterization of the bacterial-community structures of the rhizosphere for other plant members (*Thymus vulgaris*, *T. citriodorus*, *T. zygis*, *Santolina chamaecyparissus*, *Lavandula dentata*, and *Salvia miltiorrhiza*) of the Lamiaceae family showed that Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Acidobacteria, and Gemmatimonadetes were among the most abundant bacterial phyla [5,52–56].

Lastly, the capability to establish biofilms was assessed for all 60 bacterial isolate strains from the *M. spicata* and *R. officinalis* plants and both treatments, with no differences highlighting the essential role of biofilm development in bacterial survival and physiology [36]. We determined that most of the isolates (66.67% in *R. officinalis* and 80% in *M. spicata*) had weak capacity (CV-OD595) to form a biofilm; a smaller proportion were able to produce a strong biofilm for both *M* plants and both treatments. In an aqueous environment, such as a hydroponic system, biofilm establishment follows other mechanisms that are not yet characterized. Authors should discuss the results and how they can be interpreted from the perspective of previous studies and working hypotheses. The findings and their implications should be discussed in the broadest context possible. Future research directions may also be highlighted.

5. Conclusions

In this study, we showed that the substitution of inorganic fertilizer by vermicompost leachate in a hydroponic system allows for us to maintain or increase the production of two crop plants with agricultural importance (*M. spicata* (mint), and *R. officinalis* (rosemary)). Furthermore, we determined that this fertilizer substitution modifies neither the bacterial communities for both plants nor their ability to form biofilms. Through time, the vermicompost leachate tendency showed an increase in relative abundance, which is important to consider for future studies. Therefore, we propose the use of vermicompost leachate fertilizer as a feasible replacement for inorganic fertilizer in hydroponic systems to achieve sustainable and ecofriendly agricultural production, in agreement with our results and recent research conducted on open-field cultures, to face the challenge of a growing population and pollution derived from the use of inorganic fertilizers.

Author Contributions: A.L.-M.: conceptualization, methodology, formal analysis, investigation, writing—original draft. A.B.: writing—review and editing. G.C.-C.: writing—review and editing. E.T.-D.: supervision and writing-review. B.M.-A.: Funding acquisition. A.N.-G.: conceptualization, formal analysis, writing—review and editing, funding acquisition, project administration. G.L.-V.: methodology. All authors have read and agreed to the published version of the manuscript.

Funding: The current investigation was supported by CONACYT/Mexico through the Ciencia Básica SEP-CONACYT grant No. 236240, and funds provided to Centro de Investigaciones Biologicas del Noroeste S.C. (CIBNOR) and Consejo Sudcalifornio de Ciencia y Tecnología grant 20403.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available for transparency at www.cibnor.gob.mx (anieto04@cibnor.mx, aloera04@cibnor.mx) (accessed on 27 April 2021).

Acknowledgments: Current investigations from the group are supported by CONACYT/México. We thank the technical assistance to all the team of the experimental field of the Agriculture Program in Arid Zones, Saúl Edel Briseño Ruiz, Pedro Luna García, Adrián Jordán Castro, and Raymundo Ceseña Nuñez. We also thank the technical assistance of Lidia Hirales-Lucero of Fitotecnia Laboratory and Angel Edgardo Carrillo García of Microbial Molecular Ecology Laboratory.

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

References

1. Gupta, R.; Bisaria, V.S.; Sharma, S. Effect of agricultural amendments on *Cajanus cajan* (*Pigeon pea*) and its rhizospheric microbial communities—A comparison between chemical fertilizers and bioinoculants. *PLoS ONE* **2015**, *10*, e0132770. [CrossRef]
2. Hartman, K.; Tringe, S.G. Interactions between plants and soil shaping the root microbiome under abiotic stress. *Biochem. J.* **2019**, *476*, 2705–2724. [CrossRef] [PubMed]
3. Wang, G.; Kong, F.; Zhang, S.; Meng, X.; Wang, Y.; Meng, Q. A tomato chloroplast-targeted DnaJ protein protects Rubisco activity under heat stress. *J. Exp. Bot.* **2015**, *66*, 3027–3040. [CrossRef]
4. Gruda, N.S. Increasing sustainability of growing media constituents and stand-alone substrates in soilless culture systems. *Agronomy* **2019**, *9*, 298. [CrossRef]
5. Lee, S.; Lee, J. Beneficial bacteria and fungi in hydroponic systems: Types and characteristics of hydroponic food production methods. *Sci. Hortic.* **2015**, *195*, 206–215. [CrossRef]
6. Savvas, D.; Gruda, N. Application of soilless culture technologies in the modern greenhouse industry—A review. *Eur. J. Hortic. Sci.* **2018**, *5*, 280–293. [CrossRef]
7. Gruda, N. *Advances in Horticultural Soilless Culture*; Burleigh Dodds Science Publishing Limited: Cambridge, UK, 2021; 442p, ISBN 9781786764355.
8. NOSB. *Hydroponic and Aquaponic Task Force Report*; National Organic Standards Board (NOSB): Washington, DC, USA, 2016; 75p.
9. Kiyasudeen, K.S.; Ibrahim, M.H.; Quaik, S.; Ismail, S.A. *Prospects of Organic Waste Management, and the Significance of Earthworms*; Springer: Berlin/Heidelberg, Germany, 2016; 254p, pp. 201–230, Chapter 9 Vermicompost, Its Applications and Derivatives.
10. Garg, V.K.; Suthar, S.; Yadav, A. Management of food industry waste employing vermicomposting technology. *Bioresour. Technol.* **2012**, *126*, 437–443. [CrossRef]
11. Hanc, A.; Boucek, J.; Svehla, P.; Dreslova, M.; Tlustos, P. Properties of vermicompost aqueous extracts prepared under different conditions. *Environ. Technol.* **2017**, *38*, 1428–1434. [CrossRef]
12. Grantina-Ievina, L.; Andersone, U.; Berkolde-Pīre, D.; Nikolajeva, V.; Ievinsh, G. Critical tests for determination of microbiological quality and biological activity in commercial vermicompost samples of different origins. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 10541–10554. [CrossRef]
13. Zhang, H.; Tan, S.N.; Wong, W.S.; Ng, C.Y.L.; Teo, C.H.; Ge, L.; Chen, X.; Yong, J.W.H. Mass spectrometric evidence for the occurrence of plant growth promoting cytokinins in vermicompost tea. *Biol. Fertil. Soils* **2014**, *50*, 401–403. [CrossRef]
14. Wang, L.M.; Zhang, Y.M.; Lian, J.J.; Chao, J.Y.; Gao, Y.X.; Yang, F.; Zhang, L.Y. Impact of fly ash and phosphatic rock on metal stabilization and bioavailability during sewage sludge vermicomposting. *Bioresour. Technol.* **2013**, *136*, 281–287. [CrossRef]
15. Gutiérrez-Miceli, F.A.; García-Gómez, R.C.; Rincón, R.R.; Abud-Archila, M.; Oliva, L.M.A.; Guillen, M.J.; Dendooven, L. Formulation of a liquid fertilizer for sorghum (*Sorghum bicolor* (L.) Moench) using vermicompost leachate. *Bioresour. Technol.* **2008**, *99*, 6174–6180. [CrossRef] [PubMed]
16. Churilova, E.V.; Midmore, D.J. Vermiliquer (Vermicompost Leachate) as a Complete Liquid Fertilizer for Hydroponically-Grown Pak Choi (*Brassica chinensis* L.) in the Tropics. *Horticulturae* **2019**, *5*, 26. [CrossRef]

17. Pathma, J.; Sakthivel, N. Microbial diversity of vermicompost bacteria that exhibit useful agricultural traits and waste management potential. *Springerplus* **2012**, *1*, 26. [CrossRef] [PubMed]
18. Olanrewaju, O.S.; Glick, B.R.; Babalola, O.O. Mechanisms of action of plant growth promoting bacteria. *World J. Microbiol. Biotechnol.* **2017**, *33*, 197. [CrossRef]
19. Karkanis, A.; Lykas, C.; Liava, V.; Bezou, A.; Petropoulos, S.; Tsiropoulos, N. Weed interference with peppermint (*Mentha* x *piperita* L.) and spearmint (*Mentha spicata* L.) crops under different herbicide treatments: Effects on biomass and essential oil yield. *J. Sci. Food Agric.* **2018**, *98*, 43–50. [CrossRef]
20. Trivedi, P.; Leach, J.; Tringe, S.; Sa, T.; Singh, B.K. Plant-microbiome interactions: From community assembly to plant health. *Nat. Rev. Microbiol.* **2020**, *18*, 607–621. [CrossRef]
21. Koohakan, P.; Ikeda, H.; Jeanaksorn, T.; Tojo, M.; Kusakari, S.I.; Okada, K.; Sato, S. Evaluation of the indigenous microorganisms in soilless culture: Occurrence and quantitative characteristics in the different growing systems. *Sci. Hortic.* **2004**, *101*, 179–188. [CrossRef]
22. Andrade, J.M.; Faustino, C.; Garcia, C.; Ladeiras, D.; Reis, C.P.; Rijo, P. *Rosmarinus officinalis* L.: An update review of its phytochemistry and biological activity. *Future Sci. OA* **2018**, *4*, FSO283. [CrossRef] [PubMed]
23. Aghaei, Y.; Hossein-Mirjalili, M.; Nazeri, V. Chemical diversity among the essential oils of wild populations of Stachys lavandulifolia Vahl (Lamiaceae) from Iran. *Chem. Biodivers.* **2013**, *10*, 262–273. [CrossRef]
24. Perrino, E.V.; Valerio, F.; Gannouchi, A.; Trani, A.; Mezzapesa, G. Ecological and plant community implication on essential oils composition in useful wild Officinal species: A pilot case study in Apulia (Italy). *Plants* **2021**, *10*, 574. [CrossRef] [PubMed]
25. García-Caparrós, P.; Llanderal, A.; Rodríguez, J.C.; Maksimovic, I.; Urrestarazu, M.; Lao, M. Rosemary growth and nutrient balance: Leachate fertigation with leachates versus conventional fertigation. *Sci. Hortic.* **2018**, *242*, 62–68. [CrossRef]
26. Samperio, R.G. *Hidroponía Básica*; Editorial Diana: Ciudad de México, Mexico, 1997; 176p.
27. Gunadi, B.; Blount, C.; Edwards, C.A. The growth and fecundity of *Eisenia fetida* (Savigny) in cattle solids pre-composted for different periods. *Pedobiologia* **2002**, *46*, 15–23. [CrossRef]
28. García-Galindo, E.; Nieto-Garibay, A.; Troyo-Diéguez, E.; Lucero-Vega, G.; Murillo-Amador, B.; Ruiz-Espinoza, F.H.; Fraga-Palomino, H.C. Germination of *Salicornia bigelovii* (Torr.) under Shrimp Culture Effluents and the Application of Vermicompost Leachate for Mitigating Salt Stress. *Agronomy* **2021**, *11*, 424. [CrossRef]
29. Hunt, R. *Plant Growth Analysis. The Institute of Biology´s Studies in Biology*; Edward Arnold: London, UK, 1978; No 96.
30. Yang, D.Q.; Dong, W.H.; Luo, Y.L.; Song, W.T.; Cai, T.; Li, Y.; Yin, Y.P.; Wang, Z.L. Effects of nitrogen application and supplemental irrigation on canopy temperature and photosynthetic characteristics in winter wheat. *J. Agric. Sci.* **2018**, *156*, 13–23. [CrossRef]
31. Strain, H.H.; Svec, W.A. Extraction, separation, estimation, and isolation of the chlorophylls. In *The Chlorophylls*; Vernon, L.P., Seely, G.R., Eds.; Academic Press: London, UK, 1966; pp. 21–66.
32. Sambrook, J.; Russell, D.W. *Molecular Cloning a Laboratory Manual*, 3rd ed.; Science Press: Beijing, China, 2002; pp. 461–471.
33. López-Gutiérrez, J.C.; Henry, S.; Hallet, S.; Martin-Laurent, F.; Catroux, G.; Philippot, L. Quantification of a novel group of nitrate-reducing bacteria in the environment by real-time PCR. *J. Microbiol. Methods* **2004**, *57*, 399–407. [CrossRef] [PubMed]
34. Bogino, P.; Abod, A.; Nievas, F.; Giordano, W. Water-limiting conditions alter the structure and biofilm-forming ability of bacterial multispecies communities in the alfalfa rhizosphere. *PLoS ONE* **2013**, *8*, e79614. [CrossRef] [PubMed]
35. Stach, J.E.; Bathe, S.; Clapp, J.P.; Burns, R.G. PCR-SSCP comparison of 16S rDNA sequence diversity in soil DNA obtained using different isolation and purification methods. *FEMS Microbiol. Ecol.* **2001**, *36*, 139–151. [CrossRef] [PubMed]
36. Labrie, J.; Pelletier-Jacques, G.; Deslandes, V.; Ramjeet, M.; Auger, E.; Nash, J.H.; Jacques, M. Effects of growth conditions on biofilm formation by *Actinobacillus pleuropneumoniae*. *Vet. Res.* **2010**, *41*, 3. [CrossRef] [PubMed]
37. Peng, Y.; Yan-zhi, G.; Ling, Q. Effects of ozone-treated domestic sludge on hydroponic lettuce growth and nutrition. *J. Integr. Agric.* **2018**, *17*, 593–602.
38. Van Diepeningen, A.D.; de Vos, O.J.; Korthals, G.W.; van Bruggen, A.H.G. Effects of organic versus conventional management on chemical and biological parameters in agricultural soils. *Appl. Soil Ecol.* **2006**, *31*, 120–135. [CrossRef]
39. Mendoza-Hernández, D.; Fornes, F.; Belda, R.M. Compost and vermicompost of horticultural waste as substrates for cutting rooting and growth of rosemary. *Sci. Hortic.* **2014**, *178*, 192–202. [CrossRef]
40. Rinaldi, S.; De Lucia, B.; Salvati, L.; Rea, E. Understanding complexity in the response of ornamental rosemary to different substrates: A multivariate analysis. *Sci. Hortic.* **2014**, *176*, 218–224. [CrossRef]
41. García-Gómez, R.C.; Dendooven, L.; Gutiérrez-Miceli, F.A. Vermicomposting leachate (Worm Tea) as liquid fertilizer for maize (*Zea mays* L.) forage production. *Asian J. Plant Sci.* **2008**, *7*, 360–367.
42. Singh, R.; Gupta, R.K.; Patil, R.T.; Sharma, R.R.; Asrey, R.; Kumar, A.; Jangra, K.K. Sequential foliar application of vermicompost leachates improves marketable fruit yield and quality of strawberry (*Fragaria* x *ananassa* Duch.). *Sci. Hortic.* **2010**, *124*, 34–39. [CrossRef]
43. Lazcano, C.; Revilla, P.; Malvar, R.A.; Domínguez, J. Yield and fruit quality of four sweet corn hybrids (*Zea mays*) under conventional and integrated fertilization with vermicompost. *J. Sci. Food Agric.* **2011**, *91*, 1244–1253. [CrossRef] [PubMed]
44. Tharmaraj, K.; Ganesh, P.; Kolanjinathan, K.; Suresh-Kumar, R.; Anandan, A. Influence of vermicompost and vermiwash on physicochemical properties of rice cultivated soil. *Curr. Bot.* **2011**, *2*, 18–21.
45. Emperor, G.N.; Kumar, K. Microbial population and activity on vermicompost of *Eudrilus eugeniae* and *Eisenia fetida* in different concentrations of tea waste with cow dung and kitchen waste mixture. *Int. J. Curr. Microbiol. Appl. Sci.* **2015**, *4*, 496–507.

46. Allard, S.M.; Walsh, C.S.; Wallis, A.E.; Ottesen, A.R.; Brown, E.W.; Micallef, S.A. *Solanum lycopersicum* (tomato) hosts robust phyllosphere and rhizosphere bacterial communities when grown in soil amended with various organic and synthetic fertilizers. *Sci. Total Environ.* **2016**, *573*, 555–563. [CrossRef] [PubMed]
47. Sheridan, C.; Depuydt, P.; De Ro, M.; Petit, C.; Van Gysegem, E.; Delaere, P.; Dixon, M.; Stasiak, M.; Aciksöz, S.B.; Frossard, E.; et al. Microbial community dynamics and response to Plant Growth-Promoting Microorganisms in the rhizosphere of four common food crops cultivated in hydroponics. *Microb. Ecol.* **2017**, *73*, 378–393. [CrossRef] [PubMed]
48. Neher, D.A.; Weicht, T.R.; Bates, S.T.; Leff, J.W.; Fierer, N. Changes in bacterial and fungal communities across compost recipes, preparation methods, and composting times. *PLoS ONE* **2013**, *8*, e79512. [CrossRef] [PubMed]
49. Lazcano, C.; Gómez-Brandón, M.; Domínguez, J. Comparison of the effectiveness of composting and vermicomposting for the biological stabilization of cattle manure. *Chemosphere* **2008**, *72*, 1013–1019. [CrossRef] [PubMed]
50. Pascual, J.; Blanco, S.; García-López, M.; García-Salamanca, A.; Bursakov, S.A.; Genilloud, O.; Bills, G.F.; Ramos, J.L.; van Dillewijn, P. Assessing bacterial diversity in the rhizosphere of *Thymus zygis* growing in the Sierra Nevada National Park (Spain) through culture-dependent and independent approaches. *PLoS ONE* **2016**, *11*, e0146558. [CrossRef]
51. Checcucci, A.; Maida, I.; Bacci, G.; Ninno, C.; Bilia, A.R.; Biffi, S.; Firenzuoli, F.; Flamini, G.; Fani, R.; Mengoni, A. Is the plant-associated microbiota of *Thymus* spp. adapted to plant essential oil? *Res. Microbiol.* **2017**, *168*, 276–282. [CrossRef]
52. Armada, E.; Leite, M.F.A.; Medina, A.; Azcón, R.; Kuramae, E.E. Native bacteria promote plant growth under drought stress condition without impacting the rhizomicrobiome. *FEMS Microbiol. Ecol.* **2018**, *94*. [CrossRef]
53. Chen, H.; Wu, H.; Yan, B.; Zhao, H.; Liu, F.; Zhang, H.; Sheng, Q.; Miao, F.; Liang, Z. Core microbiome of medicinal plant *Salvia miltiorrhiza* seed: A rich reservoir of beneficial microbes for secondary metabolism? *Int. J. Mol. Sci.* **2018**, *19*, 672. [CrossRef] [PubMed]
54. Ali, K.; Wang, X.; Riaz, M.; Islam, B.; Khan, Z.H.; Shah, F.; Munsif, F.; Ijaz Ul Haq, S. Biochar: An eco-friendly approach to improve wheat yield and associated soil properties on sustainable basis. *Pak. J. Bot.* **2019**, *51*, 1255–1261. [CrossRef]
55. Perrino, E.V.; Ladisa, G.; Calabrese, G. Flora and plant genetic resources of ancient olive groves of Apulia (southern Italy). *Genet. Resour. Crop Evol.* **2014**, *61*, 23–53. [CrossRef]
56. Rani, A.J.; Murugan, P.P. Suggestions for the increase use efficiency of eco-friendly agricultural practices for sustainable paddy cultivation. *Agric. Update* **2010**, *5*, 103–105.

horticulturae

MDPI

Article

Calcium Carbonate Can Be Used to Manage Soilless Substrate pH for Blueberry Production

Michael J. Schreiber and Gerardo H. Nunez *

Horticultural Sciences Department, University of Florida, Gainesville, FL 32611, USA; schreiberm@ufl.edu
* Correspondence: g.nunez@ufl.edu

Abstract: Blueberry (*Vaccinium corymbosum* interspecific hybrids) production in soilless substrates is becoming increasingly popular. Soilless substrates have low pH buffering capacity. Blueberry plants preferentially take up ammonium, which acidifies the rhizosphere. Consequently, soilless substrates where blueberry plants are grown exhibit a tendency to get acidified over time. Agricultural lime ($CaCO_3$) is commonly used to raise soil and substrate pH in other crops, but it is rarely used in blueberry cultivation. We hypothesized that substrate amendment with low rates of agricultural lime increases substrate pH buffering capacity and provides nutritional cations that can benefit blueberry plants. We tested this hypothesis in a greenhouse experiment with 'Emerald' southern highbush blueberry plants grown in rhizoboxes filled with a 3:1 mix of coconut coir and perlite. We found that substrate amendment with $CaCO_3$ did not cause high pH stress. This amendment maintained substrate pH between 5.5 and 6.5 and provided Ca and Mg for plant uptake. When blueberry plants were grown in $CaCO_3$-amended substrate and fertigated with low pH nutrient solution (pH 4.5), they exhibited greater biomass accumulation than plants grown in unamended substrates. These results suggest that low rates of $CaCO_3$ could be useful for blueberry cultivation in soilless substrates.

Keywords: *Vaccinium corymbosum*; container; ammonium uptake; southern highbush blueberry

Citation: Schreiber, M.J.; Nunez, G.H. Calcium Carbonate Can Be Used to Manage Soilless Substrate pH for Blueberry Production. *Horticulturae* **2021**, *7*, 74. https://doi.org/10.3390/horticulturae7040074

Academic Editors: Nazim Gruda and Juan A. Fernández

Received: 2 March 2021
Accepted: 2 April 2021
Published: 7 April 2021

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

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Cultivation in containers filled with soilless substrates is rapidly becoming a popular growing system for blueberry (*Vaccinium corymbosum* interspecific hybrids) production. Soilless substrates based on sphagnum peat moss or coconut coir are generally acidic [1] and have high water holding capacity [2]. These substrate characteristics promote blueberry nutrient uptake and support vigorous growth [3,4]. As this growing system becomes widespread [5], there is a need for research focused on fertilization and management practices for substrate-grown blueberry.

Sphagnum peat moss and coconut coir have low pH buffering capacity [6,7]. Consequently, pH changes of up to 1 unit per month are not uncommon [3,4,8,9]. While blueberry roots exhibit limited ability to change the rhizosphere pH through H^+ extrusion [10], ammonium uptake can lead to rapid rhizosphere acidification [11,12]. Considering blueberry growth and N content are enhanced by ammonium-based fertilization [11,13], substrate acidification appears inevitable in this production system.

Calcitic ($CaCO_3$) and dolomitic [$CaMg(CO_3)_2$] lime are commonly used to raise soilless substrate pH, but amendment rates and effects are crop-specific (reviewed in [14]). The carbonate moiety in lime acts as a buffer that maintains the rhizosphere approximately at pH 6.4 [14]. The cations in lime are nutritionally relevant Ca and Mg. Substrates used for cultivation of other acid-loving plants are routinely amended with lime to limit substrate pH change [15,16]. Nevertheless, the effects of lime amendments in substrate-grown blueberry remain understudied.

Lime is rarely used in soil-based blueberry cultivation because high liming rates can raise soil pH excessively and cause plant stress. When grown in high pH soils, blueberry

plants exhibit nutritional deficiencies, stunted growth, and lower yields [17–19]. Lime is only used in situations where soil pH is very low to deliver Ca and Mg [20]. Hence, lime amendments must be meticulously used to avoid stressing blueberry plants.

This research investigates the effect of substrate amendment with $CaCO_3$ on the substrate pH, growth, and nutrition of southern highbush blueberry. We hypothesized that substrate amendment with low rates of agricultural lime increases substrate pH buffering capacity and provides nutritional cations that can benefit blueberry plants. We tested this hypothesis in a greenhouse experiment with plants grown in rhizoboxes.

2. Materials and Methods

Rooted cuttings of 'Emerald' southern highbush blueberry (SHB; rooting volume = 3 cm^3, average dry weight = 1.15 g, average height = 12 cm) were acquired from a commercial micropropagation nursery (Agristarts LLC, Apopka, FL, USA) and transplanted to benchtop rhizoboxes as per [21]. Rhizoboxes were built using two 35.56 cm × 35.56 cm plexiglass panels spaced 1.9 cm apart using wood inserts. Each rhizobox contained approximately 1.7 L of substrate and was irrigated or fertigated by two 1.89 $l{\cdot}h^{-1}$ pressure-regulating emitters, spaced approximately 15.25 cm apart. Custom-made rhizobox stands kept roots in the dark at 33° inclination. There was one plant per rhizobox. Rhizoboxes were used as a tool to study root growth patterns in response to substrate amendment and fertigation pH treatments.

Rhizoboxes were filled with a 3:1 mixture of coconut coir (SpongEase™, Enroot Products LLC, Cromwell, CT, USA) and horticultural grade perlite (American Garden Perlite, LLC, Lake Wales, FL, USA) pre-treated to deliver two substrate amendment treatments. In one treatment, substrate was amended with $CaCO_3$ (Garden Lime, Austinville Limestone, Austinville, VA, USA) at a rate of 6.18 $Kg{\cdot}m^{-3}$. This rate corresponds to half of the rate used in [22] where lime amendments were used to stress azalea (*Rhododendron* spp.). In the other treatment, substrate was amended with Ca-containing fertilizer produced from neutralized $CaCO_3$ (Calexin ®, Miller Chemical & Fertilizer Corporation, Hanover, PA, USA) at a rate of 100.3 $L{\cdot}m^{-3}$. Guaranteed analysis and product density information were used to calculate a Calexin application rate that delivered the same amount of Ca as the $CaCO_3$ amendment. Both amendments were incorporated into moist substrate 7 days before transplant.

Fertigation solution pH was a second variable in the experiment. Plants were fertigated with a solution containing 0.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2PO_4, 1.0 mM $MgSO_4$, 0.5 mM $CaCl_2$, 0.045 mM H_3BO_3, 0.01 mM $MnSO_4$, 0.01 mM $ZnSO_4$ with 0.3 mM $CuSO_4$, 0.2 mM Na_2MoO_4, and 45 mM Fe provided as Sequestrene 330 (10% iron(III)-diethylenetriamine pentaacetic acid) (Becker Underwood, Inc., Ames, IA, USA). Ammonium was the only form of N provided, in agreement with industry practices [20]. The low N rate was selected because blueberry microcuttings exhibited ammonium toxicity when fertigated with higher N rates in a preliminary experiment. Fertigation solution was buffered using 5.0 mM 2-(4-morpholino)-ethane sulfonic acid to pH 4.5 or pH 6.5 using HCl or KOH. These fertigation pH treatments are referred to as low pH and high pH respectively in relation to fertigation pH used in previous studies [3]. There were 21 fertigation/irrigation events per week. Each plant received 1.75 L of fertigation solution (delivered through 7 events) and 3.96 L of irrigation water per week (delivered through 14 events). Fertigation events preceded irrigation events. Fertigation and irrigation volumes were measured with graduated cylinders connected directly to emitters.

Substrate samples were collected at the start (day 0) and end (days 75–77) of the experiment and submitted for analysis at a commercial laboratory (Waters Agricultural Laboratory, Camila, GA, USA). Ca, Mg, and K concentrations in the substrate were determined using inductively coupled plasma mass spectrometry [23]. Cation exchange capacity (CEC) was calculated from K, Ca, Mg, and H concentrations as per [24]. Substrate pH was measured in a 1:1 substrate:deionized water slurry [25].

Substrate pH and electrical conductivity (EC) were monitored using the pour-through method [26]. Deionized water samples that eluted through the substrate (hereon, leachate) were collected on a weekly basis (n = 3 per treatment). Rhizoboxes were removed from the stand and placed vertically on top of plastic trays (one rhizobox per tray) approximately 2 h after the last fertigation event. Then, 500 mL of deionized water were slowly poured on top of the substrate. Leachate was collected in the plastic tray for approximately 20 min. Then, leachate volume was measured with a graduate cylinder and 50 mL aliquots were transported to the laboratory for immediate measurement of leachate pH (Accumet AP110 Portable pH Meter, Thermo Fisher Scientific, Hampton, NH, USA) and EC (Accumet Excel Conductivity Meter, XL30, Thermo Fisher Scientific, Hampton, NH, USA) using standardized electrodes. In this manuscript and elsewhere [26], it is assumed that leachate pH and EC represent rhizosphere conditions. Leaf greenness was measured on the youngest fully expanded leaf of each plant using a SPAD-502 meter (Konica Minolta, Inc., Ramsey, NJ, USA).

Rhizoboxes were scanned using a flatbed scanner (LX1100, Seiko Epson Corp., Tokyo, Japan) at a resolution of 1000 dots per inch (dpi). The scanner was held at an inclination of 30° during scanning to avoid substrate loss. Rhizoboxes were scanned on a weekly basis starting on week 2 of the experiment. Rhizobox images were used to measure root system convex hull area using ImageJ version 1.51 [27]. Convex hull area is the area of the polygon formed by lines connecting the most distal root tips in a plant. Root system spread was computed as the ratio of the convex hull area to root dry weight.

Rhizoboxes were disassembled 75 to 77 days after the start of the experiment. Roots were washed clean of substrate using tap water. A subset of the root systems (n = 4 per treatment except for $CaCO_3$ + pH 6.5 where one root image was lost due to human error) were scanned floating in water using the transparency unit of the flatbed scanner at 1000 dpi. Images were divided into 5 tiles using ImageJ. Then, total root length was determined using WinRhizo Pro 2013b (Regent Instruments, Quebec, QC, Canada). Organ and whole plant fresh weight were measured. Leaves were laid flat and photographed at a distance of 48.25 cm from the lens using mobile phone cameras (iPhone 7 and iPhone X, Apple Inc., Cupertino, CA, USA) on a white background with a scale bar of known size. Total leaf area was measured using ImageJ. Plant tissues were weighted after drying at 72 °C for a week. Dry tissue was ground until it passed through a size-20 mesh (sieve opening = 0.841 mm). Then, tissue was submitted for elemental analysis at a commercial laboratory (Waters Agricultural Laboratory, Camila, GA, USA).

The experiment was conducted in a greenhouse where average temperature and relative humidity were 22.53 °C and 70.19%, respectively. The experiment followed a completely randomized design with treatments in a 2 × 2 factorial arrangement. There were 10 single-plant replications per amendment × pH combination. Unless otherwise stated, n = 10 per treatment. Treatment effects on biomass accumulation, leaf area, substrate characteristics, elemental content, and root traits were assessed using two-way analysis of variance (R package agricolae, [28]). Where significant effects were identified, pairwise comparisons were made using the least significant difference method. Leachate pH and EC data were analyzed through linear mixed-effect analysis (R package lme4, [29]). Fertigation solution pH, substrate amendment, and their interaction were considered fixed effects. Repeated measures per plant and week were considered random sources of error. Leachate pH and EC were response variables analyzed in separate models. Statistical significance was determined by likelihood ratio tests comparing the full model against a model without the effect being investigated. All statistical analyses were conducted in R version 3.6.2 [30]. Data were illustrated using ggplot 2 [31].

3. Results

At the start of the experiment, substrates amended with $CaCO_3$ exhibited higher pH, percentage base saturation, Mg content, and K content than substrates amended with Calexin (Table 1). Substrate CEC and Ca content were not different between the amend-

ment treatments. At the end of the experiment, substrate pH was not different among treatments (Table 2). Substrates amended with $CaCO_3$ exhibited higher CEC, percentage base saturation, Ca concentration, and Mg concentration than substrates amended with Calexin. Substrates that were fertigated at pH 4.5 exhibited lower K concentration than substrates that were fertigated at pH 6.5. The interaction of substrate amendment and fertigation pH did not affect substrate characteristics ($p \geq 0.158$).

Table 1. Substrate characteristics before transplant. A substrate composed of a 3:1 mixture of coconut coir and perlite was amended with $CaCO_3$ or a Ca-containing fertilizer (Calexin) 7 days before transplanting 'Emerald' southern highbush blueberry.

Amendment	pH	Cation Exchange Capacity (meq·100 g^{-1} Substrate)	Base Saturation (%)	Ca ($mg \cdot Kg^{-1}$)	Mg ($mg \cdot Kg^{-1}$)	K ($mg \cdot Kg^{-1}$)
$CaCO_3$	6.4	9.50	71.77	932.17	158.67	325.67
Calexin	4.4	10.97	57.27	1089.00	43.84	194.00
p value [z]	<0.001	0.084	0.021	0.283	<0.001	0.009

[z] Treatments were compared using ANOVA.

Table 2. Substrate characteristics after 75–77 days of growing 'Emerald' southern highbush blueberry with contrasting substrate amendments and fertigation pH.

Treatment	Substrate pH	Cation Exchange Capacity (meq·100 g^{-1} Substrate)	Base Saturation (%)	Ca ($mg \cdot Kg^{-1}$)	Mg ($mg \cdot Kg^{-1}$)	K ($mg \cdot Kg^{-1}$)
$CaCO_3$	4.9	8.05	57.38	1298.33	307.50	99.17
Calexin	4.7	6.73	34.60	601.83	169.67	95.83
p value [z]	0.094	0.009	<0.001	<0.001	0.003	0.596
pH 6.5	4.8	7.30	48.95	958.33	248.67	164.67
pH 4.5	4.8	7.48	43.03	941.83	228.50	30.33
p value	0.999	0.643	0.144	0.901	0.563	<0.001

[z] Data were analyzed by two-way ANOVA. The interaction of fertigation pH and substate amendment did not affect substrate characteristics ($p \geq 0.158$).

Substrate amendments and fertigation pH created contrasting leachate pH and EC during most of the experiment (Figure 1). Leachate pH gradually decreased in all treatments ($\chi^2 = 11.74$, df = 1, $p \leq 0.001$, estimate = −0.42). Amendment with $CaCO_3$ ($\chi^2 = 93.34$, df = 1, $p < 0.001$) and high pH fertigation ($\chi^2 = 28.69$, df = 1, $p < 0.001$) led to high leachate pH. The interaction of substrate amendment and fertigation pH did not affect leachate pH ($\chi^2 = 2.57$, df = 3, $p = 0.11$). Amendment with $CaCO_3$ led to higher leachate EC ($\chi^2 = 5.26$, df = 1, $p = 0.02$). Fertigation pH ($\chi^2 = 1.13$, df = 1, $p = 0.29$), time ($\chi^2 = 0.22$, df = 1, $p = 0.63$), and the interaction of substrate amendment and fertigation pH ($\chi^2 = 7.33$, df = 3, $p = 0.06$) did not affect leachate EC.

Substrate amendments and fertigation pH affected plant biomass accumulation (Table 3). Plants grown with a combination of Calexin amendment and low pH fertigation solution exhibited lower cane, leaf, and total dry weight than plants grown with $CaCO_3$ amendments. Within a substrate amendment, fertigation pH did not affect biomass accumulation. Plants grown in substrates amended with $CaCO_3$ exhibited larger root systems than plants grown in substrates amended with Calexin. Leaf area followed the same trends as leaf dry weight (data not shown). Leaf greenness was not affected by the treatments (average = 24.68, $p = 0.23$).

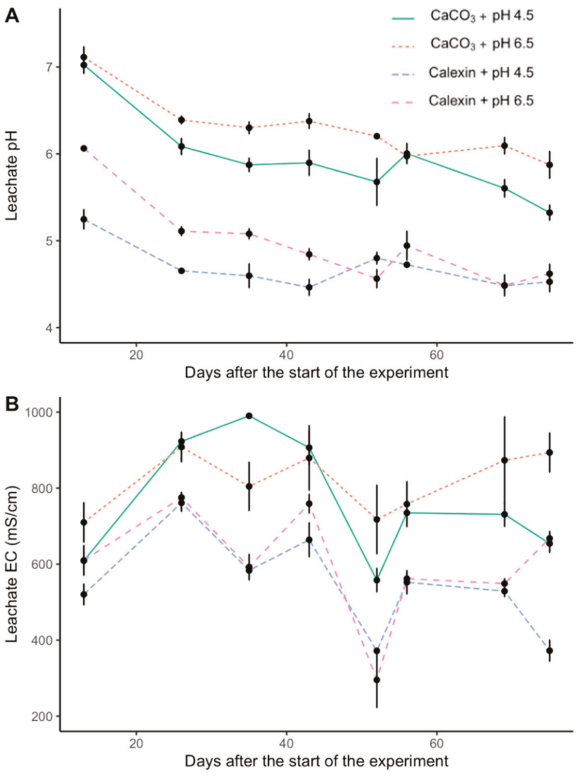

Figure 1. Leachate pH (**A**) and electrical conductivity (EC) (**B**) collected from rhizoboxes where 'Emerald' southern highbush blueberry grew with contrasting substrate amendments ($CaCO_3$ or Ca-containing fertilizer Calexin) and fertigation pH (pH 4.5 and pH 6.5).

Table 3. Biomass accumulation of 'Emerald' southern highbush blueberry plants grown in rhizoboxes with contrasting substrate amendments and fertigation pH.

Amendment	Fertigation pH	Root Dry Weight (g)	Cane Dry Weight (g)	Leaf Dry Weight (g)	Total Dry Weight (g)
$CaCO_3$	6.5	2.38 a	3.40 ab	5.06 ab	10.84 ab
	4.5	2.38 a	4.91 a	6.99 a	14.32 a
Calexin	6.5	1.16 b	2.33 bc	4.01 bc	7.50 bc
	4.5	0.58 b	1.33 c	2.59 c	4.2 c
Effect [z]					
Amendment		<0.001	<0.001	<0.001	<0.001
Fertigation pH		0.272	0.574	0.672	0.947
Amendment x pH		0.208	0.009	0.008	0.017

[z] Data were analyzed by two-way ANOVA. Means followed by the same letter were not significantly different according to Tukey LSD at $\alpha = 0.05$.

Substrate amendment and fertigation pH also affected root system characteristics. Root systems of plants grown with low pH fertigation and $CaCO_3$ amendments exhibited larger convex hull areas than all other treatment combinations between weeks 3 and 9 (Figure 2A). Root systems of plants grown with low pH fertigation and Calexin amendments had smaller convex hull area than all other treatments initially (weeks 3 and 4). Plants grown with low pH fertigation and $CaCO_3$ amendment exhibited higher total root length than plants grown with high pH fertigation and $CaCO_3$ amendments and plants grown with low pH fertigation and Calexin (Figure 2B). High pH fertigation so-

lution (274.53 $cm^2 \cdot g^{-1}$ vs. 191.66 $cm^2 \cdot g^{-1}$) and $CaCO_3$ amendments (338.97 $cm^2 \cdot g^{-1}$ vs. 127.22 $cm^2 \cdot g^{-1}$) reduced root system spread ($p < 0.008$ in all cases). Root system spread was not affected by the interaction of substrate amendment and fertigation solution pH ($p = 0.29$).

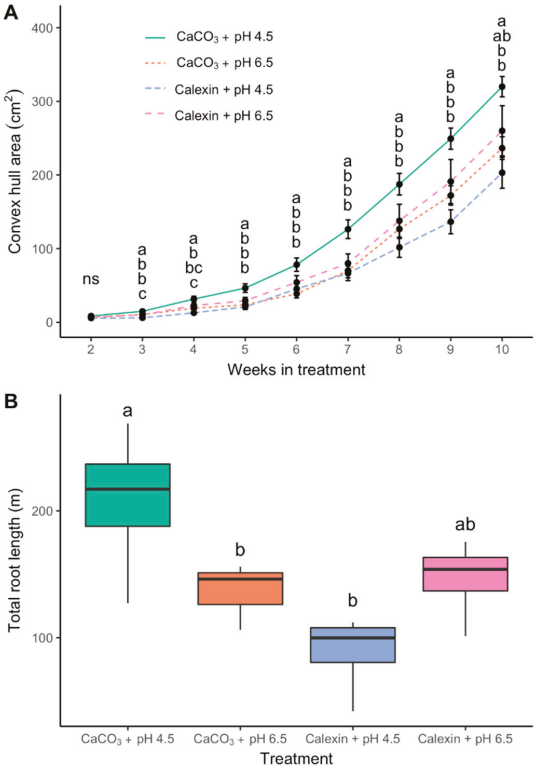

Figure 2. Root system characteristics of 'Emerald' southern highbush blueberry grown with contrasting substrate amendments and fertigation pH. (**A**) Convex hull area during the treatment period. (**B**) Total root length after 77 days of cultivation. Means followed by the same letter were not significantly different according to Tukey LSD at $\alpha = 0.05$.

Substrate amendment and fertigation pH affected root and leaf nutrient concentrations (Table S1). High pH fertigation decreased N, Zn, and Cu concentrations and increased Ca concentration in roots. Substrate amendment with $CaCO_3$ decreased K, S, B, and Cu concentrations and increased Fe concentrations in roots. Other elements were not affected. The interaction of fertigation pH and substate amendment did not affect root nutrient concentrations ($p \geq 0.078$). Plants grown in substrates amended with Calexin and fertigated with low pH solution exhibited the highest leaf N, P, Ca, Mg, S, Fe, Zn, and Cu concentrations (Table S2). Plants grown in substrates amended with $CaCO_3$ generally exhibited the lowest leaf concentrations of these elements. Plants grown with low pH fertigation exhibited higher leaf Mn concentrations than plants grown with high pH fertigation. Plants grown in substrates amended with $CaCO_3$ exhibited lower leaf K, Mn, and B. With the exception of K, treatment effects on nutrient concentration did not exhibit the same trends in roots and leaves.

4. Discussion

Soilless substrates have limited pH buffering capacity [6], which allows large pH changes over the cultivation period [3,4,8,9]. In this experiment, 'Emerald' SHB plants were fertigated with a nutrient solution where ammonium was the only form of N. Ammonium uptake leads to rhizosphere acidification [11,12]. As expected, leachate pH gradually decreased in all treatments. Similar leachate acidification has been previously observed in experiments with substrate-grown blueberry [4,12] and azalea [32].

$CaCO_3$ is routinely used to raise soil or substrate pH in other crops [14–16], but not in blueberry. When blueberry and other acid-loving plants are grown in soils or substrates amended with high $CaCO_3$ rates, they exhibit high pH stress symptoms such as interveinal chlorosis and stunted growth [17–19,33]. In this experiment, $CaCO_3$ in the substrate did not cause high pH stress in 'Emerald' SHB, probably due to the low rate used. Plants grown in substrates amended with $CaCO_3$ did not exhibit Fe deficiency symptoms either, but leaf Fe concentrations were lower than published recommendations [20]. These results suggest that even though $CaCO_3$ raised substrate pH, the effect was mild enough to avoid causing high pH stress in 'Emerald' SHB. Further research will be necessary to determine if the $CaCO_3$ rate used here is appropriate for other blueberry varieties.

In this experiment, $CaCO_3$ in the substrate acted as a pH buffer that partially neutralized H^+ from ammonium uptake and the fertigation solution, maintaining leachate pH between pH 5.5 and pH 6.5 for most of the experiment. Additionally, $CaCO_3$ amendment replaced cations from the substrate adhesion sites with Ca and Mg. The combination of acidic substrate and nutritional cation availability supported vigorous growth above- and below-ground in 'Emerald' SHB, especially when $CaCO_3$ amendment was matched with low pH fertigation solution.

When the substrate did not contain carbonates, leachate pH ranged between pH 4.5 and pH 5.0 and almost half of the adhesion sites were occupied by non-nutritional cations. The lack of nutritional cations was likely caused by the abundance of H^+ and/or Calexin leaching out of the rhizoboxes. These substrate conditions affected shoot and root growth, particularly when the fertigation solution pH was low. Low pH increases Al solubility [34], which can cause Al toxicity in blueberry [35]. Perlite contains 10–15% Al_2O_3 [36]. Thus, it is possible that Al toxicity might have affected 'Emerald' SHB growth when substrate pH was extremely low. Al concentrations in the rhizosphere were not measured in this experiment. Further research will be necessary to establish if Al toxicity impacted plant responses.

Substrate characteristics affected blueberry root abundance and distribution. $CaCO_3$ amendments increased 'Emerald' SHB root dry weight and, in combination with low pH fertigation, they led to large root systems that reached most of the substrate in the rhizoboxes. Nevertheless, large root systems were not always better at taking up nutrients. Previous research has shown that $CaCO_3$ can affect nutrient uptake through pH-dependent and pH-independent effects [37]. Thus, fertilization practices might need to be adapted to maintain optimum plant nutrition in substrates amended with $CaCO_3$.

Irrigation water can contain carbonates and bicarbonates (collectively called alkalinity). Alkaline water sources are not uncommon in blueberry production areas [38], but water acidification through sulfuric acid injection or sulfur dioxide generators is routinely used to neutralize alkalinity [39]. Our results suggest that increasing substrate pH buffering capacity can be beneficial for blueberry. Thus, it is important to recognize the tradeoff between irrigation water pH and pH buffering capacity when irrigation water is acidified. Considering the substrate acidification tendency observed here and elsewhere [4,12], it is tempting to speculate on the utility of alkaline water for substrate pH management. Future research should explore this potential management strategy.

Altogether, our results indicate that substrate amendment with low rates of $CaCO_3$ is a viable tool to increase pH buffering capacity in coconut coir-based substrates used for blueberry cultivation. $CaCO_3$ neutralized H^+ and contributed Ca and Mg for plant uptake. Access to a weakly acidic substrate with abundant nutritional cations supported vigorous

growth in 'Emerald' SHB. Further research should evaluate other $CaCO_3$ amendment rates and other blueberry varieties to facilitate decision making when using $CaCO_3$ in substrate-based blueberry cultivation.

Supplementary Materials: The following are available online at https://www.mdpi.com/2311-7524/7/4/74/s1, Table S1: Nutrient concentrations in roots of 'Emerald' southern highbush blueberry, Table S2: Nutrient concentrations in leaves of 'Emerald' southern highbush blueberry.

Author Contributions: Conceptualization, G.H.N.; methodology, data collection, analysis, and visualization, writing, M.J.S. and G.H.N. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data presented in this study are available on request from the corresponding author.

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

References

1. Konduru, S.; Evans, M.R.; Stamps, R.H. Coconut husk and processing effects on chemical and physical properties of coconut coir dust. *HortScience* **1999**, *34*, 88–90. [CrossRef]
2. Abad, M.; Fornes, F.; Carrión, C.; Noguera, V.; Noguera, P.; Maquieira, A.; Puchades, R. Physical properties of various coconut coir dusts compared to peat. *HortScience* **2005**, *40*, 2138–2144. [CrossRef]
3. Kingston, P.H.; Scagel, C.F.; Bryla, D.R.; Strik, B.C. Suitability of sphagnum moss, coir, and douglas fir bark as soilless substrates for container production of highbush blueberry. *HortScience* **2017**, *52*, 1692–1699. [CrossRef]
4. Kingston, P.H.; Scagel, C.F.; Bryla, D.R.; Strik, B.C. Influence of perlite in peat- and coir-based media on vegetative growth and mineral nutrition of highbush blueberry. *HortScience* **2020**, *55*, 658–663. [CrossRef]
5. Fang, Y.; Nunez, G.H.; Silva, M.N.d.; Phillips, D.A.; Munoz, P.R. A review for southern highbush blueberry alternative production systems. *Agronomy* **2020**, *10*, 1531. [CrossRef]
6. Kithome, M.; Paul, J.W.; Kannangara, T. Adsorption isotherms of ammonium on coir. *Com. Soil Sci. Plant Anal.* **1999**, *30*, 83–95. [CrossRef]
7. Rippy, J.F.M.; Nelson, P.V. Cation exchange capacity and base saturation variation among Alberta, Canada, moss peats. *HortScience* **2007**, *42*, 349–352. [CrossRef]
8. Ramirez, S.V.; Altland, J. Minding Your pHs and Qs. *Grow. Talks* **2018**, *81*, 68–69.
9. Taylor, M.D.; Nelson, P.V.; Frantz, J.M. Substrate acidification by geranium: Light effects and phosphorus uptake. *J. Am. Soc. Hort. Sci.* **2008**, *133*, 515–520. [CrossRef]
10. Nunez, G.H.; Olmstead, J.W.; Darnell, R.L. Rhizosphere acidification is not part of the strategy I iron deficiency response of Vaccinium arboreum and the Southern Highbush Blueberry. *HortScience* **2015**, *50*, 1064–1069. [CrossRef]
11. Imler, C.S.; Arzola, C.I.; Nunez, G.H. Ammonium uptake is the main driver of rhizosphere pH in southern highbush blueberry. *HortScience* **2019**, *54*, 955–959. [CrossRef]
12. Tamir, G.; Zilkah, S.; Dai, N.; Shawahna, R.; Cohen, S.; Bar-Tal, A. Combined effects of $CaCO_3$ and the proportion of N-NH_4^+ among the total applied inorganic N on the growth and mineral uptake of Rabbiteye blueberry. *J. Soil Sci. Plant Nutr.* **2020**, *21*, 35–48. [CrossRef]
13. Merhaut, D.J.; Darnell, R.L. Ammonium and nitrate accumulation in containerized southern highbush blueberry plants. *HortScience* **1995**, *30*, 1378–1381. [CrossRef]
14. Altland, J.E.; Jeong, K.Y. Dolomitic lime amendment affects pine bark substrate pH, nutrient availability, and plant growth: A review. *HortTechnology* **2016**, *26*, 565–573. [CrossRef]
15. Ownley, B.H.; Benson, D.M.; Bilderback, T.E. Physical properties of container media and relation to severity of Phytophthora root rot of rhododendron. *J. Am. Soc. Hort. Sci.* **1990**, *115*, 564–570. [CrossRef]
16. Scagel, C.F.; Bi, G.; Fuchigami, L.H.; Regan, R.P. Nutrient uptake and loss by container-grown deciduous and evergreen Rhododendron nursery plants. *HortScience* **2011**, *46*, 296–305. [CrossRef]
17. Finn, C.E.; Luby, J.J.; Rosen, C.J.; Ascher, P.D. Blueberry germplasm screening at several soil pH regimes. I. plant survival and growth. *J. Am. Soc. Hort. Sci.* **1993**, *118*, 377–382. [CrossRef]
18. Finn, C.E.; Rosen, C.J.; Luby, J.J.; Ascher, P.D. Blueberry germplasm screening at several soil pH regimes. II. plant nutrient composition. *J. Am. Soc. Hort. Sci.* **1993**, *118*, 383–387. [CrossRef]

19. Jiang, Y.; Zeng, Q.; Wei, J.; Jiang, J.; Li, Y.; Chen, J.; Yu, H. Growth, fruit yield, photosynthetic characteristics, and leaf microelement concentration of two blueberry cultivars under different long-term soil pH treatments. *Agronomy* **2019**, *9*, 357. [CrossRef]
20. Hart, J.; Strik, B.; White, L.; Yang, W. Nutrient management for blueberries in Oregon. *Or. State Univ. Ext. Serv.* **2006**, EM8918.
21. Nunez, G.H.; Rodríguez-Armenta, H.P.; Darnell, R.L.; Olmstead, J.W. Toward marker-assisted breeding for root architecture traits in southern highbush blueberry. *J. Am. Soc. Hort. Sci.* **2016**, *141*, 414–424. [CrossRef]
22. Susko, A.Q.; Rinehart, T.A.; Bradeen, J.M.; Hokanson, S.C. An evaluation of two seedling phenotyping protocols to assess pH adaptability in deciduous azalea (*Rhododendron* sect. Pentanthera G. Don). *HortScience* **2018**, *53*, 268–274. [CrossRef]
23. US Environmental Protection Agency. *Determination of Trace Elements in Waters and Wastes by Inductively Coupled Plasma—Mass Spectrometry*; Office of Research and Development US Environmental Protection Agency: Cincinnati, OH, USA, 1994; pp. 200.8-1–200.8-51.
24. Reganold, J.P.; Harsh J., B. Expressing cation exchange capacity in milliequivalents per 100 grams and in SI units. *J. Agron. Educ.* **1985**, *14*, 84–90. [CrossRef]
25. Schofield, R.K.; Taylor, A.W. The measurement of soil pH. *Soil Sci. Soc. Am. J.* **1955**, *19*, 164–167. [CrossRef]
26. Cavins, T.J.; Whipker, B.E.; Fonteno, W.C.; Harden, B.; McCall, I.; Gibson, J.L. *Monitoring and Managing Ph and Ec Using the Pourthru Extraction Method*; Horticulture Information Leaflet, No. 590; North Carolina State University: Raleigh, NC, USA, 2000.
27. Rueden, C.T.; Schindelin, J.; Hiner, M.C.; DeZonia, B.E.; Walter, A.E.; Arena, E.T.; Eliceiri, K.W. ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinform.* **2017**, *18*, 529. [CrossRef]
28. De Mendiburu, F. Agricolae: Statistical Procedures for Agricultural Research. Available online: https://www.cran.r-project.org/web/packages/agricolae/index.html. (accessed on 7 June 2020).
29. Bates, D.; Maechler, M.; Bolker, B.; Walker, S. Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **2015**, *67*, 48. [CrossRef]
30. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2017. Available online: http://www.R-project.org/ (accessed on 7 June 2020).
31. Wickham, H. *Ggplot2: Elegant Graphics for Data Analysis*, 2nd ed.; Springer: Dordrecth, The Netherlands, 2016.
32. Demasi, S.; Caser, M.; Handa, T.; Kobayashi, N.; De Pascale, S.; Scariot, V. Adaptation to iron deficiency and high pH in evergreen azaleas (*Rhododendron* spp.): Potential resources for breeding. *Euphytica* **2017**, *213*, 148. [CrossRef]
33. Turner, A.J.; Arzola, C.I.; Nunez, G.H. High pH Stress Affects Root Morphology and Nutritional Status of Hydroponically Grown Rhododendron (*Rhododendron* spp.). *Plants* **2020**, *9*, 1019. [CrossRef] [PubMed]
34. Driscoll, C.T.; Schecher, W.D. The chemistry of aluminum in the environment. *Environ. Geochem. Health* **1990**, *12*, 28–49. [CrossRef]
35. Carcamo, M.P.; Reyes-Díaz, M.; Rengel, Z.; Alberdi, M.; Omena-Garcia, R.P.; Nunes-Nesi, A.; Inostroza-Blancheteau, C. Aluminum stress differentially affects physiological performance and metabolic compounds in cultivars of highbush blueberry. *Sci. Rep.* **2019**, *9*, 11275. [CrossRef] [PubMed]
36. Bush, A.L. Construction materials: Lightweight aggregates. In *Encyclopedia of Materials: Science and Technology*, 2nd ed.; Elsevier: Oxford, UK, 2001. [CrossRef]
37. Brito, L.M.; Monteiro, J.M.; Mourão, I.; Coutinho, J. Organic lettuce growth and nutrient uptake response to lime, compost and rock phosphate. *J. Plant Nutr.* **2014**, *37*, 1002–1011. [CrossRef]
38. Smith, E.; Porter, W.; Hawkins, G.; Harris, G., Jr. *Blueberry Irrigation Water Quality*; University of Georgia Cooperative Extension: Athens, GA, USA, 2016.
39. Bryla, D.R.; Strik, B.C. Nutrient requirements, leaf tissue standards, and new options for fertigation of northern highbush blueberry. *HortTechnology* **2015**, *25*, 464–470. [CrossRef]

horticulturae

MDPI

Article

Organic Fertilization of Growing Media: Response of N Mineralization to Temperature and Moisture

Patrice Cannavo [1,*], Sylvie Recous [2], Matthieu Valé [3], Sophie Bresch [4], Louise Paillat [1], Mohammed Benbrahim [5] and René Guénon [1]

[1] Institut Agro, EPHOR, 49000 Angers, France; louisepaillat@gmail.com (L.P.); rene.guenon@agrocampus-ouest.fr (R.G.)
[2] Université de Reims Champagne Ardenne, INRAE, FARE, UMR A 614, 51097 Reims, France; sylvie.recous@inrae.fr
[3] AUREA AGROSCIENCES, 45160 Ardon, France; m.vale@aurea.eu
[4] CDHR Centre-Val de Loire, Domaine de Cornay, 45590 Saint-Cyr-en-Val, France; sophie.bresch@astredhor.fr
[5] RITTMO Agroenvironnement, ZA Biopôle, 37 rue de Herrlisheim, CS 80023, CEDEX, 68025 Colmar, France; mohammed.benbrahim@rittmo.com
* Correspondence: patrice.cannavo@agrocampus-ouest.fr

Abstract: Managing plant fertilization is a major concern of greenhouse growers when it comes to sustainable production on growing media. Organic fertilization is popular, but more difficult to control since organic compounds first need to be mineralized by microbes. The objective of this study was to characterize the time course of N mineralization by different fertilizer–growing media pairs, in the absence of plants. Several incubations were carried out at four temperatures (4, 20, 28, and 40 °C) and three suction potentials (−3.2, −10, and −31.6 kPa) on four growing media under two organic fertilization conditions to study the dynamics of NH_4^+ and NO_3^- production. The results showed that the release of mineral N was strongly dependent on growing media, temperature, humidity, and fertilizer nature, varying from 10.7% to 71.3% of the N fertilizer applied. A temperature action law was established for the four growing media. The Q10 value of the growing media was 1.13, lower than the average Q10 value of arable soils. On the other hand, the specific behavior of the growing media did not yield a single humidity action law. Nevertheless, the nitrification process, evaluated by analyzing the ratio of NO_3^- to total mineral N, showed a humidity-dependent relationship common to the four growing media and comparable to admitted observations on soils. Nitrification was optimal when growing media humidity was higher than 0.46 *v/v*.

Keywords: NH_4^+; NO_3^-; nitrification; Q10; modeling

Citation: Cannavo, P.; Recous, S.; Valé, M.; Bresch, S.; Paillat, L.; Benbrahim, M.; Guénon, R. Organic Fertilization of Growing Media: Response of N Mineralization to Temperature and Moisture. *Horticulturae* **2022**, *8*, 152. https://doi.org/10.3390/horticulturae8020152

Academic Editors: Nazim Gruda and Juan A. Fernández

Received: 12 December 2021
Accepted: 7 February 2022
Published: 10 February 2022

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

Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Consumers are concerned about food quality and the environmental impact of its production. The subject is thorny in horticulture, particularly in soilless production which consumes resources (water and other inputs). As a consequence, producers are moving toward agro-ecological practices such as organic fertilization and the development of growing media from renewable organic materials [1]. Indeed, organic fertilization introduces a recycling concept in agroecosystems, and the non-use of synthetic inorganic N fertilizers considerably reduces the CO_2 emissions produced during the industrial N_2 fixation.

In conventional soilless production (cultivation in pots and containers), the plant grows in a finite volume of a growing medium with limited buffering capacity for water, temperature, and pH in particular [2]. The physical, chemical, and, to a lesser extent, biological properties of growing media materials have been investigated over the last 40 years, but practical considerations have been relatively little investigated [3]. Professionals have good knowledge of the physicochemical properties of the growing media, allowing for the control of irrigation and mineral fertilization. Introducing organic fertilizers requires

adapted practices because organic fertilizers first have to be mineralized by the microbiota of the growing media before being assimilated by plants.

Matching the rate of nutrient release by micro-organisms to the plant demands is essential [4,5]. Although microbial communities are widely used in growing media, few studies have characterized them. The authors of [6,7] studied microbial communities in peat, coir, and wood fiber growing media. They showed that organic growing media display specific activities and microbial structures depending on their origin and manufacturing process. Organic nutrient sources may be single or blended, and they may come from plant or animal byproducts or allowable mined sources [5]. Solid organic fertilizers are often unbalanced in their nutrient content, especially in their nitrogen, phosphorus, and potassium ratios, and a delay in shoot growth can result from their use [8,9]. The authors of [10] studied microbial activities involved in organic C, N, P, and S availability and the release of mineral forms in different growing media made of organic fertilizer combinations. Specific responses were observed, showing the complexity of the mineralization process. In particular, the mineralization rate varied greatly from one growing media to another.

Mineral nitrogen is the nutrient most used by plants, and it is often the most limiting element for plant growth [11]. The N preference of plants is variable and closely related to environmental conditions. For example, the N preference of plants changes from NO_3^- to NH_4^+ from drier to wetter sites [12], while the preference shifts from NH_4^+ under an acidic environment to NO_3^- at alkaline locations [13]. Moreover, NO_3^- is more accessible to plants because it reaches the roots by mass flow, whereas ammonium reaches them by diffusion [14]. Nitrogen deficiency results in symptoms such as reduced growth, and yellowing of leaves occurs very fast after the onset of deficiency [15]. Conversely, nutrient excess due to too rapid mineralization of organic fertilizer or the presence of unwanted ions such as SO_4^{2-}, sodium, or chlorine at high concentrations can result in salinization of the organic growing media [16]. It is difficult for growers to match the availability of dissolved growing media nutrients with plant demands at different stages of their developmental cycle [17]. Because a large proportion of organic nutrients are mineralized within the first few weeks and can leach out of containers, substrates containing organic fertilizers are typically used as the sole fertilizer source only for short-term crops. For long-term crops, substrates containing fertilizers are typically not enough to supply plant needs throughout the crop cycle and must be supplemented by top-dressing, side-dressing, or the use of liquid fertilizers in irrigation water [1].

The microbe-mediated N mineralization rate and the microbial community composition are highly variable and dependent on several factors such as growing media temperature, air porosity, and moisture content, as well as on the nature of the organic fertilizer source and the growing media composition (particle size and composition) [17–19]. The C:N ratio of growing media can also impact organic fertilizer mineralization. Substrates with C:N ratios exceeding 30:1 tend to immobilize N due to microbial decomposition of available C, which requires N [20]. Wood components such as composted barks, hammer milled, wood materials, and sawdust can have C:N ratios of 300:1 or more, and they have a high potential to immobilize N from applied fertilizers. Non-wood components with high C:N ratios, such as coir fiber, can also immobilize N [21].

N mineralization and nitrification have been thoroughly studied in soils, but knowledge gaps persist with regard to growing media; as they present low biodegradability, one can wonder whether indigenous microbial communities are suitable for organic N mineralization and nitrification, depending on temperature and moisture conditions. More generally, the transposition of mineralization and nitrification knowledge from soils to growing media is questioned. Thus, understanding the drivers of organic fertilizer mineralization and nitrification in horticultural growing media is necessary for a better prediction of mineral N availability for plants. The objectives of this study were to characterize the dynamics of NH_4^+ and NO_3^- production, and to evaluate the impacts of temperature and humidity on the N dynamics in the growing media. The ambition was to set up temperature and moisture functions to be ultimately used for modeling the N dynamics

in fertilized growing media under fluctuating conditions of moisture and temperature, as met by producers. A laboratory incubation experiment was conducted to characterize the organic N fertilizer mineralization of four commercial growing media under different growing media moisture and temperature regimes. We hypothesized that the action laws for temperature and moisture established for soils would be applicable to the growing media, whatever the growing media.

2. Materials and Methods

2.1. Materials

Four marketed growing media (GM1 to GM4) were studied. They were selected to be representative of the mostly used growing media (frequency and commercial volume, growing media producers survey, results not shown). Their properties are presented in Table 1. GM1 was made of black peat and composted plant fibers (80–20 vol.%), GM2 contained blond and black peat, coconut fiber, and composted plant fibers (50–20–10–20 vol.%), GM3 contained blond peat, coconut fiber, and composted bark (70–20–10 vol.%), and GM4 contained blond and black peat, coconut fiber, and green waste compost (60–10–20–10 vol.%). The properties of GM3 were somewhat different from those of the other growing media, with a coarser particle size, a higher OM content, a higher C:N ratio, and a lower bulk density. Three fertilizer modalities were studied: no fertilization (F0), organic fertilizer of an animal-based origin (F1), and organic fertilizer of a plant-based origin (F2). The fertilizer compositions are presented in Table 2, but commercial names were kept confidential.

Table 1. Physicochemical properties of the growing media.

	GM1	GM2	GM3	GM4
Professional Use	**Container/Aromatic and Flowering Plants**	**Market Garden Plants in Plugs or Trays**	**Container/Tree and Shrub**	**Market Garden Plants in Plugs or Trays**
Particle size distribution				
Size fraction >4 mm (%)	6.1	4.7	43.3	5.4
Size fraction 2–4 mm (%)	10.1	12.7	11.6	9.3
Size fraction <2 mm (%)	83.8	82.6	45.1	85.3
Bulk density ($g \cdot cm^{-3}$) [1]	0.18	0.20	0.12	0.18
Total porosity (*v/v*) [2]	89.1	88.3	92.0	88.0
EAW (*v/v*) [3]	0.38	0.41	0.23	0.40
AFP (*v/v*) [4]	0.17	0.03	0.47	0.04
pH water [5]	6.8	6.7	7.3	6.5
EC ($mS \cdot cm^{-1}$) [6]	0.7	0.7	0.6	0.6
OM ($g\ dw \cdot kg^{-1}$) [7]	687.3	690.6	908.5	712.6
Org N ($g\ dw \cdot kg^{-1}$)	11.1	11.1	6.9	13.1
C:N ratio	30.9	31.0	65.9	27.2
Total N ($g\ dw \cdot kg^{-1}$) [8]	11.5	11.5	6.9	13.4
AmoA ($log\ nb\_seq \cdot g^{-1}$)	8.06	7.96	6.60	8.02
Basal respiration ($\mu g\ C\text{-}CO_2 \cdot g^{-1}\ dw \cdot h^{-1}$) [9]	0.30 ± 0.1	0.80 ± 0.32	0.85 ± 0.1	0.99 ± 0.2

[1] Bulk density ($g \cdot cm^{-3}$) was determined following [22], and [2] total porosity (*v/v*) was determined following [22], [3] EAW: easy available water (%, *v/v*) and [4] AFP air-filled porosity (%, *v/v*) were calculated from water retention curves determined using a tension table draining at pressure potentials ranging from −1 to −10 kPa [22]; [5] pH was determined following [23]; [6] EC: electrical conductivity was determined following [24]; [7] OM (organic matter; % dry mass) was determined by loss of ignition (550 C, 7 h); [8] total N was determined by dry ignition according to [25]; [9] basal respiration was obtained by the Microresp™ method with growing media maintained at 60% of the water holding capacity at 25 °C for 1 week.

2.2. Experimental Design

The four growing media were incubated at four temperatures (4, 20, 28, and 40 °C) and three matric suctions (humidity maintained at −3.2, −10.0, and −31.6 kPa corresponding to pF1.5, pF2.0, and pF2.5, respectively), with or without added fertilizer, in the dark, in the absence of plants. Destructive samples were used to measure NH_4^+-N and NO_3^--N contents after 3, 7, 14, 28, and 49 days. They consisted of 90 mL vials filled with growing media depending on its bulk density (Table 1), i.e., 16, 18, 11, and 17 g dw per vial for GM1, GM2, GM3, and GM4, respectively. Vials were destroyed at each date for measurements.

They were placed in trays with a lid on each vial, and the growing media water content was maintained by weighing control. The organic fertilizer was applied at a rate of 55 g $N \cdot kg^{-1}$ growing media dw. Caps were placed on the vials, unsealed to permit air circulation but limit fast water evaporation. The amount of applied fertilizer was calculated on the basis of usual producers' practices (200 g fertilizer $N \cdot m^{-3}$). The growing media were analyzed for their NH_4^+-N and NO_3^--N contents at the beginning and after the start of the experiment. Three replicates were prepared per modality.

Table 2. Fertilizer composition.

	F1	F2
OM ($g \cdot kg^{-1}$)	559.5	636.7
C ($g \cdot kg^{-1}$)	279.8	318.4
Org N ($g \cdot kg^{-1}$)	59.5	54.1
Total N ($g \cdot kg^{-1}$)	67.4	55.1
Total P ($mg \cdot kg^{-1}$)	35.4	18.6
Total K ($mg \cdot kg^{-1}$)	52.3	40.8
Total Mg ($mg \cdot kg^{-1}$)	6.4	5.2
Total Mn ($mg \cdot kg^{-1}$)	50.8	258.8
C:N ratio	3.8	5.1
C:P ratio	7.9	17.1
N:P ratio	1.9	3.0
pH	6.8	6.9

OM was determined following [26]; inorganic N was extracted with 1 M KCl (1:5 *v/v* ratio). Ammonium and nitrate concentrations were determined by colorimetry using a continuous flow analyzer (Skalar Analytical). Total P, K, Mg, and Mn were extracted following [27] and measured following [28]; pH was measured following [23].

2.3. Analysis

NH_4^+-N and NO_3^--N were extracted with deionized water (1:1.5 vol.) for 1 h. Concentrations were determined by colorimetry using a continuous flow analyzer (Skalar Analytical). To obtain a growing media water suction curve, samples were saturated with distilled water for 48 h, with three replicates per growing media. Then, they were gradually dried using sand suction tables [29] with potentials equivalent to 0, −3.2, and −10 kPa. A ceramic pressure press was used for suction equivalent to −31.6 kPa [30,31]. When equilibrium was reached (2–3 days), the samples were dried in an oven at 105 °C for 48 h and weighed.

Nitrifying bacteria were quantified as follows: total nucleic acids were extracted from growing media samples using a Qiagen DNeasy PowerSoil kit (Cat No./ID: 12888-100). Then, quantitative polymerase chain reaction was performed using primers 968 R and 1401 R [32] for total bacteria and primers amoA-1F and amoA-2R [33] for nitrifying bacteria.

2.4. Data Treatments

2.4.1. Ammonium and Nitrate Concentrations

N mineralization was estimated by measuring NH_4^+-N and NO_3^--N concentrations, and their sum was used as the total mineral N concentration at each sampling time. Since growing media already contained mineral N (Table 1), NH_4^+-N and NO_3^--N concentrations were expressed by subtracting each respective initial mineral N content. Nitrate was the final product of N mineralization we monitored; hence, we expressed NO_3^- production as the relative proportion of total mineral N (i.e., NO_3^- + NH_4^+) released from fertilizer degradation at each timepoint.

2.4.2. Abiotic Factors

The temperature action law was determined in two steps, as described below.

First, it corresponded to a ratio of the mineralization rate at a given temperature over the mineralization rate at a reference temperature. Second, this ratio was plotted against

growing media temperature, and modeled using the STICS crop model temperature action law dedicated to the simulation of organic matter decomposition [34].

$$f(T) = \frac{\left([N]_{i,fin} - [N]_{i,init}\right)}{\left([N]_{ref,fin} - [N]_{ref,init}\right)} = \frac{B}{1 + C \times exp(-k \times T)}, \quad (1)$$

where T is the temperature (°C), $[N]_{i,fin}$ and $[N]_{i,init}$ are the total mineral N on days 49 and 0 at a given temperature T_i, respectively, and $[N]_{ref,fin}$ and $[N]_{ref,init}$ are the total mineral N on days 49 and 0 at the reference temperature T_{ref} = 20 °C, respectively. This reference temperature was made to be close to that of the STICS model (i.e., 15 °C). B was a dimensionless adjusted parameter, and k was an adjusted parameter ($°C^{-1}$). C was a parameter and was recovered by solving the following equation:

$$C = (B - 1) \times \exp\left(k \times T_{ref}\right), \quad C = (B - 1) \times \exp\left(k \times T_{ref}\right). \quad (2)$$

The water content action law was also determined in two steps, as described below.

First, it corresponded to a ratio of the mineralization rate at a given growing media matric suction over the mineralization rate at a reference matric suction. Second, this ratio was plotted against growing media moisture, and modeled using the STICS crop model moisture action law dedicated to the simulation of organic matter decomposition [34].

$$f(H) = \frac{\left([N]_{i,fin} - [N]_{i,init}\right)}{\left([N]_{ref,fin} - [N]_{ref,init}\right)} = \frac{H - H_{wp} \times H_{fc}}{\left(H_{fc} - H_{wp}\right) \times H_{fc}}, \quad (3)$$

where H is the volumetric water content of the growing medium (*v/v*), $[N]_{i,fin}$ and $[N]_{i,init}$ are the total mineral N on days 49 and 0 at a given water content H_i, respectively, and $[N]_{ref,fin}$ and $[N]_{ref,init}$ are the total mineral N on days 49 and 0 at the reference water content corresponding to water suction −10 kPa, respectively. H_{wp} is the volumetric water content at the wilting point (water suction −100 kPa), and H_{fc} is the volumetric water content at field capacity (water suction −1 kPa).

To further understand the temperature and moisture interactions, the ratio of NO_3^- to total mineral N was calculated as a mean ratio for the whole incubation period. This allowed us to identify abiotic conditions that may have slowed down or favored the nitrification process.

2.5. Statistical Analyses

We used three-way repeated-measures ANOVA (rmANOVA) to test the interaction of the growing medium type, the fertilizer type (Fert), temperature (Temp), and matric water suction (ψ) on NO_3^-, NH_4^+, and $NH_4^+ + NO_3^-$ concentrations following fertilization. We analyzed these effects separately depending on the significant interactions. We present the results for each growing medium, comparing temperatures or matric water suctions, and only with or without addition of fertilizers 1 or 2 to simplify the viewing of these effects. Significant differences were tested by the least significance difference test (LSD, $p < 0.05$). Correlations were tested using Pearson correlations ($p < 0.05$). When data seemed to present segmented regressions, we tested piecewise regressions with SegReg free software.

3. Results

3.1. Dynamics of Growing Media N Mineral Content

We did not find a significant four-level interaction among growing medium, fertilizer type (Fert), temperature (Temp), and humidity (Hum) over time as hypothesized (Table S1, four-way repeated-measures ANOVA). Instead, we did find significant three-way interactions such as GM × Temp × Hum. This interaction was the most powerful one ($F > 3.9$, $p < 0.001$, within effect) and was also confirmed independently of time

(GM × Temp × Hum: F > 1.7, $p < 0.001$, between effect). As a result, we focused on these interaction factors to present our results (i.e., without considering the fertilizer type, even though we detected some minor modularity of the results per fertilizer type compared to GM, Temp and Hum).

Temperature significantly controlled the total mineral N content (Figure 1), as well as NH_4^+ and NO_3^- contents (Figures S1 and S2, respectively), in the four growing media. The pattern of total mineral N differed depending on the growing media. We generally observed a plateau between 28 and 49 days modulated by temperature, except GM1 and GM2 that displayed a linear increase in mineral N content at 40 °C. GM1 and GM2 presented the best mineral N content at 40 °C after 49 days of incubation (877 and 807 mg N·kg^{-1} dw growing media, respectively, Tables S2 and S3). The temperature increase from 4 to 20 °C was always significant, whereas 20 °C and 28 °C tended to have similar effects on GM1 and GM2. For GM3 and GM4, 28 °C gave the best mineral N content, whereas 40 °C gave a lower content. In the absence of organic fertilization, the rates were close to zero except for GM1 and GM2, where total mineral N significantly increased at 40 °C; moreover, a negative N content was found in GM2 and GM4, especially at 28 °C, corresponding to organization of the initial mineral N content. At 28 °C, GM3 contained the highest mineral N content reached in these incubations (1053 mg N·kg^{-1} dw growing media, Table S4).

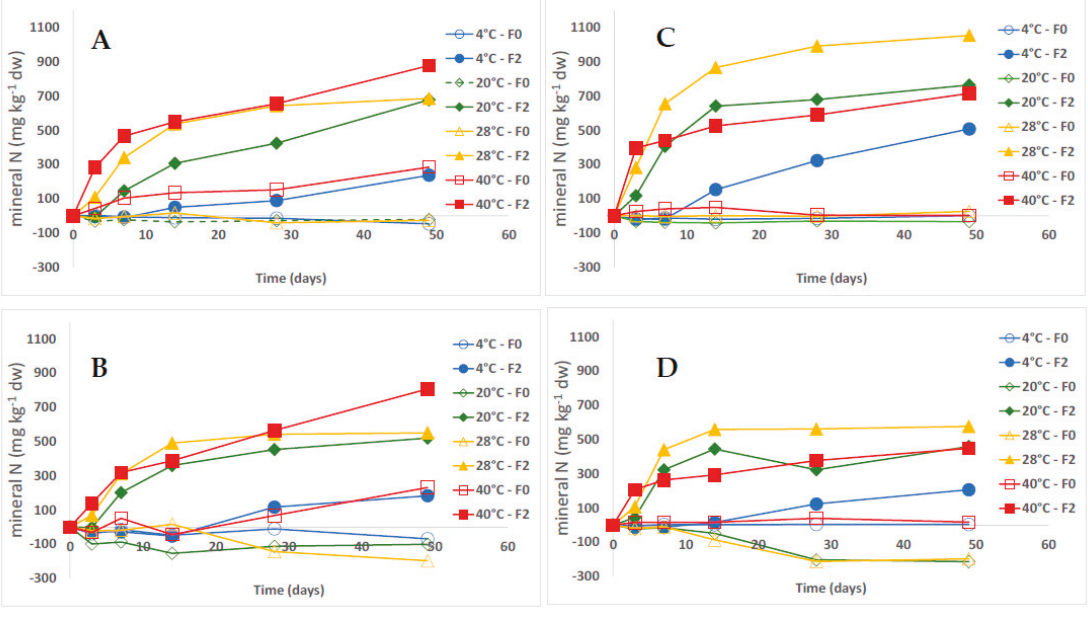

Figure 1. Influence of temperature on net N mineralization at −10 kPa water matric suction, for (**A**) GM1, (**B**) GM2, (**C**) GM3, and (**D**) GM4, with fertilizer F2 or without fertilizer (F0).

Humidity significantly controlled the mineral N content (Table S1, Figure 2), as well as NH_4^+ and NO_3^- contents (Figures S3 and S4, respectively), in the four growing media, but less markedly so than temperature. We observed similar trends, with a decreasing mineral N content between 28 and 49 days of incubation. GM1 showed the best mineral N content at −3.2 kPa (the highest humidity rate) and the lowest one at −31.6 kPa (the lowest humidity rate), whereas GM2 presented its best mineral N content at −31.6 kPa. GM3 showed very contrasted mineral N dynamics from 0 to 28 days, and finally reached the same level of mineral N content after 49 days of incubation whatever the humidity level (Figure 2). GM4 showed the highest contrasts between humidity levels, with −31.6 kPa giving the highest mineral N content and −3.2 kPa giving the lowest one (712 mg N·kg^{-1} dw GM

and 378 mg N·kg^{-1} dw growing media, respectively, Figure 2; Table S5). In the absence of fertilization, GM1 provided mineral N at −3.2 kPa (151 mg N·kg^{-1} dw growing media), while GM2 provided a similar content at −31.6 kPa, indicating that humidity did not drive the mineral N content in the same way as in GM1. In GM4, we observed a strong organization of the mineral N content, with no significant effect of humidity (Figure 2, Table S5).

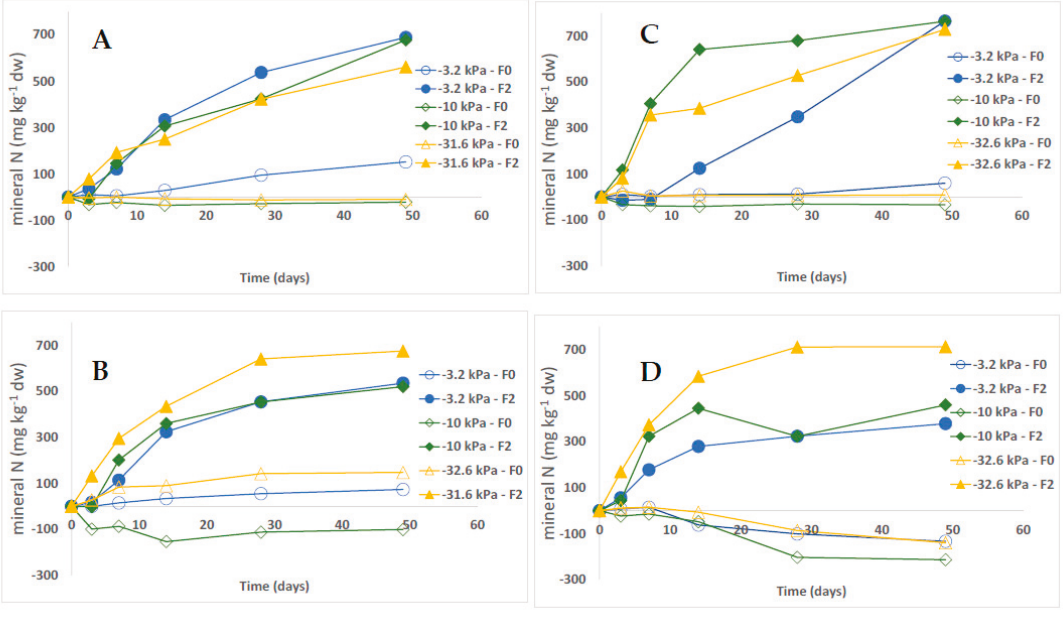

Figure 2. Influence of water matric suction on net N mineralization at 20 °C, for (**A**) GM1, (**B**) GM2, (**C**) GM3, and (**D**) GM4, with fertilizer F2 or without fertilizer (F0).

3.2. Fertilizer Mineralization

An increase in temperature from 4 °C induced an increase in the percentage of fertilizer N mineralized (Table 3), with slightly contrasting results depending on the growing medium, the fertilizer type, and humidity leading to 20, 28, or 40 °C with the highest percentage mineralization of the applied fertilizer N. Whatever the factor, F2 mineralized faster than F1 (10.7–69.2% vs. 14.7–71.3%). GM1 and GM4 reached the highest percentage of fertilizer mineralization at −31.6 kPa, but at different temperatures (40 °C and 20 °C, respectively). GM2 and GM3 reached the highest percentage of fertilizer mineralization at −10 kPa and 28 °C.

Table 3. Total mineralized fertilizer F1 and F2 as a percentage of applied N on day 49 (i.e., at the end of the experiment) (n = 3, SD = standard deviation).

		GM1				GM2				GM3				GM4			
		F1		F2		F1		F2		F1		F2		F1		F2	
suction (kPa)	T	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
−3.2	4	10.7	1.2	14.7	1.3	31.7	2.7	36.5	3.3	20.3	1.4	35.0	0.5	14.5	0.8	35.0	8.2
	20	28.2	0.9	43.1	3.7	32.3	1.6	42.5	0.6	29.2	2.7	37.5	0.5	26.1	1.4	43.0	2.5
	28	25.0	0.3	42.7	0.7	24.2	1.1	39.9	0.6	32.7	1.4	40.0	1.3	39.7	0.8	27.8	1.4
	40	30.9	1.0	28.8	0.8	17.0	1.8	29.7	0.9	22.3	1.1	31.0	0.8	38.1	1.6	45.6	1.4
−10	4	22.3	1.8	22.6	1.9	18.5	0.8	23.1	1.4	17.1	3.5	25.2	0.7	12.7	1.6	17.0	1.2
	20	44.9	0.9	55.7	1.1	56.9	1.2	57.0	0.3	36.2	0.7	39.7	1.3	46.2	1.4	56.3	0.6
	28	45.2	0.4	56.6	0.4	52.5	0.2	68.5	0.4	32.4	2.2	51.2	0.3	44.2	2.6	64.7	0.3
	40	39.1	1.6	47.1	0.8	42.5	0.4	52.7	0.3	26.8	2.0	35.4	1.0	28.3	1.0	36.2	1.4
−31.6	4	19.1	2.4	21.1	0.4	16.6	3.3	32.1	2.7	13.8	0.8	17.8	0.5	26.2	2.0	34.3	0.7
	20	34.0	1.2	45.5	2.2	40.8	1.8	48.4	1.4	29.2	2.5	35.8	4.6	69.2	0.9	71.3	0.8
	28	50.5	1.2	57.4	1.5	29.2	1.1	35.6	1.3	36.9	4.8	43.5	0.5	59.5	1.0	61.8	0.5
	40	36.3	1.8	59.8	2.0	50.7	1.3	57.5	0.7	20.3	0.9	25.3	0.7	47.4	1.8	50.7	2.4

3.3. Relative Proportion of NO_3^- to Total Mineral N

We observed four patterns for the relative proportion of nitrate to total mineral N depending on the GM type, temperature, and humidity (Figure 3). GM1 was affected by a decrease in humidity (i.e., a suction decrease), with a weak influence of temperature. GM2 was affected mostly at 40 °C and in the driest and wettest conditions (−3.2 and −31.6 kPa, respectively). GM3 was affected by temperature but not by humidity; the ratio was almost the same for all temperatures. The very low values of the ratio revealed that NH_4^+ accumulated substantially in this growing medium type, especially at 4 °C. GM4 was the least affected growing medium, with a slow but linear decrease in the values of the ratio as humidity decreased. These decreases were constant, but more pronounced at 20 and 28 °C than at 40 and 4 °C.

3.4. Temperature and Humidity Action Laws

A temperature action law was established for all growing media, all humidity levels, and by combining fertilizers F1 and F2 (Figure 4A). The model (Equation (1)) fitted the observed data well. It was calibrated for all humidity levels taken together. Table 4 presents the calibrated parameters and statistical performances (RMSE, R^2). The lowest RMSE and the best R^2 corresponded to the model adjustment with humidity at −10 kPa (Figure 4B).

The humidity action law f(H) is presented in Figure 5, combining fertilizers F1 and F2. Different patterns were observed depending on the growing medium, and they also changed according to temperature. At 28 °C, f(H) presented less variation for all growing media, with values around 1 in most cases (Figure 5B). This was almost the same at 40 °C, except in GM4 (Figure 5C). However, strong variations of f(H) were observed at 4 °C, except in GM1 where it was around 1 whatever the H-to-Hcc ratio (Figure 5A).

No correlation was found between the amount of mineralized N and the humidity level whatever the growing medium, but a relationship was established between the ratio of NO_3^-N to total mineral N and the humidity content H (Figure 6). The ratio first increased with increasing H, whatever the temperature and considering all growing media, with a breakpoint of the slope when H reached 0.46 *v/v*, and a plateau thereafter. The segmented regression gave a very good correlation coefficient (R^2 = 0.83, $p < 0.001$).

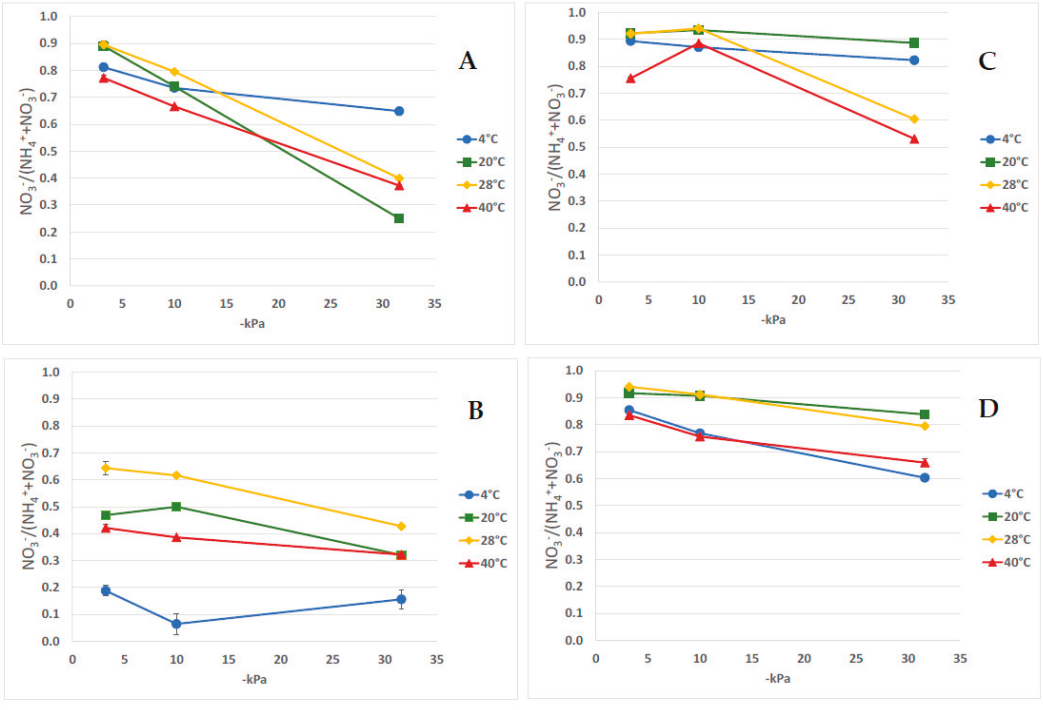

Figure 3. Relative proportion of nitrate to total mineral N (ratio: NO_3^- / total min N) depending on humidity and temperature in GM1 (**A**), GM2 (**B**), GM3 (**C**), and GM4 (**D**) fertilized with F2. Bars represent standard deviations (n = 3).

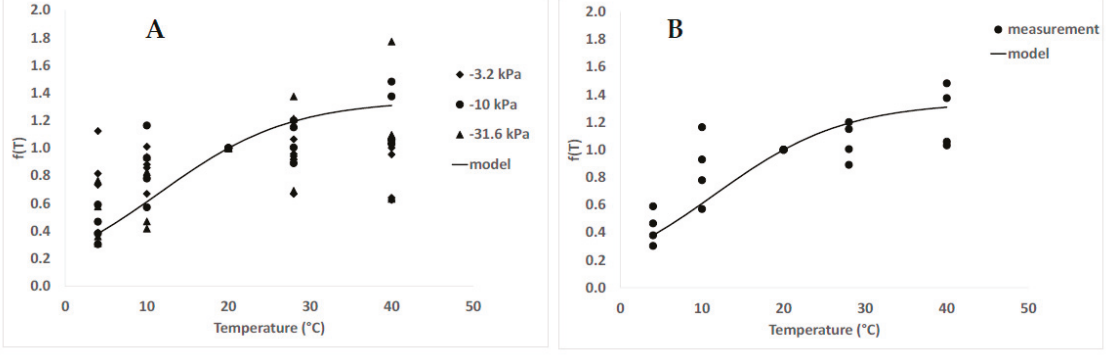

Figure 4. (**A**) Temperature response during N mineralization for all growing media incubated at −3.2, −10, and −31.6 kPa; (**B**) temperature response of all growing media incubated at −10 kPa. Data are the means of six replicates (i.e., three each with fertilizers F1 and F2). The temperature action law f(T) was calculated according to Equation (1).

Table 4. Adjusted parameters B and k and statistical performance of the temperature action law model.

	−3.2 kPa	−10 kPa	−31.6 kPa	All Suction Treatments
B	1.00	1.20	1.16	1.35
k	0.25	0.12	0.12	0.12
RMSE	0.21	0.14	0.26	0.24
R^2	0.30 ns	0.91 ***	0.70 ***	0.66 ***

*** $p < 0.001$, ns: nonsignificant.

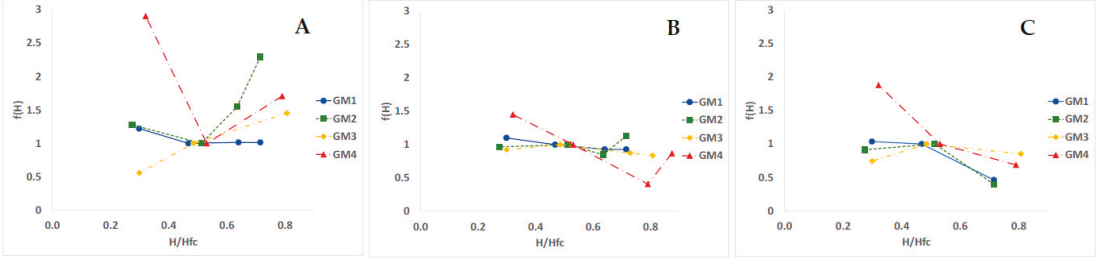

Figure 5. Water content action law at (**A**) 4 °C, (**B**) 28 °C, and (**C**) 40 °C for the four growing media. Data are the means of six replicates.

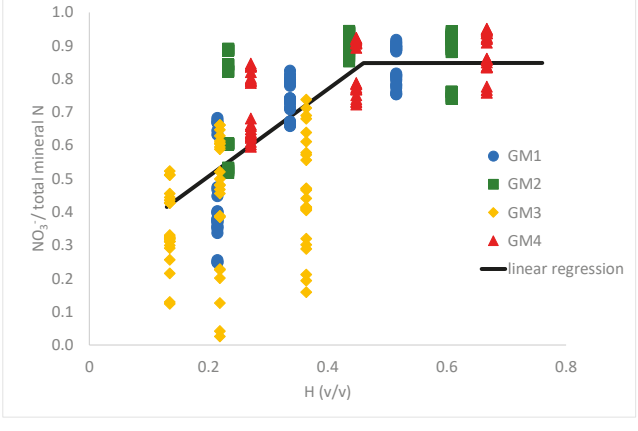

Figure 6. Effect of growing media volumetric water content on the average ratio of NO_3^- to total mineral N. The dataset compiles all temperatures.

4. Discussion

In soils, it has been widely demonstrated that N mineralization is particularly dependent on temperature [35], humidity [36], and texture [37,38]. Growing media are made of organically stable compounds that strongly limit biological activity in the absence of fertilization [10]. Compared to soils, growing media do not provide available nutrients, especially nitrogen, and they usually require mineral or organic fertilization for biological activation of microbes and plant growth. Studies on growing media are mostly focused on physical properties such as hydrodynamic parameters and aeration, or they lay the emphasis on chemical properties such as water pH, electrical conductivity, or cationic exchange capacity [39–42]. These properties are very important and easily monitored, but they weakly reflect the biological aspects and nutrient availability needed for organically fertilized crops. Growers attempt to maintain these properties steadily throughout cropping, but alteration of growing media compounds and root growth can modify them [43,44].

This study was focused on the monitoring of the N mineralization process—which depends on many bio-physicochemical interactions—in four commercial organic growing media with similar physicochemical properties (Table 1). We tested different temperature and moisture levels and compared the responses of the four growing media types when added with two different organic fertilizers and when no fertilizer was added.

The four growing media were relatively similar in terms of physicochemical properties even if they were made from different materials and for different cropping purposes. We rather focused the discussion on the biochemical and microbial aspects addressed in the body of the manuscript.

4.1. Growing Media Type

The organic growing media were biochemically stable and characterized by high C-to-nutrient ratios, much higher than the C:N:P stoichiometry of microbial biomass [45], which ranges between 42:6:1 and 60:7:1 according to [46] and [47], respectively. Due to their homeostatic stoichiometry, microbes are extremely constrained by the low resource availability in the growing medium; the growing media are considered as biologically inactive, such that microbes strongly respond to organic fertilization (Figure 1) [10]. Consequently, N immobilization can occur in growing media with a C:N ratio exceeding 30:1 due to microbial decomposition of available C—a process requiring N [48]. In the present study, the C:N ratios were similar: around 30 for three growing media types (GM1, GM2, and GM4), and twice higher in GM3 (Table 1). Thus, high N immobilization (i.e., a greater microbial demand) was expected in the four growing media, with the strongest effects in GM3. However, the time course of N mineralization did not confirm these expectations whatever the growing media or the temperature and humidity conditions when fertilizer was added. Even so, GM3 presented the best performances in terms of mineral N release (Figure 1C). Net N immobilization (i.e., negative net N mineralization rates due to gross N organization higher than gross N mineralization) occurred and was only observed in the absence of fertilization and in all growing media; it was the highest in GM2 and GM4, not in GM3 as expected (Figure 1). Net N immobilization was only observed in growing media that already contained mineral N and plant-based compost (i.e., GM2 and GM4). Consequently, its intensity appeared to be limited by the very low initial mineral N content of GM3. Significant N mineralization occurred at 40 °C in the absence of fertilization. However, we failed to distinguish whether it resulted from the activation of microbes mineralizing growing media compounds or from dead cells, since this temperature can be critical for some microbial populations such as nitrifiers [49] (Supplementary Materials Table S6). These results could be confirmed by decreased N mineralization rates at this temperature in the fertilized treatments, but further investigations are required. N immobilization occurred in all four commercial growing media, especially because they received a compost fraction during formulation that produced mineral nitrogen before they were used. However, this immobilization effect was easily outperformed by organic fertilization. On the contrary, the raw materials showed no such immobilization effect and even no biological activity or very weak activity in the absence of organic fertilization [10].

4.2. Fertilizer Type

F1 and F2 were commercial animal-based and plant-based fertilizers, respectively. Due to confidentiality rules, the fertilizer compositions were unavailable, but we analyzed them for pH, elemental composition, and C:N:P stoichiometry (Table 2). We detected significant differences between the two organic fertilizers (Table S1). However, the patterns obtained with either fertilizer were very close, and we only showed curves of unfertilized versus F2-fertilized growing media to clarify illustrations (Figures 1 and 2). We previously showed that the huge C:N and C:P ratios of different growing media were the most important drivers of organic fertilizer mineralization and microbial activity, and they constrained the autochthonous microbial communities through C and nutrient availability [10]. The results of the current study confirm these effects. The C:N ratios of the growing media

indeed largely overcame (Table 1) the different C:N ratios of the two fertilizers, and this explained the small difference in their N mineralization response. The C:N ratio of an organic fertilizer is usually considered as a good predictor of the N mineralization or immobilization balance following fertilizer incorporation in soil [50,51]. However, in growing media where nutrient availability is low, fertilizers have a smaller impact than growing media on N mineralization, and this raises the question of the importance of growing media formulation. Granular fertilizers present similar patterns of nutrient release, but other organic fertilizers such as raw or thermally treated horn can have a huge impact on the control of nitrogen release, while remaining driven by the unbalanced stoichiometry of the growing media types [10]. Since C cannot be decoupled from the N and P cycles [10], we hypothesized that the lower P content in F2 (Table 2) may also have increased the microbial mining effect [52] to access P compared to F1, leading to overall faster fertilizer mineralization. Further investigations on coupling microbial C, N, and P functions [10] would be necessary to confirm this assumption.

We also expressed total mineral N at the end of incubation as a percentage of N fertilizer addition and detailed the patterns according to temperature and humidity, as a function of the two fertilizers (F1 and F2, Table 3). However, these values were not cumulative because we only measured the mineral N content at different timepoints of incubation. Thus, at the end of the experiment, the mineral N content did not express the total mineral N produced from the organic fertilizers, but the total mineral N content as a fraction of fertilizer-added N. Only a minimum value of what was really mineralized from the organic fertilizers was expressed. We cannot rule out that some of the mineral N came from (i) microbial turnover [53], especially at 40 °C, which can be critical for some microbial populations, and (ii) growing media biodegradation. However, growing media biodegradation is believed to be very low in growing media and would necessitate specific C inputs to trigger a priming effect [54].

4.3. Temperature Effects and Action Law

Temperature influences transformation rates through the responses of microorganisms and enzymatic activities. We tested four temperatures frequently met during plant growth in horticulture. Specifically, 20 and 28 °C are classical temperatures in the greenhouse and supposed to be optimal, whereas 4 and 40 °C are extreme temperatures affecting nutrient availability by slowing down microbial activity; 40 °C potentially affects the microbes themselves. We observed maximum nitrification at 28 °C, close to the soil optimum of 30 °C [55]. However, 40 °C sometimes gave the best mineral N content depending on the growing media, suggesting that this temperature provided for the highest mineralization rates. Delving deeper into the ammonification and nitrification processes (Figure S2), this mineralization was not sustainable since the NH_4^+ content was higher (Figure S1) than at the other temperatures while the NO_3^- content decreased, indicating that nitrifiers were probably affected [55,56]. This unbalance between ammonification and nitrification was also analyzed by studying the relative proportion of NO_3^- content over total mineral N (Figure 3): 40 °C and mostly 4 °C consistently resulted in bad conditions, and even critical ones for GM3.

N mineralization increases exponentially within the range of temperatures met in farmed soils (0–40 °C) and can be successfully modeled with numerous functions [57]. This study showed that the formalism of the temperature action law proposed by the STICS model for soils is also adapted to organic growing media mineralization. This is a first modeling of the effect of temperature on N mineralization rates applied to growing media. Modeling performance was best when using incubations were run at −10 kPa, as this modality was most adequate to reveal fertilizer N mineralization. A common way to express temperature sensitivity is to use the Q10 function. A Q10 of 2, for example, means that the rate of a particular process doubles when the temperature increases by 10 °C [58]. Using 20 °C as the reference temperature, the Q10 value of the growing media

was 1.13, lower than the average Q10 value of arable soils, but within the large range of values reported in the literature (from 0.55 to 11.9 [59]).

4.4. Humidity Effect and Action Law

Matric suction (ψ) was used to study the effect of humidity (H). However, due to their composition, the growing media had a specific H at a given ψ value, and this made it more difficult to analyze the results (Table 5). H might have been a better parameter choice in the experimental design, even though ψ affected organic nitrogen mineralization statistically. The choice of ψ made agronomic sense in terms of water-filled porosity; a ψ of −1 kPa is equivalent to the retention capacity of a growing media (H*fc*), a ψ of −10 kPa corresponds to the temporary wilting point, and a ψ of −100 kPa corresponds to the permanent wilting point [60].

Table 5. Volumetric water content (*v/v*) and water-filled pore space (WFPS, %) values according to the growing media and ψ modalities.

	GM1	GM2	GM3	GM4
θ at 0 kPa (*v/v*)	0.891	0.883	0.920	0.880
θ at −3.2 kPa (*v/v*)	0.515	0.608	0.364	0.667
θ at −10 kPa (*v/v*)	0.337	0.436	0.219	0.448
θ at −31.6 kPa (*v/v*)	0.215	0.233	0.135	0.271
WFPS −3.2 kPa (%)	57.8	68.9	39.6	75.8
WFPS −10 kPa (%)	37.8	49.4	23.8	50.9
WFPS −31.6 kPa (%)	24.1	26.4	14.7	30.8

Water is necessary for microbial activity, and its content has to be balanced with the oxygen required for root and microbial respiration [61]. Aerobic microbial activity is optimal at a humidity volumetric content ranging between 50% and 70% of the water holding capacity (WHC) [62,63] corresponding to water and oxygen availability in good equilibrium. Other studies estimate the maximum microbial activity (respiration and nitrification) in soils around 60% of the total porosity occupied by water (WFPS, "water-filled pore space") [64,65]. A major influence of the water content has been shown on microorganism activity in different organic growing media, with higher microbial respiration at 63% WFPS compared to 73% and 83% WFPS [66]. According to the theory mentioned above, fertilizer mineralization should be optimal around −3.2 kPa, as in GM1 and GM3. Yet, the mineralization rates of GM2 and GM4 were highest at −31.6 kPa, i.e., the driest conditions of this study. As a result, oxygen could be more important than water availability. Suction of 31.6 kPa is supposed to be too low for plant survival and growth. The ratio of NO_3^- to (NH_4^+ + NO_3^-) as a function of growing media humidity showed a similar trend to that observed for soils, regardless of temperature [67], i.e., a progressive increase with humidity up to 0.46 *v/v* (corresponding to a WFPS of 50–52% depending on the growing medium), followed by a plateau up to 0.67 *v/v* (corresponding to a WFPS of 73–76% depending on the growing media) (Figure 6). Thus, the optimal humidity for nitrification in the growing media was similar to that of soils. However, we failed to establish a humidity action law on the basis of experimental data. Indeed, at a given temperature each growing media had a specific response curve to H/H*fc* (Figure 5); when H/H*fc* increased, f(H) decreased for some growing media and then increased with increasing H/H*fc* values. For other growing media, f(H) progressively increased, or even reached a plateau. At another temperature, the growing media behaved again in a different way, making it difficult to establish a generic humidity action law.

Thus, an interaction between growing media temperature and moisture did occur, and this process is commonly encountered in soils [68,69]. Several humidity action laws have been established for arable soils and expressed as a function of the soil water content, the WFPS, or the matric potential. The need to improve the representation of this relationship in models has been highlighted. The authors of [70] presented a data-driven analysis of soil humidity–respiration relations based on 90 soils. They showed how the relationship

between soil heterotrophic respiration and different soil humidity levels is consistently affected by soil properties. On the basis of the proportional response of soil respiration (PRSR) related to a 0.01 increase in soil humidity as the central unit for analysis, they found little or no effect of soil properties on the PRSR in organic soils (i.e., a soil organic content higher than 300 $g \cdot kg^{-1}$). Thus, due to their nature and their specific behavior, the formalisms known today are not adapted to growing media; research work has to be developed in this area.

4.5. Management Implications

In terms of professional applications, growing media and fertilizer type need to be considered at the same time to determine the rate of N release adequate for plant growth as precisely as possible. Depending on plant requirements, professionals could select a growing media according to its use (but physical characteristics would need to be checked) and use an organic fertilizer to provide nutrients. Our results tend to show that GM4 supplied slow nutrient release that quickly reached a plateau at the lowest level in this study, whereas GM3 supplied slowly the highest nutrient level. These results also indicate that detecting the plateau would have required a longer incubation time than 49 days for GM3 (Figure 1). Moreover, all four growing media presented a linear increase in total mineral N at 40 °C indicating that extreme temperature can cause fast N release with potential N loss if not synchronized with plant needs, whereas the process would be best controlled at a temperature maintained between 20 and 28 °C. Peat is a reference material in soilless production. Nevertheless, its use is questioned because exploiting peatland implies depleting a recognized carbon (C) sink. Thus, efforts and the immediate need for peat reduction in horticulture are a strong challenge for the future. Growing media tested in this study were peat-based, but combined with other alternative materials. We were able to show here that growing media with only 50% or 60% peat (GM2 and GM4, respectively) mineralized as much fertilizer as GM1 (80% of peat). The results are, therefore, encouraging and demonstrate that it is possible to progressively free ourselves from peat.

Mineralized F2 induced a high N release and appeared to be interesting for short- or medium-term crop cultivation, while the fertilizer dose could be defined accordingly. The temperature and ψ conditions that promoted the highest N fertilizer mineralization were 28 °C and −10 kPa (for GM2 and GM3) or −31.6 kPa (for GM1 and GM4). To go further, growing media–fertilizer combinations should be tested in actual growth experiments since the plant uptake could reveal inadequate growing media–fertilizer combinations or too limiting ones in terms of nutrient supply, as suspected with GM4. Indeed, synchronizing nutrient supply with the nutrient requirements of plants is a major issue for increasing nutrient use efficiency. While nitrogen is essential and often the most limiting element for plant growth, plants can be subjected to multiple nutrient limitations, especially colimitation of N and P [71]. A depressive effect of organic versus mineral fertilization is frequently observed [72,73]. For example, higher ammonification over nitrification rates is the main explanation for the lower performances of organically grown basil plant because roots are exposed to high levels of NH_4^+ without supplying enough NO_3^- [74,75].

5. Conclusions

The N mineralization dynamics of two organic fertilizers in four growing media types at different temperature and humidity conditions showed a strong impact of the different treatments on NH_4^+ and NO_3^- release. Under optimal conditions of temperature (20 °C) and humidity (−10 kPa), 32% to 57% of the applied fertilizer was mineralized after 49 days depending on the growing media. These results constitute major food for thought on fertilizer application strategies during crop itineraries. The introduction of plants in the system will have an impact on the mineralization process, which we plan to study in the future. We attempted to adapt temperature and humidity action laws, whose formalisms are derived from work on soils, to growing media. We succeeded in describing the effect of temperature with an action law common to the four growing media, but the response of

the growing media to humidity greatly varied among growing media and in a temperature-dependent manner. Therefore, we failed to establish an action law for humidity, although a satisfactory relationship between nitrification and humidity was demonstrated. Research is needed to further investigate the effect of humidity and temperature-humidity interactions on the mineralization of organic N from fertilizers. In addition, the present results need to be refined using other growing media–fertilizer pairs. This work will allow for the short-term development of a prediction model of mineralization of organic N from fertilizers in soilless growing media production because such a model is lacking at present.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae8020152/s1: Figure S1. Influence of temperature on NH_4^+-N at −10 kPa water matric suction, for (A) GM1, (B) GM2, (C) GM3, and (D) GM4; Figure S2. Influence of temperature on NO_3^--N content at −10 kPa water matric suction, for (A) GM1, (B) GM2, (C) GM3, and (D) GM4; Figure S3. Influence of water matric suction on NH_4^+-N content at 20 °C, for (A) GM1, (B) GM2, (C) GM3, and (D) GM4; Figure S4. Influence of water matric suction on NO_3^--N content at 20 °C, for (A) GM1, (B) GM2, (C) GM3, and (D) GM4; Figure S5. NO_3^- to total N min ratio, depending on matric suction and temperature and with fertilizer F1, in (A) GM1, (B) GM2, (C) GM3, and (D) GM4. Bars represent standard deviation (n = 3); Table S1. Results of three-way repeated-measures ANOVA with growing media, fertilizer (Fert), temperature (Temp), and matric water suction (ψ) as between subject and time (t) after fertilizer addition as within subject, on total mineralized N, NH_4^+, and NO_3^- contents; Table S2. NO_3^-, NH_4^+, and total mineral N in GM1, depending on ψ, temperature, and fertilizers modalities (n = 3, F0 = without fertilizer); Table S3. NO_3^-, NH_4^+, and total mineral N in GM2, depending on ψ, temperature, and fertilizer modalities (n = 3, F0 = without fertilizer); Table S4. NO_3^-, NH_4^+, and total mineral N in GM3, depending on ψ, temperature, and fertilizer modalities (n = 3, F0 = without fertilizer); Table S5. NO_3^-, NH_4^+, and total mineral N in GM4, depending on ψ, temperature, and fertilizer modalities (n = 3, F0 = without fertilizer); Table S6. AmoA content (log nb_seq·g^{-1} dw growing media) at −3.2 and −31.6 kPa, and temperatures of 20 and 40 °C, during the 49 day incubation (n = 3).

Author Contributions: Conceptualization, S.B., M.V., P.C., R.G. and M.B.; methodology, S.B., M.V., P.C., R.G. and M.B.; validation, S.B., M.V., P.C., R.G., M.B. and S.R.; formal analysis, P.C., R.G., S.R., M.V. and M.B., investigation, P.C., R.G., S.R., M.V., S.B. and M.B.; writing—original draft preparation, P.C., R.G. and S.R.; writing—review and editing, P.C., R.G., S.R., M.V., L.P., S.B. and M.B.; visualization, P.C., R.G., S.R., M.V., L.P., S.B. and M.B.; supervision, P.C., R.G. and S.R.; project administration, S.B.; funding acquisition, S.B., M.V., P.C., R.G., M.B. and S.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by CASDAR, OptiFaz project, grant number 5746.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data sharing not applicable.

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

References

1. Burnett, S.E.; Mattson, N.S.; Williams, K.A. Substrates and fertilizers for organic container production of herbs, vegetables, and herbaceous ornamental plants grown in greenhouses in the United States. *Scientia Hortic.* **2016**, *208*, 111–119. [CrossRef]
2. Raviv, M.; Wallach, R.; Silber, A.; Bar-Tal, A. Substrates and their analysis. In *Hydroponic Production of Vegetables and Ornamentals*; Savvas, D., Passam, H., Eds.; Embryo Publications: Athens, Greece, 2002; pp. 25–102.
3. Barrett, G.E.; Alexander, P.D.; Robinson, J.S.; Bragg, N.C. Achieving environmentally sustainable growing media for soilless plant cultivation systems-A review. *Scientia Hortic.* **2016**, *21*, 220–234. [CrossRef]
4. Caldwell, B.A. Enzyme activities as a component of soil biodiversity: A review. *Pedobiologia* **2005**, *49*, 637–644. [CrossRef]
5. Treadwell, D.D.; Hochmuth, G.J.; Hochmuth, R.C.; Simonne, E.H.; Davis, L.L.; Laughlin, W.L.; Li, Y.; Olczyk, T.; Sprenkel, R.K.; Osborne, L.S. Nutrient management in organic greenhouse herb production: Where are we now? *Hortic. Technol.* **2007**, *17*, 461–466. [CrossRef]

6. Montagne, V.; Capiaux, H.; Barret, M.; Cannavo, P.; Charpentier, S.; Grosbellet, C.; Lebeau, T. Bacterial and fungal communities vary with the type of organic substrate: Implications for biocontrol of soilless crops. *Environ. Chem. Lett.* **2017**, *15*, 537–545. [CrossRef]
7. Montagne, V.; Charpentier, S.; Cannavo, P.; Capiaux, H.; Grosbellet, C.; Lebeau, T. Structure and activity of spontaneous fungal communities in organic substrates used for soilless crops. *Scientia Hortic.* **2015**, *192*, 148–157. [CrossRef]
8. Jack, A.L.H.; Rangarajan, A.; Culman, S.W.; Sooksa-Nguan, T.; Thies, J.E. Choice of organic amendments in tomato transplants has lasting effects on bacterial rhizosphere communities and crop performance in the field. *Appl. Soil Ecol.* **2011**, *48*, 94–101. [CrossRef]
9. Voogt, W.; Cuijpers, W.J.M.; de Visser, P.H.E.; van de Burgt, G.J.H.M.; van Winkel, A. Nutrient management in organic greenhouse production; Navigation between constraint. *Acta Hortic.* **2011**, *915*, 75–82. [CrossRef]
10. Paillat, L.; Cannavo, P.; Barraud, F.; Huché-Thélier, L.; Guénon, R. Growing Medium Type Acts Organic Fertilizer Mineralization and CNPS Microbial Enzyme Activities. *Agronomy* **2020**, *10*, 1955. [CrossRef]
11. Zhang, J.; Wang, J.; Müller, C.; Cai, Z. Ecological and practical significances of crop species preferential N uptake matching with soil N dynamics. *Soil Biol. Biochem.* **2016**, *103*, 63–70. [CrossRef]
12. Houlton, B.Z.; Sigman, D.M.; Schuur, E.A.G.; Hedin, L.O. A climate-driven switch in plant nitrogen acquisition within tropical forest communities. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 8902–8906. [CrossRef] [PubMed]
13. Hawkins, B.J.; Robbins, S. pH affects ammonium, nitrate and proton fluxes in the apical region of conifer and soybean roots. *Physiol. Plant.* **2010**, *138*, 238–247. [CrossRef] [PubMed]
14. Schimel, J.P.; Jackson, L.E.; Firestone, M.K. Spatial and temporal effects on plant-microbial competition for inorganic nitrogen in a California annual grassland. *Soil Biol. Biochem.* **1989**, *21*, 1059–1066. [CrossRef]
15. Benton Jones, J. *Plant Nutrition Manual*; CRC Press: Boca Raton, FL, USA, 1998.
16. Gravel, V.; Dorais, M.; Pepin, S. Soil salinization of organically-grown greenhouse tomato. In *Volume 1 Organic Crop Production, Proceedings of the 17th IFOAM Organic World Congress, Organic is Life: Knowledge for tomorrow, Third Scientific Conference of the International Society of Organic Agriculture Research (ISOFAR), Seoul, South Korea, 28 September–1 October 2011*; International Society of Organic Agriculture Research (ISOFAR): Rennes, France, 2011; pp. 436–440.
17. Gaskell, M.; Smith, R. Nitrogen Sources for Organic Vegetable Crops. *Hortic. Technol.* **2007**, *17*, 431–441. [CrossRef]
18. Casadesus, J.; Caceres, R.; Marfa, O. Dynamics of CO_2 efflux from the substrate root system of container-grown plants associated with irrigation cycles. *Plant Soil* **2007**, *300*, 71–82. [CrossRef]
19. Handreck, K.A. Rapid assessment of the rate of nitrogen immobilisation in organic components of potting media: I. Method development. *Commun. Soil Sci. Plant Anal.* **1992**, *23*, 201–215. [CrossRef]
20. Harris, C.N.; Dickson, R.W.; Fisher, P.R.; Jackson, B.E.; Poleatewich, A.M. Evaluating Peat Substrates Amended with Pine Wood Fiber for Nitrogen Immobilization and Effects on Plant Performance with Container-grown Petunia. *Hortic. Technol.* **2020**, *30*, 107–116. [CrossRef]
21. Boyer, C.R.; Fain, G.B.; Gilliam, C.H.; Gallagher, T.V.; Torbert, H.A.; Sibley, J. Description of cleanchip residual forest harvest and its availability for horticultural uses in the southeastern United States. *Hortic. Technol.* **2012**, *22*, 381–387.
22. AFNOR Amendements du Sol et Supports de Culture—Détermination des Propriétés Physiques—Masse Volumique Apparente Sèche, Volume D'air, Volume d'eau, Valeur de Rétraction et Porosité Totale. NF EN 13040. U44-307. 2000. Available online: https://www.boutique.afnor.org/normes (accessed on 10 March 2017).
23. AFNOR Amendements du Sol et Supports de Culture—Détermination du pH. NF EN 13037. U44-308. 2000. Available online: https://www.boutique.afnor.org/normes (accessed on 10 March 2017).
24. AFNOR Amendements du Sol et Supports de Culture—Détermination de la Conductivité Electrique. NF EN 13038. U44-309. 2000. Available online: https://www.boutique.afnor.org/normes (accessed on 10 March 2017).
25. AFNOR Qualité du Sol—Détermination de la Teneur Totale en Azote par Combustion Sèche. NF ISO 13878. X31-418. 1998. Available online: https://www.boutique.afnor.org/normes (accessed on 10 March 2017).
26. AFNOR Amendements du Sol et Supports de Culture—Détermination de la Teneur en Matieres Organiques. NF EN 13039. U44-304. 2000. Available online: https://www.boutique.afnor.org/normes (accessed on 10 March 2017).
27. AFNOR Amendements du sol et Supports de Culture—Extraction D'éléments Solubles Dans L'eau Régale NF EN 13650. 2002. Available online: https://www.boutique.afnor.org/normes (accessed on 10 March 2017).
28. AFNOR Qualité de L'eau-Dosage D'éléments Choisis Par Spectroscopie D'émission Optique Avec Plasma Induit Par Haute Fréquence (ICP-OES). NF EN ISO 11885. 2009. Available online: https://www.boutique.afnor.org/normes (accessed on 10 March 2017).
29. De Boodt, M.; Verdonck, O.; Cappaert, I. Method for measuring the water-release curve of organic substrates. *Acta Hortic.* **1974**, *37*, 2054–2062. [CrossRef]
30. Richards, L.A. A pressure-membrane extraction apparatus for soil solution. *Soil Sci.* **1941**, *51*, 377–386. [CrossRef]
31. AFNOR Qualité du Sol— Détermination De La Caractéristique De La Rétention en eau-Méthodes De Laboratoire. NF ISO 11274. 2019. Available online: https://www.boutique.afnor.org/normes (accessed on 10 March 2017).
32. Felske, A.; Akkermans, A.D.L. Spatial homogeneity of abundant bacterial 16S rRNA molecules in grassland soils. *Microb. Ecol.* **1998**, *36*, 31–36. [CrossRef] [PubMed]

33. Rotthauwe, J.H.; Witzel, K.P.; Liesack, W. The ammonia monooxygenase structural gene amoA as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl. Environ. Microbiol.* **1997**, *63*, 4704–4712. [CrossRef] [PubMed]
34. Brisson, N.; Launay, M.; Mary, B.; Beaudoin, N. *Conceptual Basis, Formalisations and Parameterization of the STICS Crop Model*; Editions Quae: Versailles, France, 2008; p. 301.
35. Griffins, T.S.; Honeycutt, C.W.; He, Z. Effects of temperature, soil water status, and soil type on swine slurry nitrogen transformations. *Biol. Fertil. Soils.* **2001**, *36*, 442–446.
36. Drury, K.F.; Zhang, T.Q.; Kay, B.D. The non-limiting and least limiting water ranges for soil nitrogen mineralization. *Soil Sci. Soc. Am. J.* **2003**, *67*, 1388–1404. [CrossRef]
37. Sørensen, P.; Jensen, E.S. Mineralization of carbon and nitrogen from fresh and anaerobically stored sheep manure in soils with different texture and water contents. *Biol. Fertil. Soils* **1995**, *19*, 29–35. [CrossRef]
38. Thomsen, I.K.; Schoenning, P.; Christensen, B.T. Mineralization of 15N-labelled sheep manure in soils of different texture and water contents. *Biol. Fertil. Soils* **2003**, *37*, 295–301. [CrossRef]
39. Depardieu, C.; Prémont, V.; Boily, C.; Caron, J. Sawdust and bark-based substrates for soilless strawberry production: Irrigation and electrical conductivity management. *PLoS ONE* **2016**, *11*, e0154104. [CrossRef]
40. Savvas, D.; Gruda, N. Application of soilless culture technologies in the modern greenhouse industry—A review. *Eur. J. Hortic. Sci.* **2018**, *83*, 280–293. [CrossRef]
41. Schulker, B.A.; Jackson, B.E.; Fonteno, W.C.; Heitman, J.L.; Albano, J.P. Comparison of Water Capture Efficiency through Two Irrigation Techniques of Three Common Greenhouse Soilless Substrate Components. *Agronomy* **2020**, *10*, 1389. [CrossRef]
42. Durand, S.; Jackson, B.E.; Fonteno, W.C.; Michel, J.-C. The Use of Wood Fiber for Reducing Risks of Hydrophobicity in Peat-Based Substrates. *Agronomy* **2021**, *11*, 907. [CrossRef]
43. Naasz, R.; Michel, J.C.; Charpentier, S. Water repellency of organic growing media related to hysteretic water retention properties. *Eur. J. Soil Sci.* **2008**, *59*, 156–165. [CrossRef]
44. Cannavo, P.; Michel, J.C. Effect of growing medium particle size on spatial root distribution: Consequences on the evolution of hydraulic properties in the medium. *Sci. Hortic.* **2013**, *151*, 11–21. [CrossRef]
45. Grunert, O.; Reheul, D.; Van Labeke, M.; Perneel, M.; Hernandez-Sanabria, E.; Vlaeminck, S.E.; Boon, N. Growing media constituents determine the microbial nitrogen conversions in organic growing media for horticulture. *Microb. Biotechnol.* **2016**, *9*, 389–399. [CrossRef] [PubMed]
46. Cleveland, C.C.; Liptzin, D. C: N: P stoichiometry in soil: Is there a "Redfield ratio" for the microbial biomass? *Biogeochemistry* **2007**, *85*, 235–252. [CrossRef]
47. Xu, X.; Thornton, P.E.; Post, W.M. A global analysis of soil microbial biomass carbon, nitrogen and phosphorus in terrestrial ecosystems. *Glob. Ecol. Biogeogr.* **2013**, *22*, 737–749. [CrossRef]
48. Bunt, A.C. Some physical and chemical characteristics of loam-less pot-plant substrates and their relation to plant growth. In Proceedings of the Ist Symposium on Artificial Media in Horticulture, Gent, Belgium, 10 September 1973; Acta Horticulturae. International Society for Horticultural Science (ISHS): Leuven, Belgium, 1973; Volume 37, pp. 1954–1965.
49. Taylor, A.; Giguere, A.; Zoebelein, C.; Myrold, D.D.; Bottomley, P.J. Modeling of soil nitrification responses to temperature reveals thermodynamic differences between ammonia-oxidizing activity of archaea and bacteria. *ISME J.* **2017**, *11*, 896–908. [CrossRef]
50. Enwezor, W.O. The mineralization of nitrogen and phosphorus in organic materials of varying C: N and C: P ratios. *Plant Soil* **1976**, *44*, 237–240. [CrossRef]
51. Nannipieri, P.; Paul, E.A. The chemical and functional characterization of soil N and its biotic components. *Soil Biol. Biochem.* **2009**, *41*, 2357–2369. [CrossRef]
52. Allison, S.D.; Weintraub, M.N.; Gartner, T.B.; Waldrop, M.P. Evolutionary-economic principles as regulators of soil enzyme production and ecosystem function. In *Soil Enzymology, Soil Biology*; Shukla, G., Varma, A., Eds.; Springer: Berlin/Heidelberg, Germany, 2011; pp. 229–243.
53. Brookes, P. The soil microbial biomass: Concept, measurement and applications in soil ecosystem research. *Microbes Environ.* **2001**, *16*, 131–140. [CrossRef]
54. Hamer, U.; Marschner, B. Priming effects of sugars, amino acids, organic acids and catechol on the mineralization of lignin and peat. *J. Plant Nutr. Soil Sci.* **2002**, *165*, 261–268. [CrossRef]
55. Lang, H.J.; Elliott, G.C. Influence of Ammonium: Nitrate Ratio and Nitrogen Concentration on Nitrification Activity in Soilless Potting Media. *J. Am. Soc. Hortic. Sci.* **1991**, *116*, 642–645. [CrossRef]
56. Fisk, L.M.; Maccarone, L.D.; Barton, L.; Murphy, D.V. Nitrapyrin decreased nitrification of nitrogen released from soil organic matter but not amoA gene abundance at high soil temperature. *Soil Biol. Biochem.* **2015**, *88*, 214–223. [CrossRef]
57. Dessureault-Rompré, J.; Zebarth, B.J.; Georgallas, A.; Burton, D.L.; Grant, C.A.; Drury, C.F. Temperature dependence of soil nitrogen mineralization rate: Comparison of mathematical models, reference temperatures and origin of the soils. *Geodermatology* **2010**, *157*, 97–108. [CrossRef]
58. Kirschbaum, M.U.F. The temperature-dependence of soil organic matter decomposition, and the effect of global warming on soil organic C storage. *Soil Biol. Biochem.* **1995**, *27*, 753–760. [CrossRef]
59. Liu, Y.; Wang, C.; He, N.; Wen, X.; Gao, Y.; Li, S.; Niu, S.; Butterbach-Bahl, K.; Luo, Y.; Yu, G. A global synthesis of the rate and temperature sensitivity of soil nitrogen mineralization: Latitudinal patterns and mechanisms. *Glob. Chang. Biol.* **2017**, *23*, 455–464. [CrossRef]

60. Kämäräinen, A.; Simojoki, A.; Lindén, L.; Jokinen, K.; Silvan, N. Physical growing media characteristics of *Sphagnum* biomass dominated by *Sphagnum fuscum* (Schimp.) Klinggr. *Mires Peat* **2018**, *21*, 1–16.
61. Tiemann, L.K.; Billings, S.A. Changes in variability of soil moisture alter microbial community C and N resource use. *Soil Biol. Biochem.* **2011**, *43*, 1837–1847. [CrossRef]
62. Harris, R.F. Effect of water potential on microbial growth and activity. In *Water Potential Relations in Soil Microbiology*; Parr, J.F., Gardner, W.R., Elliott, L.F., Eds.; American Society of Agronomy: Madison, WI, USA, 1981; pp. 23–95.
63. Devêvre, O.C.; Horwáth, W.R. Decomposition of rice straw and microbial carbon use efficiency under different soil temperatures and moistures. *Soil Biol. Biochem.* **2000**, *32*, 1773–1785. [CrossRef]
64. Linn, D.M.; Doran, J.W. Effect of water-filled pore space on carbon dioxide and nitrous oxide production in tilled and non-tilled soils. *Soil Sci. Soc. Am. J.* **1984**, *48*, 1267–1272. [CrossRef]
65. Robertson, G.P.; Groffman, P.M. Nitrogen transformations. In *Soil Microbiology, Ecology, and Biochemistry*, 4th ed.; Paul, E.A., Ed.; Academic Press: Cambridge, MA, USA, 2015; pp. 341–364.
66. Van Gerrewey, T.; Ameloot, N.; Navarrete, O.; Vandecruys, M.; Perneel, M.; Boon, N.; Geelen, D. Microbial activity in peat-reduced plant growing media: Identifying influential growing medium constituents and physicochemical properties using fractional factorial design of experiments. *J. Clean. Prod.* **2020**, *256*, 120323. [CrossRef]
67. Lellei-Kovács, E.; Kovács-Láng, E.; Botta-Dukát, Z.; Kalapos, T.; Emmett, B.; Beier, C. Thresholds and interactive effects of soil moisture on the temperature response of soil respiration. *Eur. J. Soil Biol.* **2011**, *47*, 247–255. [CrossRef]
68. Zhou, W.P.; Shen, W.J.; Li, Y.E.; Hui, D.F. Interactive effects of temperature and moisture on composition of the soil microbial community. *Eur. J. Soil Sci.* **2017**, *68*, 909–918. [CrossRef]
69. Rodrigo, A.; Recous, S.; Neel, C.; Mary, B. Modelling temperature and moisture effects on C–N transformations in soils: Comparison of nine models. *Ecol. Model.* **1997**, *102*, 325–339. [CrossRef]
70. Moyano, F.E.; Vasilyeva, N.; Bouckaert, L.; Cook, F.; Craine, F.; Curiel Yuste, J.; Don, A.; Epron, D.; Formanek, P.; Franzluebbers, A.; et al. The moisture response of soil heterotrophic respiration: Interaction with soil properties. *Biogeosciences* **2012**, *9*, 1173–1182. [CrossRef]
71. Reich, M. Chapter 4-The significance of nutrient interactions for crop yield and nutrient use efficiency. In *Plant Macronutrient Use Efficiency*; Hossain, M.A., Kamiya, T., Burritt, D.J., Tran, L.-S.P., Fujiwara, T., Eds.; Academic Press: Cambridge, MA, USA, 2017; pp. 65–82.
72. Succop, C.E.; Newman, S.E. Organic Fertilization of Fresh Market Sweet Basil in a Greenhouse. *Hortic. Technol.* **2004**, *14*, 235–239. [CrossRef]
73. Bufalo, J.; Cantrell, C.L.; Astatkie, T.; Zheljazkov, V.D.; Gawde, A.; Boaro, C.S.F. Organic versus conventional fertilization effects on sweet basil (Ocimum basilicum L.) growth in a greenhouse system. *Ind. Crop. Prod.* **2015**, *74*, 249–254. [CrossRef]
74. Kiferle, C.; Maggini, R.; Pardossi, A. Influence of nitrogen nutrition on growth and accumulation of rosmarinic acid in sweet basil (Ocimum basilicum L.) grown in hydroponic culture. *Aust. J. Crop. Sci.* **2013**, *7*, 321–327.
75. Frerichs, C.; Daum, D.; Pacholski, A.S. Ammonia and Ammonium Exposure of Basil (*Ocimum basilicum L.*) Growing in an Organically Fertilized Peat Substrate and Strategies to Mitigate Related Harmful Impacts on Plant Growth. *Front. Plant Sci.* **2020**, *10*, 1696. [CrossRef]

horticulturae

MDPI

Article

Basil as Secondary Crop in Cascade Hydroponics: Exploring Salinity Tolerance Limits in Terms of Growth, Amino Acid Profile, and Nutrient Composition

Denisa Avdouli [1], Johannes F. J. Max [2], Nikolaos Katsoulas [1] and Efi Levizou [1,*]

[1] Department of Agriculture Crop Production and Rural Environment, University of Thessaly, Fytokou, Nea Ionia, 38446 Volos, Greece; avdouli.denisa@gmail.com (D.A.); nkatsoul@uth.gr (N.K.)
[2] Faculty of Horticulture and Food Technology, Hochschule Weihenstephan-Triesdorf, University of Applied Sciences, Am Staudengarten 14, 85354 Freising, Germany; johannes.max@hswt.de
* Correspondence: elevizou@uth.gr

Abstract: In a cascade hydroponic system, the used nutrient solution drained from a primary crop is directed to a secondary crop, enhancing resource-use efficiency while minimizing waste. Nevertheless, the inevitably increased EC of the drainage solution requires salinity-tolerant crops. The present study explored the salinity-tolerance thresholds of basil to evaluate its potential use as a secondary crop in a cascade system. Two distinct but complemented approaches were used; the first experiment examined basil response to increased levels of salinity (5, 10 and 15 dS m^{-1}, compared with 2 dS m^{-1} of control) to identify the limits, and the second experiment employed a cascade system with cucumber as a primary crop to monitor basil responses to the drainage solution of 3.2 dS m^{-1}. Growth, ascorbate content, nutrient concentration, and total amino acid concentration and profile were determined in both experiments. Various aspects of basil growth and biochemical performance collectively indicated the 5 dS m^{-1} salinity level as the upper limit/threshold of tolerance to stress. Higher salinity levels considerably suppressed fresh weight production, though the total concentration of amino acids showed a sevenfold increase under 15 dS m^{-1} and 4.5-fold under 5 and 10 dS m^{-1} compared to the control. The performance of basil in the cascade system was subject to a compromise between a reduction of fresh produce and an increase of total amino acids and ascorbate content. This outcome indicated that basil performed well under the conditions and the system employed in the present study, and might be a good candidate for use as a secondary crop in cascade-hydroponics systems.

Keywords: cascade hydroponics; basil; salinity; amino acids; nutrients; ascorbic acid

Citation: Avdouli, D.; Max, J.F.J.; Katsoulas, N.; Levizou, E. Basil as Secondary Crop in Cascade Hydroponics: Exploring Salinity Tolerance Limits in Terms of Growth, Amino Acid Profile, and Nutrient Composition. *Horticulturae* **2021**, *7*, 203. https://doi.org/10.3390/horticulturae7080203

Academic Editors: Nazim Gruda and Juan A. Fernández

Received: 3 June 2021
Accepted: 18 July 2021
Published: 21 July 2021

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

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Enhanced soil salinity is a worldwide and expanding problem posing serious threats to crop production [1]. It is an inherent problem of intensive cultivation systems and of semi-arid zones, which are characterized by the imbalance between precipitation and evapotranspiration. Nevertheless, increased salinity affects soilless cultivation systems as well—either open or closed [2]. Especially in the latter, where the nutrient solution re-circulates more than once in the crop lines, the increased salt accumulation in the root zone is inevitable. This entails risks regarding impaired plant function and performance, which negatively affect crop yield [3,4]. Additionally, in both open and closed soilless systems, the discharge of used nutrient solutions to the environment further deteriorates soil quality, causing severe environmental degradation and a waste of resources [5]. The ultimate result of such management practices is a reduced sustainability of soilless systems, although their implementation has considerable advantages in terms of crop productivity, space utilization, and nutrient-use efficiency [6,7]. Toward mitigating the environmental impacts of the discharge of waste nutrient solutions, a new concept in closed systems has

been recently proposed that includes a transformation of the classical system into a cascade system; i.e., the used nutrient solution drained from a primary crop is subsequently directed to a secondary crop, and its drainage solution to a tertiary crop [5,8]. This exhaustive re-use of the same nutrient solution confers great advantages in resource-use efficiency while minimizing waste, and thus enhances the sustainability of cascade cultivation systems. Apparently, the suitability of certain crops to be used as secondary and tertiary crops should be carefully considered in terms of salt tolerance; it becomes a crucial characteristic due to increased salinity of the drained nutrient solution [5,9].

Plants grown under increased soil or water salinity are exposed mainly to three constraints; i.e., water deficit, ion imbalance, and ion toxicity [10,11]. The consequent physiological and metabolic disturbances collectively affect gas exchange rates, as well as morphological and biochemical characteristics of plants, and hence compromise crop growth and yield [3,12]. Plant species exhibit differential potentials to tolerate salinity, ranging from non-tolerant glycophytes—among them most cultivated plants—to salt-tolerant halophytes, which are adapted to thrive in saline environments. Interestingly, though different in tolerance, plants employ the same basic mechanisms to respond and acclimate to salt stress [10]. Among them are the control of cell water balance, ion homeostasis mechanisms, and scavenging of toxic compounds, all of which are deployed to various extents by different genotypes [11]. All the above-mentioned mechanisms include the activation of certain pathways of the secondary metabolism of plants, which result in production of antioxidants and accumulation of compatible osmoprotectants such as proline and glycine betaine [11]. Thus, the effort of the plant to cope with salt stress results in the enhancement of bioactive compounds, which are defined as phytochemicals that can modulate metabolic processes in humans and promote better health [13]. This effect is desirable from the human diet perspective, representing the "bright side" of salt stress. The promotion of bioactive compounds production by plants under stress is a new, intensive, and promising line of research [13].

The selection of crops that may efficiently cope with salt stress will optimize the use of the available resources of low quality, such as saline soil and irrigation water. The fundamental step in this process is to determine the salinity thresholds for both productivity and quality of the specific crops. There is often a trade-off between yield in terms of biomass production and quality characteristics in terms of marketable plant products of high-added value; e.g., health-promoting bioactive compounds and essential oils [4,14,15]. This trade-off reflects of course the balance between primary and secondary plant metabolism, and is usually challenged by imposing abiotic stress to crops [16]. Yet, given the adverse effects of salt stress on crop function and growth, it is crucial to consider and fine-tune the balance between yield, nutritional value, and bioactivity of the given crop species [17].

It is well documented that aromatic plants can tolerate moderate salinity, and thus can be used as alternative crops in salt-degraded soils without significant yield loss [18,19]. Among them, sweet basil (*Ocinum basilicum* L.) has a high commercial value because of its vast variety of uses [20]. Apart from culinary and ornamental use, basil has antimicrobial and medicinal properties that add potential to its further utilization and increase its commercial value [21]. The aim of the present study was two-fold: (i) the exploration of the salinity tolerance thresholds of basil toward the best compromise between yield and the content of ascorbic acid and amino acids, combined with (ii) the evaluation of basil as a candidate for cascade hydroponics. Here, we report the implementation of two separate experiments, the first determining the tolerance thresholds of basil exposed to three salinity levels, through its response in terms of growth, antioxidant capacity, nutrient concentration, and amino acid profile; and the second examining the same response variables in an experimental set-up in which basil was the secondary crop irrigated by the drainage solution of a primary crop; i.e., cucumber, a commercial high-value crop that is commonly cultivated in soilless systems.

2. Materials and Methods

2.1. Plant Material and Experimental Design

This study was conducted in the greenhouse premises of Hochschule Geisenheim University in Geisenheim, Germany during the summer months of 2018. The mean monthly temperature during the experimental period ranged from 22.4 to 27.0 °C, and crops were grown under ambient light conditions, with 517 μmol m^{-2} s^{-1} average light intensity. Basil seeds of the Genovese (Eowyn) variety were sown in 3 L pots containing a mixture of peat and perlite (2:1). After two weeks, basil plants had reached the two true leaves stage. For the first experiment, a total of 40 pots with 25 plants each were selected. The pots were divided into four treatments with 10 replicates each, and were irrigated daily with a nutrient solution of four salinity levels; i.e., 2 dS m^{-1} (control), 5 dS m^{-1} (T5), 10 dS m^{-1} (T10), and 15 dS m^{-1} (T15). Commercial fertilizers were used to build up the selected salinity levels. Table 1 summarizes the elemental composition and concentration of each nutrient in the irrigation solution used for the four treatments. The pots were arranged according to the randomized complete block design, and frequent rotation (every 10 days) was performed to minimize the impact of the microenvironment. The experimental period lasted five weeks, during which two harvests were performed, 15 and 35 days after commencement of the salinity treatment.

Table 1. Nutrient concentrations in the irrigation solution used for each treatment, expressed in mmol L^{-1} for macronutrients and μmol L^{-1} for micronutrients.

	Control	T5	T10	T15
NO_3^-	13.6	47.4	93.4	140.8
NH_4^+	5.4	14.8	29.2	44.0
Ca^{2+}	1.7	11.8	23.3	35.2
P	0.2	1.2	2.4	3.6
K^+	1.7	5.4	10.6	15.9
Mg^{2+}	0.4	1.9	3.7	5.6
S	0.4	2.4	4.7	7.0
Fe	12.3	31.6	62.3	94.0
Cu	1.5	4.0	7.8	11.8
Mn	7.1	18.3	36.2	54.5
Zn	3.7	9.6	19.0	28.6
B	22.7	58.3	115.0	173.4
Mo	0.4	1.1	2.1	3.1

In the second experiment, a cascade system was established with cucumber as a primary crop grown in hydroponics, the drainage of which was driven to basil grown in pots as a secondary crop. A total of 36 cucumber plants were planted in six rows, each composed of two rock wool slabs (Grodan, Roermond, the Netherlands; length: 1 m; volume: 11.25 L) planted with three cucumber plants each. The primary crop plants were allocated to three groups, with 12 plants each. The drainage of each group was driven to a tray upon which 12 pots were placed and received this capillary irrigation, with no additional watering throughout the experimental period. Each pot contained 25 basil plants, as described above. Thus, three replicates of the drainage solution treatment were formed while the control group of basil (also 12 pots) received fresh nutrient solution, the same that was prepared for cucumber. The latter was a standard nutrient solution for cucumber grown in open hydroponic systems, with the following composition: 3.0 mM K^+, 6.0 mM Ca^{2+}, 2.0 mM Mg^{2+}, 1.0 mM NH_4^+, 11.5 mM NO_3^-, 1.5 mM $H_2PO_4^-$, 3.5 mM SO_4^{2-}. The electrical conductivity (EC) was set at 2.1 dS m^{-1} and pH 5.7. The EC range of the drainage solution that irrigated the treated basil plants was 3.2 ± 0.3 dS m^{-1}, and its composition was 4.1 ± 0.3 mM K^+, 7.1 ± 1.0 mM Ca^{2+}, 2.2 ± 0.6 mM Mg^{2+}, 13.2 ± 2.8 mM N, 1.7 ± 0.3 mM P^+ (average ± SD from three measurements during the experimental period). The same experimental duration and harvest times as for the first experiment were applied.

The following methods and sampling protocols for the growth and biochemical parameters determination apply to both experiments described above.

2.2. Plant Growth

Plant height was measured at intermediate and final harvest. At the same time-points, projected leaf area was determined through capturing photographs from the same height above plants and subsequently using the free software ImageJ (open-source software, ImageJ.net/ver. ImageJ 1.51j) to estimate the green area of the plants. At the intermediate and final harvests, five random plants were selected from each pot. Leaves and stems were separated, the fresh weight was measured immediately, and all the samples were oven dried for four days at 55 °C and weighed for biomass assessment.

2.3. Nutrient Element Analysis

After the determination of dry weight, 0.25 g of leaf tissue from each sample was used to conduct the nutrient elemental analysis. A modified Kjeldahl extraction was used for the mineralization of all nutrients. Each leaf sample was extracted with 4.4 mL of the digestion solution, which included 1.94 mL concentrated sulfuric acid, 2.82 mg Se, 82.13 mg Li_2SO_4, and 1.94 mL 30% H_2O_2. The samples were digested for two hours at 30 °C, then left to reach room temperature, and finally diluted up to 50 mL with distilled water before proceeding to elemental analysis. The concentrations of N, P, K, Ca, Mg, Fe, Zn, Mn, and Cu were determined by ICP-OES (Spectro Arcos EOS 12 ICP—OES Spectrometer, SPECTRO Analytical Instruments GmbH, Kleve, Germany) and flow injection analysis (Foss Tecator FIAStar 5000, FOSS, Hilleroed, Denmark). The concentration of macronutrients (N, P, K, Ca, Mg) and micronutrients (Fe, Zn, Mn, Cu) are expressed in % and mg/kg of leaf dry weight, respectively.

2.4. Ascorbic Acid Content

For each treatment, 20 individual plants were used to constitute five samples. For each plant, all the leaves were removed and grounded in liquid nitrogen. The ascorbic acid content was determined according to Pegg et al. (2007) [22]. First, 100–200 mg of tissue per sample was homogenized using 5 mL of 80% ethanol. The homogenate was mixed threefold using a vortex for 5 s. The samples were submerged in an iced ultrasound bath for 15 min. After that, the samples were centrifuged at 1792× *g* for 15 min at 0 °C, then 1 mL of supernatant was transferred into cryogenic Eppendorf vials and stored at –80 °C. The ascorbic acid content (mg AsA/g of dry tissue) was determined based on photochemoluminescence (PCL) with the PHOTOCHEM Antioxidant Analyzer (Analytik Jena GmbH, Jena, Germany).

2.5. Amino Acids

The sample preparation followed the procedure described above for the ascorbic acid determination. Concerning the extraction, 100–200 mg of tissue per sample were homogenized using 2 mL of polyvinylpyrrolidone (PVP) 100 buffering solution. The homogenate was mixed using a vortex for 5 s. The samples were submerged in an ultrasound bath for 15 min. After that, the samples were centrifuged at 1792× *g* for 15 min at 4 °C. The supernatant was filtered through a 0.2 nm cellulose filter to sealable glass vials. The concentration of amino acids (mg/kg of fresh tissue) was determined using an automatic Amino Acid Analyzer (SYKAM s433, Sykam GmbH, Fürstenfeldbruck, Germany).

2.6. Statistics

Statistical analysis was performed with IBM SPSS Statistics v.26 software, using one-way ANOVA, followed by Tukey's HSD post hoc tests, and confidence intervals for $p \leq 0.05$.

3. Results and Discussion

3.1. Exploration of Salinity-Tolerance Thresholds of Basil

In the first experiment, we evaluated the productivity as well as quality parameters of basil exposed to various levels of salt stress to explore its tolerance thresholds. Moreover,

we performed an evaluation with two time-points, including an intermediate harvest before the final one to identify crucial patterns of response and the course of growth performance.

Basil's growth response to increased salinity confirmed its moderate potential to cope with this stress condition. Plant height reduction was evident at both intermediate and final harvest (Figure 1A). In the latter, T5 slightly reduced plant height, but with statistical significance, while T10 and T15 severely affected it, resulting in a decrease of 46 and 62%, respectively, compared to the control. After only two weeks of exposure to stress, the treated groups began to differentiate from the control plants, and these differences were magnified in the final harvest. The same profile was followed by another indicative growth parameter, the projected leaf area, as shown in Figure 1B. The between-treatment differences over the course of the experiment were clearly reflected in both intermediate and final harvest values of projected leaf area, showing a significant stepwise reduction with increasing salinity levels (Figure 1B). The time-point of 15 days seemed to be crucial for all growth responses of basil, since it marked the establishment of the first statistically significant differences compared to the control. This applied not only to height and leaf area, but also to the plant fresh weight and dry biomass production. Figure 2A presents the fresh weight as determined in the intermediate and final harvest. After 18 days of exposure, T5 caused a small but significant decrease of 18% compared to the control, and this was maximized to 47% at the final harvest. T10 and T15 considerably suppressed fresh weight production, reaching a remarkable 72% and 87% reduction of control values, respectively, at the end of the experiment. Similar severe reduction was evidenced in basil dry biomass accumulation (Figure 2B). In the relevant literature, there are some studies on basil indicating that certain varieties are tolerant to salinity levels even higher than those examined in the present study [20]. Nevertheless, most similar works emphasize the limited potential of basil to cope with salinities higher than 5 dS m^{-1}, corroborating our results [23,24]. Indeed, increased salinity was found to negatively affect basil height [12] and have a detrimental effect on basil's canopy area [25,26], while Caliskan et al. (2017) [27] indicated a negative correlation between the accumulation of dry matter and increased salinity. The various aspects of basil growth performance in the present study collectively indicated the 5 dS m^{-1} salinity level as the upper limit/threshold of tolerance to stress, and 15 days of treatment as the critical point for the appearance of salinity symptoms on growth. It is well documented that during the early phase of salinity stress (first days), the growth reduction is ascribed to decreased leaf emergence and expansion [28,29]. The underlying mechanisms are related to osmotic stress, which affects the availability of water to the plant body with profound effects on stomatal conductance, cell cycle, and cell expansion. Apart from the rapidly occurring water stress, the evolution of oxidative stress by uncontrolled production of ROS, as well as nutrient imbalances, may account for the compromised growth under enhanced salinity [26,30]. Accordingly, the time frame of 15 days (intermediate harvest) and, moreover, 30 days (final harvest) in the present experiment may be adequate for these stresses to be developed. In an article demonstrating both the water stress imposed and the antioxidant response of salt-affected basil, Barbieri et al. (2012) [31] reported that the constitutively reduced stomatal density improved the acclimatization of the more tolerant basil variety to salinity stress, along with the efficient production of antioxidants.

The total concentration of amino acids in basil leaves showed a sevenfold increase under 15 dS m^{-1} and 4.5-fold under 5 and 10 dS m^{-1} compared to the control at the final harvest (Figure 3). Statistically significant but smaller differences between the treatments were also recorded in the intermediate harvest. It was noteworthy that the total amino acid content of the control plants remained virtually unchanged between the intermediate and final harvests, while saline treatments induced a three- to fourfold increase. An accumulation of free amino acids has been usually reported in various plants exposed to abiotic stress [32,33]. Neto et al. (2019) [21] measured the total content of amino acids of two basil varieties grown under 80 mM NaCl and reported a marginal increase in both leaves and roots. There is a tight relationship between amino acid metabolism and plant response to stress, due to the multiple roles of certain amino acids in stress mitigation; i.e., osmoprotectants, ROS scavengers, N source and storage, and

as alternative substrates for mitochondrial respiration [32–34]. Whether from a direct salinity-induced effect or basil's response in the adaptation process, the increase of total amino acid content indicated metabolic adjustments and was particularly ascribed to specific compounds. To the best of our knowledge, this is the first report of salinity effects on the detailed amino acid profile of basil. Notably, it was obvious in the individual amino acid concentrations (Table 2) that glutamine and arginine showed a significant increase at both intermediate and final harvests in all salinity levels. At the final harvest, the asparagine was also responsive to the imposed stress in a salinity level-dependent manner. In fact, the above-mentioned amino acids presented an eight- to 12-fold increase in T15 compared to the control, substantially contributing to the enhanced levels of total amino acid content shown in Figure 3. The results presented are in accordance with other authors, who suggested that amino acids such as asparagine, arginine, and glutamine, as well as proline, function as compatible solutes combating osmotic stress within plant cells [32,33]. Asparagine accumulation may also play a role in nitrogen remobilization and ammonia detoxification during abiotic stress [35], while the role of arginine as precursor of the stress-induced polyamines is well documented [32].

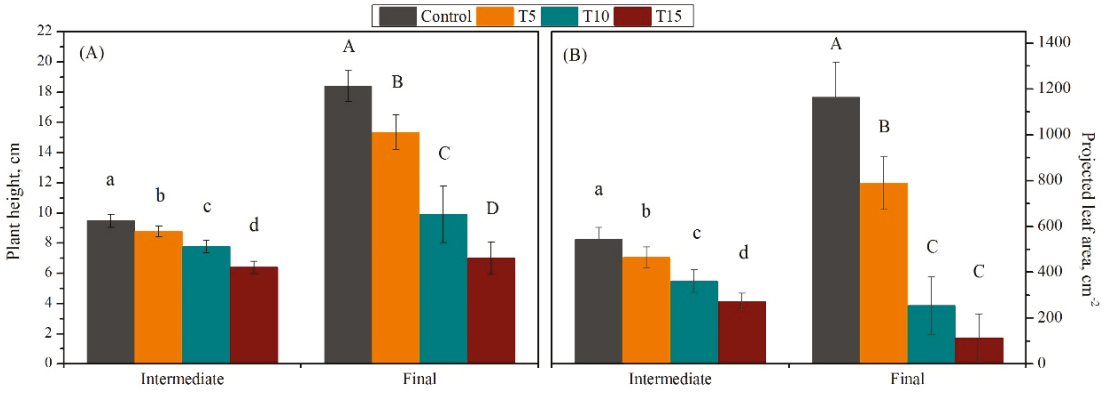

Figure 1. Growth characteristics of basil leaves grown under the various salinity treatments: plant height at the intermediate and final harvests (**A**); projected leaf area at the intermediate and final harvests (**B**). Values are expressed as mean ± standard deviation (n = 50 for plant height and n = 10 for the PLA). Different letters indicate statistically significant differences between treatments at each harvest (small letters refer to the intermediate and capital ones to the final harvest, $p < 0.05$).

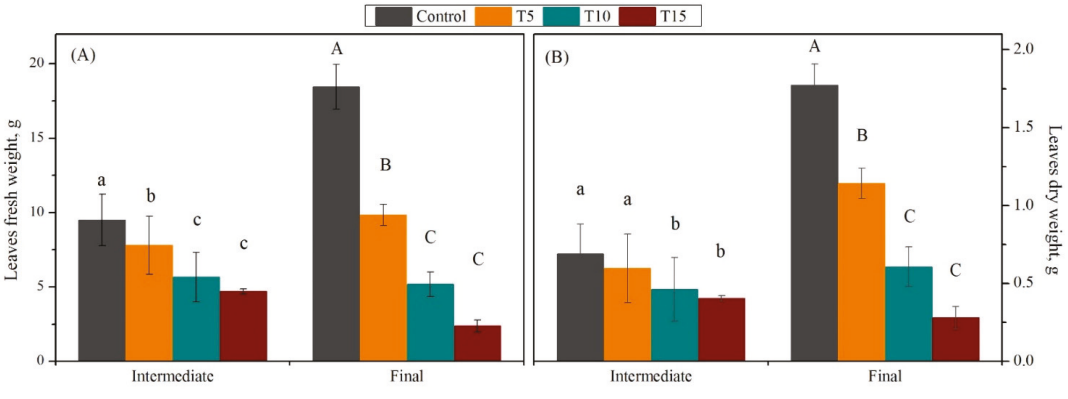

Figure 2. Leaves fresh weight (**A**) and leaves biomass (**B**) grown under the various salinity treatments at the intermediate and final harvests. Values are expressed as mean ± standard deviation (n = 10). Different letters indicate statistically significant differences between treatments at each harvest (small letters refer to the intermediate and capital ones to the final harvest, $p < 0.05$).

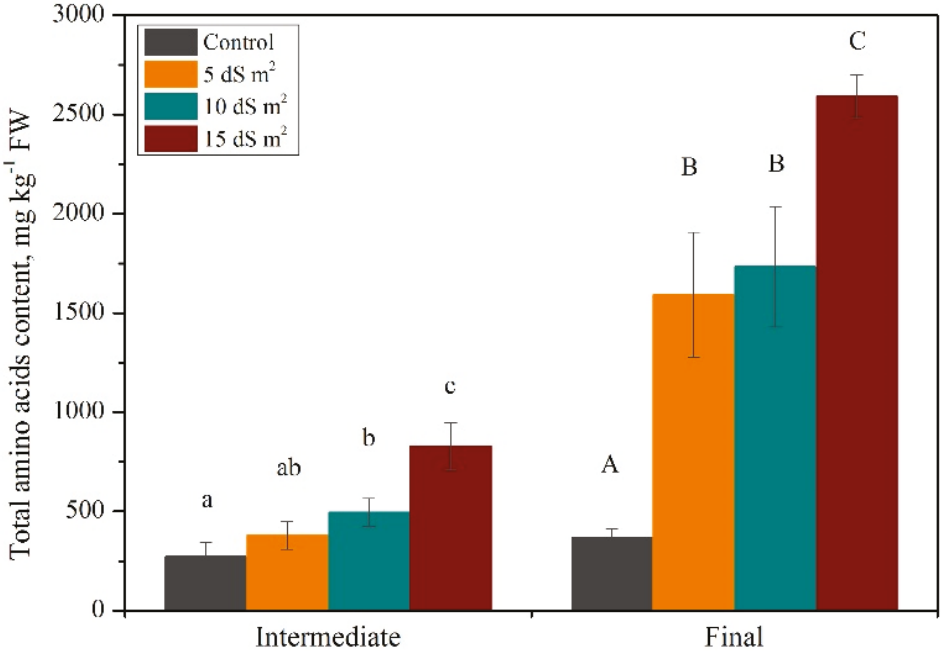

Figure 3. Total amino acid content of basil leaves grown under the various salinity treatments at the intermediate and final harvests. Values in all layers are expressed as mean ± standard deviation (n = 5). Different letters indicate statistically significant differences between treatments at each harvest (small letters refer to the intermediate and capital ones to the final harvest, $p < 0.05$).

Ascorbic acid (AsA) is directly involved in salinity stress protection, and particularly in basil, it has been identified as a good indicator of the total antioxidant capacity [36]. The AsA content of T15 plants was severely suppressed at the final harvest, although at the intermediate harvest showed a statistically significant increase compared to all other treatments (Figure 4). Only T5 plants displayed similar AsA concentration with the control group. High levels of AsA effectively maintain low levels of H_2O_2, which may prevent the H_2O_2-mediated stress responses and can therefore contribute to overcome saline stress [31]. The enhanced AsA content at the intermediate harvest in the T15 group may reflect this process. Nevertheless, the decreased levels of AsA after prolonged salinity stress in all treated plants may have multiple explanations, pointing to the parallel and overlapping mechanisms that control and modulate physiological responses to stress. Possibly the extensive utilization of AsA for the detoxification of H_2O_2, accompanied by the inefficient regeneration of ascorbate, as proposed by Barbieri et al. (2012) [31], may explain the decreased concentration at the end of the growth period. Overall, after 15 days of saline treatment, the protection that AsA confers to basil plants may be considered insufficient. It should be noted here that although the AsA concentration is correlated with salt tolerance, it is obviously not the only responsible substance, since other physiological mechanisms and metabolites, not determined in the present study, may also contribute.

Table 2. Individual amino acid concentration in basil leaves for the various salinity treatments at the intermediate and final harvests, expressed as mg kg^{-1} FW. Values in all layers are expressed as mean ± standard deviation ($n = 5$). Different letters indicate statistically significant differences between treatments at each harvest (small letters refer to the intermediate and capital ones to the final harvest, $p < 0.05$).

	Intermediate Harvest				Final Harvest			
	Control	**T5**	**T10**	**T15**	**Control**	**T5**	**T10**	**T15**
alanine	15.18 ± 2.43 a	19.54 ± 0.40 ab	22.47 ± 3.41 b	23.49 ± 3.19 b	13.30 ± 2.9 AB	17.41 ± 3.77 A	13.31 ± 2.71 A	9.87 ± 1.00 B
arginine	48.55 ± 22.03 a	81.53 ± 41.92 a	96.92 ± 37.05 a	258.26 ± 65.20 b	85.02 ± 24.38 A	742.44 ± 186.1 B	790.47 ± 144.2 B	1107.78 ± 37.4 C
asparagine	15.02 ± 9.93 a	14.80 ± 8.30 a	9.07 ± 2.91 a	31.50 ± 18.98 a	51.87 ± 7.69 A	171.59 ± 51.02 B	220.80 ± 81.25 B	428.67 ± 72.07 C
aspartic acid	9.29 ± 2.08 a	11.41 ± 1.41 a	17.82 ± 1.58 b	19.82 ± 1.99 b	11.74 ± 1.14 A	17.90 ± 3.40 B	16.25 ± 2.9 AB	15.04 ± 0.9 AB
b-alanine	0.32 ± 0.12 ab	0.31 ± 0.08 a	0.24 ± 0.03 a	0.55 ± 0.18 b	0.43 ± 0.10 A	1.34 ± 0.34 B	1.50 ± 0.42 B	2.47 ± 0.31 C
b-amino-isobutyric acid	0.30 ± 0.07 ab	0.28 ± 0.11 a	0.51 ± 0.08 ab	0.57 ± 0.24 b	0.11 ± 0.02 A	0.26 ± 0.06 B	0.33 ± 0.19 C	0.54 ± 0.22 D
citrulline	19.58 ± 5.33 a	26.55 ± 2.14 ab	30.75 ± 2.08 b	46.11 ± 7.56 c	12.94 ± 3.69 A	68.15 ± 24.34 B	73.16 ± 16.20 B	100.24 ± 2.00 B
g-aminobutyric acid	5.14 ± 0.39 a	10.71 ± 3.74 ab	11.72 ± 4.17 b	7.51 ± 1.89 ab	9.19 ± 2.04 A	13.43 ± 4.37 A	8.86 ± 2.22 A	6.60 ± 2.18 A
glutamic acid	38.29 ± 5.87 a	41.59 ± 4.05 a	47.34 ± 8.24 a	44.58 ± 7.38 a	43.96 ± 5.08 A	45.22 ± 11.56 A	37.61 ± 7.18 A	28.95 ± 2.37 A
glutamine	71.27 ± 11.95 a	117.16 ± 12.50 a	190.17 ± 42.23 b	292.82 ± 53.92 c	66.09 ± 13.01 A	343.46 ± 78.23 B	387.21 ± 69.89 B	654.43 ± 106.3 C
glycine	4.60 ± 0.87 a	7.53 ± 1.28 ab	8.95 ± 0.46 ab	13.91 ± 6.37 b	1.29 ± 0.25 A	7.62 ± 2.94 B	8.50 ± 1.47 B	3.10 ± 1.75 A
histidine	8.93 ± 4.27 a	10.19 ± 2.93 a	15.22 ± 2.03 a	27.52 ± 3.83 b	18.56 ± 6.01 aA	70.36 ± 15.64 B	73.57 ± 13.66 B	112.98 ± 6.42 C
isoleucine	1.75 ± 0.61 a	1.10 ± 0.75 a	1.12 ± 0.15 a	1.82 ± 0.32 a	4.24 ± 0.37 A	6.70 ± 0.91 B	6.04 ± 1.7 AB	4.81 ± 0.5 AB
leucine	1.78 ± 0.68 ab	1.46 ± 0.28 ab	1.25 ± 0.21 a	2.12 ± 0.24 b	4.13 ± 0.45 A	7.22 ± 0.93 B	6.59 ± 1.78 B	5.98 ± 0.8 AB
lysine	3.03 ± 0.99 a	3.51 ± 0.77 a	3.20 ± 0.42 a	5.71 ± 0.69 b	4.89 ± 1.07 A	19.49 ± 3.33 B	19.96 ± 3.70 B	29.02 ± 0.85 C
methionine	0.13 ± 0.04 a	0.10 ± 0.01 a	0.19 ± 0.06 b	0.21 ± 0.02 b	0.13 ± 0.04 A	0.21 ± 0.12 B	0.25 ± 0.12 B	0.77 ± 0.22 B
ornithine	1.61 ± 0.38 a	2.18 ± 0.99 b	2.57 ± 0.67 b	4.82 ± 0.82 b	1.56 ± 0.27 A	10.10 ± 2.66 B	10.75 ± 3.53 B	14.67 ± 2.35 B
phenylalanine	2.54 ± 1.57 a	1.71 ± 0.38 a	1.25 ± 0.17 a	1.70 ± 0.33 a	5.23 ± 2.13 A	5.94 ± 0.44 A	5.88 ± 2.27 A	7.07 ± 1.44 A
proline	0.91 ± 0.23 a	1.36 ± 0.37 a	2.18 ± 0.64 a	4.83 ± 1.36 b	0.96 ± 0.70 A	3.61 ± 1.3 B	2.48 ± 0.9 AB	2.63 ± 0.9 AB
serine	10.30 ± 1.83 a	13.21 ± 1.54 a	19.81 ± 1.29 b	23.45 ± 2.75 b	8.92 ± 1.57 A	22.47 ± 2.66 B	17.51 ± 3.14 C	20.30 ± 1.31 BC
threonine	5.17 ± 1.43 a	5.28 ± 0.31 a	5.61 ± 0.47 ab	7.01 ± 0.48 ab	6.88 ± 0.22 A	10.41 ± 0.95 B	9.36 ± 1.82 B	8.72 ± 0.8 AB
tryptophan	4.44 ± 1.97 a	3.29 ± 0.95 a	3.01 ± 0.37 a	4.00 ± 0.81 a	7.85 ± 2.88 A	9.50 ± 0.76 A	7.97 ± 2.35 A	10.33 ± 2.87 A
tyrosine	1.33 ± 0.64 a	1.04 ± 0.15 ab	0.31 ± 0.13 b	0.51 ± 0.18 b	2.56 ± 0.35 A	3.22 ± 0.52 A	2.42 ± 0.91 A	1.97 ± 0.11 A
valine	3.56 ± 1.56 a	3.04 ± 0.50 a	3.37 ± 0.29 a	5.54 ± 0.43 b	7.66 ± 0.88 A	13.77 ± 1.54 B	13.11 ± 3.23 B	14.19 ± 1.20 B

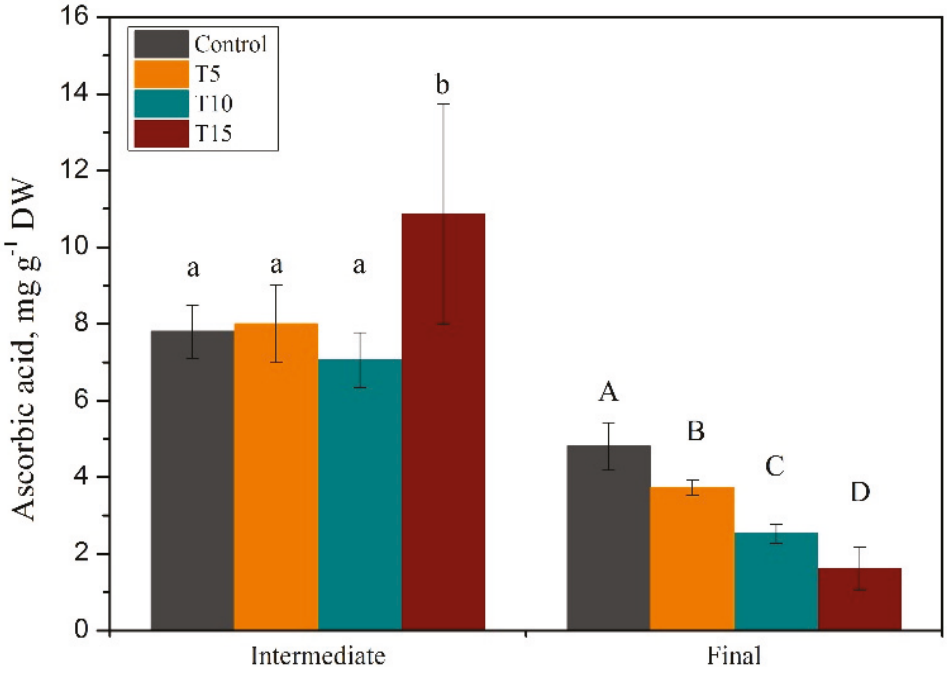

Figure 4. Ascorbic acid content of basil leaves grown under the various salinity treatments at the intermediate and final harvests. Values are expressed as mean ± standard deviation ($n = 5$). Different letters indicate statistically significant differences between treatments at each harvest (small letters refer to the intermediate and capital ones to the final harvest, $p < 0.05$).

High salinity interferes with uptake and assimilation of certain nutrients [37], mainly through alterations in related enzyme activity. The source of salinity; i.e., the composition and concentration of salts in the irrigation water or nutrient solution, significantly shapes the type and magnitude of nutrient-related problems; deficiencies, ion toxicities, and altered ion balance and competition may differentially arise due to various salinity sources [38]. In salinity-related research, the use of NaCl predominates, yet there are many other sources of excessive salts that may impact crops, and their result in plant nutritional response may be different [12,39]. In the context of cascade hydroponics, using nutrient solutions of increasing elemental concentration, thus increasing EC, is a more realistic approach compared to NaCl addition. The enhanced EC substantially modified the nutrient absorption and content. Nutrient imbalances were found in basil plants exposed to salinity in both harvests of the current study (Table 3), with the effect being more pronounced at the intermediate harvest (15 days). At the final harvest, the leaf elemental concentration may reflect the trade-off between enhanced nutrient availability in the irrigation solution and the salinity effects on plant function and metabolism; accordingly, we followed the interference of imposed salinity to nutrient status. Under T5, T10, and T15, N content in leaf tissues was increased at both harvest dates compared to the control. The opposite trend has also been reported by Elhindi et al. (2017) [23], but the differences may be ascribed to their use of NaCl for imposing salt stress, the longer duration of their experiment (57 days), and possibly to the different developmental stage of basil plants at their final harvest, since flowering alters nutrient allocation patterns. Indeed, NaCl-imposed salinity stress has profound effects on N concentration due to inhibition of NO_3-transport systems [40].

Corroborating our results, Scagel et al. (2017) [39] found increased N content in basil leaves exposed to either NaCl- or $CaCl_2$-induced salinity. N concentrations in basil in the present experiment, except for the increased availability in the irrigation solution, may also be related to the enhanced concentration of certain amino acids mentioned above. Indeed, the induction of glutamine and asparagine synthesis during stress has been linked to storage of organic nitrogen and transport within plants [33]. Phosphorus uptake was gradually decreased along increasing salinity (Table 3), a result that has been also found in NaCl-challenged basil [20,23]. According to Scagel et al. (2017) [39], the source of salinity determines the mechanism of P reduction, being either limited availability of phosphate ions or competition with other ions for binding sites within roots. Apparently, in the case of P in the current experiment, the salinity effect outweighed the increasing P supply by irrigation solution. Potassium content in leaves exhibited an interesting profile. T10 and T15 plants showed significantly lower K content compared to the control and T5 at the intermediate harvest, although their irrigation solution permitted increased K availability. An increase was evident during the last days, resulting in similar K levels in all treatments at the final harvest. This increase may be due to the role of K in osmoregulation, since it is considered, along with Cl, among the inorganic solutes with a greater contribution to the osmotic adjustment in basil [21]. Similar regulatory involvement in osmotic stress may be ascribed to Ca, the accumulation of which was induced by the two higher salinity levels at the early phase of stress. Ca content enhancement in leaves has been ascribed to its altered allocation under salt stress [40], which has been evidenced also in basil [39] as an increased translocation from root to shoot. The micronutrients determined in the present study showed distinct profiles along treatments and harvests (Table 3). A general trend was evident for lower values under salinity at the final compared to the intermediate harvest. Fe accumulation was suppressed under saline conditions, while Cu and Zn concentrations did not respond consistently. Nevertheless, the variation of Mn content was significant; at the intermediate harvest, increasing salinity induced a stepwise increase compared to the control, with almost doubled values under 15 dS m^{-1}. The opposite effect was recorded at the final harvest, where an apparent suppression of Mn uptake and/or translocation to leaves was imposed by all salinity levels, resulting in decreased concentration. The salt-affected micronutrient content of basil leaves has been rarely determined. A recent study by Elhindi et al. (2017) [23] reported a general decline in concentration of all micronutrients when plants were exposed to 6 and 12 dS m^{-1}. Scagel et al. (2019) [20] found that 5, 10, and 20 dS m^{-1} did not significantly alter Fe, Mn, and Zn concentrations. However, in an earlier work by the same authors [39], a substantially increased uptake of Cu and Zn was found, along with a reduced uptake of Fe with either NaCl- or $CaCl_2$-imposed stress. Of course, the direct comparison with other works may be misleading, since it is documented that differences in salt source used and salinity tolerance among basil cultivars may account for specific effects on leaf nutrient composition [12,39].

In conclusion, the various aspects of basil growth and biochemical performance collectively indicated the 5 dS m^{-1} salinity level as the upper limit/threshold of tolerance to stress. Additionally, the results of the first experiment indicated the first 15 days of treatment as a critical point for the process of salinity-symptom appearance in growth performance, as well as mineral composition.

Table 3. Nutrient concentrations in basil leaves for various salinity treatments (1st experiment) as determined at intermediate and final harvests. Different letters indicate statistically significant differences between treatments at each harvest (small letters refer to the intermediate and capital ones to the final harvest, $p < 0.05$). Values in all layers are expressed as mean ± standard deviation ($n = 10$).

Intermediate Harvest									
Treatment	**N (%)**	**P (%)**	**K (%)**	**Ca (%)**	**Mg (%)**	**Fe (mg/kg)**	**Zn (mg/kg)**	**Mn (mg/kg)**	**Cu (mg/kg)**
Control	5.18 ± 0.16 a	0.94 ± 0.05 a	5.31 ± 0.29 a	2.32 ± 0.16 a	0.47 ± 0.04 a	136.39 ± 13.59 a	97.50 ± 4.98 a	293.29 ± 23.55 a	12.05 ± 1.12 ab
T5	5.62 ± 0.06 b	0.88 ± 0.03 b	5.12 ± 0.12 a	2.62 ± 0.08 a	0.43 ± 0.03 a	143.01 ± 16.17 a	100.49 ± 8.60 a	323.06 ± 24.20 a	13.54 ± 1.78 a
T10	5.56 ± 0.18 b	0.70 ± 0.05 c	4.23 ± 0.25 b	4.02 ± 0.50 b	0.57 ± 0.11 b	132.82 ± 22.12 a	100.69 ± 11.62 a	462.32 ± 54.44 b	11.23 ± 2.18 b
T15	5.71 ± 0.14 b	0.58 ± 0.04 d	4.09 ± 0.26 b	4.76 ± 0.35 c	0.51 ± 0.07 ab	89.24 ± 7.42 b	108.18 ± 13.89 a	540.10 ± 90.89 c	9.46 ± 1.66 b
Final Harvest									
Treatment	**N (%)**	**P (%)**	**K (%)**	**Ca (%)**	**Mg (%)**	**Fe (mg/kg)**	**Zn (mg/kg)**	**Mn (mg/kg)**	**Cu (mg/kg)**
Control	5.16 ± 0.14 A	0.77 ± 0.02 A	5.04 ± 0.28 A	3.04 ± 0.18 A	0.64 ± 0.05 A	152.46 ± 11.62 A	93.76 ± 4.80 A	234.17 ± 19.15 A	14.77 ± 2.32 A
T5	6.16 ± 0.18 B	0.69 ± 0.03 B	5.18 ± 0.21 A	2.91 ± 0.17 A	0.55 ± 0.04 B	109.91 ± 14.74 B	75.82 ± 6.58 B	182.71 ± 17.37 B	13.44 ± 1.79 A
T10	6.40 ± 2.86 B	0.66 ± 0.30 B	5.33 ± 2.38 A	3.31 ± 1.48 A	0.57 ± 0.26 AB	108.20 ± 48.30 B	90.81 ± 4.69 AC	223.65 ± 100.18 AC	13.49 ± 6.04 A
T15	6.49 ± 0.30 B	0.67 ± 0.02 B	4.92 ± 0.25 A	3.28 ± 0.69 A	0.59 ± 0.09 AB	94.98 ± 28.05 B	80.47 ± 2.52 BC	186.65 ± 17.24 BC	13.38 ± 0.53 A

3.2. Is Basil Suitable as a Secondary Crop in a Cascade Hydroponics System?

The second experiment was established to evaluate the suitability of basil as a secondary crop in a cascade hydroponics system. The inevitably moderate to high electrical conductivity of the solution that drains from the primary crop to the secondary one challenges the growth and functional performance and depends on the salinity-tolerance thresholds of the latter. In the current experiment, basil grown in pots directly received the drainage solution of cucumber grown in hydroponics without any further treatment.

The growth response of basil clearly correlated with the low salinity level of the first experiment analyzed above. Even though the EC of the solution that was channeled to basil never exceeded 3.5 dS m^{-1}, a reduction of growth was evident, notably at the final harvest (Figure 5). All aspects of growth were affected by salinity to a different extent, ranging from 20% reduction of the projected leaf area to 47% and 42% reduction of the fresh and dry weights, respectively (Figure 5B–D). Similar growth restrictions were also recorded in the T5 plants in the first experiment (Figures 1 and 2). Elvanidi et al. (2020) [5] reported similar reductions of basil grown in cascade hydroponics in which basil received only 40% of cucumber drainage complemented with typical irrigation water.

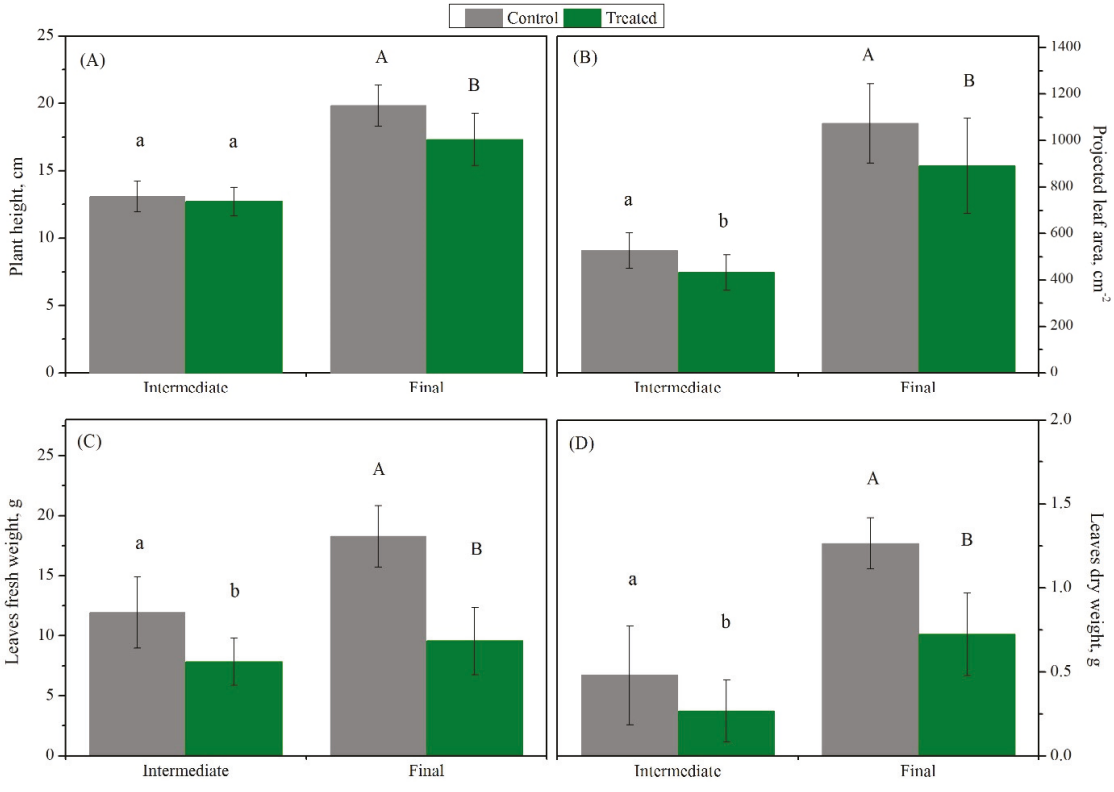

Figure 5. Growth characteristics of basil leaves grown as a secondary crop in the second experiment: plant height (**A**), projected leaf area (**B**), leaves fresh weight (**C**), and leaves biomass (**D**) at the intermediate and final harvests. Values are expressed as mean ± standard deviation (n = 50 for plant height and n = 10 for all the others). Different letters indicate statistically significant differences between treatments at each harvest (small letters refer to the intermediate and capital ones to the final harvest, $p < 0.05$).

The total amino acid content of basil that received the drainage solution from cucumber (hereinafter referred to as "treated plants") displayed a trend for higher values compared

to the control only at the final harvest (Figure 6). Accumulation of amino acids is usually connected to a stress-induced protein breakdown as mentioned above; nevertheless, plants may actively synthesize specific amino acids that play a distinct and beneficial role in stress response [33]. In this line, the 67% increase of glutamic acid concentration at the final harvest (Table 4) may be correlated with its use as a precursor to essential amino acids or its newly reported signaling role toward increased activities of antioxidative enzymes [41]. A trend toward increase in citrulline concentration in treated plants may be ascribed to its function as a compatible solute involved in the maintenance of cellular osmolarity [42]. Overall, the profile of amino acids of the treated plants was in accordance with the T5 basil plants in the first experiment. However, there was a significant difference in magnitude of certain amino acid responses between the T5 and the second experiment. For example, while glycine, ornithine, and proline had a 40–60% increase in treated plants compared to the control in the second experiment, their increase in T5 was two- to fivefold of the control values. Additionally, two- to sevenfold increases in asparagine, glutamine, and arginine of T5 plants were not found in the second experiment. The above-mentioned distinct responses indicated that other factors apart from EC might also act as drivers of the regulation of free amino acid homeostasis and control the dynamic amino acid pool. We may speculate that these factors were related to cucumber root exudates that enriched the drainage solution, and affected the basil plants' response, but they were not determined in the present study. Further and targeted experiments on exudate composition and their detailed metabolomic profile are needed to validate this hypothesis.

The ascorbic acid content of treated plants showed an increase compared to the control plants, but was statistically significant only in the intermediate harvest. The same trend was observed at the final harvest, but it was marginally non-significant (Figure 7). This finding was slightly different compared to the AsA concentration of T5 plants presented above (Figure 4). It seemed that the drainage solution from cucumber triggered the antioxidant machinery, and the response was more pronounced during the acclimation process compared to the first experiment. Apparently, other antioxidants, not determined in the present study, may also play a role in this process.

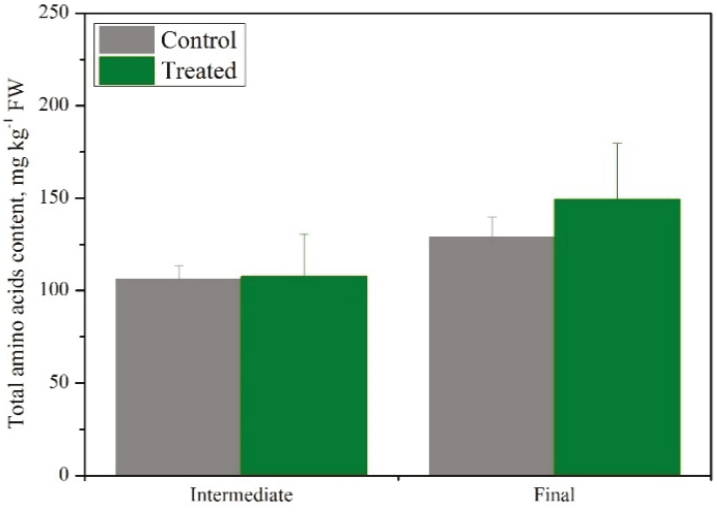

Figure 6. Total amino acid content of basil leaves grown as a secondary crop in the second experiment. The absence of letters indicates no statistically significant differences between treatments at both harvests ($p < 0.05$). Values are expressed as mean ± standard deviation ($n = 5$).

Table 4. Individual amino acid concentrations in basil leaves for the various salinity treatments at the intermediate and final harvests, expressed as mg kg^{-1} FW. Different letters indicate statistically significant differences between treatments at each harvest (small letters refer to the intermediate and capital ones to the final harvest, $p < 0.05$). Values are expressed as mean ± standard deviation ($n = 5$).

	Intermediate Harvest		Final Harvest	
	Control	**Treated**	**Control**	**Treated**
alanine	13.27 ± 1.88 a	13.13 ± 3.39 a	9.14 ± 0.71 A	12.83 ± 2.69 B
arginine	2.44 ± 0.64 a	1.56 ± 0.64 b	13.07 ± 3.65 A	9.48 ± 16.25 A
asparagine	2.48 ± 0.68 a	1.97 ± 0.53 a	11.41 ± 3.84 A	8.86 ± 5.29 A
aspartic acid	3.92 ± 0.40 a	4.29 ± 1.30 a	8.34 ± 1.44 A	10.44 ± 2.54 A
b-alanine	0.04 ± 0.00 a	0.05 ± 0.02 a	0.09 ± 0.04 A	0.10 ± 0.04 A
b-amino-isobutyric acid	0.03 ± 0.02 a	0.13 ± 0.08 b	0.05 ± 0.02 A	0.09 ± 0.05 A
citrulline	3.16 ± 0.51 a	3.09 ± 0.96 a	0.82 ± 0.22 A	1.80 ± 1.32 A
g-aminobutyric acid	9.18 ± 1.87 a	6.88 ± 2.49 a	8.72 ± 2.04 A	6.61 ± 1.32 B
glutamic acid	31.31 ± 1.29 a	38.05 ± 6.52 b	28.91 ± 3.74 A	48.20 ± 7.30 B
glutamine	25.49 ± 3.61 a	22.67 ± 7.62 a	30.87 ± 6.19 A	31.28 ± 8.60 A
glycine	2.09 ± 0.33 a	1.83 ± 0.48 a	0.84 ± 0.22 A	1.80.51 A
histidine	0.84 ± 0.20 a	0.79 ± 0.16 a	1.67 ± 0.47 A	1.49 ± 0.74 A
isoleucine	0.33 ± 0.05 a	0.34 ± 0.08 a	0.87 ± 0.21 A	0.87 ± 0.37 A
leucine	0.37 ± 0.04 a	0.34 ± 0.07 a	0.90 ± 0.23 A	0.93 ± 0.41 A
lysine	2.08 ± 0.48 a	2.20 ± 0.98 a	2.57 ± 0.54 A	2.60 ± 0.68 A
methionine	0.05 ± 0.01 a	0.03 ± 0.02 b	0.02 ± 0.02 A	0.03 ± 0.02 A
ornithine	0.30 ± 0.10 a	0.39 ± 0.17 a	0.13 ± 0.05 A	0.19 ± 0.10 A
phenylalanine	0.30 ± 0.03 a	0.42 ± 0.09 b	0.68 ± 0.18 A	0.85 ± 0.38 A
proline	0.37 ± 0.07 a	0.38 ± 0.20 a	0.27 ± 0.16 A	0.42 ± 0.17 A
serine	5.09 ± 0.64 a	5.16 ± 1.37 a	4.07 ± 0.63 A	5.07 ± 1.31 A
threonine	1.68 ± 0.10 a	1.74 ± 0.36 a	2.66 ± 0.35 A	3.23 ± 0.76 A
tryptophan	0.33 ± 0.13 a	0.69 ± 0.35 b	0.51 ± 0.16 A	0.56 ± 0.29 A
tyrosine	0.07 ± 0.04 a	0.12 ± 0.10 a	0.35 ± 0.18 A	0.32 ± 0.15 A
valine	1.11 ± 0.10 a	1.27 ± 0.41 a	1.73 ± 0.27 A	1.93 ± 0.50 A

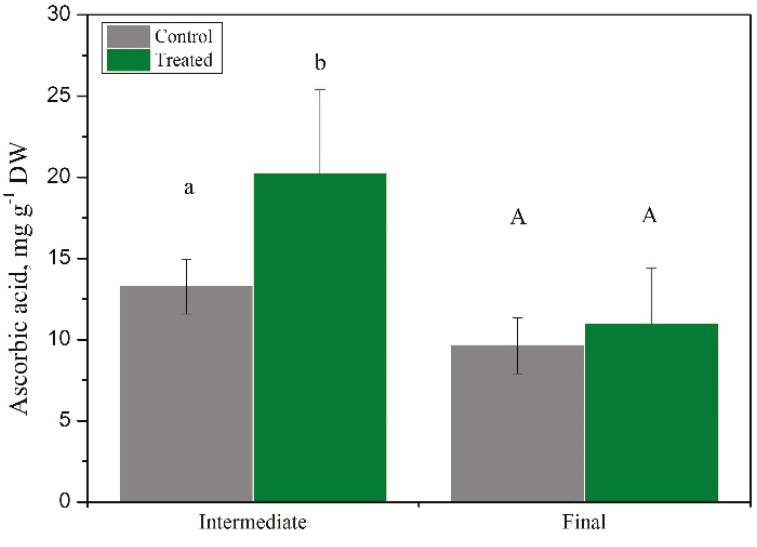

Figure 7. Ascorbic acid content of basil leaves grown as a secondary crop in the second experiment at the intermediate and final harvests. Values are expressed as mean ± standard deviation ($n = 5$). Different letters indicate statistically significant differences between treatments at each harvest (small letters refer to the intermediate and capital ones to the final harvest, $p < 0.05$).

Table 5 summarizes the macro- and micro-nutrient content of basil plants in the second experiment. Noticeable impacts of treatment were recorded in P concentration, with 40% and 52%, and Mn with 74% and 60% reductions in the intermediate and final harvests, respectively; and in K, which showed a significant 45% reduction at the end of the experiment. On the contrary, Mg concentration was increased by 108% at the final harvest.

Table 5. Nutrient concentrations in basil leaves of the control and cascade hydroponics treatments (2nd experiment) as determined at the intermediate and final harvests. Different letters indicate statistically significant differences between treatments at each harvest (small letters refer to the intermediate and capital ones to the final harvest, $p < 0.05$). Values in all layers are expressed as mean ± standard deviation ($n = 10$).

Intermediate Harvest									
Treatment	**N (%)**	**P (%)**	**K (%)**	**Ca (%)**	**Mg (%)**	**Fe (mg/kg)**	**Zn (mg/kg)**	**Mn (mg/kg)**	**Cu (mg/kg)**
Control	5.54 ± 0.64 a	0.91 ± 0.21 a	5.97 ± 0.45 a	2.66 ± 0.68 a	0.44 ± 0.13 a	132.62 ± 30.72 a	87.11 ± 7.02 a	175.75 ± 17.69 a	17.49 ± 2.93 a
Treated	4.82 ± 0.32 b	0.55 ± 0.05 b	4.87 ± 0.65 b	2.74 ± 0.23 a	0.59 ± 0.09 b	146.36 ± 25.92 a	57.27 ± 8.31 b	46.36 ± 10.19 b	16.92 ± 2.87 a
Final Harvest									
Treatment	**N (%)**	**P (%)**	**K (%)**	**Ca (%)**	**Mg (%)**	**Fe (mg/kg)**	**Zn (mg/kg)**	**Mn (mg/kg)**	**Cu (mg/kg)**
Control	4.74 ± 0.13 A	1.06 ± 0.05 A	7.11 ± 0.46 A	2.23 ± 0.13 A	0.33 ± 0.02 A	172.69 ± 30.86 A	91.49 ± 5.46 A	198.76 ± 10.19 A	19.22 ± 2.21 A
Treated	4.75 ± 0.17 A	0.50 ± 0.05 B	3.89 ± 0.46 B	2.95 ± 0.14 B	0.69 ± 0.06 B	148.13 ± 17.79 B	58.24 ± 4.61 B	46.36 ± 12.01 B	14.69 ± 2.42 B

The performance of basil under the conditions and the system in which the present experiment was carried out proved to be promising for its use as a secondary crop in cascade hydroponic systems. Obviously, there are numerous aspects of basil biochemistry, complementary to those measured in the present study, that might be determined in future studies and complete the picture of basil performance. Among them, the impact of the drainage solution for various primary crops on concentrations and profiles of secondary metabolites, especially those responsible for aroma, would be worth studying.

The concept of cascade cropping systems is new; thus, few studies have explored their potential in ornamental and horticultural production and delineated their advantages and drawbacks [5,8,43,44]. The main constraint seems to be the increased salinity in the root zone of the secondary and tertiary crops, a problem that may be overcome by various levels of dilution of the primary crop leachates with water of low electrical conductivity [5,8,45]. Additionally, the use of salt-tolerant or even halophytic species, which can successfully grow under conditions of increased salinity, may be a feasible idea. Future experiments are expected to focus on this latter group, i.e., halophytes, some of which have recently been domesticated and included in human diet. Therefore, halophytes may be excellent candidates for their use as tertiary crops in cascade hydroponics.

4. Conclusions

The present study explored the salinity-tolerance thresholds of basil to evaluate its potential use as a secondary crop in a cascade hydroponics system. We used two distinct but complemented approaches to address our target; the first experiment tested several aspects of basil's response to increasing levels of salinity in order to identify the tolerance limits, while the second experiment employed a cascade system to monitor the responses, with cucumber grown in hydroponics as the primary crop, the drainage solution of which irrigated basil grown in pots, a setup comparable to the first experiment. The various aspects of basil growth and biochemical performance collectively indicated the 5 dS m^{-1} salinity level as the upper limit/threshold of tolerance to stress. Additionally, the results of

the first experiment indicated the first 15 days of treatment as a critical point for the process of salinity-symptom appearance on growth performance, as well as mineral composition. The use of basil as a secondary crop, which inevitably faces increased EC of the drainage solution of the primary crop, is subject to a compromise between fresh produce reduction and an increase in specific biochemical attributes related to basil quality. The increase of total amino acids under enhanced EC in both experiments and the trend for higher levels of the antioxidant AsA, as a surrogate of the antioxidant pool of basil, may compromise the 40% reduction in fresh produce yield in the cascade system. Another important aspect that should be considered is the benefit of re-using the drainage solution from the primary crop, which results in combined production of more than one crop and the optimization of the environmental footprint. Comparing the two experiments reported in the current study, we should highlight certain different responses of basil's biochemical parameters when exposed to drainage solution in the cascade system. This finding may indicate that other factors, except for the increased EC, may also act as drivers of plant response, and this must be confirmed in future experiments to reach deeper insights. We concluded after both experiments that basil performed well under the specific conditions and in the system employed in the present study and might be a good candidate for use as a secondary crop in cascade hydroponics systems.

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

Funding: This research has been co-financed by the European Union and Greek national funds through the national action "Bilateral and Multilateral E&T Cooperation Greece-Germany" (project code: T2DGE-0893); and for the German action, by the German Federal Ministry of Education and Research.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

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

References

1. Plaut, Z.; Edelstein, M.; Ben-Hur, M. Overcoming Salinity Barriers to Crop Production Using Traditional Methods. *Crit. Rev. Plant Sci.* **2013**, *32*, 250–291. [CrossRef]
2. Katsoulas, N.; Voogt, W. Recent trends in salinity control for soilless growing systems managemenT. *Acta Hortic.* **2014**, 433–442. [CrossRef]
3. Shahbaz, M.; Ashraf, M.; Al-Qurainy, F.; Harris, P.J.C. Salt Tolerance in Selected Vegetable Crops. *Crit. Rev. Plant Sci.* **2012**, *31*, 303–320. [CrossRef]
4. Gruda, N.; Savvas, D.; Colla, G.; Rouphael, Y. Impacts of genetic material and current technologies on product quality of selected greenhouse vegetables—A review. *Eur. J. Hortic. Sci.* **2018**, *83*, 319–328. [CrossRef]
5. Elvanidi, A.; Reascos, C.B.; Gourzoulidou, E.; Kunze, A.; Max, J.; Katsoulas, N. Implementation of the Circular Economy Concept in Greenhouse Hydroponics for Ultimate Use of Water and Nutrients. *Horticulturae* **2020**, *6*, 83. [CrossRef]
6. Gruda, N.S. Increasing Sustainability of Growing Media Constituents and Stand-Alone Substrates in Soilless Culture Systems. *Agronomy* **2019**, *9*, 298. [CrossRef]
7. Savvas, D.; Gruda, N. Application of soilless culture technologies in the modern greenhouse industry—A review. *Eur. J. Hortic. Sci.* **2018**, *83*, 280–293. [CrossRef]
8. Garcia-Caparros, P.; Llanderal, A.; Maksimovic, I.; Lao, M.T. Cascade Cropping System with Horticultural and Ornamental Plants under Greenhouse Conditions. *Water* **2018**, *10*, 125. [CrossRef]
9. Katsoulas, N.; Elvanidi, A.; Zagari, E.; Bartzanas, T.; Kalaitzis, P.; Kittas, C. Detection of salinity stress in soilless tomato based on crop reflectance. *Acta Hortic.* **2019**, *1242*, 723–728. [CrossRef]
10. Cabot, C.; Sibole, J.V.; Barceló, J.; Poschenrieder, C. Lessons from crop plants struggling with salinity. *Plant Sci.* **2014**, *226*, 2–13. [CrossRef] [PubMed]
11. Shahid, M.A.; Sarkhosh, A.; Khan, N.; Balal, R.M.; Ali, S.; Rossi, L.; Gómez, C.; Mattson, N.; Nasim, W.; Garcia-Sanchez, F. Insights into the Physiological and Biochemical Impacts of Salt Stress on Plant Growth and Development. *Agronomy* **2020**, *10*, 938. [CrossRef]

12. Tarchoune, I.; Degl'Innocenti, E.; Kaddour, R.; Guidi, L.; Lachaâl, M.; Navari-Izzo, F.; Ouerghi, Z. Effects of NaCl or Na_2SO_4 salinity on plant growth, ion content and photosynthetic activity in *Ocimum basilicum* L. *Acta Physiol. Plant.* **2011**, *34*, 607–615. [CrossRef]
13. Rouphael, Y.; Kyriacou, M. Enhancing Quality of Fresh Vegetables Through Salinity Eustress and Biofortification Applications Facilitated by Soilless Cultivation. *Front. Plant Sci.* **2018**, *9*, 1254. [CrossRef] [PubMed]
14. Bahcesular, B. Seed Priming with Melatonin Effects on Growth, Essential Oil Compounds and Antioxidant Activity of Basil (*Ocimum Basilicum* L.) under Salinity Stress. *Ind. Crops Prod.* **2020**, *146*, 112165. [CrossRef]
15. Selmar, D.; Kleinwächter, M. Influencing the product quality by deliberately applying drought stress during the cultivation of medicinal plants. *Ind. Crop. Prod.* **2013**, *42*, 558–566. [CrossRef]
16. Boestfleisch, C.; Papenbrock, J. Changes in secondary metabolites in the halophytic putative crop species *Crithmum maritimum* L., *Triglochin maritima* L. and *Halimione portulacoides* (L.) Aellen as reaction to mild salinity. *PLoS ONE* **2017**, *12*, e0176303. [CrossRef] [PubMed]
17. Gruda, N. Do Soilless Culture Systems Have an Influence on Product Quality of Vegetables? *J. Appl. Bot. Food Qual.* **2009**, *82*, 141–147.
18. Tanaka, H.; Yamada, S.; Masunaga, T.; Yamamoto, S.; Tsuji, W.; Murillo-Amador, B. Comparison of nutrient uptake and antioxidative response among four Labiatae herb species under salt stress condition. *Soil Sci. Plant Nutr.* **2018**, *64*, 589–597. [CrossRef]
19. Petropoulos, S.A.; Levizou, E.; Ntatsi, G.; Fernandes, Â.; Petrotos, K.; Akoumianakis, K.; Barros, L.; Ferreira, I.C. Salinity effect on nutritional value, chemical composition and bioactive compounds content of *Cichorium spinosum* L. *Food Chem.* **2017**, *214*, 129–136. [CrossRef] [PubMed]
20. Scagel, C.F.; Lee, J.; Mitchell, J.N. Salinity from NaCl changes the nutrient and polyphenolic composition of basil leaves. *Ind. Crop. Prod.* **2019**, *127*, 119–128. [CrossRef]
21. Neto, A.D.D.A.; Menezes, R.V.; Gheyi, H.R.; Silva, P.C.C.; Cova, A.M.W.; Ribas, R.F.; Ribeiro, M.D.O. Salt-induced changes in solutes, pigments and essential oil of two basil (*Ocimum basilicum* L.) genotypes under hydroponic cultivation. *Aust. J. Crop. Sci.* 2019, *13*. [CrossRef]
22. Pegg, R.B.; Amarowicz, R.; Naczk, M.; Shahidi, F. PHOTOCHEM® for Determination of Antioxidant Capacity of Plant Extracts. In *Antioxidant Measurement and Applications*; ACS Symposium Series; American Chemical Society: Washington, DC, USA, 2007; Volume 956, pp. 140–158, ISBN 978-0-8412-7428-0.
23. Elhindi, K.M.; Al-Amri, S.M.; Abdel-Salam, E.; Al-Suhaibani, N.A. Effectiveness of salicylic acid in mitigating salt-induced adverse effects on different physio-biochemical attributes in sweet basil (*Ocimum basilicum* L.). *J. Plant Nutr.* **2017**, *40*, 908–919. [CrossRef]
24. Gohari, G.; Alavi, Z.; Esfandiari, E.; Panahirad, S.; Hajihoseinlou, S.; Fotopoulos, V. Interaction between hydrogen peroxide and sodium nitroprusside following chemical priming ofOcimum basilicumL. against salt stress. *Physiol. Plant.* **2019**, *168*, 361–373. [CrossRef] [PubMed]
25. Attia, H.; Ouhibi, C.; Ellili, A.; Msilini, N.; Bouzaïen, G.; Karray, N.; Lachaâl, M. Analysis of salinity effects on basil leaf surface area, photosynthetic activity, and growth. *Acta Physiol. Plant.* **2010**, *33*, 823–833. [CrossRef]
26. Tarchoune, I.; Sgherri, C.; Izzo, R.; Lachaal, M.; Ouerghi, Z.; Navari-Izzo, F. Antioxidative responses of Ocimum basilicum to sodium chloride or sodium sulphate salinization. *Plant Physiol. Biochem.* **2010**, *48*, 772–777. [CrossRef] [PubMed]
27. Caliskan, O.; Kurt, D.; Temizel, K.E.; Odabas, M.S. Effect of Salt Stress and Irrigation Water on Growth and Development of Sweet Basil (*Ocimum basilicum* L.). *Open Agric.* **2017**, *2*, 589–594. [CrossRef]
28. Berger, B.; de Regt, B.; Tester, M. Trait Dissection of Salinity Tolerance with Plant Phenomics. In *Plant Salt Tolerance: Methods and Protocols*; Shabala, S., Cuin, T.A., Eds.; Methods in Molecular Biology; Humana Press: Totowa, NJ, USA, 2012; pp. 399–413, ISBN 978-1-61779-986-0.
29. Awlia, M.; Nigro, A.; Fajkus, J.; Schmoeckel, S.M.; Negrao, S.; Santelia, D.; Trtílek, M.; Tester, M.; Julkowska, M.M.; Panzarová, K. High-Throughput Non-destructive Phenotyping of Traits that Contribute to Salinity Tolerance in Arabidopsis thaliana. *Front. Plant Sci.* **2016**, *7*, 1414. [CrossRef] [PubMed]
30. Morano, G.; Amalfitano, C.; Sellitto, M.; Cuciniello, A.; Maiello, R.; Caruso, G. Effects of Nutritive Solution Electrical Conductivity and Plant Density on Growth, Yield and Quality of Sweet Basil Grown in Gullies by Subirrigation. *Adv. Hortic. Sci.* **2017**, *31*, 25–30. [CrossRef]
31. Barbieri, G.; Vallone, S.; Orsini, F.; Paradiso, R.; De Pascale, S.; Zakharov, F.; Maggio, A. Stomatal density and metabolic determinants mediate salt stress adaptation and water use efficiency in basil (*Ocimum basilicum* L.). *J. Plant Physiol.* **2012**, *169*, 1737–1746. [CrossRef]
32. Batista-Silva, W.; Heinemann, B.; Rugen, N.; Nunes-Nesi, A.; Araújo, W.L.; Braun, H.-P.; Hildebrandt, T.M. The role of amino acid metabolism during abiotic stress release. *Plant Cell Environ.* **2019**, *42*, 1630–1644. [CrossRef]
33. Hildebrandt, T.M. Synthesis versus degradation: Directions of amino acid metabolism during Arabidopsis abiotic stress response. *Plant Mol. Biol.* **2018**, *98*, 121–135. [CrossRef]
34. Fuertes-Mendizábal, T.; Bastías, E.I.; González-Murua, C.; González-Moro, M.B. Nitrogen Assimilation in the Highly Salt- and Boron-Tolerant Ecotype *Zea mays* L. *Amylacea. Plants* **2020**, *9*, 322. [CrossRef]
35. Oddy, J.; Raffan, S.; Wilkinson, M.; Elmore, J.S.; Halford, N.G. Stress, nutrients and genotype: Understanding and managing asparagine accumulation in wheat grain. *CABI Agric. Biosci.* **2020**, *1*, 1–14. [CrossRef]

36. Neocleous, D.; Ntatsi, G. Seasonal variations of antioxidants and other agronomic features in soilless production of selected fresh aromatic herbs. *Sci. Hortic.* **2018**, *234*, 290–299. [CrossRef]
37. Arif, Y.; Singh, P.; Siddiqui, H.; Bajguz, A.; Hayat, S. Salinity induced physiological and biochemical changes in plants: An omic approach towards salt stress tolerance. *Plant Physiol. Biochem.* **2020**, *156*, 64–77. [CrossRef] [PubMed]
38. Shannon, M.; Grieve, C. Tolerance of vegetable crops to salinity. *Sci. Hortic.* **1998**, *78*, 5–38. [CrossRef]
39. Scagel, C.F.; Bryla, D.; Lee, J. Salt Exclusion and Mycorrhizal Symbiosis Increase Tolerance to NaCl and $CaCl_2$ Salinity in 'Siam Queen' Basil. *HortScience* **2017**, *52*, 278–287. [CrossRef]
40. Maathuis, F.J.M. The role of monovalent cation transporters in plant responses to salinity. *J. Exp. Bot.* **2005**, *57*, 1137–1147. [CrossRef] [PubMed]
41. Teixeira, W.F.; Fagan, E.B.; Soares, L.H.; Umburanas, R.C.; Reichardt, K.; Neto, D.D. Foliar and Seed Application of Amino Acids Affects the Antioxidant Metabolism of the Soybean Crop. *Front. Plant Sci.* **2017**, *8*, 327. [CrossRef]
42. Song, Q.; Joshi, M.; DiPiazza, J.; Joshi, V. Functional Relevance of Citrulline in the Vegetative Tissues of Watermelon During Abiotic Stresses. *Front. Plant Sci.* **2020**, *11*, 512. [CrossRef]
43. García-Caparrós, P.; Espino, C.; Lao, M. Comparative Behavior of *Dracaena marginata* Plants Integrated into a Cascade Cropping System with the Addition of Hydrogen Peroxide. *Agronomy* **2021**, *11*, 218. [CrossRef]
44. García-Caparrós, P.; Llanderal, A.; Velasquez, C.; Lao, M. Water and Nutrient Balance in an Ornamental Cascade Cropping System. *Agronomy* **2021**, *11*, 1251. [CrossRef]
45. Plaza, B.M.; Soriano, F.; Jiménez-Becker, S.; Lao, M.T. Nutritional responses of Cordyline fruticosa var. 'Red Edge' to fertigation with leachates vs. conventional fertigation: Chloride, nitrogen, phosphorus and sulphate. *Agric. Water Manag.* **2016**, *173*, 61–66. [CrossRef]

horticulturae

MDPI

Article

Composting Spent Mushroom Substrate from *Agaricus bisporus* and *Pleurotus ostreatus* Production as a Growing Media Component for Baby Leaf Lettuce Cultivation under *Pythium irregulare* Biotic Stress

Daniel Hernández [1], Margarita Ros [1,*], Francisco Carmona [2], José Antonio Saez-Tovar [3] and Jose Antonio Pascual [1]

[1] Conservación de Suelos y Agua y Manejo de Residuos Orgánicos, CEBAS-CSIC, Campus de Espinardo, 30100 Murcia, Spain; dhernandez@cebas.csic.es (D.H.); jpascual@cebas.csic.es (J.A.P.)
[2] Symborg SL Polígono Industrial Cabezo Cortado, Av. Jesús Martinez Cortado, 51, 30100 Murcia, Spain; franciscocarmona@symborg.com
[3] Agroquímica y Medioambiente, Universidad Miguel Hernández, UMH-EPSO, ctra. Beniel Km 3.2, 03312 Orihuela, Spain; jose.saezt@umh.es
* Correspondence: margaros@cebas.csic.es; Tel.: +34-968-396-200

Abstract: Composts of spent mushrooms substrates can be an alternative for the partial replacement of peat as growing media in horticulture. Three mature composts from *Agaricus bisporus* (Ag), *Pleurotus ostreatus* (Pl), and 70% Ag:30% Pl (AgPl) production were used as partial components of peat growing media, used at a 1:4 compost:peat ratio for growing red baby leaf lettuce. They showed higher yields, between 3 and 7 times more than that for peat itself, even under the pressure of the plant pathogen *Pythium irregulare*. AgPl showed the higher suppressiveness (50%) against *Pythium irregulare* than Ag- (38%) or Pl- (15%) supplemented media. The combination of these raw materials and a suitable composting process is important for obtaining mature compost for use as a partial component of peat-based growing media.

Keywords: suppressiveness; *Trichoderma harzianum*; peat; compost; substrate

Citation: Hernández, D.; Ros, M.; Carmona, F.; Saez-Tovar, J.A.; Pascual, J.A. Composting Spent Mushroom Substrate from *Agaricus bisporus* and *Pleurotus ostreatus* Production as a Growing Media Component for Baby Leaf Lettuce Cultivation under *Pythium irregulare* Biotic Stress. *Horticulturae* **2021**, *7*, 13. https://doi.org/10.3390/horticulturae7020013

Received: 31 December 2020
Accepted: 21 January 2021
Published: 25 January 2021

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

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

At the present time, there is an increasing demand for proteins of plant origin, which cost less and are healthier than the proteins from animal sources [1]. Edible mushrooms belonging to the Basidiomycetes are an interesting alternative due to their high concentrations of proteins and vitamins. *Agaricus bisporus* (*A. bisporus*) and *Pleurotus ostreatus* (*P. ostreatus*) are the most commonly cultivated mushroom species.

Worldwide mushroom production is greater than 25 MT per year [2], producing an average 5 kg of spent mushroom substrate (SMS) per kilogram of mushroom. Accumulation of this waste over time has a negative impact on the environment [3,4], generating leachates that can contaminate the soil and surrounding water [5]. After mushroom harvest, SMS still holds high levels of organic matter and nutrients and could be of potential use in agriculture, horticulture, or disease management [4]. However, SMS requires stabilization for using in agriculture, due to the amount of labile organic matter, assuring at the same time the elimination of mushroom mycelia that invade the SMS [6]. The stabilization of SMS through a composting process could offer a sustainable alternative for agriculture [7,8]. The composting process involves the succession of microorganisms, which is directly affected by various factors such as the specific mix of raw materials, temperature, aeration, moisture, C/N ratio, and pH, among others [9,10].

Lettuce (*Lactuca sativa* L.) is the most common of the salad leaf crops and is mainly consumed fresh. Among lettuces types, baby leaf red lettuce has popularity, due to its easier and faster processing and high content of phytochemicals with health beneficial effects.

The successful production of lettuce in soilless culture with a minimal level of pest control depends on uniform, high-quality seedling germination and growth in a substrate [11].

Peat is the main component of growing media for lettuce production, because of its ideal characteristics for cropping such as constant chemical and physical properties [12]. Nevertheless, peat is a non-renewable resource whose harvest produces a negative impact on global climate change, and which is susceptible to soilborne pathogens such as *Pythium irregulare* (*P. irregulare*) (causing damping-off diseases), characterized as virulent and fast spreading in baby leaf lettuce crops in Mediterranean areas [11].

Composts from SMS can be partial components of growing media [13,14]. Moreover, some have shown potential suppressive activity against plant pathogens [11–15]. There are different mechanisms involved in pathogen suppression, including nutrient and space competition, antibiosis, and mycoparasitism [16], and the induction of systemic resistance to biotic stresses such as disease and abiotic stresses [17].

Our hypothesis is that the use, as a component of plant growing media, of compost made from spent mushroom substrate (SMS) after culture of *A. bisporus* (Ag), or *P. ostreatus* (Pl), or a combination combination of 70% *A. bisporus* and 30% *P. ostreatus* (AgPl) mixed with peat (1:4; compost:peat) would increase germination and plant biomass production and reduce the effects of *P. irregulare* in red baby leaf lettuce grown under soilless conditions compared to peat alone as growing media. To test this hypothesis, several experiments were carried out with the following objectives: (1) to evaluate the composting process of SMS from Ag, Pl, and AgPl; (2) to evaluate whether the composts could be used as a component of soilless growing media (1:4; compost:peat) to produce red baby leaf lettuce; (3) to evaluate the suppressive capacity of the composts under biotic stress of *P. irregulare*; and, (4) to evaluate whether the suppressiveness of SMS compost from AgPl inoculated with the biocontrol agent *Trichoderma harzianum* (AgPl + T) as a component of soilless growing media could be increased.

2. Materials and Methods

2.1. Raw Materials

Spent mushroom substrates from *A. bisporus* culture and *P. ostreatus* culture were produced after 3–4 mushroom harvests. The substrate for *A. bisporus* production was principally made using cereal straw, poultry manure, calcium sulphate ($CaSO_4 \cdot 2H_2O$) and water to reach 70% humidity. Limestone gravel (high-purity calcium carbonate) was added to buffer the pH to 7.5, and the compost reached temperatures around 70 °C and was turned 3–4 times. The substrate for *P. ostreatus* production was principally made using straw, 70% humidity, and was not composted. Both substrates for *A. bisporus* or *P. ostreatus* production were packed in plastic bags for mushroom production and they were distributed to the production sites, 30–40 farms in a radius of 20 km from the substrate production site. Once they were spent, they were moved to a recycling plant for their management, which involved removing the plastic, homogenizing the SMS, and placing in piles for composting. For this study, SMS was collected from the compost recycling plant Sustratos de la Rioja located in Pradejón (La Rioja, Spain). The main characteristics of the Ag SMS and Pl SMS can be found in Table 1.

2.2. The Composting Process

Three composting piles of 2500 tons were set up: 100% Ag SMS (Pile Ag), 100% Pl SMS (Pile Pl), and a mix of 70% Ag SMS and 30% Pl SMS (*v*/*v*) (Pile AgPl). The piles showed an initial water holding capacity of 70%, which was maintained to 50–60% by regular turning when the temperature was higher than 65 °C. The composting processes lasted around 130 days, including 50 days for the bio-oxidative phase and a maturation phase of 80 days. Sampling was performed throughout the composting process at 0, 20, 35, 90, and 130 days from the beginning, from three sites on each pile and mixed to obtain a representative sample.

Table 1. Physicochemical and chemical properties during composting process.

	Temperature °C	pH	EC mS/cm	C/N	TOC	TN	P	K	S	Ca	Mg	Na	Fe
					g/100 g								
Agaricus bisporus composting pile (Pile Ag)													
I (0) [y]	41.8 ± 0.21	7.01 ± 0.42	6.44 ± 0.15	16.60	37.9 ± 0.1	2.28 ± 0.02	0.43 ± 0.01	1.78 ± 0.06	2.24 ± 0.03	4.97 ± 0.02	0.69 ± 0.01	0.25 ± 0.01	1.07 ± 0.01
T (20)	48.3 ± 0.16	7.51 ± 0.33	6.61 ± 0.07	15.10	35.2 ± 0.1	2.32 ± 0.03	0.52 ± 0.03	1.84 ± 0.10	2.64 ± 0.17	5.71 ± 0.38	0.74 ± 0.05	0.28 ± 0.01	1.48 ± 0.04
E (35)	54.3 ± 0.17	7.54 ± 0.22	7.96 ± 0.10	13.10	35.0 ± 0.1	2.66 ± 0.01	0.62 ± 0.00	2.61 ± 0.03	3.43 ± 0.02	7.65 ± 0.12	0.97 ± 0.02	0.34 ± 0.01	1.78 ± 0.03
M (90)	50.4 ± 0.12	7.65 ± 0.12	8.19 ± 0.05	11.40	33.4 ± 0.1	2.92 ± 0.00	0.61 ± 0.02	2.40 ± 0.02	2.89 ± 0.11	6.50 ± 0.13	0.97 ± 0.00	0.36 ± 0.01	1.78 ± 0.06
F (130)	32.2 ± 0.11	7.62 ± 0.23	7.67 ± 0.10	10.90	29.5 ± 0.1	2.69 ± 0.01	0.64 ± 0.03	2.30 ± 0.11	2.81 ± 0.15	6.01 ± 0.23	0.92 ± 0.00	0.28 ± 0.02	1.90 ± 0.09
Pleurotus ostreatus composting pile (Pile Pl)													
I (0)	37.5 ± 0.09	6.03 ± 0.22	5.66 ± 0.44	50.50	41.0 ± 0.4	0.81 ± 0.05	0.08 ± 0.00	1.51 ± 0.03	0.49 ± 0.01	1.71 ± 0.03	0.24 ± 0.01	0.08 ± 0.00	1.14 ± 0.03
T (20)	43.6 ± 0.12	7.73 ± 0.12	5.42 ± 0.19	33.50	38.8 ± 0.2	1.16 ± 0.01	0.10 ± 0.01	1.67 ± 0.11	0.51 ± 2.04	2.04 ± 0.11	0.26 ± 0.02	0.11 ± 0.01	1.37 ± 0.01
E (35)	47.8 ± 0.09	7.87 ± 0.23	5.15 ± 0.10	33.70	39.1 ± 0.1	1.16 ± 0.01	0.10 ± 0.01	2.33 ± 0.04	0.69 ± 0.00	2.37 ± 0.03	0.31 ± 0.01	0.14 ± 0.00	1.64 ± 0.01
M (90)	45.3 ± 0.11	8.12 ± 0.08	5.46 ± 0.09	26.00	38.1 ± 0.1	1.47 ± 0.01	0.14 ± 0.00	2.42 ± 0.02	0.74 ± 0.01	2.67 ± 0.08	0.41 ± 0.01	0.18 ± 0.00	1.78 ± 0.02
F (130)	33.1 ± 0.21	7.88 ± 0.21	7.11 ± 0.86	17.90	32.8 ±0.3	1.84 ± 0.00	0.42 ± 0.02	2.35 ± 0.21	1.89 ± 0.34	4.48 ± 0.30	0.62 ± 0.06	0.22 ± 0.02	2.37 ± 259
70%. *A. bisporus* and 30% *P. ostreatus* composting pile (Pile AgPl)													
I (0)	47.3 ± 0.11	7.49 ± 0.25	6.09 ± 0.15	23.50	39.0 ± 0.2	1.66 ± 0.05	0.27 ± 0.01	1.68 ± 0.05	1.25 ± 0.03	3.13 ± 0.08	0.46 ± 0.01	0.13 ± 0.00	1.10 ± 0.04
T (20)	50.2 ± 0.09	7.49 ± 0.11	7.81 ± 0.11	19.20	34.7 ± 0.1	1.81 ± 0.02	0.20 ± 0.03	1.84 ± 0.23	1.13 ± 0.13	2.8 ± 0.32	0.33 ± 0.04	0.19 ± 0.02	1.21 ± 0.16
E (35)	53.6 ± 0.14	7.3 ± 0.13	8.01 ± 0.03	13.50	34.6 ± 0.1	2.56 ± 0.00	0.55 ± 0.02	3.01 ± 0.11	2.74 ± 0.11	6.66 ± 0.30	0.87 ± 0.03	0.31 ± 0.01	2.61 ± 0.09
M (90)	50.5 ± 0.15	7.69 ± 0.24	8.13 ± 0.24	11.70	32.8 ± 0.2	2.79 ± 0.01	0.49 ± 0.00	3.1 ± 0.11	2.25 ± 0.02	5.91 ± 0.07	0.85 ± 0.04	0.34 ± 0.02	2.02 ± 0.07
F (130)	31.4 ± 0.11	7.57 ± 0.09	7.56 ± 0.53	12.00	30.2 ± 0.5	2.52 ± 0.03	0.46 ± 0.00	2.45 ± 0.00	2.96 ± 0.32	4.42 ± 0.05	0.66 ± 0.01	0.22 ± 0.00	2.70 ± 0.24

Mean value ± standard errors. EC, electrical conductivity; TOC, total organic carbon; TN, total nitrogen. [y] Days of composting in brackets I: initial phase; T: thermophilic phase; E: end of bio-oxidative phase; M: maturity phase; F: final compost.

2.3. Assessment of Composts as a Component of Growing Media for Red Baby Leaf Lettuce Cultivation and as a Suppressive Growing Media under P. irregulare Biotic Stress

A pot experiment was performed to assess the different composts obtained after the composting process as compost growing media for red baby leaf lettuce cultivation. Treatments were Ag, Pl, and AgPl composts mixed with commercial peat 315 (Blond/black 60/40 Turbas y Coco Mar Menor S.L.) at a 1:4 (*w*/*w*; compost:peat) ratio. This ratio was selected as optimal for avoiding seed germination inhibition. Peat alone was used as the control treatment. The main physicochemical and chemical characteristics of the peat were as follows: pH 5.6; electrical conductivity (EC) 1 mS cm^{-1}; total C 466 g kg^{-1}; total N 9.4 g kg^{-1}; total P 0.3 g kg^{-1}; and total K 0.9 g kg^{-1}. Red baby leaf lettuce "Ligier RZ84-14" (Rijk Zwaan, De Lier, The Netherlands) was selected as the assayed crop and *P. irregulare* as the pathogen to evaluate compost suppressiveness.The pathogen (*P. irregulare*) was isolated in potato dextrose agar medium (PDA, Sharlau, Spain) culture from lettuce plants showing disease symptoms in a lettuce field, then selected based on phenotypic appearance, and re-cultured on PDA to ensure identity The *P. irregulare* inoculum was produced by mixing and blending 4-day-old mycelia onto PDA with 200 mL of sterile distilled water. Thirty replicate pots were prepared from each treatment: half (15) were not inoculated with the pathogen and were used to evaluate the effect of compost as a growing media; the other half were infected with the pathogen (6.75 mL) before planting, equivalent to 8.23 log copies of internally transcribed spacers (ITS) g^{-1} growing media.

For germination, the pots were placed in a growth chamber at 18 ± 1 °C at 80% relative humidity (RH) and in darkness for 48 h. After that, the pots were randomly distributed in a growth chamber at 24/18 °C day/night with a RH range of 60–70% for 25 days. The germination percentage was measured six days after sowing and was calculated as the ratio of germinated seeds divided by total seeds, multiplied by 100. The lettuce plants were collected 25 days after planting, and the fresh plant biomass was weighed.

2.4. Assessment of Composts Inoculated with T. harzianum as a Component of Growing Media and a Suppressive Growing Media under P. irregulare Biotic Stress for Red Baby Leaf Lettuce Cultivation

A pot experiment was performed with two treatments: AgPl and AgPl + T. The latter was inoculated with *T. harzianum* (CEBAS collection) to achieve a final concentration of 6.75 log copies ITS g^{-1} growing media. *T. harzianum* was produced and immobilized in bentonite (1:9) [18]. The experiment was set up as described in the prior section.

2.5. Chemical and Microbiological Properties

The pH and electrical conductivity (EC) were measured in a 1:10 (*w*/*v*) aqueous extract of the substrate media. The total organic carbon (TOC) and total nitrogen (N) were measured using a LECO TruSpec C/N Elemental Analyzer. P, K, Na, Ca, Mg, Fe, and heavy metals were determined by inductively coupled plasma-mass spectrophotometry (ICP-MS PQExCell, VG-Thermo Elemental, Winsford, Cheshire, UK), after HNO3/HClO4 high-pressure digestion. Total organic carbon (TOC) loss due to mineralization was calculated from the initial (X1) and final (X2) ash contents according to the following equation [19]:

$$\text{TOC loss (\%)} = 100 - 100\,[X1\,(100 - X2)/X2\,(100 - X1)]$$

The suppressiveness index was calculated according to the formulae of disease suppressiveness describe by Veeken et al. [20]. The abundance of *P. irregulare* and *T. harzianum* inoculated was measured in a real-time PCR system by quantitative 7500 Fast real-time PCR (qPCR), following the protocol described by Giménez et al. [11] with the specific primers for *P. irregulare* and *T. harzianum* previously described by López-Mondéjar et al. [21].

2.6. Statistical Analysis

Data were analysed using the IMB Statistics SPSS 26 software, and an ANOVA test was performed. When the F-statistic was significant, the differences between treatments

were determined using Tukey's test at $\alpha = 0.05$, or Duncan's multiple range test for non-homogeneous values at $p = 0.05$. Normality and homogeneity of the variances were checked using the Shapiro–Wilk and Levene tests, respectively.

3. Results

3.1. The Composting Process

The temperature in the piles increased until it reached values ranging from 47.76 to 54.32 °C; these temperatures were maintained for 55 days (thermophilic phase). The temperatures then decreased during the cooling and maturation phase (Table 1). The thermophilic phase duration for Pile Ag was 64 days, for Pile AgPl 52 days, and for the Pile Pl only 44 days. Both piles with Ag (Pile Ag and Pile AgPl) showed higher temperatures (>50 °C) than Pile Pl.

The variations in physicochemical and chemical parameters during the composting process are shown in Table 1. In general, the pH and EC increased during the composting process in the three piles. After 130 days, the pH reached values of 7.62 (Pile Ag), 7.57 (Pile AgPl), and 7.88 (Pile Pl), while the EC reached values of 7.67 (Pile Ag), 7.56 (Pile AgPl), and 7.11 (Pile Pl). The C/N ratios and total carbon (TC) of the three piles diminished during the composting process, although the C content was higher in Pile Pl during composting than in the other two piles. The highest percentage of TOC loss occurred in Pile AgPl (45%), followed by Pile Ag (30%) and Pile Pl (23%) (Figure 1). Inversely to C content, the total nitrogen (TN) content increased during the composting process, and Piles AgPl and Ag showed the highest TN content throughout the process (Table 1). In general, total P, K, Mg, and, especially, Ca also significantly increased during the composting process, with Pile Ag showing the highest values at the end of composting process. Total Cd, Cr, Mn, Zn, Cu, Cr, Pb, and Ni showed a similar trend to the other measured minerals, also increasing during composting (Table 2). Composts did not show evidence of *Salmonella* spp., *Listeria* spp., or *Escherichia coli*. Moreover, no animal pathogens were detected in the substrates before use in mushroom cultivation (data not shown).

3.2. Composts as a Growing Media Component

The percentage of red baby leaf lettuce seed germination in the different composts was significantly higher than the germination rate of plants grown in peat alone, and no significant differences between the composts were observed (Figure 2A). The fresh shoot weight of red baby leaf lettuce grown in the composts was also significantly higher than that grown in peat. Comparing the three composts, the highest fresh shoot weight was obtained for Ag (Figure 2B).

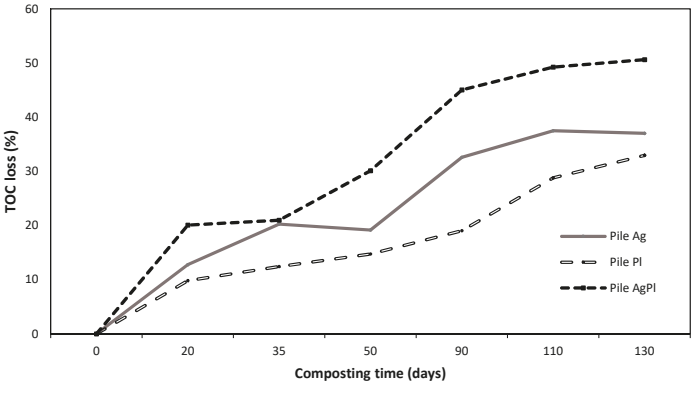

Figure 1. Percentage of total organic carbon losses during the composting process of the different piles. 100% *A. bisporus* (Pile Ag), 100% *P. ostreatus* (Pile Pl), and 70% *A. bisporus:* 30% *P. ostreatus* (Pile AgPl).

Table 2. Heavy metal concentration of 100% *A. bisporus* (Ag), 100% *P. ostreatus* (Pl), and 70% *A. bisporus:* 30% *P. ostreatus* (AgPl) composts.

Compost	Cu (mg/kg)	Zn (mg/kg)	Cd (mg/kg)	Cr (mg/kg)	Pb (mg/kg)	Ni (mg/kg)
Ag	43 ± 2.06 [z]	258 a ± 12.93	<1 ± 0.01	7 ± 0.28	2 ± 0.11	4 ± 0.17
Pl	36 ± 2.43	169 b ± 9.00	<1 ± 0.00	9 ± 1.37	2 ± 0.16	4 ± 0.60
AgPl	41 ± 0.11	167 ± 1.4	<1 ± 0.01	9 ± 0.33	3 ± 0.10	4 ± 0.05
Spanish framework [y]	400	1000	3	300	200	100

[z] Mean value ± standard error. [y] Limits permitted in the current Spanish legal framework (Real Decreto 506/2013). For each growing media, values with different letter differ significantly according to Tukey's test ($\alpha < 0.05$).

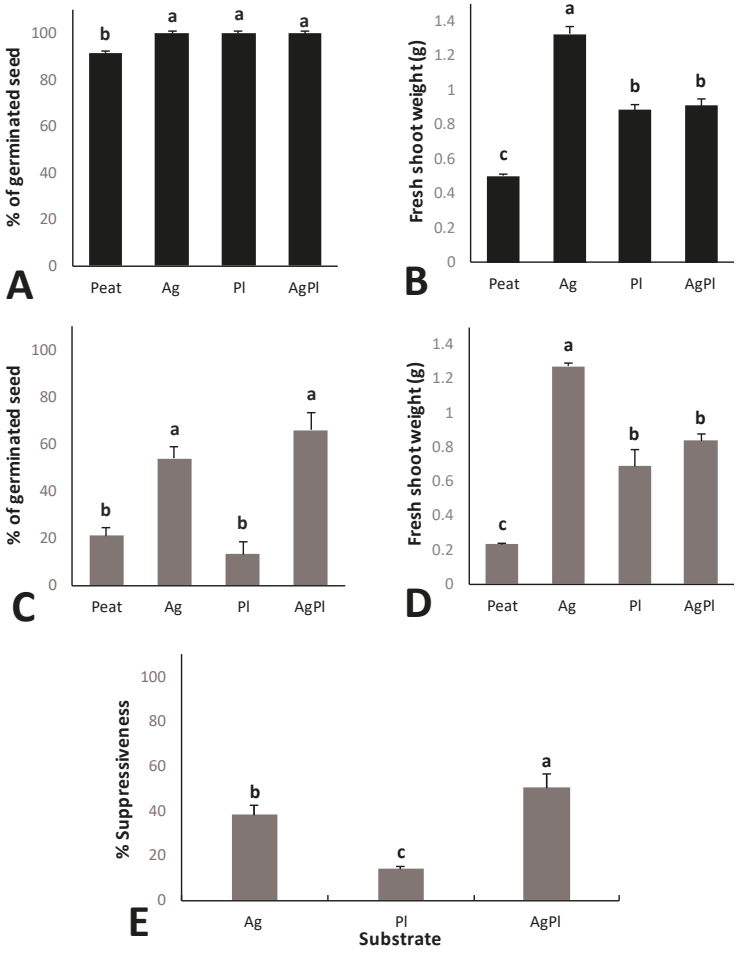

Figure 2. Germinated seed percentage (**A**) and fresh shoot weight (**B**) of red baby leaf lettuce plants without pathogen. Germinated seed percentage with *P. irregulare* (**C**) and fresh shoot weight (**D**) of red baby leaf lettuce plants with *P. irregulare*. Suppressiveness index (%) against *P. irregulare* (**E**). Error bars represent the standard errors. Values with the same letter do not differ significantly according to Tukey's test ($\alpha < 0.05$). Compost growing media of 100% peat, and peat with 100% *A. bisporus* (Ag), 100% *P. ostreatus* (Pl), or 70% *A. bisporus*: 30% *P. ostreatus* (AgPl) added at a 1:4 compost:peat ratio.

3.3. Composts as a Component of Suppressive Growing Media against P. irregulare

Under *P. irregulare* pressure, red baby leaf lettuce seed germination was between 20% and 70%. The Ag and AgPl media showed significantly higher seed germination rates than Pl and peat (Figure 2C). Moreover, the fresh shoot weight was significantly higher in all compost growing media than in peat alone. Comparing the composts, the fresh shoot weight for Ag was significantly higher than the others, which did not differ (Figure 2D).

The suppressiveness index made it possible to separate suppression against the pathogen from the nutritional and biostimulant effects of the composts. AgPl showed the highest suppressiveness index against *P. irregulare* (Figure 2E). Both AgPl and Ag showed greater suppressiveness than Pl and peat. Differences were not observed in final *P. irregulare* concentration (Table 3).

Table 3. Amount of *P. irregulare* in the different compost growing media.

Composts [z]	***P. irregulare*** **Log Copies ITS g^{-1}**		
Experiment 1			
Peat	7.14 a [y]	±	0.12
Ag	6.61 b	±	0.02
Pl	6.17 b	±	0.08
AgPl	6.73 b	±	0.09
Experiment 2			
Peat	6.17	±	0.12
Peat + T	5.73	±	0.11
AgPl	5.90	±	0.09
AgPl + T	5.92	±	0.08

[z] 100% peat (Peat); peat with *A. bisporus* (Ag), *P. ostreatus* (Pl), or 70% *A. bisporus:* 30% *P. ostreatus* (AgPl) added at a 1:4 compost:peat ratio. Peat + *T. harzianum* (Peat + T), (70% *A. bisporus* and 30% *P. ostreatus*) (AgPl); (70% *A. bisporus* and 30% *P. ostreatus*) + *T. harzianum* (AgPl + T). ITS, internally transcribed spacer. [y] Mean value ± standard errors. For each growing media, values with different letters differ significantly according to Tukey's test ($\alpha < 0.05$).

3.4. Composts Amended with T. harzianum as a Component of Growing Media

AgPl showed the best suppressiveness index and germination under *P. irregulare* biotic stress and a good value for fresh plant biomass weight. This compost was inoculated with *T. harzianum* (AgPl + T) in order to evaluate the possibility of increasing the effects against *P. irregulare.* Red baby leaf lettuce grown in AgPl and AgPl + T showed significantly higher germination rates and fresh shoot weights than lettuce grown in Peat and Peat + T (Figure 3A,B). No significant differences were observed between compost growing media either with or without *T. harzianum* (Figure 3A,B).

3.5. Composts Amended with T. harzianum as a Component of Suppressive Growing Media against P. irregulare: Effects on Red Baby Leaf Lettuce Seed Germination, Growth, and the Suppressiveness Index

Lettuce seed germination was significantly lower in Peat than in Peat + T and in both AgPl and AgPl + T (Figure 3C). No significant differences were observed between AgPl and AgPl + T. Both compost growing media also showed significantly higher fresh shoot weight than Peat and Peat + T (Figure 3D). *T. harzianum* did not increase the fresh shoot weight compared to its non-inoculated treatment. With respect to the suppressiveness index, *T. harzianum* was not found in either compost growing media or in peat under *P. irregulare* pressure (data not shown). Moreover, there were no differences between the amount of *T. harzianum* in Peat and AgPl showing, 4.44 and 4.51 log copies ITS g^{-1}, respectively (Table 3).

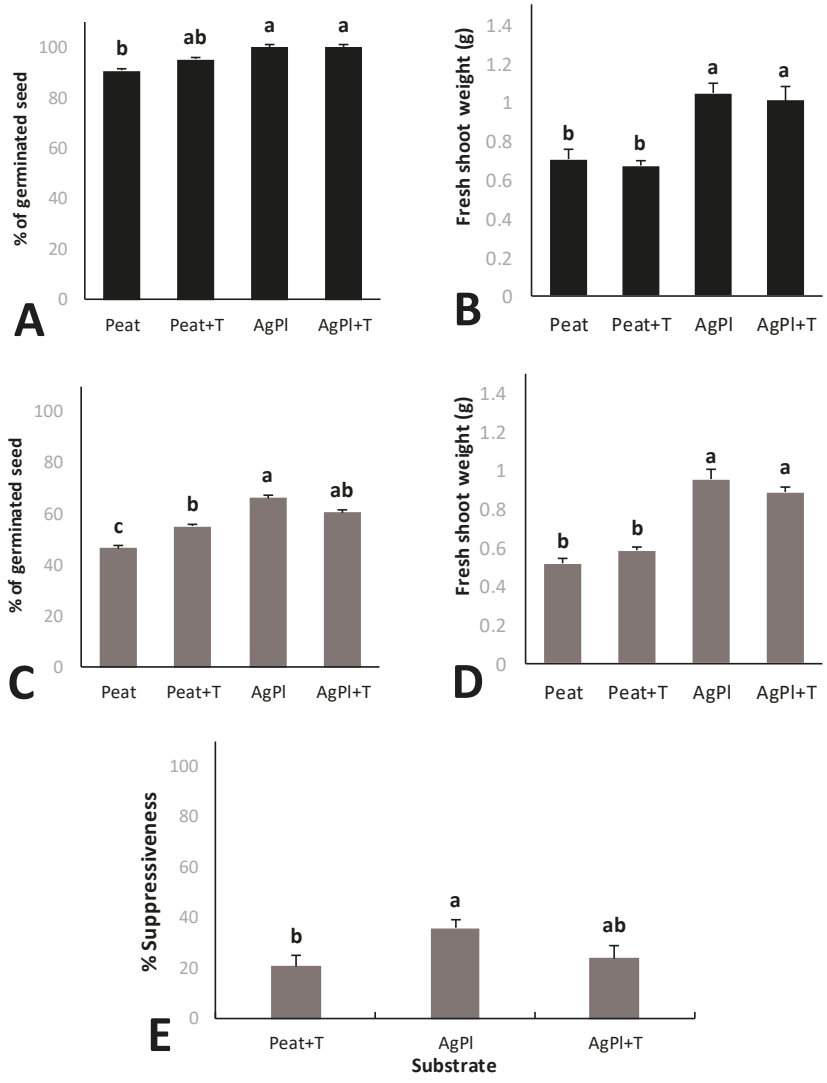

Figure 3. Germinated seed percentage (**A**) and fresh shoot weight of baby red lettuce plants (**B**) without pathogen. Germinated seed percentage (**C**) and fresh shoot weight of baby red lettuce plants with *P. irregulare* (**D**). Suppressiveness index (%) against *P. irregulare* (**E**). Error bars represent the standard errors. Values with the same letter do not differ significantly according to Tukey's post hoc test ($\alpha < 0.05$). Peat; Peat + *T. harzianum* (Peat + T), (70% *A. bisporus* and 30% *P. ostreatus*) + *T. harzianum* (AgPl + T).

4. Discussion

Composting has gained significant attention as an environmentally friendly way to dispose of utilized organic wastes, rather than sending them to a landfill [14]. However, it is necessary to develop adequate composting processes. The temperature profile, C/N ratio, and the evolution of the total organic C are three of the main parameters that indicate the progress of a composting process [22,23]. The temperatures profiles of the compost piles followed the stages frequently observed in the composting process. These stages included

a thermophilic phase (>45 °C) resulting from the intense aerobic microbial metabolism that leads to the rapid breakdown of organic matter by microbes producing heat as an exothermic reaction, and a maturation stage with a temperature decrease (down to 35 °C) as the organic matter is stabilized and consequently microbial activity drops [24]. The length and temperatures of the thermophilic phase depend on the composition of the raw materials. Pile Ag and Pile AgPl showed higher temperatures (54 °C) than Pile Pl (48 °C), probably due to the fact that the *A. bisporus* SMS contained labile components, especially nitrogen, to reactivate the microbial biomass during the composting. This would increase the temperature to a greater extent and maintain it for longer than in Pile Pl [25].

During composting, the amount of organic matter tends to drop due to mineralization and carbon loss in the form of carbon dioxide. The highest TOC losses were found in both piles with *A. bisporus* (Pile Ag (30%) and Pile AgPl (45%)), probably due to the higher amounts of most labile components in the organic matter from *A. bisporus* SMS. In contrast to the C losses, the TN level increased during the composting process; this usually occurs in the composting process when organic matter loss is greater than ammonium loss [23] or nitrate leaching. The higher TN levels in Pile Ag and Pile AgPl could be due to the chicken manure, rich in organic nitrogen [3–14]. Similar results were also observed by González-Marcos et al. [3], who found a TOC reduction of 50% when composting a mix of *A. bisporus* SMS and by-products from a winery. During the composting process, the C/N ratio diminished significantly due to the C losses, and the piles reached values below 15–20, indicative of high-quality mature compost [14,26]. Both pH and EC are important factors that influence seed germination and plant growth rates. The pH values of the three final composts ranged between 7.57 and 7.88, adequate for use in agriculture. Nevertheless, a lower range for growing media (5.2–7.0) is recommended [9]. The ECs of the three SMSs assayed were also higher than those found in other agroindustrial wastes (>4 dS m^{-1}) [9]. Furthermore, during composting, the mineralization of organic matter contributes to EC increases [27], reaching values ranging from 7.11 to 7.67. These EC values are not recommended in growing media [28], and some strategy must be applied to make the composts more suitable for use. One of those is the use of smaller ratios of compost as growing media. We used composts in at a 1:4 compost:peat ratio. As a result, both the EC and pH levels reached values within the range recommended. The composts displayed some characteristics ideal for agricultural application: [29] N > 1 g/100 g, P > 0.43 g/100 g, K > 0.41 g/100 g, Ca > 1.4 g/100 g, Mg > 0.2 g/100 g. The heavy metal content also increased in the three composts due to the composting process, although the levels were within the limits permitted in the current Spanish legal framework [30].

The use of these three composts as a growing media component for baby leaf lettuce cultivation increased the germination percentage and fresh plant weight over peat alone, mainly due to the nutrient content and a possible biostimulant effect [10]. Ag and AgPl resulted in the highest plant weights, even in presence of the *P. irregulare* pathogen. These characteristics make the three composts (Ag, AgPl, and Pl) attractive as at least a partial component of growing media, not only for their effect but also for the homogeneity of the raw materials. Moreover, spent mushroom composts from *A. bisporus* and *P. ostreatus* production would assure the same characteristics of the final composts, which is an important aspect of growing media materials, which should not result in differences in production from one batch to another [10]. Properties such as suppressiveness against certain plant diseases make it possible to reduce the use of chemical pesticides in agriculture. The disease-suppressive effects of composts of different origins and compositions have been widely studied, and different results have been obtained according to the compost type, pathogen to be controlled, environmental conditions, etc. [15]. The three compost growing media (Ag, Pl, AgPl) also showed a suppressive capacity against *P. irregulare.* AgPl followed by Ag showed the highest suppressiveness index. Disease suppression by composts is mainly attributed to the biotic factor [31], where beneficial microorganisms recolonize the compost [32]. The suppressive effects of composts are associated with the organic matter–microorganism–root consortia that occur in the plant rhizosphere. There are two

main types of mechanisms via which composts help suppress plant pathogens: direct and indirect. In our assay, as no effect on the pathogen interaction was observed, the suppressive effect should be therefore mainly attributed to an indirect effect through the plant rather than through a direct interaction with the pathogen. Indirect mechanisms include the activation of plant disease-resistance genes or the improvement of plant nutrition and vigour, allowing the plant to grow in the presence of the pathogen and not be affected [10,11].

The difference observed between the suppressiveness of AgPl and Ag could be due to the presence in the combined AgPl compost of plant growth-promoting rhizobacteria (PGPR) and endophyte microorganisms, rending the host more resistant or tolerant to disease [33]. This would explain the fact that there was a suppressive effect when Ag and Pl were combined but not with Ag alone. The suppressiveness of composts has been studied in depth, and it can be generally concluded that the raw materials from which a given compost is prepared are crucial to the development of suppressive microbiota within it [33]. Kumbhar [34] observed, for instance, that compost from *A. bisporus* showed a beneficial effect in controlling some pests and diseases such as damping off, root rot of creeping grass, *Verticillium* disease, and *Fusarium* wilt in tomato.

The incorporation of *T. harzianum* into composts is a method used to induce or increase the natural suppressiveness of growing media [35]. The incorporation of *T. harzianum* into the AgPl compost did not appear to increase the compost's natural suppressiveness, while the incorporation in peat was effective. It could be due to the raw materials in the composts or the addition of biocontrol microorganisms against *T. harzianum*, that did not permit *T. harzianum* growth. It is well documented that some species of *Trichoderma* are mushroom pathogens [36], and this forces mushroom growers to control them by using biocontrol microorganisms such as *Bacillus* spp. [37]. These could have been well established in the spent composts and therefore be part of their potential natural suppressiveness, yet they would not permit *T. harzianum* establishment.

5. Conclusions

We conclude that the composting process of spent mushroom substrates from *A. bisporus*, *P. ostreatus*, and a mix of 70%:30% mixture, respectively (Pile Ag, Pile Pl, and Pile AgPl) may produce quality, stabilized composts. The compost may be reintroduced into a production system and be a promising partial component (1:4, compost:peat) of organic growing media that could produce higher red baby leaf lettuce yields and provide some suppressive activity against *P. irregulare*. The compost obtained from the combination of both *A. bisporus* and *P. ostreatus* showed the highest suppressiveness against *P. irregulare* although the incorporation of *T. harzianum* did not increase the suppressiveness. A study of a compost microbial community before adding *T. harzianum* would be recommended to evaluate the establishment of the *T. harzianum*.

Author Contributions: Investigation, D.H., F.C., and J.A.P.; funding acquisition, J.A.P.; writing—original draft preparation, D.H.; writing—review and editing, D.H., M.R., F.C., J.A.S.-T., and J.A.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Spanish Ministry of Economy and Competitiveness (AGL2017-84085-C3-3-R); the Centre for the Development of Industrial Technology (CDTI) funding the project "Biotechnological management of organic waste for obtaining new bioproducts of new generation" code 99193 and the "Fundación Seneca de la Region de Murcia" within the Research Groups of Excellence programme of the Region of Murcia (19896/GERM/15).

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

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

Data Availability Statement: The data presented in this study were obtained from red baby leaf lettuce (*Lactuca sativa* L., Ligier RZ84-14, Rijk Zwaan, De Lier, The Netherlands) culture.

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

Abbreviations

SMS	spent mushroom substrate
Ag	SMS of *Agaricus bisporus*
Pl	SMS of *Pleurotus ostreatus*
AgPl	mix of 70% SMS of *Agaricus bisporus* and 30% SMS of *Pleurotus ostreatus*

References

1. Jerzak, M.A.; Śmiglak-Krajewska, M. Globalization of the Market for Vegetable Protein Feed and Its Impact on Sustainable Agricultural Development and Food Security in EU Countries Illustrated by the Example of Poland. *Sustainability* **2020**, *12*, 888. [CrossRef]
2. Phan, C.W.; Sabaratnam, V. Potential Uses of Spent Mushroom Substrate and Its Associated Lignocellulosic Enzymes. *Appl. Microbiol. Biotechnol.* **2012**, *96*, 863–873. [CrossRef] [PubMed]
3. González-Marcos, A.; Alba-Elías, F.; Martínez-de-Pisón, F.J.; Alfonso-Cendón, J.; Castejón-Limas, M. Composting of Spent Mushroom Substrate and Winery Sludge. *Compost Sci. Util.* **2015**, *23*, 58–65. [CrossRef]
4. Lau, K.L.; Tsang, Y.Y.; Chiu, S.W. Use of Spent Mushroom Compost to Bioremediate PAH-Contaminated Samples. *Chemosphere* **2003**, *52*, 1539–1546. [CrossRef]
5. Stewart, D.P.C.; Cameron, K.C.; Cornforth, I.S. Inorganic-N Release from Spent Mushroom Compost under Laboratory and Field Conditions. *Soil Biol. Biochem.* **1998**, *30*, 1689–1699. [CrossRef]
6. García-Delgado, C.; Yunta, F.; Eymar, E. Methodology for Polycyclic Aromatic Hydrocarbons Extraction from Either Fresh or Dry Spent Mushroom Compost and Quantification by High-Performance Liquid Chromatography–Photodiode Array Detection. *Commun. Soil Sci. Plant Anal.* **2013**, *44*, 817–825. [CrossRef]
7. López-González, J.A.; Suárez-Estrella, F.; Vargas-García, M.C.; López, M.J.; Jurado, M.M.; Moreno, J. Dynamics of Bacterial Microbiota during Lignocellulosic Waste Composting: Studies upon Its Structure, Functionality and Biodiversity. *Bioresour. Technol.* **2015**, *175*, 406–416. [CrossRef] [PubMed]
8. Medina, J.; Monreal, C.; Barea, J.M.; Arriagada, C.; Borie, F.; Cornejo, P. Crop Residue Stabilization and Application to Agricultural and Degraded Soils: A Review. *J. Waste Manag.* **2015**, *42*, 41–54. [CrossRef] [PubMed]
9. Khater, E.S.G. Some physical and chemical properties of compost. *Int. J. Waste Resour.* **2015**, *5*, 1–5. [CrossRef]
10. Pascual, J.A.; Ceglie, F.; Tuzel, Y.; Koller, M.; Koren, A.; Hitchings, R.; Tittarelli, F. Organic Substrate for Transplant Production in Organic Nurseries. A Review. *Agron. Sustain. Dev.* **2018**, *38*, 35. [CrossRef]
11. Giménez, A.; Fernández, J.A.; Pascual, J.A.; Ros, M.; López-Serrano, M.; Egea-Gilabert, C. An Agroindustrial Compost as Alternative to Peat for Production of Baby Leaf Red Lettuce in a Floating System. *Sci. Hortic. (Amst.)* **2019**, *246*, 907–915. [CrossRef]
12. Pane, C.; Spaccini, R.; Piccolo, A.; Scala, F.; Bonanomi, G. Compost Amendments Enhance Peat Suppressiveness to *Pythium Ultimum, Rhizoctonia Solani* and *Sclerotinia Minor*. *Biol. Control* **2011**, *56*, 115–124. [CrossRef]
13. Medina, E.; Paredes, C.; Pérez-Murcia, M.D.; Bustamante, M.A.; Moral, R. Spent Mushroom Substrates as Component of Growing Media for Germination and Growth of Horticultural Plants. *Bioresour. Technol.* **2009**, *100*, 4227–4232. [CrossRef] [PubMed]
14. Paula, F.S.; Tatti, E.; Abram, F.; Wilson, J.; O'Flaherty, V. Stabilisation of Spent Mushroom Substrate for Application as a Plant Growth-Promoting Organic Amendment. *J. Environ. Manag.* **2017**, *196*, 476–486. [CrossRef]
15. Blaya, J.; Lloret, E.; Ros, M.; Pascual, J.A. Identification of Predictor Parameters to Determine Agro-Industrial Compost Suppressiveness against Fusarium Oxysporum and *Phytophthora Capsici* Diseases in Muskmelon and Pepper Seedlings. *J. Sci. Food Agric.* **2015**, *95*, 1482–1490. [CrossRef]
16. Benhamou, N.; Chet, I. Cellular and Molecular Mechanisms Involved in the Interaction between *Trichoderma Harzianum* and *Pythium Ultimum*. *Appl. Environ. Microbiol.* **1997**, *63*, 2095–2099. [CrossRef]
17. Shoresh, M.; Harman, G.E.; Mastouri, F. Induced Systemic Resistance and Plant Responses to Fungal Biocontrol Agents. *Annu. Rev. Phytopathol.* **2010**, *48*, 21–43. [CrossRef]
18. Zohar-Perez, C.; Chernin, L.; Chet, I.; Nussinovitch, A. Structure of dried cellular alginate matrix containing fillers provides extra protection for microorganisms against UVC radiation. *Radiat. Res.* **2003**, *1*, 198–204. [CrossRef]
19. Paredes, C.; Roig, A.; Bernal, M.P.; Sánchez-Monedero, M.A.; Cegarra, J. Evolution of Organic Matter and Nitrogen during Co-Composting of Olive Mill Wastewater with Solid Organic Wastes. *Biol. Fertil. Soils* **2000**, *32*, 222–227. [CrossRef]
20. Veeken, A.H.M.; Blok, W.J.; Curci, F.; Coenen, G.C.M.; Termorshuizen, A.J.; Hamelers, H.V.M. Improving Quality of Composted Biowaste to Enhance Disease Suppressiveness of Compost-Amended, Peat-Based Potting Mixes. *Soil Biol. Biochem.* **2005**, *37*, 2131–2140. [CrossRef]
21. López-Mondéjar, R.; Antón, A.; Raidl, S.; Ros, M.; Pascual, J.A. Quantification of the Biocontrol Agent *Trichoderma Harzianum* with Real-Time TaqMan PCR and Its Potential Extrapolation to the Hyphal Biomass. *Bioresour. Technol.* **2010**, *101*, 2888–2891. [CrossRef] [PubMed]
22. Bustamante, M.A.; Paredes, C.; Marhuenda-Egea, F.C.; Pérez-Espinosa, A.; Bernal, M.P.; Moral, R. Co-Composting of Distillery Wastes with Animal Manures: Carbon and Nitrogen Transformations in the Evaluation of Compost Stability. *Chemosphere* **2008**, *72*, 551–557. [CrossRef] [PubMed]

23. Bernal, M.P.; Alburquerque, J.A.; Moral, R. Composting of Animal Manures and Chemical Criteria for Compost Maturity Assessment. A Review. *Bioresour. Technol.* **2009**, *100*, 5444–5453. [CrossRef] [PubMed]
24. Gao, M.; Liang, F.; Yu, A.; Li, B.; Yang, L. Evaluation of Stability and Maturity during Forced-Aeration Composting of Chicken Manure and Sawdust at Different C/N Ratios. *Chemosphere* **2010**, *78*, 614–619. [CrossRef]
25. Paredes, C.; Medina, E.; Moral, R.; Pérez-Murcia, M.D.; Moreno-Caselles, J.; Bustamante, M.A.; Cecilia, J.A. Characterization of the Different Organic Matter Fractions of Spent Mushroom Substrate. *Commun. Soil Sci. Plant Anal.* **2009**, *40*, 150–161. [CrossRef]
26. Bernal, M.P.; Sánchez-Monedero, M.A.; Paredes, C.; Roig, A. Carbon Mineralization from Organic Wastes at Different Composting Stages during Their Incubation with Soil. *Agric. Ecosyst. Environ.* **1998**, *69*, 175–189. [CrossRef]
27. Farrell, M.; Jones, D.L. Critical Evaluation of Municipal Solid Waste Composting and Potential Compost Markets. *Bioresour. Technol.* **2009**, *100*, 4301–4310. [CrossRef] [PubMed]
28. Noguera, P.; Abad, M.; Puchades, R.; Maquieira, A.; Noguera, V. Influence of Particle Size on Physical and Chemical Properties of Coconut Coir Dust as Container Medium. *Commun. Soil Sci. Plant. Anal.* **2003**, *34*, 593–605. [CrossRef]
29. Debertoldi, M.; Vallini, G.; Pera, A. The Biology of Composting: A Review. *Waste Manag. Res.* **1983**, *1*, 157–176. [CrossRef]
30. *Real Decreto 506/2013, de 28 de Junio, Sobre Productos Fertilizantes*; Ministerio de la Presidencia: Madrid, Spain, 2013.
31. Noble, R.; Roberts, S.J. Eradication of Plant Pathogens and Nematodes during Composting: A Review. *Plant Pathol.* **2004**, *53*, 548–568. [CrossRef]
32. Alabouvette, C.; Olivain, C.; Steinberg, C. Biological Control of Plant Diseases: The European Situation. *Eur. J. Plant. Pathol.* **2006**, *114*, 329–341. [CrossRef]
33. Castaño, R.; Borrero, C.; Avilés, M. Organic Matter Fractions by SP-MAS 13C NMR and Microbial Communities Involved in the Suppression of Fusarium Wilt in Organic Growth Media. *Biol. Control.* **2011**, *58*, 286–293. [CrossRef]
34. Kumbhar, A.S. Assessment of Growth Promotion and Disease Suppressing Ability of Spent Mushroom Substrate. Master Thesis, Dr. Panjabrao Deshmukh Krishi Vidyapeeth University, Akola, India, June 2012.
35. Blaya, J.; López-Mondéjar, R.; Lloret, E.; Pascual, J.A.; Ros, M. Changes Induced by *Trichoderma Harzianum* in Suppressive Compost Controlling Fusarium Wilt. *Pestic. Biochem. Physiol.* **2013**, *107*, 112–119. [CrossRef] [PubMed]
36. Velázquez-Cedeño, M.A.; Farnet, A.M.; Ferré, E.; Savoie, J.M. Variations of Lignocellulosic Activities in Dual Cultures of *Pleurotus Ostreatus* and *Trichoderma Longibrachiatum* on Unsterilized Wheat Straw. *Mycologia* **2004**, *96*, 712–719. [CrossRef]
37. Colavolpe, M.B.; Mejía, S.J.; Albertó, E. Efficiency of treatments for controlling *Trichoderma* spp. during spawning in cultivation of lignicolous mushrooms. *Braz. J. Microbiol.* **2014**, *45*, 1263–1270. [CrossRef]

horticulturae

MDPI

Article

Coir, an Alternative to Peat—Effects on Plant Growth, Phytochemical Accumulation, and Antioxidant Power of Spinach

Rui M. A. Machado [1,*], Isabel Alves-Pereira [2], Rui Ferreira [2,*] and Nazim S. Gruda [3]

[1] MED—Mediterranean Institute for Agriculture, Environment and Development, Departamento de Fitotecnia, Escola de Ciências e Tecnologia, Universidade de Évora, 7006-554 Évora, Portugal
[2] MED—Mediterranean Institute for Agriculture, Environment and Development, Departamento de Química, Escola de Ciências e Tecnologia, Universidade de Évora, 7000-671 Évora, Portugal; iap@uevora.pt
[3] Division of Horticultural Sciences, University of Bonn, Auf dem Hügel 6, 53121 Bon, Germany; ngruda@uni-bonn.de
* Correspondence: rmam@uevora.pt (R.M.A.M.); raf@uevora.pt (R.F.)

Abstract: The effects of four commercial substrates, a peat-based substrate, and three coir types (coir pith, coir chips, and coir pith + fibers) on yield, phytochemical accumulation, and antioxidant activity were evaluated in *Spinacia oleracea* L. cv. 'Manatee'. Soil-blocked spinach seedlings were transplanted into Styrofoam planting boxes filled with the substrate. Each planting box was irrigated daily by drip with a complete nutrient solution, and the irrigation scheduling was optimized to the peat. Leaf area and fresh yield in coir pith and coir pith + fiber were similar to those obtained in peat. However, shoot dry weight accumulation and leaf chlorophyll were lower in plants grown in coir. Substrate type did not affect leaf carotenoids. Total flavonoid content was higher in plants grown in the different types of coir. Total phenols and antioxidant activity (DPPH) were higher in plants grown in coir pith. This indicates that the different coir types, mainly coir pith, may provide an alternative to peat since they allowed a high fresh yield to be reached and the total flavonoids to be increased. In contrast, the levels of other phytochemicals and antioxidant activity were usual for spinach. However, further research is necessary to analyze the effects of irrigation scheduling and the nutrient solution adjusted to each growing medium on yield and phytochemical accumulation.

Keywords: *Spinacia oleracea*; substrates; soilless culture systems; photosynthetic pigments; phenols; flavonoids; ascorbic acid; DPPH; FRAP

Citation: Machado, R.M.A.; Alves-Pereira, I.; Ferreira, R.; Gruda, N.S. Coir, an Alternative to Peat—Effects on Plant Growth, Phytochemical Accumulation, and Antioxidant Power of Spinach. *Horticulturae* **2021**, *7*, 127. https://doi.org/10.3390/horticulturae7060127

Academic Editor: Pietro Santamaria

Received: 12 May 2021
Accepted: 26 May 2021
Published: 28 May 2021

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

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Peat alone or mixed with other constituents is the most used material in horticultural production. However, peat is a nonrenewable resource. Its exploration has negative environmental and ecological impacts [1,2], being classified as the growing medium with the greatest impact on climate change and resources [3]. Coir, also known as coir dust, coir meal, coir pith, and coir fibers, may provide an alternative to peat since it is a biodegradable and renewable by-product. Social and ecological questions concerning child labor, inadequate wastewater management, and transportation should be additionally considered. From the perspective of substrate properties, coir pith has high water capacity and easily available water. It contains more lignin and less cellulose than peat, thus being more resistant to microbial breakdown. It is also easily rewettable, which improves the water absorption of substrate mixtures and water distribution in the growing medium [2,4]. All those properties make coir pith a good peat alternative growing medium. The use of coir enabled high yields in spinach [5,6]. However, nowadays, in addition to yield, the nutritional quality of vegetables is essential. A further increase in bioactive compounds is desirable and an object of diverse research projects worldwide.

Spinach is one of the healthiest vegetables for the human diet due to its high concentration of nutrients and health-promoting compounds [7]. Among vegetable crops, it has one of the highest aggregate nutrient density index values [8,9].

According to [10], the nutrient level in plants is strongly affected by nutrient solution characteristics, such as the nutrient concentration, chemical forms of the elements, the temperature of the nutrient solution, pH, and irrigation scheduling (dose and frequency). On the other hand, substrate characteristics and irrigation interaction influence wetting and salt patterns in the root medium, easily available water content, leaching fraction, and nutrient and water availability. This affects the water and nutrient uptake by plants, which may lead to a greater or lesser degree of abiotic stress related to the water deficit, nutrient deficiency, salinity, and the combination of these factors.

Abiotic stress affects phytochemical accumulation and antioxidant activity. In response to water deficit, plants typically accumulate phytochemicals of low molecular weight and enzymes for scavenging the reactive oxygen species (ROS) induced by stress. [11,12].

The synthesis and accumulation of secondary metabolites may be associated with changes in nutritional status [13,14]. Thus, nitrogen, potassium, and phosphorus deficiency affect phytochemical accumulation in spinach [15]. Salinity affects the bioactivity of various fruits and vegetables and could be considered a sustainable and low-cost approach towards this direction [14]. Cultural practices that involve either low fertilizer levels or slight and moderate salt stress could reduce the yield but improve the nutritional value of vegetables [10,16], including spinach [15]. According to Shimomachi et al. [17], salt stress increased polyphenol contents in spinach. However, [18] reported that moderate levels of nutrient solution concentration (1.2 and 1.7 dS m^{-1}) did not affect total phenols, ascorbic acid, chlorophyll a and b, carotenoids, and ascorbate peroxidase content. It could be concluded that the response of phytochemical accumulation to salinity is not always linear [19] and clear [20].

The physicochemical properties of coir in the market differ significantly from peat [21]. This is due to different levels of fiber, which may affect water and plant nutrition, creating a greater or lesser abiotic degree of stress. Therefore, we hypothesize that coir can replace peat, but it is necessary to know their effects on yield and nutritional quality of the produce.

Therefore, this study aimed to evaluate the effects of different coir types on plant growth and nutritional quality, such as phytochemical composition, antioxidant enzyme levels, and antioxidant activity of spinach grown during late winter and early spring in unheated greenhouses.

2. Materials and Methods

2.1. Growth Conditions and Substrates

The experiment was conducted in a greenhouse located at the "Herdade Experimental da Mitra" (38°31′52″ N; 8°01′05″ W), University of Évora, Portugal. The greenhouse was covered with polycarbonate and had no supplemental lighting or heating. Diurnal changes in air temperature inside the greenhouse at the plant canopy level ranged from 8 to 27 °C. Solar radiation ranged from 34 to 248 $W \cdot m^{-2} \cdot d^{-1}$.

Our experiment used four commercial substrates: peat (70% black peat + 30% white peat) and three different types of coir from Projar Group (Table 1). According to the manufacturer, coir chips, coir pith, and coir pith + fiber had 0, 7, and 20% fiber, respectively.

Spinach (*Spinacia oleracea* L. cv. Manatee) seedlings were produced in soil blocks with six seedlings per block 18 days after emergence. Soil blocks were obtained from a commercial nursery. They were transplanted into Styrofoam plant boxes on 16 February 2017. The boxes (100 × 25 × 10 cm) were filled with 14 L substrate at the height of approx. 7 cm. The blocks were spaced 12.5 cm in two rows per box and 10 cm between rows with a plant density of 384 plants m^{-2}. Treatments were arranged in a complete randomized block design with five replicates. Each planting box was irrigated using 4 $L \cdot h^{-1}$ pressure-compensating and antidrain emitters. The emitters were attached to 4 fine tubes with 70 cm

length and 5 mm diameter, inserted into the substrate along the center of the Styrofoam box. Thus, 8 water emission points were used per box.

Table 1. Physicochemical properties of substrates.

Substrate	Peat	Coir Pith	Coir Chips	Coir Pith + Fiber
Composition	70% black peat + 30% white peat	100%	100%	93% coir pith + 7% fiber
pH *	5.5–6.0	5.5–6.0	5.5–6.2	5.5–6.2
EC ($dS \cdot m^{-1}$) *	1.5–1.8	<1.9	≥1.5	≥1.5
CEC (meq/100g)*	100–190	60–120	20–40	40–80
N ($mg\ L^{-1}$) *	50–300			
P ($mg\ L^{-1}$) *	35–131			
K ($mg\ L^{-1}$) *	60–330			
Total porosity (v/v, %) *		95		
Granulometry (mm) *		0–10	10–15	2–4
Air (v/v, %) *	-	25	40	30
Water holding capacity (v/v, %) *	-	70	54	65
Mass wetness (g water/g substrate) **	6.07 † c	7.84 b	5.75 d	8.65 a
Moisture content (w/w, %) **	82.6 ab	84.68 a	71.10 b	84.63 a
Bulk density ($g \cdot cm^{-3}$) **	0.127 a	0.103 a	0.070 b	0.081 a

* According to the manufacturer. ** Determined following the methods described in [22]. Moisture content: The percent moisture found in a sample on a wet mass basis. This is calculated by ((wet weight − dry weight)/wet weight) × 100. Mass wetness the water content of a sample on a dry mass basis. This is calculated by (wet weight − dry weight)/dry weight. † Means followed by different letters within a line are significantly different at $p < 0.05$.

The irrigation schedule was optimized for peat. It was based on substrate volumetric water content at Styrofoam box control (peat), measured using a soil moisture probe (SM105T delta devices England), and the volume of water drained.

The nutrient solution was applied three to seven times per day, depending on climatic conditions, and averaged 15 to 30% drainage, i.e., leaching fraction, for each application. The leaching fraction was controlled through a relay level connected to an electric valve that stopped watering when the level of leached water was within 10 to 25% of the applied water. Excepting the first irrigation to moisten the growing mediums, the nutrient solution was applied continuously from transplanting to the day before harvesting.

The fresh tap water had an electrical conductivity (EC) of 0.4–0.5 $dS \cdot m^{-1}$ and a pH of 7–7.4 and contained 0.10–0.30 $mol \cdot L^{-1}$ NO_3, 1 $mol \cdot L^{-1}$ Ca, 1 $mol \cdot L^{-1}$ Mg, 2.1 $mmol \cdot L^{-1}$ Cl^-, 0.7 $mol \cdot L^{-1}$ Na, 0.53 $\mu mol \cdot L^{-1}$ Fe, and 0.16 $\mu mol \cdot L^{-1}$ Mn. The nutrient solution initially contained 7.21 $mol \cdot L^{-1}$ NO_3, 2.32 $mmol \cdot L^{-1}$ NH_4, 0.59 $mmol \cdot L^{-1}$ P, 3.38 $mmol \cdot L^{-1}$ K, 2.55 $mmol \cdot L^{-1}$ Ca, 1.35 $mmol \cdot L^{-1}$ Mg, 0.80 $mmol \cdot L^{-1}$ S, 46 $\mu mol \cdot L^{-1}$ B, 7.86 $\mu mol \cdot L^{-1}$ Cu, 8.95 $\mu mol \cdot L^{-1}$ Fe, 18.3 $\mu mol \cdot L^{-1}$ Mn, 1 $\mu mol \cdot L^{-1}$ Mo, 2 $\mu mol \cdot L^{-1}$ Zn, 2.1 $mmol \cdot L^{-1}$ Cl^-, and 0.7 $mmol \cdot L^{-1}$ Na.

At 26 DAT, in order to reduce the nitrate concentration in the leaves, the nutrient concentrations and the NO_3/NH4 ratio in the nutrient solution were adjusted to 4.26 $mmol \cdot L^{-1}$ NO_3, 4.11 $mmol \cdot L^{-1}$ NH^4, 0.67 $mmol \cdot L^{-1}$ P, 2.84 $mmol \cdot L^{-1}$ K, 2.13 $mmol \cdot L^{-1}$ Ca, 0.88 $mmol \cdot L^{-1}$ Mg, 0.47 $mmol \cdot L^{-1}$ S, 46 $\mu mol \cdot L^{-1}$ B, 7.86 $\mu mol \cdot L^{-1}$ Cu, 8.95 $\mu mol \cdot L^{-1}$ Fe, 18.3 $\mu mol \cdot L^{-1}$ Mn, 1 $\mu mol \cdot L^{-1}$ Mo, 2 $\mu mol \cdot L^{-1}$ Zn, 2.1 $mmol \cdot L^{-1}$ Cl^-, and 0.7 $mmol \cdot L^{-1}$ Na.

2.2. Measurements

The pH, EC, and the concentration of NO_3 of the drainage water from each box were measured weekly using a potentiometer (pH Micro 2000 Crison), a conductivity meter (LF 330 WTW, Weilheim, Germany), and an ion-specific electrode (Crison Instruments, Barcelona, Spain), respectively, following the procedures outlined in [23].

The plants were harvested at 40 DAT. The shoots of the plants were cut off at 1 cm above the substrate surface. The shoots of five representative plants from each box were washed, oven-dried at 70 °C for 2–3 days, weighed, and ground.

Samples of 1.000 g of spinach leaf-blade from four treatments and five replicates were macerated in a mortar and homogenized in 8 mL of methanol/water solution (90:10 (*v*/*v*), MW90 extract) for 1 min and then centrifuged at 4 °C at 6440× *g* for 5 min. The methanol extracts were stored in aliquots at −20 °C for later use [24]. Total chlorophyll, chlorophyll a (Chl a) and b (Chl b), and total carotenoids (Cc) were determined in MW90 extract by the method of [24] using the following equations:

$$\text{Chl a } (\mu g/mL) = 16.82\ A665.2 - 9.28\ A652.4;$$

$$\text{Chl b } (\mu g/mL) = 36.92\ A652.4 - 16.54\ A665.2;$$

$$\text{Cc } (\mu g/mL) = (1000\ A470 - 1.91\text{Chl a} - 95.15\text{Chl b})/225,$$

where A = absorbance, Chl a = chlorophyll a, Chl b = chlorophyll b, and Cc = carotenoids.

Samples of 1.000 g of spinach leaf-blade were macerated in a mortar and homogenized in 8 mL of methanol/water solution (80:20 (*v*/*v*), MW80 extract) for 1 min and then centrifuged at 4 °C at 6440× *g* for 5 min. The methanol extracts were stored in aliquots at −20 °C for later use.

Content of total phenolic compounds (TPCs) was determined using Folin–Ciocalteau phenol reagent described earlier [25], reading the absorbance at 760 nm. TPC content expressed as milligrams of gallic acid equivalent (GAE) per 100 g of fresh weight (FW) was calculated using a calibration curve (GAE, *n* = 6 concentrations from 0 to 50 mg/L).

For determination of flavonoid contents, 100 µL of MW80 extract was mixed with 20 µL of 10% $AlCl_3$ (*w*/*v*), 500 µL of 1 M potassium acetate, and 380 µL of distilled water and incubated at 25 °C for 30 min. Total flavonoid content was determined by reading the absorbance at 420 nm, using an extinction coefficient of 0.004 $\mu M^{-1}\ cm^{-1}$, and expressed in mg of quercetin equivalent (QE) per 100 g of fresh weight [26].

Total anthocyanin content was determined by mixing 500 µL of MW80 extract with 500 µL of 50% ethanol (*v*/*v*) and 84 µL of 37% HCl. After incubation at 60 °C for 30 min, the absorbance was measured at 530, 620, and 650 nm, and the absorbance of cyanidin-3-glycoside was calculated using the following equation:

$$\text{Aant} = (A530 - A620) - 0.1\ (A650 - A620).$$

Total anthocyanin content was calculated using a molar extinction coefficient of 34,300 $M^{-1}cm^{-1}$ and a molecular weight of 449.2 $gmol^{-1}$ and expressed in mg of cyanidin-3-glycoside equivalent (C3GE) per 100 g of fresh weight [27].

Ascorbic acid (AsA) content was determined by the method of [28], incubating the sample (extracts or standard suitably diluted) in a mixture containing 5% TCA in ethanol, 0.4% H_3PO_4, 0.5% β-phenanthroline in ethanol, and 0.03% $FeCl_3$ in ethanol, warmed at 30 °C, for 90 min. The absorbance of Fe (II)–β-phenanthroline complex formed was read at 534 nm. AsA concentration was calculated using a calibration curve (ascorbic acid, *n* = 6 concentrations from 0 to 30 mg/L).

Free Pro levels of MW80 extract were determined using the acid ninhydrin reaction [29], reading the absorbance of yellow-orange chromophore formed 546 nm. Pro concentration was calculated using a calibration curve (L-proline, *n* = 6 concentrations between 0 and 20 mg/L).

The 2,2-diphenyl-1-picrylhydrazyl free radical scavenging antioxidant power (DPPH) was determined by measuring the ability of plant MW80 extracts to capture the stable organic radical DPPH• (2, 2-diphenyl-1-picryl-hydrazyl, violet) and its conversion into a stable product, DPPH-H (diphenyl-picryl hydrazine, yellow). Aliquots of an extemporaneous methanol solution of 0.03 g/L DPPH•, kept in the dark, were added to a known volume of sample or standard solution. The reduction of DPPH• to DPPH-H was followed

by reading the absorbance at 515 nm, at 25 °C, for 180 s. Antioxidant power reported as milligrams of GAE per 100 g of FW was calculated using a calibration curve (GAE, $n = 8$ concentrations from 0 to 200 mg L^{-1}) [30].

Ferric reducing antioxidant power (FRAP) was determined by the method of [25]. In sum, the FRAP reagent was prepared freshly by mixing 300 mM acetate buffer pH 3.6 and 10 mM TPTZ solution in 40 mM HCl and 20 mM iron (III) chloride solution (10:1:1, $v/v/v$) and warmed to 37 °C before use. Then, 0.050 mL of the sample (suitably diluted MW80 extracts or standard) was mixed with 0.950 mL of FRAP reagent. Absorbance change was read at 593 nm at 37 °C, for 180 s. The reducing power of iron present in the samples reported as milligrams of Trolox equivalent per 100 g of FW was calculated using a calibration curve (Trolox solution, $n = 8$ concentrations from 0 to 1120 mg L^{-1}). For all previous determinations, a Genesys10S UV/Vis spectrophotometer was used.

Samples of 1.000 g of spinach leaf blade were macerated in liquid N_2 and homogenized in 5 mL of 0.12 mM phosphate buffer pH 7.2. The obtained supernatant using the centrifugation of this extract for 15 min at 15,000× g at 4 °C was collected and stored in aliquots at −20 °C (PB extract) for further use [31].

Glutathione (GSH) was assayed by the method of [32], based on the reaction of o-phthalaldehyde (OPT) as a fluorescent reagent with GSH at pH 8 present in the PB extract. The fluorescence of products was determined at 420 nm with the excitation at 350 nm, at 25 °C, using GSH as a standard in a single-beam Shimadzu RF-5001PC fluorimeter.

Glutathione reductase (GR) enzyme activity was determined by the method of [33] in a reaction mixture containing 15 mM EDTA, 635 mM GSSG, and a suitable volume of leaf-blade PB extract (0.5–0.2 mg mL^{-1} protein) in 0.12 mM phosphate buffer pH 7.2. The reaction was started with the addition of 9.6 mM NADPH. The oxidation of NADPH was determined by reading the absorbance at 340 nm for 360 s. At 37 °C, GR activity was calculated based on the slope of the reaction curves, using an extinction coefficient value of 6.22 mM^{-1} cm^{-1} for NADPH. GR activity was expressed in terms of nmol min^{-1}/mg protein.

Peroxidase enzyme activity (POD) was determined by the method of [34] in a reaction mixture containing 1% p-phenylenediamine, 1.5% hydrogen peroxide, and a suitable volume of leaf-blade PB extract (0.5 mg/mL of protein) in 0.2 M potassium phosphate buffer pH 6.5. The oxidation of p-phenylenediamine was determined by reading the absorbance at 485 nm for 10 min, at 25 °C. POD activity was calculated based on the slope of the reaction curves using the value of the extinction coefficient of 2.1 × 10^4 M^{-1} cm^{-1} for p-phenylenediamine. For all enzyme determinations, a double-beam Hitachi-U2001 spectrophotometer with temperature control was used.

The protein content of the PB extract was determined by the method of [35], using a calibration curve (bovine serum albumin (BSA); $n = 6$ concentrations from 0 to 200 μg mL^{-1}).

Data were analyzed using the analysis of variance using SPSS Statistics 25 software (Chicago, IL, USA). Means were separated at the 5% level using Duncan's new multiple range test. Bivariate correlation analysis between parameters was realized using Pearson's bilateral correlation coefficient.

3. Results and Discussion

3.1. Drainage Water

Nitrate and H_3O^+ concentration in the drainage water were affected by the substrate (Figure 1). Nitrate concentration in the drainage water was higher in peat until 15 DAP and in coir-chips during the crop cycle than in the other treatments (Figure 1). Coir chips had the highest drainage volume (data not presented) and the lowest wetness mass (5.75 g water/g substrate). In general, the H_3O^+ concentration was lower in peat and coir chips than in the other coir types. The differences could be due to the different cation exchange capacities of the substrates that contribute to the adsorption of the hydronium ions. The differences in nitrate leaching can also affect the form of nitrogen uptake by plants, affecting

hydronium and hydroxide concentration in the root medium. The EC of the drainage water was not significantly affected by substrate type.

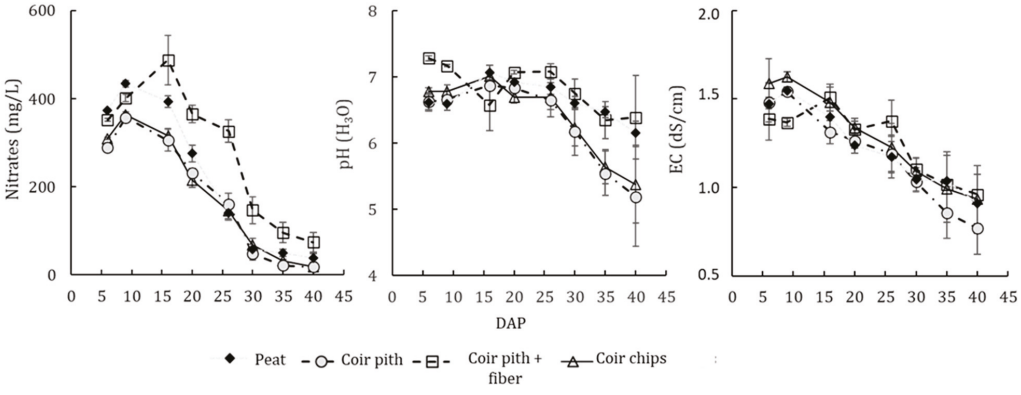

Figure 1. The NO_3 and H_3O^+ concentrations and EC in the drainage water. Each symbol represents the mean of five replicates, and the error bars represent ± 1SE.

NO_3^- concentration and the EC in the drainage water on the last three sampling dates, as compared with the NO_3^- and the EC in the nutrient solution, decreased significantly (Figure 1) due to the decrease in nitrate applied to the nutrient solution (264 mg NO_3^- L^{-1}) and due to high nutrient uptake by spinach plants.

3.2. Plant Growth and Yield

Leaf area and spinach fresh yield in coir pith and coir pith + fiber did not differ significantly from those obtained in peat (Figure 2). The coir pith and coir-pith + fiber yields were high, ranging from 3.79 to 4.32 kg·m^2. These findings are consistent with those obtained in [5,6]. The use of coir, a growing medium, enables the achievement of high yields. The fresh yield in coir chips (2.64 kg m^{-2}) was lower than in the other substrates.

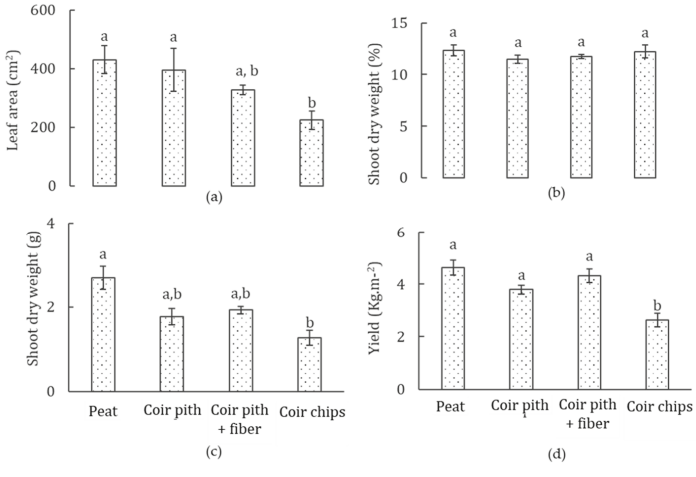

Figure 2. Leaf area (**a**), shoot dry weight (%) (**b**), shoot dry weight per plant (**c**), and fresh yield (**d**) of spinach. Means with different letters are significantly different at $p < 0.05$. Each bar represents the mean of five replicates, and the error bars represent ± 1SE.

Plants grown in coir accumulated less shoot biomass than those grown in peat. This could be due to the physiochemical properties of coir, with lower nutrient and water holding capacity than peat. Moreover, the initial content of nutrients in peat (Table 1) and the substrates' interaction and irrigation frequency affect substrate water content and nutrient availability [36]. Shoot biomass accumulation was positively correlated to leaf K ($r = 0.75$, $p > 0.01$) and N ($r = 0.58$ $p > 0.01$) content, with a higher level in plants grown in peat than in plants grown in coir (Table 2). Biomass accumulation and crop growth are related to crop N accumulation [37]. In spinach, shoot biomass decreased in response to deficit irrigation [38], and it was higher in the plants grown in coir, mainly in coir chips.

3.3. Leaf Nutrients

Leaf nutrient concentrations of the plants grown in coir pith, coir pith + fiber, and peat did not differ significantly, except for the concentrations of potassium and calcium. Leaf N, K, Mn, and Zn concentrations in plants grown in coir chips were lower than those grown in the other substrates (Table 2). However, leaf Ca and Mg concentrations were higher in plants grown in coir chips than in plants grown in the other substrates. That could be related to the low bulk density of the substrate (Table 1), which may have allowed for high root branching and Ca and Mg uptake primarily occurring in the new roots [39].

Despite some differences, the concentrations of macronutrients, except nitrogen, were within the sufficiency ranges (Table 2). Leaf nitrogen average values in coir pith + fiber and coir chips were slightly lower than the lower end of the sufficiency range (4%). However, the plant shoot dry weight of the plants grown in peat was higher, and plant nutrient uptake may also have increased. Shoot nutrient uptake in spinach increased with dry shoot matter in plants with the same leaf nutrient content [14].

Leaf Fe, B, Cu, and Mn concentrations were unaffected by substrate type. Leaf Zn content was well above the recommended range (Table 2), which could be due to the NH_4^+ concentration in the nutrient solution, which was high from 26 DAT until the harvest. In lettuce, Savvas et al. (2006) [40] reported an increase in leaf Zn as ammonium supply increased. These Zn concentrations are higher than the sufficiency range (100 μg g^{-1} DM [41] and 75 μg g^{-1} DM [42]). However, none of the plants in the treatments showed visual symptoms of excess Zn. Zinc in excess can cause chlorosis in leaves due to a reduction in chlorophyll [43]. According to [44], leaf Zn concentrations of up to 100–700 mg kg^{-1} DM can be achieved without yield loss, which can be advantageous since Zn is a desirable nutrient for human health.

Table 2. Nutrient concentration in fully expanded spinach leaves.

Substrate	Leaf Macronutrients (%)					Leaf Micronutrients (μg·g^{-1})				
	N	P	K	Ca	Mg	Fe	B	Cu	Mn	Zn
Peat	4.48 a [Z]	0.38	6.88 a	0.94 c	0.70 b	89.2	34.0	31.2	105.2 a	198.2 a
Coir pith	4.18 a	0.36	5.96 b	1.00 b	0.64 b	112.6	33.2	39.4	104.8 a	211.0 a
Coir pith + fiber	3.98 ab	0.32	6.26 ab	1.04 ab	0.72 b	77.0	35.2	25.4	104.8 a	210.0 a
Coir chips	3.48 b	0.32	5.14 bc	1.20 a	0.82 a	65.0	30.0	29.6	73.0 b	115.2 b
Recommended range										
[41]	4.00–6.00	0.30–0.60	5.00–8.00	0.70–1.20	0.60–1.00	60–200	25–60	5–25	30–250	25–100
[42]	4.00–6.00	0.30–0.50	3.00–8.00	1.00–1.50	0.40–1.00	50–200	25–60	5–15	25–200	20–75

[Z] Means followed by different letters within a column are significantly different at $p \leq 0.05$.

3.4. Photosynthetic Pigments

Leaf total chlorophyll (chl a + chl b) (Figure 3a) and chlorophyll b (Figure 3c) were higher in plants grown in peat and coir chips than in plants grown in coir pith and coir pith + fiber. Total chlorophyll in a plant grown in peat (79.82 mg/100 g FW) was similar to that recorded in [45] (65.4 mg/100 g FW) and [46]. However, it was lower than those reported in [47] (96.2 to 301.8 mg/100 g FW). Conversely, the ratio of chlorophyll a to chlorophyll b (Figure 3d) was higher in plants grown in coir pith and coir pith + fiber. The differences in

chlorophyll could be due to the water availability; salinity in the root media; and nutrient uptake for nitrogen, potassium, and zinc. These factors or their combination may affect chlorophyll biosynthesis.

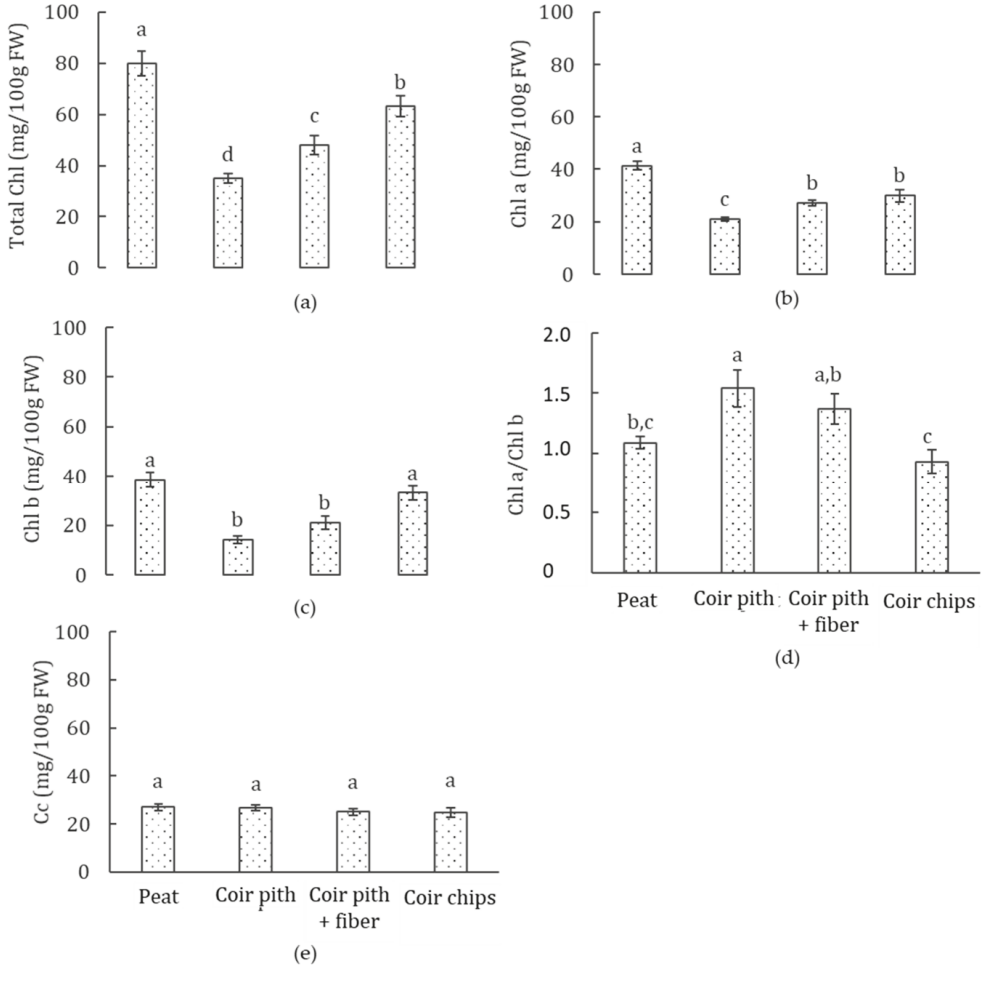

Figure 3. Accumulation of photosynthetic pigments (total chlorophyll (**a**), Chl a (**b**), Chl b (**c**), and carotenoids (Cc) (**e**)) and Chl a/Chl b ratio (**d**). Means with different letters are significantly different at $p < 0.05$; FW—fresh weight. Each bar represents the mean of five replicates, and the error bars represent ± 1SE.

Abiotic stresses have negative influences on chlorophyll biosynthesis [48]. Salinity reduces the contents of photosynthetic pigments [49,50]. Average levels of chlorophyll were lower in plants with average values of Zn > 200 μg g^{-1}, that is, in plants grown in coir pith and coir pith + fiber. As previously mentioned, high levels of Zn in spinach can decrease the chlorophyll content.

Leaf-blade Cc content was unaffected by substrate type (Figure 3e). According to [51], leaf Cc of strawberries was not also affected by substrate type. Carotenoid levels ranged from an average of 25 to 30 mg/100 g FW (Figure 3e). These concentrations were in the ranges reported in [14,52,53] (17 to 40 mg/100 g FW) for spinach grown in soil.

3.5. Proline Accumulation

Leaf-blade proline (Pro) ranged on average from 3.22 to 4.27 mg/100 g FW (Figure 4). These values are lower than those recorded in [54] (4.66–43.15 mg/100 g FW) and are within the range recorded in [55] (2.74–7.2 mg/100 g FW) for spinach grown in the greenhouse and the open air. The proline concentration was higher in plants grown in coir pith than in those grown in the other substrates (Figure 4). Proline concentration is closely related to abiotic stress, such as water and nutrient deficiency and salinity [56–59]. This indicates that plants grown in coir pith may be subject to stress conditions. However, in the present study, proline did not correlate with growth parameters, such as leaf area and plant dry weight, as reported for young plants of tomatoes and lettuces in [60,61]. On the other hand, proline was negatively correlated to FRAP ($r = -0.628$, $p < 0.001$), which increases when the leaf extract's ability to reduce ferric iron decreases. The plant produces Pro to compensate for the oxidizing role of Fe, preventing the formation of reactive oxygen species (ROS). In chickpea, [62] also reported a negative correlation between proline content and FRAP.

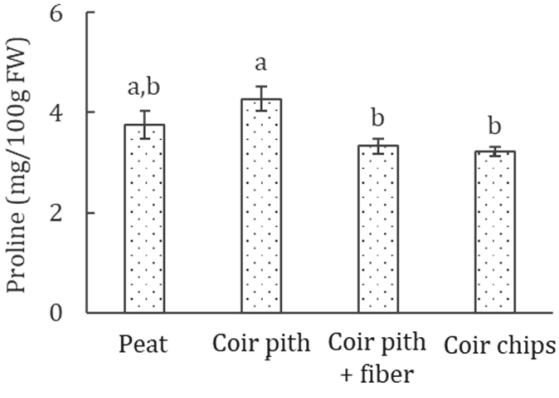

Figure 4. Proline content in the leaf blade. Means with different letters are significantly different at $p < 0.05$; FW—fresh weight. Each bar represents the mean of five replicates, and the error bars represent ± 1SE.

3.6. Phytochemical Accumulation

Leaf-blade TPCs, flavonoids, and GSH were higher in plants grown in coir pith than in those grown in the peat and the other coir types (Figure 5a,b,e).

Leaf-blade TPCs in plants grown in coir pith, peat, coir pith + fiber, and coir chips were 329, 263, 220, and 213 mg GAE/100 g FW, respectively (Figure 5a). TPC concentrations were next to the high end of the range reported by other authors (71–320 mg GAE/100 g FW) [6,63–65].

It is worth mentioning that total flavonoid content was significantly higher in plants grown in the different types of coir than in those grown in peat (Figure 5b). This could be due to the differences in leaf nutrient contents, shoot nutrient uptake, water availability of the substrate, irrigation scheduling, or interaction. Flavonoid biosynthesis is affected by nutrient and water availability and salinity [66,67]. The total flavonoids in the plants grown in different coir types ranged from an average of 6.58 to 7.14 mg/100 g FW. These values were higher than those recorded in [68] (1.45 to 4.47 mg/100 g FW) for 27 varieties of spinach grown organically and conventionally and were slightly lower than those recorded in [45] (8.25 mg/100 g FW). However, they were much lower than those reported in [69] (100 mg/ 100 g FW) and [70] (185 to 375 mg/100 g FW). The high variation might result from different genotypes investigated [68,71], maturation [70], etc.

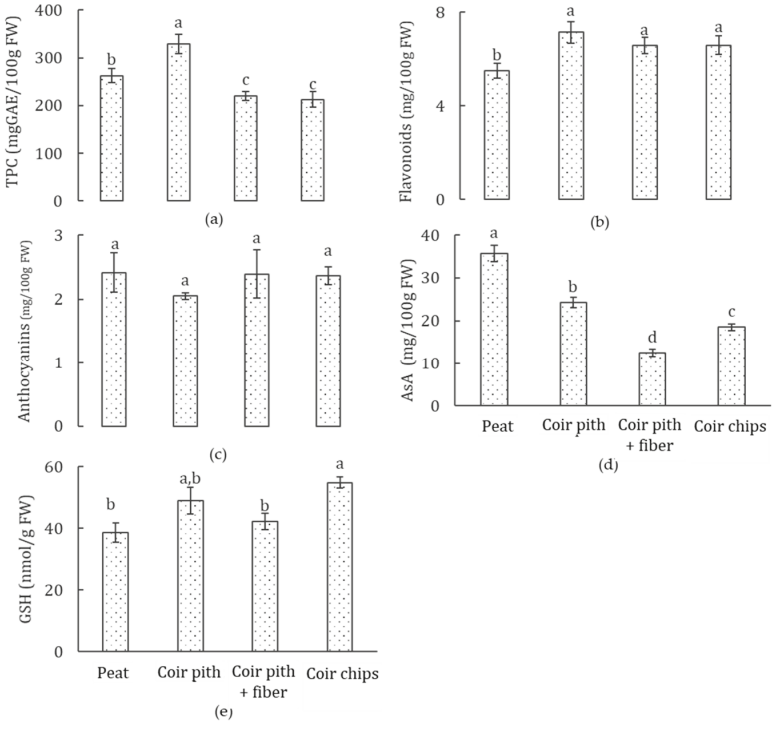

Figure 5. Contents of total phenolic compounds (TPCs) (**a**), flavonoids (**b**), anthocyanins (**c**), ascorbic acid (AsA) (**d**), and glutathione (GSH) (**e**) in the leaf blade. Means with different letters are significantly different at $p < 0.05$; FW—fresh weight. Each bar represents the mean of five replicates, and the error bars represent ± 1SE.

As a compound of the flavonoids group, the anthocyanin content ranged from an average of 2.05 to 2.42 mg/100 g FW. These values were similar to those recorded in [12] (15 to 38 mg/100 g DW), considering that the dry weight percentage of the spinach leaf-blade is close to 12%. However, the content was not significantly affected by the treatments (Figure 5c). This indicates that the higher antioxidant protection mediated by flavonoids in the plants grown in coir was affected by other flavonoid types.

AsA content of spinach grown in different growing media fell within the range reported by other authors (11 to 130 mg AsA/100 g FW) [14,68,72,73]. It was higher in leaf-blades of plants grown in peat (36 mg/100 g/FW) than in those grown in coir pith + fiber (13 mg/100 g/FW), coir pith (24 mg/100 g/FW), and coir chips (19 mg/100 g/FW) (Figure 5d). The differences could be related to leaf nitrogen content and/or plant nitrogen uptake since the nitrogen amount [74–76] and its form can affect AsA [53,77]. However, AsA was slightly correlated to leaf N ($r = 0.483$, $p < 0.05$). This may be due to the maintenance of ascorbic acid synthesis requiring a moderate amount of N [78].

Glutathione (GSH) content was higher in plants grown in coir than in those grown in peat. This indicates that plants grown in coir may have higher availability of antioxidant activity modulated by the SH group of cysteine [79]. ROS are scavenged by low-molecular-weight antioxidative metabolites like glutathione [80]. Leaf-blade GSH ranged from an average of 40 to 54 nmol/g FW (Figure 5e). These values are lower than those reported for spinach grown in soil in [81] (114–136 nmol/g FW), which could be related to the level of oxidative stress [79].

3.7. Antioxidant Enzyme (GR and POD) Activities

Leaf-blade GR activity was unaffected significantly by substrate type (Figure 6a). This means that the substrate type did not influence the glutathione tripeptide regeneration capacity of the spinach leaf blade. GR activity in the present study was approximately half of that reported in [82] for spinach (16.85 μmol min^{-1}/mg prot).

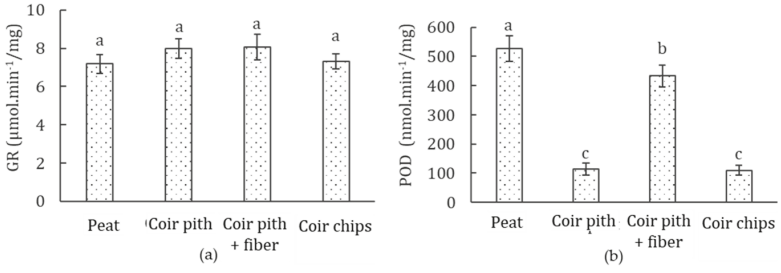

Figure 6. Glutathione reductase (GR) (**a**) activity and peroxidase (POD) (**b**) activity in the leaf blade. Means with different letters are significantly different at $p < 0.05$. Each bar represents the mean of five replicates, and the error bars represent ± 1SE.

POD activity was significantly higher in plants grown in peat than those grown in coir substrates (Figure 6b), reaching 527 nmol.min^{-1}/mg in peat, 433 nmol.min^{-1}/mg in coir pith + fiber, and 114 nmol.min^{-1}/mg in coir pith and coir chips. This could be related to the influence of substrates on plant nutrition and water uptake, as reported in [83]. The lower POD activity can be advantageous since peroxidases are the enzymes responsible for the browning of vegetable tissues [84]. Thus, spinach plants grown in coir substrate, mainly in coir pith and coir chips, may present a longer shelf life than those grown in peat. This is important in leafy vegetables since they are highly susceptible to enzymatic browning, shriveling, microbial growth, and loss of nutrients [85].

3.8. Antioxidant Activity

Leaf-blade FRAP was higher in plants grown in coir pith + fiber (32 mg Trolox/g FW) than in plants grown in peat (30 mg Trolox/g FW), coir chips (23 mg Trolox/g FW), and coir pith (10 Trolox/g FW) (Figure 7a). Generally, FRAP concentrations in our study were higher than those reported by other authors, which ranged from 2.67 to 13.8 Trolox/g FW [6,86,87]. The authors of [88] reported an increase in FRAP in basil as potassium increased in the nutrient solution. However, in the present study, FRAP was not correlated to leaf K. K concentration in nutrient solution affected total phenols, flavonoids, and antioxidant activity (FRAP, DPPH) in *Lavandula angustifolia* (Mill.). However, FRAP response to leaf K was not linear [89]. In the present study, despite K concentration in the nutrient solution being equal, leaf K was affected, but FRAP was not correlated to leaf K.

Leaf-blade DPPH was higher in plants grown in peat (38 mg GAE/100 g FW) than in plants grown in the coir types. Leaf-blade DPPH in plants grown in coir pith and coir chips ranged from 29 to 31 GAE/100 g FW (Figure 7b).

The free radical-scavenging activity estimated by DPPH has a strong positive correlation with AsA ($r = 0.656$, $p < 0.01$), indicating that ascorbic acid level contributes to the scavenging capacity of the leaf extract.

The differences in dry weight accumulation, phytochemical accumulation, antioxidant enzyme activities, and antioxidant power could be due to the characteristics of the substrates and/or effects of irrigation scheduling optimized to peat in water and nutrient uptake. Despite this, the findings indicate that the different types of coir, mainly coir pith, may provide a promising substitute for peat since it allowed reaching a high yield and increased total flavonoid content. The other phytonutrient contents and antioxidant activi-

ties were within the range of values reported in the literature for spinach. The adaptation of cultural management to the specific substrate and crop demand can further improve the quality of horticultural products [10,14,90]. Therefore, further research is needed to evaluate the response of spinach grown in different coir types with optimized irrigation and nutrient solution.

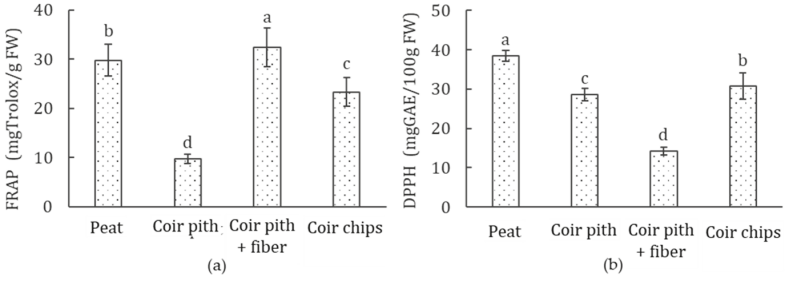

Figure 7. FRAP (**a**) and DPPH (**b**) in the leaf blade. Means with different letters are significantly different at $p < 0.05$; FW—fresh weight. Each bar represents the mean of five replicates, and the error bars represent ± 1SE.

4. Conclusions

Coir pith and coir pith + fiber may provide an alternative to peat. Plants grown in these substrates had a similar fresh yield but a higher total flavonoid content than plants cultivated in peat. The levels of other phytochemicals and the antioxidant activity (FRAP and DPPH) in plants grown in coir were within the usual ranges for spinach. However, further research will be necessary to analyze the effects of adjusting the irrigation scheduling and nutrient solution characteristics for each coir type, for instance, in coir chips, on spinach yield and product quality.

Author Contributions: R.M.A.M. conceived and designed the experiments; performed the experiments; analyzed and interpreted the data; contributed reagents, materials, analysis tools, or data; and wrote the paper. I.A.-P. and R.F. performed the experiments; analyzed and interpreted the data; contributed reagents, materials, analysis tools, or data; and wrote the paper. N.S.G. reviewed, corrected, and edited the paper. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Foundation for Science and Technology (FCT) as part of Project UIDB/05183/2020.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

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

References

1. Gruda, N.; Bisbis, M.; Tanny, J. Impacts of protected vegetable cultivation on climate change and adaptation strategies for cleaner production—A review. *J. Clean. Prod.* **2019**, *225*, 324–339. [CrossRef]
2. Gruda, N. Increasing sustainability of growing media constituents and stand-alone substrates in soilless culture systems. *Agronomy* **2019**, *9*, 298. [CrossRef]
3. QUANTIS. *Comparative Life Cycle Assessment of Horticultural Growing Media Based on Peat and Other Growing Media Constituents*; EPAGMA Final Report: Lausanne, Switzerland, 2012; pp. 1–156.
4. Gruda, N.; Brag, N. Developments in alternative organic materials as growing media in soilless culture systems. Chapter 3. In *Advances in Horticultural Soilless Culture*; Gruda, N., Ed.; Burleigh Dodds Science Publishing Limited: Cambridge, UK, 2021; ISBN 13 9781786764355.
5. Barcelos, C.; Machado, R.M.; Alves Pereira, I.; Ferreira, R.; Bryla, D.R. Effects of substrate type on plant growth and nitrogen and nitrate concentration in spinach. *Int. J. Plant Biol.* **2016**, *7*, 44–47. [CrossRef]

6. Machado, R.M.A.; Alves-Pereira, I.; Ferreira, R.M.A. Plant growth, phytochemical accumulation and antioxidant activity of substrate-grown spinach. *Heliyon* **2018**, *4*, e00751. [CrossRef]
7. Lester, G.E.; Makus, D.J.; Hodges, D.M.; Jifon, J.L. Summer (subarctic) versus winter (subtropic) production affects spinach (*Spinacia oleracea* L.) leaf bionutrients: Vitamins (C, E, Folate, 425 K1, provitamin A), lutein, phenolics, and antioxidants. *J. Agric. Food Chem.* **2013**, *61*, 7019–7027. [CrossRef]
8. Dias, J.S. Nutritional quality and effect on disease prevention of vegetables. *Food Nutr. Sci.* **2019**, *10*, 369. [CrossRef]
9. Barker, A.V.; Stratton, M.L. Nutrient density of fruit crops as a function of soil fertility. In *Fruit Crops. Diagnosis and Management of Nutrient Constraints*; Srivastava, A.K., Hu, C., Eds.; Elsevier: Amsterdam, The Netherland, 2020; pp. 13–31.
10. Gruda, N. Do soilless culture systems have an influence on product quality of vegetables? *J. Appl. Bot. Food Qual.* **2009**, *82*, 141–147.
11. Sofo, A.; Dichio, B.; Xiloyannis, C.; Masia, A. Antioxidant defences in olive trees during drought stress: Changes in activity of some antioxidant enzymes. *Funct. Plant Biol.* **2005**, *32*, 45–53. [CrossRef]
12. Hasanuzzaman, M.; Bhuyan, M.H.M.B.; Zulfiqar, F.; Raza, A.; Mohsin, S.M.; Mahmud, J.A.; Fujita, M.; Fotopoulos, V. Reactive Oxygen Species and Antioxidant Defense in Plants under Abiotic Stress: Revisiting the Crucial Role of a Universal Defense Regulator. *Antioxidants* **2020**, *9*, 681. [CrossRef] [PubMed]
13. Gruda, N.; Savvas, D.; Colla, G.; Rouphael, Y. Impacts of genetic material and current technologies on product quality of selected greenhouse vegetables—A review. *Eur. J. Hortic. Sci.* **2018**, *83*, 319–328. Available online: https://www.pubhort.org/ejhs/83/5/5/index.htm (accessed on 5 March 2021). [CrossRef]
14. Rouphael, Y.; Giordano, M.; Cardarelli, M.; Cozzolino, E.; Mori, M.; Kyriacou, M.C.; Bonini, P.; Colla, G. Plant- and seaweed-based extracts increase yield but differentially modulate nutritional quality of greenhouse spinach through biostimulant action. *Agronomy* **2018**, *8*, 126. [CrossRef]
15. Xu, C.; Mou, B. Responses of spinach to salinity and nutrient deficiency in growth, physiology, and nutritional value. *J. Am. Soc. Hortic. Sci.* **2016**, *141*, 12–21. [CrossRef]
16. Gruda, N. Impact of environmental factors on product quality of greenhouse vegetables for fresh consumption. *Crit. Rev. Plant Sci.* **2005**, *24*, 227–247. [CrossRef]
17. Shimomachi, Y.; Kawahara, C.; Kobashigawa, E.; Omoda, K.; Hamabe, K.T. Effect of residual salinity on spinach growth and nutrient contents in polder soil. *Acta Hortic.* **2008**, *797*, 419–424. [CrossRef]
18. Machado, R.; Alves-Pereira, I.; Robalo, M.; Ferreira, R. Effects of municipal solid waste compost supplemented with inorganic nitrogen on physicochemical soil characteristics, plant growth, nitrate content, and antioxidant activity in Spinach. *Horticulturae* **2021**, *7*, 53. [CrossRef]
19. Telesiński, A.; Nowak, J.; Smolik, B.; Dubowska, A.; Skrzypiec, N. Effect of soil salinity on activity of antioxidant enzymes and content of ascorbic acid and phenols in bean [*Phaseolus vulgaris* L.] plants. *J. Elem.* **2008**, *13*, 401–409.
20. Ferreira, J.F.; Sandhu, D.; Liu, X.; Halvorson, J.J. Spinach (*Spinacea oleracea* L.) response to salinity: Nutritional value, physiological parameters, antioxidant capacity, and gene expression. *Agriculture* **2018**, *10*, 163.
21. Abad, M.; Noguera, P.; Puchades, R.; Maquieira, A.; Noguera, V. Physico-chemical and chemical properties of some coconut coir dusts for use as a peat substitute for containerised ornamental plants. *Bioresour. Technol.* **2002**, *82*, 241–245. [CrossRef]
22. Fonteno, W.C.; Harden, C.T. Procedures for determining physical properties of horticultural substrates using the NCS-Porometer. In *Horticultural Substrates Laboratory*; North Carolina State University: Raleigh, NC, USA, 2003. Available online: https://www.ncsu.edu/project/hortsublab/pdf/porometer_manual.pdf (accessed on 21 March 2020).
23. Prazeres, A.O. *Comparação de Metodologias Laboratoriais para Determinação de Azoto Nítrico e Amoniacal em Solos e Águas*; Programa e Livro de Resumos do 1° Congresso Nacional e Rega e Drenagem: Beja, Portugal, 2005; pp. 59–60.
24. Lichtenthaler, H.K.; Buschmann, C. Chlorophylls and carotenoids: Measurement and characterization by UV-VIS spectroscopy. *Curr. Protoc. Food Anal. Chem.* **1987**, *4*. [CrossRef]
25. Bouayed, J.; Hoffmann, L.; Bohn, T. Total phenolics, flavonoids, anthocyanins and antioxidant activity following simulated gastro-intestinal digestion and dialysis of apple varieties. Bioaccessibility and potential uptake. *Food Chem.* **2011**, *128*, 14–21. [CrossRef]
26. Pourmorad, F.; Hosseinimehr, S.J.; Shahabimajd, N. Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. *Afr. J. Biotechnol.* **2006**, *5*, 1142–1145. Available online: http://www.academicjournals.org/AJB50 (accessed on 21 January 2021).
27. Paiva, E.A.S.; Isaias, R.M.D.S.; Vale, F.H.A.; Queiroz, C.G.D.S. The influence of light intensity on anatomical structure and pigment contents of *Tradescantia pallida* (Rose) Hunt. cv. purpurea Boom (Commelinaceae) leaves. *Braz. Arch. Biol. Technol.* **2003**, *46*, 617–624. [CrossRef]
28. Cai, W.M.; Tang, Z.C. Plant tolerance physiology. In *Experimental Guide for Modern Plant Physiology*, 1st ed.; Tang, Z.C., Ed.; Science Press: Beijing, China, 1999; pp. 315–316. [CrossRef]
29. Bates, L.S. Rapid determination of free proline for water stress studies. *Plant Soil* **1973**, *39*, 205–207. [CrossRef]
30. Brand-Williams, W.; Cuvelier, M.E.; Berse, C. Use of a free radical method to evaluate antioxidant activity. *Lwt-Food Sci. Technol.* **1995**, *28*, 25–30. [CrossRef]
31. Lake, B. Preparation and characterization of microsomal fractions for studies of xenobiotic metabolism. In *Biochemical Toxicology: A Practical Approach*; Snell, K., Mullock, B., Eds.; IRL Press: Oxford, UK, 1987; pp. 183–215.

32. Hissin, A.; Hilf, P.A. Fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal. Biochem.* **1976**, *74*, 214–226. [CrossRef]
33. Goldberg, D.; Spooner, R. Gluathione reductase. In *Methods of Enzymatic Analysis*, 3rd ed.; Bergmeyer, H.-U., Ed.; Academic Press: Weinheim, Germany, 1987; pp. 258–265.
34. Hernández, A.; Cano, M.P. High-pressure and temperature effects on enzyme inactivation in tomato puree. *J. Agric. Food Chem.* **1998**, *46*, 266–270. [CrossRef] [PubMed]
35. Lowry, O.H.; Roseburg, N.J.; Farr, A.L.; Randell, R.J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275. [CrossRef]
36. Silber, A.; Bar-Tal, A. Nutrition of substrate-grown plants. In *Soilless Culture: Theory and Practice*; Elsevier: San Diego, CA, USA, 2008; pp. 291–339.
37. Gastal, F.; Lemaire, G. N uptake and distribution in crops: An agronomical and ecophysiological perspective. *J. Exp. Bot.* **2002**, *53*, 789–799. [CrossRef] [PubMed]
38. Zhang, J.; Liang, Z.; Jiao, D.; Tian, X.; Wang, C. Different water and nitrogen fertilizer rates effects on growth and development of spinach. *Commun. Soil Sci. Plan.* **2018**, *49*, 1922–1933. [CrossRef]
39. Russell, R.S.; Clarkson, D.T. Ion transport in root systems. *Perspect. Exp. Biol.* **1976**, *2*, 401–411.
40. Savvas, D.; Passam, H.C.; Olympios, C.; Nasi, E.; Moustaka, E.; Mantzos, N.; Barouchas, P. Effects of ammonium nitrogen on lettuce grown on pumice in a closed hydroponic system. *HortScience* **2006**, *41*, 1667–1673. [CrossRef]
41. Mills, H.A.; Jones, J.R. *Plant Analysis Handbook II. A Practical Sampling, Preparation Analysis and Interpretation Guide*; Micro Macro International, Inc.: Athens, GA, USA, 1996; ISBN 1878148001.
42. Campbell, C. *Reference Sufficiency Ranges for Plant Analysis in the Southern Region of the United States*; Southern Cooperative Series Bulletin #394; Southern Cooperative Series Bulletin: Raleigh, NC, USA, 2000; ISBN 1-58161-394-6. Available online: www.ncagr.gov/agronomi/saaesd/scsb394.pdf (accessed on 10 March 2020).
43. Lopez-Mosquera, M.E.; Moiron, C.; Carral, E. Use of dairy-industry sludge as fertiliser for grasslands in northwest Spain: Heavy metal levels in the soil and plants. *Resour. Conserv. Recycl.* **2000**, *30*, 95–109. [CrossRef]
44. White, P.J.; Broadley, M.R. Physiological limits to zinc biofortification of edible crops. *Front. Plant Sci.* **2011**, *2*, 80. [CrossRef] [PubMed]
45. Hussain, P.R.; Suradkar, P.; Javaid, S.; Akram, H.; Parvez, S. Influence of postharvest gamma irradiation treatment on the content of bioactive compounds and antioxidant activity of fenugreek (*Trigonella foenum-graceum* L.) leaves. *Innov. Food Sci. Emerg.* **2016**, *33*, 268–281. [CrossRef]
46. Machado, R.M.A.; Alves-Pereira, I.; Ferreira, R.M.A. Effects of plant density and the number of emitters by styrofoam box on plant growth and nitrate concentration in spinach cultivated in substrate. *Acta Hortic.* **2019**, *1242*, 847–853. [CrossRef]
47. Batziakas, K.G.; Rivard, C.L.; Stanley, H.; Batziakas, A.G.; Pliakoni, E.D. Reducing preharvest food losses in spinach with the implementation of high tunnels. *Sci. Hortic.* **2020**, *265*, 109268. [CrossRef]
48. Sharma, A.; Kumar, V.; Shahzad, B.; Ramakrishnan, M.; Sidhu, G.P.S.; Bali, A.S.; Handa, N.; Kapoor, D.; Yadav, P.; Khanna, K.; et al. Photosynthetic response of plants under different abiotic stresses: A review. *J. Plant Growth Regul.* **2019**, 1–23. [CrossRef]
49. Delfine, S.; Alvino, A.; Villani, M.C.; Loreto, F. Restrictions to carbon dioxide conductance and photosynthesis in spinach leaves recovering from salt stress. *Plant Physiol.* **1999**, *119*, 1101–1106. [CrossRef] [PubMed]
50. Ashraf, M.; Harris, P.J.C. Photosynthesis under stressful environments: An overview. *Photosynthetica* **2013**, *51*, 163–190. [CrossRef]
51. Ebrahimi, R.; Ebrahimi, F.; Ahmadizadeh, M. Effect of different substrates on herbaceous pigments and chlorophyll amount of strawberry in hydroponic cultivation system. *Am. Eurasian J. Agric. Environ. Sci.* **2012**, *12*, 154–158, ISSN 1818-6769.
52. Borowski, E.; Michalek, S. The effect of foliar nutrition of spinach (*Spinacia oleracea* L.) with magnesium salts and urea on gas exchange, leaf yield and quality. *Acta Agrobot.* **2010**, *63*, 77–85. [CrossRef]
53. Machado, R.M.A.; Alves-Pereira, I.; Lourenço, D.; Ferreira, R.M.A. Effect of organic compost and inorganic nitrogen fertigation on spinach growth, phytochemical accumulation, and antioxidant activity. *Heliyon* **2020**, *6*, e05085. [CrossRef]
54. Du, S.-T.; Liu, Y.; Zhang, P.; Liu, H.-J.; Zhang, X.-Q.; Zhang, R.-R. Atmospheric application of trace amounts of nitric oxide enhances tolerance to salt stress and improves nutritional quality in spinach (*Spinacia oleracea* L.). *Food Chem.* **2015**, *173*, 905–911. [CrossRef] [PubMed]
55. Aslan, M.; Sultana, B.; Anwar, F.; Munir, H. Foliar spray of selected plant growth regulators affected the biochemical and antioxidant attributes of spinach in a field experiment. *Turk. J. Agric.* **2016**, *40*, 136–145. Available online: http://journals.tubitak.gov.tr/agriculture/ (accessed on 11 May 2021). [CrossRef]
56. Rhodes, D.; Nadolska-Orczyk, A.; Rich, P. Salinity, Osmolytes and Compatible Solutes. In *Salinity: EnvironmentPlants—Molecules*; Läuchli, A., Lüttge, U., Eds.; Springer: Dordrecht, The Netherland, 2002; pp. 181–204. [CrossRef]
57. Pérez-López, U.; Sgherri, C.; Miranda-Apodaca, J.; Micaelli, F.; Lacuesta, M.; Mena-Petite, A.; Quartacci, M.F.; Muñoz-Rueda, A. Concentration of phenolic compounds is increased in lettuce grown under high light intensity and elevated CO_2. *Plant Physiol. Biochem.* **2018**, *123*, 233–241. [CrossRef] [PubMed]
58. Verbruggen, N.; Hermans, C. Proline accumulation in plants: A review. *Amino Acids* **2008**, *35*, 753–759. [CrossRef]
59. Claussen, W. Growth, water use efficiency, and proline content of hydroponically grown tomato plants as affected by nitrogen source and nutrient concentration. *Plant Soil* **2002**, *247*, 199–209. [CrossRef]

60. Gruda, N.; Schnitzler, W.H. The effect of water supply on biomorphological and plant-physiological parameters of tomato transplants cultivated in wood fiber substrate. *J. Appl. Bot.* **2000**, *74*, 233–239.
61. Gruda, N.; Schnitzler, W.H. The effect of water supply of seedlings cultivated in different substrates and raising systems on the bio-morphological and plant-physiological parameters of lettuce. *J. Appl. Bot.* **2000**, *74*, 240–247.
62. Bhagyawant, S.S.; Narvekar, D.T.; Gupta, N.; Bhadkaria, A.; Koul, K.K.; Srivastava, N. Variations in the antioxidant and free radical scavenging under induced heavy metal stress expressed as proline content in chickpea. *Physiol. Mol. Biol. Plants* **2019**, *25*, 683–696. [CrossRef]
63. Proteggente, A.R.; Pannala, A.S.; Paganga, G.; van Buren, L.; Wagner, E.; Wiseman, S.; van de Put, F.; Dacombe, C.; Rice-Evans, C.A. The antioxidant activity of regularly consumed fruit and vegetables reflects their phenolic and vitamin C composition. *Free Radic. Res.* **2002**, *36*, 217–223. [CrossRef]
64. Tavarini, S.; Cardelli, R.; Saviozzi, A.; Degl'Innocenti, E.; Guidi, L. Effects of green compost on soil biochemical characteristics and nutritive quality of leafy vegetables. *Compos. Sci. Util.* **2011**, *19*, 114–122. [CrossRef]
65. Mazzucotelli, C.A.; Gonzalez-Aguilar, G.A.; Villegas-Ochoa, M.A.; Domínguez-Avila, A.J.; Ansorena, M.R.; Di Scala, K.C. Chemical characterization and functional properties of selected leafy vegetables for innovative mixed salads. *J. Food Biochem.* **2018**, *42*, e12461. [CrossRef]
66. Di Ferdinando, M.; Brunetti, C.; Fini, A.; Tattini, M. Flavonoids as antioxidants in plants under abiotic stresses. In *Abiotic Stress Responses in Plants: Metabolism, Productivity, and Sustainability*; Ahmad, P., Prasad, M.N.V., Eds.; Springer Science + Business Media: Berlin, Germany, 2012; pp. 159–179. [CrossRef]
67. Çetinkaya, H.; Kulak, M.; Karaman, M.; Karaman, H.; Koçer, F. Flavonoid accumulation behavior in response to the abiotic stress: Can a uniform mechanism be illustrated for all plants? In *Flavonoids—From Biosynthesis to Human Health*; Justino, J.G.D., de Campos Justino, D.D., Pinheiro, P.B.P.F., Eds.; Intech Editors: Lisboa, Portugal, 2017; pp. 151–165. [CrossRef]
68. Koh, E.; Charoenprasert, S.; Mitchell, A.E. Effect of organic and conventional cropping systems on ascorbic acid, vitamin C, flavonoids, nitrate, and oxalate in 27 varieties of spinach (*Spinacia oleracea* L.). *J. Agric. Food Chem.* **2012**, *60*, 3144–3150. [CrossRef]
69. Gil, M.I.; Ferreres, F.; Tomás-Barberán, F.A. Effect of postharvest storage and processing on the antioxidant constituents (flavonoids and vitamin C) of fresh-cut spinach. *J. Agric. Food Chem.* **1999**, *47*, 2213–2217. [CrossRef]
70. Cho, M.J.; Howard, L.R.; Prior, R.L.; Morelock, T. Flavonoid content and antioxidant capacity of spinach genotypes determined by high-performance liquid chromatography/mass spectrometry. *J. Sci. Food Agric.* **2008**, *88*, 1099–1106. [CrossRef]
71. Kidmose, U.; Knuthsen, P.; Edelenbos, M.; Justesen, U.; Hegelund, E. Carotenoids and flavonoids in organically grown spinach (*Spinacia oleracea* L) genotypes after deep frozen storage. *J. Sci. Food Agric.* **2001**, *81*, 918–923. [CrossRef]
72. Proietti, S.; Moscatello, S.; Colla, G.; Battistelli, Y. The effect of growing spinach (*Spinacia oleracea* L.) at two light intensities on the amounts of oxalate, ascorbate and nitrate in their leaves. *J. Hortic. Sci. Biotechnol.* **2004**, *79*, 606–609. [CrossRef]
73. Cocetta, G.; Baldassarre, V.; Spinardi, A.; Ferrante, A. Effect of cutting on ascorbic acid oxidation and recycling in fresh-cut baby spinach (*Spinacia oleracea* L.) leaves. *Postharvest Biol. Technol.* **2014**, *88*, 8–16. [CrossRef]
74. Lee, S.K.; Kader, A.A. Technology. Preharvest and postharvest factors influencing vitamin C content of horticultural crops. *Postharvest Biol. Technol.* **2000**, *20*, 207–209. [CrossRef]
75. Salomez, J.; Hofman, G. Nitrogen nutrition effects on nitrate accumulation of soil grown greenhouse butterhead lettuce. *Commun. Soil Sci. Plant Anal.* **2009**, *40*, 620–632. [CrossRef]
76. Miceli, A.; Miceli, C. Effect of Nitrogen Fertilization on the quality of swiss chard at harvest and during storage as minimally processed produce. *J. Food Qual.* **2014**, *37*, 125–134. [CrossRef]
77. Krezel, J.; Kolota, E. Source of nitrogen affects the yield and quality of spinach cultivars grown for autumn harvest. *Acta Agric. Scand Sect. B Soil Plant Sci.* **2014**, *64*, 583–589. [CrossRef]
78. Mozafar, A. Nitrogen fertilizers and the amount of vitamins in plants: A review. *J. Plant Nutr.* **1993**, *16*, 2479–2506. [CrossRef]
79. Per, T.S.; Khan, N.A.; Reddy, P.S.; Masood, A.; Hasanuzzaman, M.; Khan, M.I.R.; Anjum, N.A. Approaches in modulating proline metabolism in plants for salt and drought stress tolerance. *Plant Physiol. Bioch.* **2017**, *115*, 126–140. [CrossRef] [PubMed]
80. Hayat, S.; Hayat, Q.; Alyemeni, M.N.; Wani, A.S.; Pichtel, J.; Ahmad, A. Role of proline under changing environments: A review. *Plant Signal. Behav.* **2012**, *7*, 1456–1466. [CrossRef] [PubMed]
81. De Kok, L.J.; Oosterhuis, F.A. Effects of frost-hardening and salinity on glutathione and sulfhydryl levels and on glutathione reductase activity in spinach leaves. *Physiol. Plant* **1983**, *58*, 47–51. [CrossRef]
82. Hodges, D.M.; Forney, C.F. The effects of ethylene, depressed oxygen and elevated carbon dioxide on antioxidant profiles of senescing spinach leaves. *J. Exp. Bot.* **2000**, *51*, 645–655. [CrossRef] [PubMed]
83. Ekinci, M.; Atamanalp, M.; Turan, M.; Alak, G.; Kul, R.; Kitir, N.; Yildirim, E. Integrated use of nitrogen fertilizer and fish manure: Effects on the growth and chemical composition of spinach. *Commun. Soil Sci. Plan.* **2019**, *50*, 1580–1590. [CrossRef]
84. Singh, B.; Suri, K.; Shevkani, K.; Kaur, A.; Kaur, A.; Singh, N. Enzymatic browning of fruit and vegetables: A review. In *Enzymes in Food Technology*; Kuddus, M., Ed.; Springer: Singapore, 2018; pp. 63–78. [CrossRef]
85. Maghoumi, M.; Gómez, P.A.; Mostofi, Y.; Zamani, Z.; Artés-Hernández, F.; Artés, F. Combined effect of heat treatment, UV-C and super atmospheric oxygen packing on phenolics and browning related enzymes of fresh-cut pomegranate arils. *LWT Food Sci. Technol.* **2013**, *54*, 389–396. [CrossRef]

86. Apack, R.; Güçlü, K.; Demirata, B.; Özyürek, M.; Çelik, S.; Bektasoğlü, B.; Berker, K.; Özyurt, D. Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. *Molecules* **2007**, *12*, 1496–1547. [CrossRef] [PubMed]
87. Sreeramulu, D.; Reddy, C.V.K.; Chauhan, A.; Balakrishna, N.; Raghunath, M. Natural antioxidant activity of commonly consumed plant foods in India: Effect of domestic processing. *Oxid. Med. Cell Longev.* **2013**, *369479*, 1–12. [CrossRef] [PubMed]
88. Salas-Pérez, L.; Fornari-Reale, T.; Preciado-Rangel, P.; García-Hernández, J.L.; Sánchez-Chávez, E.; Troyo-Diéguez, E. Cultivar variety and added potassium influence the nutraceutical and antioxidant content in hydroponically grown basil (*Ocimum basilicum*, L.). *Agronomy* **2018**, *8*, 13. [CrossRef]
89. Chrysargyris, A.; Drouza, C.; Tzortzakis, N. Optimization of potassium fertilization/nutrition for growth, physiological development, essential oil composition and antioxidant activity of *Lavandula angustifolia* Mill. *J. Soil Sci. Plant Nutr.* **2017**, *17*, 291–306. [CrossRef]
90. Massantini, F.; Favilli, R.; Magnani, G.; Oggiano, N. Soilless culture, biotechnology for high-quality vegetables. *Soil. Cult. Cult.* **1988**, *4*, 27–40.

horticulturae

MDPI

Article

First Steps toward a Test Procedure to Identify Peat Substitutes for Growing Media by Means of Chemical, Physical, and Biological Material Characteristics

Katharina Leiber-Sauheitl *, Heike Bohne and Jürgen Böttcher

Institute for Soil Science, Leibniz University Hannover, 30419 Hannover, Germany; bohne@baum.uni-hannover.de (H.B.); boettcher@ifbk.uni-hannover.de (J.B.)
* Correspondence: katharina.leiber@web.de

Abstract: Due to the major environmental impact of peat-based growing media production and the need of lowering greenhouse gas emissions in all sectors, a wider application of peat substitutes in growing media is requested. All peat substitutes under use have constraints associated with their properties. Therefore, a preliminary test procedure for identifying new raw materials as peat substitutes in growing media was developed and validated. By applying the preliminary test procedure, the potential limitations of cultivation of potential peat substitutes are indicated, and measures for cultivation regulation are recommended. For the development of the new preliminary test procedure, four raw materials were investigated: composted heather, alder, cattail, and reed. The preliminary test procedure comprises several material and technological criteria as well as aspects of plant cultivation, enabling the evaluation of the raw materials and the processed components for growing media. Results derived from the preliminary test procedure were checked and confirmed by experiments with horticultural crops in different sections of commercial horticulture. Within two years, the identification of new peat substitutes was possible by the application of the preliminary test procedure and its test criteria, which provide a structure for the systematic investigation of potential new peat substitutes starting with the raw material.

Keywords: peat substitute; growing media; decision tree; feasibility; heather; cattail; reed; alder; peat reduction

Citation: Leiber-Sauheitl, K.; Bohne, H.; Böttcher, J. First Steps toward a Test Procedure to Identify Peat Substitutes for Growing Media by Means of Chemical, Physical, and Biological Material Characteristics. *Horticulturae* **2021**, *7*, 164. https://doi.org/10.3390/horticulturae7070164

Academic Editors: Nazim Gruda and Juan A. Fernández

Received: 20 May 2021
Accepted: 22 June 2021
Published: 28 June 2021

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

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

For several decades, peat has been the most important component for growing media [1] due to its ideal chemical, physical, and biological properties in plant cultivation [2]. Globally, about 90 million m^3 of peat are produced per year from which 40 million m^3 are used in horticulture [3,4]. Starting as a by-product, it is often to be found as the only component in a growing medium. The search for peat substitutes started in the 1980s due to rising awareness of the importance of peatland conservation especially focusing on nature conservation [1]. Recently, peatlands came into focus in the light of climate conservation due to the large stocks of carbon contained in peatlands. At present, several peat substitutes are already under use such as coconut fibers, wood fibers, compost, and many other [5–10]. However, there are constraints for each of these peat substitutes that limit their usage in large quantities, or the available amounts of these peat substitutes are limited (e.g. [2,11]). Therefore, new materials for growing media have to be found, which first of all have to be tested if they meet the material requirements. Up to now, a lot of plant trials with different raw materials and different combinations of growing media components were conducted (e.g. [12]). First, these trials are cost intensive and often cannot directly be compared to each other since e.g., experimental conditions differ. Second, due to the mixture of three or more substrate components in plant trials, results cannot be clearly attributed to a single substrate component. Due to this, it is often not clear

if a raw material per se is not applicable or if it might be promising to specifically modulate some of its unfavorable characteristics by for instance another processing or mixing with other materials.

Due to these reasons, a new test procedure for raw materials focusing on material characteristics was developed, the application of which makes the trials of different materials tested as growing media components comparable. Several raw materials (heather, cattail, reed, and alder) were investigated by this new test procedure, and their suitability as a growing media component was evaluated. By using a hierarchical combination of test criteria, the resulting test procedure helps to identify completely unsuited substrate components with simple tests at an early stage of the procedure and gives enough information on problematic characteristics of more promising candidates at later stages so that it can be decided if these can be overcome technically. Various chemical, physical, and biological analyses are applied at different steps of the test procedure starting with material and simple substrate analyses of a raw material, continuing with extended substrate analyses on the chemical and physical properties of a processed raw material and ideally finishing with the plant test under practical conditions. The possible limitations of properties of potential peat substitutes, such as the water-holding capacity and N immobilization, are indicated, and measures for plant cultivation regulation are recommended. In the future, the test procedure needs to be extended by economic and ecological criteria such as availability studies, cost effectiveness, and life cycle analyses.

The selection of the raw materials under investigation created synergy effects among climate protection, nature conservation, and the interests of agriculture and horticulture. These raw materials were derived from landscape conservation of a terrestrial site (heather maintenance) and from water affected or rewetted sites (reed, cattail, alder). The new raw materials tested here were selected according to a possible cultivation on rewetted peatlands as paludiculture crop or in order to utilize material from landscape conservation. It was not possible to include availability aspects in the investigation. The replacement of peat in horticulture needs to be achieved by applying a broad set of peat substitutes of which each may be available in smaller amounts only.

The objective of our study is to demonstrate the feasibility of the first steps of a test procedure for peat substitutes by presenting the results of several raw materials applying the test criteria regarding material aspects. In the future, economic and ecological aspects need to be added to the test procedure. We hypothesize that the test procedure enables a true-proof identification of peat substitutes for growing media in horticulture.

2. Materials and Methods

2.1. Test Procedure and Test Criteria

In general, the test procedure starts with simple and quick tests and only if these are passed through successively, more complex and time-consuming tests are performed in the following steps. The aim is to assure that the materials show a high probability being a new peat substitute but also to identify and sort out unsuitable materials as early as possible. Furthermore, especially, the more complex tests will first give information regarding how to handle the material in practice. The test procedure for new components of growing media involves several consecutive test criteria, which are applied successively (Figure 1).

The investigation of a new raw material using the proposed test procedure is performed in two consecutive years or seasons, which are called stage 1 and stage 2. In stage 1, test criteria I–IV are applied to the same batch of raw material resulting in a first rating of the horticultural suitability in laboratory and greenhouse experiments. In stage 2, steps I to IV of the test procedure have to be applied again with a new batch of the raw material in order to check reproducibility of the results of stage 1. The implementation of adaptations, e.g., in the processing of a raw material or fertilization strategies are possible. Finally, stage 2 complements experiments under laboratory and greenhouse conditions by experiments under practical conditions (test criterion V).

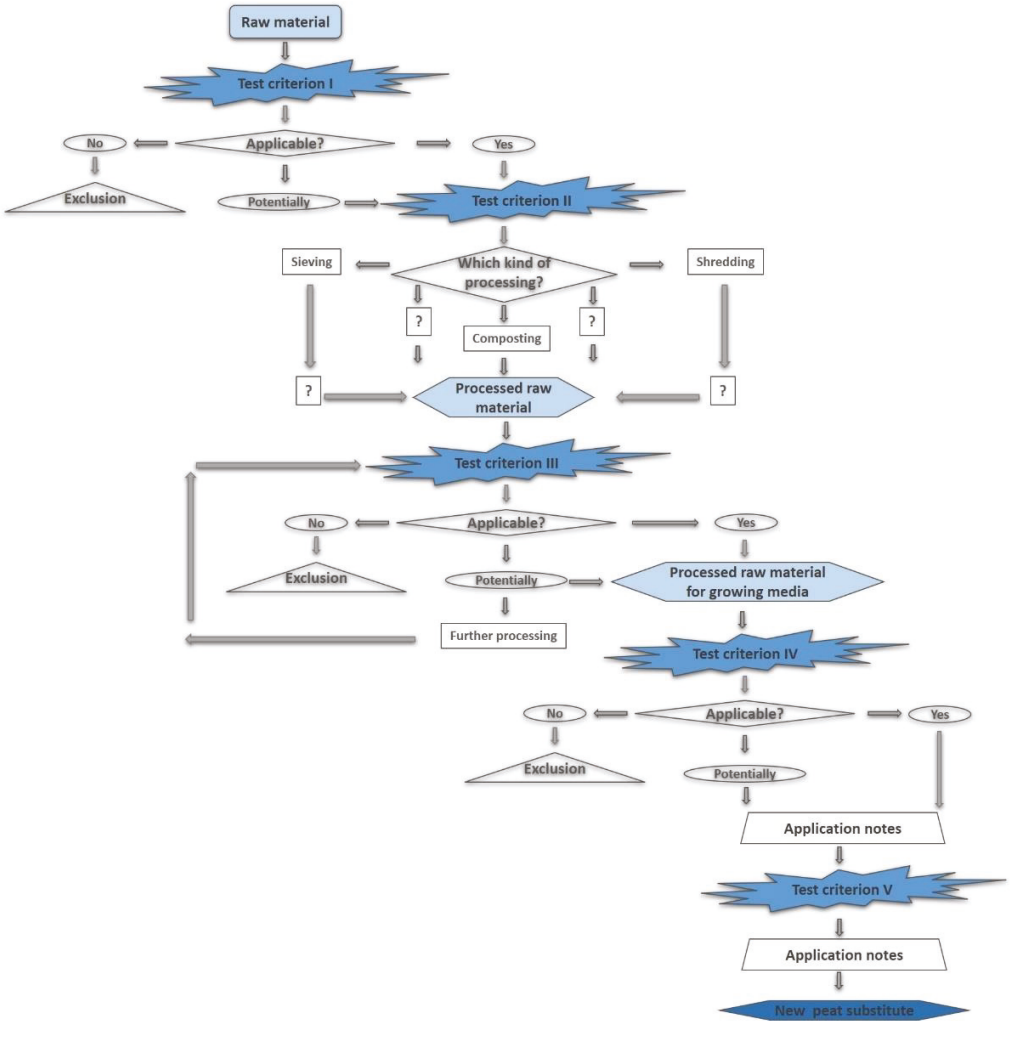

Figure 1. Decision tree for testing raw materials as potential components for growing media.

First, test criterion I (TC I) comprises material and simple substrate analyses of the not processed raw material, which include the determination of nutrient contents (% N, P, K in dry matter) as well as C/N ratio, volume weight (g L^{-1}), pH, and salt content (g KCl L^{-1}). If required due to the origin of the raw material, additional parameters, e.g., heavy metals or herbicides, are to be analysed.

If the raw material under investigation does not show any constraints, test criterion II (TC II) follows, which deals with the way of processing of the raw material. Depending on the properties of the raw material, different ways of processing are possible. At the beginning of the investigation of a new raw material, simple ways of processing are conducted such as e.g., sieving, chopping, or shredding. If some information from pre-studies on a raw material already exists, more complex methods of processing such as composting or extruding can be considered. At the end of composting, the level of maturity is determined.

Next, in test criterion III (TC III), extended substrate analyses on chemical and physical properties are applied on the processed materials; because of the processing in TC II, some properties of the processed raw material might have changed. Therefore, chemical analyses such as volume weight, pH, salt content, and nutrient contents (mineral N contents, plant available P and K, mg L^{-1}) are conducted again as well as physical analyses such as water, air capacity, and particle size distribution. In order to determine the demand on liming, the buffer curves of the processed raw material are determined.

In the following test criterion IV (TC IV), more complex substrate analyses of the potential substrate components are conducted. Stability tests of the nitrogen budget and CO_2 incubation tests are carried out. In order to attribute material characteristics for possible instabilities, water and salt-extractable C and N compounds as well as hemicellulose, cellulose, and lignin contents are analysed. Other complex analyses are standardized growing tests under controlled conditions, which summarize material characteristics and reflect them in plant growth.

In the last test criterion V (TC V), tests under practical conditions are performed in different sections of horticulture. During cultivation, substrate analyses are performed in order to check for the adaptation of fertilization, liming, or irrigation during cultivation. At the end of cultivation, plant biomass as well as N, P, and K (% in dry matter) in leaf biomass are analysed. The quality of the plants for marketable yield is also assessed.

Test criteria I–IV can be conducted within the first season or year with the same batch of raw material (stage 1). During the next season or year (stage 2), steps I to IV of the test procedure have to be applied again with a new batch of the raw material in order to check the reproducibility of the results (e.g. homogeneity of the raw material). If this run was again successful, tests under practical conditions (TC V) have to be performed considering the application notes developed from test criteria I–IV. If results from criteria I–IV are not satisfying, before continuing in the test procedure, modifications can be carried out, and their effect can be checked at different levels of the decision tree—for instance, in processing the raw materials (TC II) or in running the growing tests (TC IV). If all the test criteria are completed successfully, the test procedure results in a new peat substitute considering material aspects that can be applied in practice, possibly under consideration of application notes. In the future, economic and ecological criteria need to be added to the test procedure.

2.2. Raw Materials and Experiment Treatments

In order to confirm the test procedure, it was applied to several raw materials. In all experiments, milled white peat (fine to medium particle size; hereinafter referred to as "peat") derived from the Baltic States was used as control and for mixing. Heather (*Calluna vulgaris*) was mowed at the military training area Nordhorn in Lower Saxony, Germany. Subsequently, the heather material was chopped and composted for 8 months (September to April). After composting, it was sieved to 0–10 mm particle size. Alder (*Alnus glutinosa*) was harvested from a hedge bank near Bad Bentheim, Germany. Before chopping, the leaves were removed from the stem. Alder chaffs were sieved to 0–10 mm. In stage 1, cattail (*Typha angustifolia*) was harvested without seeds at the estuary of the river Danube, Romania, in January, and reed (*Phragmites australis*) was harvested in a nature conservation area "Untere Wuemme" near Bremen in February. After drying the plant material of reed and cattail, it was chopped and sieved to 3–6 mm particle size.

Analyses of the different raw materials in the test procedure were performed with single raw materials and their mixtures with peat. For experimental treatments of stage 1, see the following table (Table 1).

According to the results from stage 1, experimental treatments were adapted in stage 2 as well as reed was excluded from further analyses. In contrast to the first year, *Typha latifolia* was used in the experiments of the second year. Peat 100, Cal 100, Cal 50, Al 50, Al 25, Ty 50, and Ty 25 were set up as experimental treatments in stage 2 (for more details, see [13]).

Table 1. Treatments and mixing ratios in stage 1.

Treatment	Processed Raw Material I	Processed Raw Material II
Peat 100	100% (Vol.) peat	-
Cal 100	100% (Vol.) composted heather	-
Cal 50	50% (Vol.) composted heather	50% (Vol.) peat
Al 100	100% (Vol.) alder	-
Al 50	50% (Vol.) alder	50% (Vol.) peat
Ty 100	100% (Vol.) cattail	-
Ty 50	50% (Vol.) cattail	50% (Vol.) peat
Ph 100	100% (Vol.) reed	-
Ph 50	50% (Vol.) reed	50% (Vol.) peat

2.3. Analyses of Raw Materials and Treatments

The following methods were used for test criterion I. Volume weight of each treatment was determined according to VDLUFA [14]. pH was measured in $CaCl_2$ [15] and salt content in distilled water [16]. Nutrients (% N, % P, % K) of raw materials in the dry matter (d. m.) were determined. C (% in d. m.) and N (% in d. m.) were measured by oxidative burning of the sample at 1080 °C with an elementary analyzer (vario EL III, Elementar) [17]. For P (% in d. m.) and K (% in d. m.), 0.1 g of the dry matter of each raw material was ashed at 480 °C overnight, and the ash was transferred in 0.5 M HCl. P (% in d. m.) was measured at 470 nm with an UV VIS spectrometer after staining with the molybdenum yellow method and K (% in d. m.) in a CsCl matrix at 767 nm with an AAS.

For test criterion II, the technical processing of the raw materials was performed. In the case of the raw materials used in these investigation, alder, cattail, and reed were shredded and sieved. Drawing on results from previous experiments with heather, this material was composted, and the degree of rotting (Rottegrad index) was determined for heather compost [18,19].

For test criterion III, besides volume weight (g L^{-1}), pH, and salt content (g KCl L^{-1}), plant available nutrients (mineral N, P, K; mg L^{-1}) of processed raw materials and mixtures were extracted with $CaCl_2$ and DTPA (CAT extract) according to VDLUFA [20]. Mineral nitrogen (N_{min} (CAT)) was measured with a Skalar rapid flow analyzer, P in the CAT extract was measured as described in the previous section, and K (CAT) was analysed at 767 nm with an AAS.

The maximum water-holding capacity ($WHC_{max,}$ % v/v) was determined according to VDLUFA [21]. Water and air capacity (% v/v) were determined for each treatment at the pressure head levels −10 hPa, −50 hPa, and −100 hPa and the plant available water (% v/v) was calculated as the amount of water between −10 and −100 hPa [22]. Particle size distribution (% m/m) was determined for each processed raw material according to DIN EN 11540 [23]. Buffer curves were carried out for each treatment in order to determine the required amount of lime for adjusting the pH value in the subsequent experiments. For liming, $CaCO_3$ (85%) was used.

For test criterion IV, more elaborate analyses of C dynamics, standardized growing tests, and stability tests were performed. The stability of the budget of mineral N was tested according to VDLUFA [24] by adding 1000 mg of mineral N to 75 g material of each treatment (60% WHC_{max}), adjusting to pH 6, and measuring N_{min} (CAT) at the start of the incubation at 25 °C. After 20 days, the N_{min} of each treatment was extracted with CAT and determined with a Skalar flow analyzer. Differences between N_{min} (CAT) after 20 days and N_{min} (CAT) at the start provide information on the degree of N immobilization or N mobilization of a material.

To obtain a rough idea concerning the plant tolerability of the processed raw materials, standardized growing tests with Chinese cabbage under constant temperature and illumination cycles were performed [25]. First, 25 seeds of Chinese cabbage were sown in pots containing the fertilized and limed growing media mixtures of the respective treatments (5 replicates). Chinese cabbage was watered daily; seed germination was recorded daily

for 7 days, and leaf development and leaf color were observed daily from day 7 until day 21. After 21 days, plant biomass was harvested, and fresh and dry matter as well as the percentage of N, P, and K in the dry matter of the plants were determined. In stage 2, fertilization of the treatments was adjusted to the amount of immobilized N determined in the N stability test.

Degradation stability was determined by incubation experiments of each treatment at 60% of WHC_{max} and measurement of microbial-derived CO_2 [26]. This was done at the original pH value and at pH 6, a horticultural relevant pH value. Each treatment was incubated with five replications. Incubation took place at 20 °C for 13 days. At day 1, 2, 3, 6, 9, and 13 after the start of the incubation, samples were taken and titrated. The amount of released CO_2 was calculated for every sampling date according to Alef [1991; [26]], and cumulated CO_2 sums over 13 days were displayed.

For the determination of easily degradable compounds such as e.g., carbohydrates [27,28], processed raw materials were extracted with 0.5 M K_2SO_4 at 20 °C and 80 °C, respectively, according to an experimental set-up of Amha Amde [2011; [29]]. Salt-soluble C (SSC) compounds were analysed with a liquid TOC analyzer (vario TOC cube, Elementar Analysensysteme GmbH, Germany).

The amounts of lignin-derived phenols as slowly degradable organic compounds were determined for each processed raw material using alkaline Cu oxidation following the method of Hedges and Ertel (1982; [30]) modified by Dao et al. (2018; [31]). Total lignin-derived phenols (VSC) were defined as the sum of individual units vanillyl (V), syringyl (S), and cinnamyl (C).

Total contents of hemicellulose, cellulose, and lignin were analysed by determination of the amount of acid–detergent–fiber (ADF) according to an abbreviated version of the VDLUFA method [32], the amount of the neutral–detergent–fiber (aNDF) [33] and the amount of acid–detergent–lignin (ADL) [34], respectively. The C and N contents of the sample residues of each fraction were measured with an elemental analyzer (vario EL III, Elementar Analysensysteme GmbH, Germany).

Only processed raw materials and mixtures according to the treatments successfully applied TC I to TC IV were used for stage 2. Here, growing tests with treatments Peat 100, Cal 100, Cal 50, Al 50, Al 25, and an in-house growing medium were performed under practical conditions in three different companies specialized in the cultivation of vegetables, ornamentals, as well as tree nursery plants. Due to logistical delays, Ty 50 and Ty 25 could not be tested. Basil (*Ocimum basilicum*—variety Marian) was cultivated according to the rules of an organic farming association for six weeks in a greenhouse, cyclamen (*Cyclamen persicum*—variety Verano) was cultivated for four months in a greenhouse, and yew (*Taxus baccata*—variety Renkes Kleiner Grüner) was cultivated for five months in containers outdoors. Due to N immobilization in pretests, the treatments Al 50 and Al 25 were additionally fertilized during cyclamen and yew cultivation. At the end of the respective cultivation period, a rating of the color and shape of the leaves as well as the rooting intensity and root health were determined according to VDLUFA [25]. Aboveground biomass (fresh and dry) and the N, P, and K (% in d.m.) in leaves were analysed.

2.4. Statistical Analyses

All statistics were performed with R (version 4.0.2; R core team 2020).

In order to analyse differences among the different treatments, variance analyses were performed. If the data were normally distributed and variances homogeneous, ANOVA with a pairwise t test as the post hoc test was applied. If data were not normally distributed and/or variances not homogeneous, a non-parametrical Kruskal–Wallis test with a pairwise t test as post hoc test were performed. For correlation analyses, the R package "corrplot" was used, and the correlation coefficient according to Pearson was calculated. The level of significance for all analyses was $p < 0.05$. In stage 2, CO_2 emissions of the processed raw material cattail were excluded from the analyses, since it was an outlier.

3. Results

3.1. Development of the Test Procedure

For the development of the test procedure, several raw materials were investigated following the test criteria. Experiments were performed with different charges of the same raw materials in two consecutive years. Results of stage 1 and partly of stage 2 are shown in this article. Additional results of raw materials and experiments of stage 2 can be found in Leiber-Sauheitl et al. (2021; [13]).

3.1.1. TC I: Analyses of Raw Materials

The basic parameters of all raw materials show that no raw material has to be excluded (Table 2), but they also show that the C/N ratio could be an issue. Since the suitability of heather was shown in pre-studies (data not published), composted heather was used in the evaluation of the test procedure. For composted heather, the heavy metals Cu and Zn were analysed, since this raw material was derived from a military training area. With 7.6 mg Cu kg^{-1} d. m. and 28.5 mg Zn kg^{-1} d. m., both values were definitely lower than the critical value for growing media for trees used in landscaping [35]. Test criterion I was successfully applied, and therefore, processing of the raw materials was investigated in the subsequent test criterion II.

Table 2. Basic parameters of the investigated raw materials in stage 1. n = 3, variance analysis: Kruskal–Wallis (except P: ANOVA), post hoc test: pairwise t test, $p < 0.05$. Different letters indicate significant differences among raw materials. d.m.: dry matter.

Raw Material	pH ($CaCl_2$)	Salt Content [g KCl L^{-1}]	C/N	N	P	K
				[% in d. m.]		
Peat	3.2 ± 0.0 a	0.06 ± 0.00 a	60 ± 0 a	0.82 ± 0.00 a	0.03 ± 0.01 a	0.03 ± 0.01 a
Composted heather	5.2 ± 0.1 bc	0.47 ± 0.00 b	21 ± 0 b	1.44 ± 0.01 b	0.07 ± 0.02 b	0.22 ± 0.01 b
Alder	6.2 ± 0.1 d	0.21 ± 0.01 c	83 ± 0 c	0.55 ± 0.01 c	0.06 ± 0.01 b	0.17 ± 0.00 c
Cattail	5.4 ± 0.2 b	0.41 ± 0.00 d	154 ± 1 d	0.30 ± 0.00 d	0.01 ± 0.01 ac	0.23 ± 0.00 b
Reed	5.1 ± 0.0 c	0.37 ± 0.01 e	184 ± 4 e	0.25 ± 0.01 e	0.00 ± 0.00 c	0.07 ± 0.01 d

3.1.2. TC II: Processing of Raw Materials

The concept of the test procedure is to start with simple, inexpensive processing steps if no information is available of the materials to be tested. This was the case of alder, cattail, and reed. Therefore, branches and trunks of alder were shredded with bark and sieved <9 mm, and after harvest, cattail and reed were dried, shredded, and sieved to 3–6 mm. Heather was chopped after mowing and composted for several months, while temperature, water content, and aeration were checked regularly. Composting was achieved within 8 months, resulting in completed compost (Rottegrad index V), which was sieved <9 mm.

The processed raw materials (composted heather, alder, peat) were stored at 4 °C, whereas cattail and reed were stored at dry at room temperature until being analysed.

3.1.3. TC III: Extended Substrate Analyses of the Processed Materials

In TC III, extended substrate analyses of chemical and physical properties were applied with the processed materials (Table 3). Peat used as a control and mixing component showed medium volume weight and a low pH value, salt, and nutrient content. Except for cattail (36 g L^{-1}), the volume weights of all investigated raw materials were higher than the control (103–248 g L^{-1}). The pH values of composted heather, alder, cattail, and reed were significantly higher than the control peat (5.1–6.2). The salt contents of all processed raw materials under investigation did not exceed 0.5 g KCl L^{-1}. Except for N_{min}, the nutrient contents of composted heather, alder, cattail, and reed were significantly higher than the control peat (P 10–62 mg L^{-1}, K 68–608 mg L^{-1}).

Table 3. Basic parameters of the processed materials in stage 1. n = 3, variance analysis: Kruskal–Wallis, post hoc test: pairwise t test, $p < 0.05$. Different letters indicate significant differences among raw materials.

Raw Material	Volume Weight Dry [g L^{-1}]	pH ($CaCl_2$)	Salt Content [g KCl L^{-1}]	N_{min}	P	K
				[mg L^{-1}]		
Peat	78 ± 1 a	3.2 ± 0.0 a	0.06 ± 0.00 a	20 ± 1 a	1 ± 0 a	3 ± 0 a
Composted heather	248 ± 1 b	5.2 ± 0.1 bc	0.47 ± 0.00 b	28 ± 1 b	62 ± 1 b	608 ± 14 b
Alder	170 ± 1 c	6.2 ± 0.1 d	0.21 ± 0.01 c	0 ± 0 c	34 ± 1 c	231 ± 5 c
Cattail	36 ± 0 d	5.4 ± 0.2 b	0.41 ± 0.00 d	6 ± 0 d	10 ± 0 d	68 ± 2 d
Reed	103 ± 5 e	5.1 ± 0.0 c	0.37 ± 0.01 e	10 ± 0 e	15 ± 0 e	97 ± 1 e

The maximum water-holding capacity of all four potential substrate components (Cal 100, Al 100, Ty 100, Ph 100) was significantly lower than that of peat (Table 4). As a consequence, the WHC_{max} of all processed raw materials were proportionally increased by the addition of 50% peat. WHC_{max} of Cal 50 was higher than that of Al 50 followed by Ty 50 and Ph 50.

Table 4. Maximum water-holding capacity (WHC_{max}) and plant available water between −10 and −100 hPa of treatments in stage 1. WHC_{max} n = 4 and plant available water n = 16, variance analysis: Kruskal–Wallis, post hoc test: pairwise t test, $p < 0.05$. Different letters indicate significant differences among treatments. Explanation of treatment names see Table 1.

Treatment	WHC_{max} [Vol. %]	Plant Available Water [Vol. %]
Peat 100	82 ± 1 a	35 ± 3 a
Cal 100	63 ± 1 b	31 ± 3 b
Cal 50	72 ± 1 c	37 ± 2 c
Al 100	36 ± 1 d	6 ± 1 d
Al 50	57 ± 1 e	20 ± 2 e
Ty 100	15 ± 1 f	0 ± 0 f
Ty 50	48 ± 1 g	17 ± 1 g
Ph 100	15 ± 1 f	0 ± 0 f
Ph 50	45 ± 1 h	19 ± 2 eg

The water and air capacity of the different treatments were analysed in order to draw conclusions regarding the irrigation frequency and amount (Table A1) and were used to calculate the amount of plant-available water (Table 4). With about 35% Vol., Peat 100 and Cal 50 showed the highest amount of plant available water, followed by Cal 100 with approximately 30% Vol.. Al 50 and Ph 50 still showed half of the plant-available water in comparison to peat, whereas the values for Al 100, Ph 100, and Ty 100 were too low to be used as a sole component for growing media.

The particle size distribution of the different treatments confirmed the targeted particle size of the processing step (Table A2).

3.1.4. TC IV: Stability Tests, Growing Tests, and Analyses of C Dynamics

At test criterion IV, more elaborate analyses on the stability of the N budget, standardized growing tests, and analyses of C dynamics were performed.

N_{min} Budget

In order to gain information on the mineral nitrogen demand of the different raw materials in plant experiments, the N_{min} budget of the processed raw materials was determined (Figure 2). All treatments except Peat 100 immobilized mineral N over the incubation period in stage 1. According to VDLUFA, the mineral N budgets of Peat 100 and Cal 50 were stable (change of N_{min} contents lower than 50 mg N L^{-1}; [24]) followed by Cal 100 and Ty 50, which were slightly instable (change of N_{min} contents between 51 and 125 mg N L^{-1}), whereas Ty 100 and Al 50 were instable (change of N_{min} contents larger than 125 mg N L^{-1}). Al 100, Ph 100, and Ph 50 showed very high N immobilizations (larger than 250 mg N L^{-1}), which would lead to an exclusion as a component for growing media according to the defi-

nition of VDLUFA [24]. According to the principles of the test procedure, treatments were adapted in stage 2 (Al 25 and Ty 25 instead of Al 100 and Ty 100), and reed was excluded from further analyses (see also [13]). This adaptation resulted in an improvement of the N stability of Ty 50 and Ty 25 for cattail and Al 25 for alder [13].

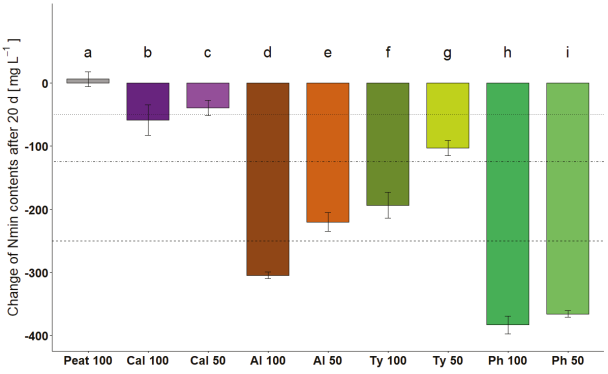

Figure 2. Change of N_{min} content of the different treatments after 20 days of incubation in stage 1, determined according to VDLUFA [24]. n = 16, variance analysis: Kruskal–Wallis, post hoc test: pairwise t test, $p < 0.05$. Lines show thresholds of the different stability levels [24]. Different letters indicate significant differences among treatments.

Standardized Growing Test

For the estimation of plant tolerability, growing tests with Chinese cabbage were performed using Peat 100 as control treatment. At the end of the growing test, Chinese cabbage biomass of Cal 100 and Cal 50 was comparable to Peat 100 (Table 5). Ty 50 showed significantly lower dry biomass than Peat 100. With Al 100, Al 50, and Ph 50, only 25–30% of the dry biomass of Peat 100 was formed. The low water-holding capacity of Ty 100 and Ph 100 impeded germination caused the absence of seedlings in both treatments. In addition, reed in comparison to cattail showed a high degree of mildew formation. For Al 100, Al 50, Ty 50, and Ty 50, a high immobilization of N was identified in the analyses of the substrates, which caused the bad performance of these processed raw materials in the growing test. In stage 2, N deficiency was compensated by a treatment-specific fertilization at the start of the experiment and an additional fertilization of single treatments if required (for details, see also [13]). With these adaptations in stage 2, Al 50, Ty 50, and Ty 25 attained 80% of the dry biomass formed with Peat 100 (for details, see also [13]).

Table 5. Dry matter and N, P, K (% in d. m.) of the cabbage biomass at the end of the experiment in stage 1. N = 4, variance analysis: Kruskal–Wallis, post hoc test: pairwise t test, $p < 0.05$. d.m.: dry matter. Explanation of treatment names see Table 1. Different letters indicate significant differences among treatments.

Treatment	Dry Matter [g pot^{-1}]	N	P	K
		[% in d. m.]		
Peat 100	3.4 ± 0.3 a	3.1 ± 0.3 a	0.52 ± 0.05 a	2.1 ± 0.3 a
Cal 100	3.0 ± 0.5 a	2.8 ± 0.3 a	0.48 ± 0.08 a	4.1 ± 0.6 b
Cal 50	3.0 ± 0.3 a	2.9 ± 0.3 a	0.49 ± 0.05 a	3.8 ± 0.3 b
Al 100	0.9 ± 0.5 b	1.1 ± 0.0 b	0.40 ± 0.10 a	2.1 ± 0.7 a
Al 50	0.8 ± 0.4 b	1.0 ± 0.1 b	0.44 ± 0.02 a	2.5 ± 0.1 a
Ty 100	-	-	-	-
Ty 50	2.2 ± 0.2 c	2.0 ± 0.1 c	0.42 ± 0.03 a	2.5 ± 0.3 a
Ph 100	-	-	-	-
Ph 50	1.0 ± 0.2 b	1.0 ± 0.0 b	0.37 ± 0.09 a	1.9 ± 0.5 a

Degradation Stability Tests

The amount of emitted CO_2 during a two-week incubation experiment served as a measure of microbial activity and therefore degradation stability of each treatment [36]. In stage 1, all treatments were incubated at their natural pH without liming (Figure 3a). Peat 100 showed the lowest CO_2 emissions due to the enrichment of stable organic compounds during peat formation and a strongly acidic pH (3.2). Cal 100 showed only slightly higher CO_2 emissions since easily degradable compounds were already degraded during composting. The highest CO_2 emissions were found at Ty 100 and Ph 100, since cattail and reed showed a low level of processing and still contained large amounts of easily degradable compounds such as hemicellulose and cellulose (Table 7). Al 100 showed a medium level of microbial activity due to its high contents of lignin in comparison to Ty 100 and Ph 100 (Table 7). A mixture with peat resulted in a significant decrease of CO_2 emissions among all processed raw materials, which may also be due to a decreasing pH value from slightly acid (Cal 100 5.2, Al 100 6.2, Ty 100 5.4, Ph 100 5.1) to stronger acidic (Cal 50 4.0, Al 50 3.9, Ty 50 3.4, Ph 50 3.4). However, correlation between pH value and CO_2 emissions over all processed raw materials and treatments was not significant ($R^2 = 0.22$, $p < 0.05$). There was also no significant correlation between the total organic carbon content and the CO_2 emissions over all treatments in stage 1 ($R^2 = 0.21$, $p < 0.05$) and in stage 2 ($R^2 = 0.13$, $p < 0.05$).

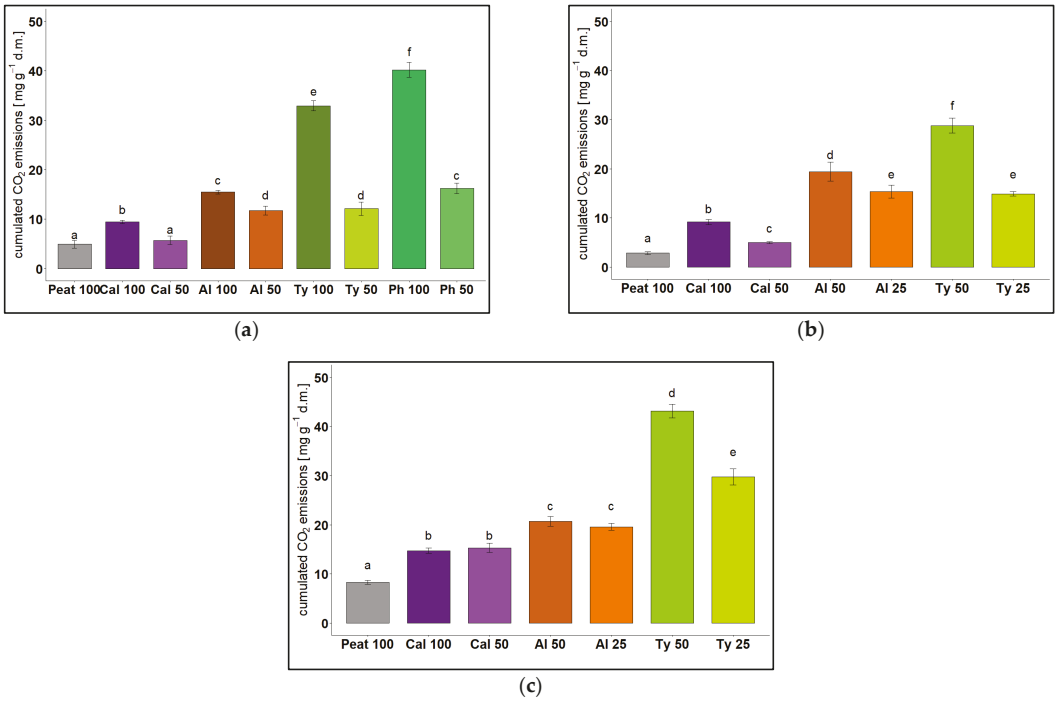

Figure 3. Cumulated CO_2 emissions of not limed processed raw materials and their mixtures over 13 days (**a**) stage 1 (n = 5, except Peat 100 n = 19) and (**b**) stage 2 (n = 5) as well as of (**c**) limed treatments (pH 6) in stage 2 (n = 5). Variance analysis: Kruskal–Wallis, post hoc tests: pairwise t test, $p < 0.05$. Different letters indicate significant differences among treatments.

In stage 2, trends of CO_2 emissions among not limed treatments were similar to stage 1 (Figure 3b). Ty 50 showed nearly doubled emissions in stage 2 in comparison to stage 1, which could be due a different cattail species. In general, the higher the proportion of peat in a treatment, the lower the CO_2 emissions of the respective processed raw material. In

order to gain information on CO_2 emissions of the treatments at a horticultural relevant pH value, incubations were repeated at pH 6 in stage 2 (Figure 3c). Despite doubled CO_2 emissions in comparison to stage 1, Peat 100 showed the lowest values of all treatments followed by Cal 100 and Cal 50 (no significant difference) and Al 50 and Al 25. CO_2 emissions of Ty 50 and Ty 25 increased by 50% and 100% in comparison to unlimed mixtures, respectively. This results in an order of degradation stability of Peat 100 > Cal 100, Cal 50 > Al 50, Al 25 > Ty 50, Ty 25.

Compound Classes Determining Degradation Stability

Several parameters such as the amount of salt-soluble carbon, the contents of hemicellulose, cellulose, (raw) lignin, and the sum of VSC units were measured in order to determine the reason for the state of stability of a processed raw material.

In stage 1, Typha 100 showed the highest amounts of SSC at 20 °C followed by Ph 100, whereas Peat 100, Cal 100, and Al 100 showed the lowest amounts (Table 6). At 80 °C, the highest amounts of SSC were again recorded for Ty 100 followed by Al 100, Peat 100, Ph 100, and Cal 100 in descending order of quantities of SSC (Table 6). At both temperatures, contents of SSC of mixtures (Cal 50, Al 50, Ty 50, Ph 50) were always between the contents of SSC of the respective processed raw material and peat. In stage 2, the SSC contents of the treatments were in a similar range than in stage 1.

Table 6. Content of salt-extractable C compounds of the different treatments at 20 and 80 °C in stage 1 and stage 2. n = 4, variance analysis: Kruskal–Wallis, post hoc test: pairwise t test, $p < 0.05$. C_{org}: organic carbon, SSC: salt-soluble carbon, d.m.: dry matter. Different letters indicate significant differences among treatments. Explanation of treatment names see Table 1.

Treatment	Stage 1			Stage 2		
	Total C_{org}	SSC 20 °C	SSC 80 °C	Total C_{org}	SSC 20 °C	SSC 80 °C
	[mg C g^{-1} d. m.]					
Peat 100	470 ± 1 a	0.9 ± 0.1 a	8.2 ± 0.3 a	499 ± 1 a	0.6 ± 0.0 a	6.4 ± 0.2 a
Cal 100	302 ± 2 b	0.9 ± 0.1 a	6.5 ± 0.6 b	220 ± 2 b	0.9 ± 0.1 a,b	7.5 ± 0.5 b
Cal 50	350 ± 2 c	0.9 ± 0.0 a	7.2 ± 0.3 c	397 ± 1 c	0.7 ± 0.1 a,b	6.4 ± 0.1 a
Al 100	455 ± 3 d	0.9 ± 0.2 a	8.8 ± 0.4 d	n.d.	n.d.	n.d.
Al 50	442 ± 1 e	1.0 ± 0.1 a	8.6 ± 0.3 a,d	477 ± 1 d	1.2 ± 0.0 b	10.7 ± 0.5 c
Al 25	n.d.	n.d.	n.d.	486 ± 1 e	0.9 ± 0.0 a,b	8.8 ± 0.2 d
Ty 100	469 ± 1 f	5.3 ± 0.1 b	14.0 ± 0.3 e	n.d.	n.d.	n.d.
Ty 50	460 ± 1 g	2.2 ± 0.1 c	9.5 ± 0.1 f	475 ± 0 d	1.9 ± 0.6 c	11.4 ± 0.6 e
Ty 25	n.d.	n.d.	n.d.	485 ± 1 e	0.9 ± 0.1 a,b	8.5 ± 0.5 d
Ph 100	468 ± f 0	2.9 ± 0.1 d	7.4 ± 0.5 c	n.d.	n.d.	n.d.
Ph 50	453 ± 1 d	2.0 ± 0.0 c	7.3 ± 0.1 c	n.d.	n.d.	n.d.

During the composting of Cal 100, hemicellulose and cellulose were degraded, resulting in a relative enrichment of lignin (Table 7). Al 100, a material from trees, showed the highest lignin contents of all not processed raw materials and medium contents of hemicellulose and cellulose. The lowest proportions of lignin and the highest of hemicellulose and cellulose were found in Ty 100 and Ph 100 (Table 7), since both are grasses of the family *Poaceae*. Medium contents of hemicellulose and lignin and high contents of cellulose were found in Peat 100 due to the plant genus and the peat-forming processes.

Due to its formation, Peat 100 and the composted heather (Cal 100) showed the lowest contents of total lignin-derived phenols (VSC units) in both stages (Table 7). During the long process of peat formation and the composting of heather material, VSC units were degraded in Peat 100 and Cal 100, respectively. Ph 100, Ty 100, and Al 100 showed significantly higher amount of VSC units in both stages. The amount of VSC units of Al 100 was 40% higher and Ty 100 was 30% lower in stage 2 compared to stage 1, which may be due to a different age, different proportion of stem to branches, or different environmental conditions [37] and different cattail species in both stages.

Table 7. Proportions of cellulose, hemicellulose, and raw lignin (n = 1) and sum of VSC units (n = 4) of each processed raw material in stage 1 and stage 2. VSC units: variance analysis: ANOVA, post hoc test: pairwise t test, $p < 0.05$. d.m.: dry matter. Different letters indicate significant differences among treatments. Explanation of treatment names see Table 1.

Treatment	Stage 1/Stage 2			
	Hemi-Cellulose	Cellulose	(Raw) Lignin	Sum of VSC Units
	[% of d. m.]			[mg g^{-1} d. m.]
Peat 100	10.0/10.9	53.0/46.6	21.1/23.0	7.6 ± 1.9 a/ 11.6 ± 1.5 a
Cal 100	3.4/10.0	8.4/8.2	73.6/65.1	17.2 ± 3.7 b/ 22.6 ± 7.3 b
Al 100	16.4/13.4	41.2/40.3	32.4/31.1	37.4 ± 8.7 c/ 61.2 ± 7.8 c
Ty 100	23.5/22.3	47.0/32.6	16.8/20.7	55.4 ± 2.8 d/ 39.8 ± 3.5 d
Ph 100	25.1/-	53.7/-	14.7/-	70.8 ± 10.0 e/-

3.1.5. TC V: Experiments under Practical Conditions

After some of the processed raw materials passed through the test procedure successfully up to TC IV, a confirmation of the developed test procedure followed in stage 2 by experiments under practical conditions in three different nurseries specialized in the cultivation of vegetables, ornamentals as well as tree nursery plants (Table A3; see also [13]). As control treatments, Peat 100 and the respective in-house growing medium were used.

Experiments under practical conditions in three different nurseries specialized in the cultivation of vegetables, ornamentals, as well as tree nursery plants were successful if an additional N fertilization was given in treatments which showed N immobilization during testing with the test procedure. The cultivation of basil was successful for the treatments without N immobilization (Cal 100, Cal 50, and in-house growing media). In treatments with N immobilization, basil cultivation was not successful, as no extra N fertilization was added to compensate for N immobilization (Table 8). The pH value of Peat 100 was by mistake 7.4 instead of 6.4, which resulted in growth deficits of basil. Cyclamen were successfully grown in all treatments due to additional N fertilization to Al 50 and Al 25 at the start and during cultivation. Fresh and dry weight of shoots of Al 25, Cal 50, and Peat 100 showed no significant differences in comparison to the in-house growing medium, whereas those of Cal 100 and Al 50 were significantly lower (Table 8). During cultivation of yew, additional N was added to compensate for N immobilization. Fresh as well as dry weight of yew were not significantly different among the treatments (Table 8).

Table 8. Experiments under practical conditions with basil, cyclamen, and yew. Aboveground biomass weights, n = 10. Variance analysis: Kruskal–Wallis (basil, yew), ANOVA (cyclamen), post hoc test: pairwise t test, $p < 0.05$. Different letters indicate significant differences among treatments. Explanation of treatment names see Table 1.

Treatment	Basil		Cyclamen		Yew	
	Fresh Weight	Dry Weight	Fresh Weight	Dry Weight	Fresh Weight	Dry Weight
	[g pot^{-1}]					
Peat 100	10 ± 2 a	1.1 ± 0.3 a	86 ± 12 a	7.2 ± 0.9 a	84 ± 15 a	31 ± 6 a
Cal 100	40 ± 7 b	3.9 ± 0.5 b	49 ± 4 b	4.1 ± 0.3 b	85 ± 10 a	31 ± 4 a
Cal 50	35 ± 1 b	3.7 ± 0.1 b	87 ± 11 a	6.9 ± 1.0 a	102 ± 10 a	38 ± 4 a
Al 50	7 ± 1 a	0.9 ± 0.2 a	46 ± 11 b	3.9 ± 0.7 b	84 ± 14 a	31 ± 4 a
Al 25	5 ± 1 a	0.7 ± 0.2 a	66 ± 11 a	5.9 ± 0.8 a	83 ± 11 a	31 ± 4 a
In-house growing media	51 ± 4 c	5.1 ± 0.3 c	89 ± 20 a	7.3 ± 1.3 a	93 ± 14 a	35 ± 5 a

4. Discussion

4.1. Evaluation of Processed Raw Materials

Having gone through the procedures of criteria I to IV in stage 1 and stage 2, the raw materials under investigation were evaluated and compared to already established peat substitutes. Peat used as the control and a mixing component showed a volume

weight, a pH value, and salt and nutrient contents as expected from literature [2] (Table 3). The salt contents of all processed raw materials under investigation did not exceed 0.5 g KCl L^{-1}, which is clearly under the critical value of 3 g KCl L^{-1} for components for growing media [35]. Nutrient contents were significantly higher than in the control peat but were according to established and material-related criteria in a convenient range (e.g., for compost [2,38]). Therefore, the basic parameters of all processed raw materials were in a range convenient for components of growing media (e.g., [2]).

Composted heather showed the best results of all raw materials under investigation. Chemical and physical analyses of composted heather were in the range of a substrate compost according to RAL ([2,38]; Tables 2–4, Table A1 and [13]). The stability of the N budget and the results in growing tests with Chinese cabbage were comparable to the control (Peat 100) for both treatments Cal 100 and Cal 50 (Figure 2, Table 5 and [13]). In tests under practical conditions, the promising results of Cal 100 and Cal 50 were confirmed in comparison to the control "in-house growing medium" (Table 8).

The results of chipped and sieved alder in the chemical tests were comparable to cattail and reed (Tables 2 and 3). The results of physical tests were moderate, but plant-available water improved with increasing amounts of peat from Al 50 to Al 25 (Table 4). In both stages, the N budgets of all treatments Al 100, Al 50, and Al 25 were highly instable, and high amounts of nitrogen were immobilized (Figure 2 and [13,24]). However, by increased N fertilization, good results were achieved in standardized growing tests [13] and also in tests under practical conditions for cyclamen and yew (Table 8 and [13]). Therefore, we suggest that alder chips need to be treated with additional N fertilization similar to wood fibers from coniferous wood [39].

For chopped and sieved cattail, especially the treatments Ty 50 and Ty 25 showed promising results in the chemical and physical analyses (Tables 2–4, Table A1 and [13]). The N budget of both treatments was slightly instable (Figure 2 and [13,24]); however, with a low N compensation, the results in standardized growing tests with Chinese cabbage yielded good results [13].

Reed showed similar results as cattail in physical and chemical analyses in stage 1 (Tables 2–4, Table A1). However, reed immobilized huge amounts of N in N budget tests (Figure 2), and therefore, growing tests without additional N showed a strongly reduced biomass of Chinese cabbage (Table 5). Due to these results and the development of mildew during standardized growing tests, reed was excluded in stage 2.

4.2. (Biological) Degradation Stability

In order to draw conclusions on the degradation stability of a processed raw material or treatment, contents of SSC, hemicellulose, cellulose, and (raw) lignin as well as the sums of VSC units were correlated to the cumulated CO_2 emissions over 13 days, respectively. In stage 2, CO_2 emissions of the processed raw material cattail were excluded from the analyses, since it was an outlier. The degradation stability of a substrate component expressed as cumulated CO_2 emissions during incubation under constant moisture and temperature conditions depends mainly on the chemical composition of the processed raw material [40,41].

In both years, CO_2 emissions showed high correlations to SSC 20 °C on a treatment level (R^2 = 0.83 stage 1, R^2 = 0.87 stage 2, or R^2 = 0.72 for stage 1 and 2, $p < 0.05$) and on the level of raw materials if stage 1 and 2 were combined (R^2 = 0.88). SSC 80 °C only showed very high correlations with CO_2 emissions (R^2 = 0.95, $p < 0.05$) but only in stage 2. Amounts of salt-soluble carbon did not depend on the total amount of organic carbon in any treatment but on extraction temperature. Therefore, SSC 20 °C should be used as a proxy to estimate the degradation stability of a processed raw material. The higher the SSC 20 °C content, the higher the degradation of the raw material. Investigations of different types of peat by Amha Amde (2011; [29]) also showed a correlation of the content of dissolved organic carbon that was extracted by water or salt solutions and microbial activity measured as long-term CO_2 evolution, which was measured by basal respiration.

The investigation of the cell wall components and the level of lignification was performed for the processed raw materials, and therefore, the data of all raw materials and both stages were combined for correlation analyses. The sum of VSC units was highly correlated to cumulated CO_2 emissions ($R^2 = 0.86$, $p < 0.05$). Among the cell wall components, hemicellulose showed the highest correlation to the cumulated CO_2 emissions ($R^2 = 0.78$, $p < 0.05$).

As a comprehensive result, SSC 20 °C, VSC, and also hemicellulose contents could be used to predict degradation stability, which were partly also used in stability indices [42]. Due to the fact that a higher C decomposition rate of a processed raw material results in a lower air volume in the growing media and poorer plant growth [43,44], composted heather showed the best results of all raw materials under investigation besides the control Peat 100.

4.3. Comparison of Investigated Processed Raw Materials to Substrate Components in Commercial Use

The chemical, physical, and biological properties of the peat material used as control and mixing components showed comparable results, as reported in the literature [2,45].

The pH, salt, and nutrient contents of composted heather are considerably lower than the threshold values for green waste composts according to RAL [2,38]. Therefore, composted heather could be used to a much higher proportion than the 40% (*v*/*v*) recommended by RAL. In addition to its beneficial stable N budget, the properties of composted heather are comparable to composted bark, which tends to a slight N immobilization [2,46]. Due to its good performance in experiments under practical conditions, composted heather could be used in more than the maximum recommended proportion of 50% (*v*/*v*) in growing media [47].

Chemical and physical parameters of the investigated reed and cattail are similar to the results for reed found by Stucki et al. (2019; [48]), which indicates similar horticultural properties of straw-based biomass. In Frangi et al. (2012; [49]), different ratios of pine bark and miscanthus (0–6 mm) and their effect on physical growing media parameters were investigated. The higher the content of *Miscanthus giganteus* was, the lower were the container capacity and the higher the air capacity, which is similar to our results for the straw-based raw materials reed and cattail. In another study, plant trials with *Prunus laurocerasus* showed stunted growth with increasing proportion of miscanthus in mixes [50] which is comparable to the high and medium N immobilization of reed and cattail in our experiments, respectively.

Chopped and sieved alder material in our experiments behaved similarly to other wood-based substrate components at the beginning of their development, showing low water capacity and high N immobilization [39]. In addition, with an adapted fertilization, the application of alder achieved promising results in distinct sectors of horticulture such as ornamental and tree nurseries. A different processing of alder such as refining it to fibers combined with an impregnation with nitrogen or also composting should be considered in future trials.

4.4. Advantages of the Test Procedure

The test procedure for new raw materials that could be used as potential substrate components consists of several consecutive test criteria regarding material aspects that are applied successively. If the investigations in all five test criteria show positive results, a new raw material can be identified as a not peat-based substrate component, which can from a material point of view be applied in practice—possibly considering information on cultivation derived from the test procedure.

The test procedure and its test criteria provide a structure for a systematical and reproducible analysis of new peat substitutes starting with the raw materials. In the first instance, simple and—depending on the results—in the following steps more elaborate analyses are applied. As an alternative to the applied methods of the Association of German Agricultural Analytic and Research Institutes (VDLUFA), the application of other methods is reasonable if they are appropriate for investigating the requested parameter and approved by the national or international scientific community. Concerning the

safety of new substrate components with regard to plant production, more elaborate analyses have to contain unconditional tests that give information on plant cultivation such as N immobilization and standardized growing tests. Analyses of C dynamics, degradation stability, and of the compounds influencing it broaden the existing scope of routine substrate analyses to properties that give additional useful information. This might become increasingly important if uncommon raw materials come into focus in the future. In case laboratory equipment is not suitable, they can be omitted in a first approach and be included if results are promising. Furthermore, it is worthwhile to check if already existing and easier applicable analyses of stability can be applied in case a correlation with the analyses used here was confirmed.

Decisions on the further procedure with a possible peat substitute can be made at an early stage. In our case, reed was provisionally excluded from further investigations in contrast to composted heather, cattail, and alder. However, a different processing for reed and a new test in the frame of the test procedure could improve the suitability of reed. By means of the test criteria, limitations of cultivation were identified which, in the case of the raw materials examined, concerned water-holding capacity and N immobilization. The limitation of cultivation "N immobilization" could be dealt with within the framework of the test procedure by an adapted N fertilization. This revealed good results in the growing tests for the N immobilizing substrates, which were confirmed in the tests under practical conditions. It could be shown that composted heather, alder, and cattail can serve as a substrate component by partly adapted N fertilization. Within 2 years, it was possible to identify new peat substitutes by means of the test procedure and its test criteria.

In the future, additional test criteria representing the field of economy and ecology have to be included in the original test procedure. The availability of a raw material, cost effectiveness, life cycle analyses, as well as environmental aspects need to be integrated. Evaluating only material aspects a raw material such as e.g., alder could also be excluded as a substrate component by the test procedure. However, if including the suitability of alder as a paludiculture crop [51] and thereby achieving benefits for the climate, alder could be considered for further investigation with a modified processing.

5. Conclusions

The preliminary test procedure with its test criteria offers a structure enabling a systematical and reproducible investigation of new possible peat substitutes regarding material aspects and starting at the level of the raw material. At first, simple and—depending on the results—more elaborate analyses are applied. Decisions on the further proceeding with a possible peat substitute can be made at an early stage. The preliminary test procedure with its test criteria is suitable to identify possible new peat substitutes within two years and to give first information on handling them in practice. This will promote the urgent search for new peat substitutes.

Our hypothesis that suitable peat substitutes for growing media can be reliably identified by the preliminary test procedure has been confirmed. Before the test procedure can be used as a standard, economic and ecological aspects need to be added in the future.

Author Contributions: Conceptualization. K.L.-S., H.B. and J.B.; methodology. K.L.-S. and H.B.; software. K.L.-S.; validation. K.L.-S.; formal analyses. K.L.-S.; investigation. K.L.-S.; resources. H.B. and J.B.; data curation. K.L.-S.; writing—original draft preparation. K.L.-S.; writing—review and editing. K.L.-S., H.B. and J.B.; visualization. K.L.-S.; supervision. K.L.-S., H.B. and J.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Deutsche Bundesstiftung Umwelt, Germany, and the Ministry of Food, Agriculture and Consumer protection of Lower Saxony, Germany. The publication of this article was funded by the Open Access Fund of the Leibniz Universität Hannover, Germany.

Data Availability Statement: The data presented in this study is contained in this article.

Acknowledgments: The author wishes to thank Gebrueder Brill GmbH for providing the raw materials and substrates and Olde Bolhaar for composting. We thank Gebrueder Brill GmbH, Gärtnerei Schulze Eckel, Baumschule zur Mühlen, and Gartenbau Kupke for cooperation during the tests under practical conditions. We are grateful to the technical staff of the Institute of Soil Science in Hannover for laboratory assistance.

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

Appendix A

Table A1. Water and air capacity of the different treatments at −10 hPa, −50 hPa, and −100 hPa in stage 1 determined according to DIN EN 13041 ([22]; n = 4. except −100 h Pa: Ty 100 n = 2. Ty 50 n = 1. Ph 50 n = 5). variance analysis: Kruskal–Wallis (except air capacity at −100 hPa: ANOVA). Post hoc test: pairwise t test. $p < 0.05$. At −100 hPa Ty 100 and Ty 50 were excluded from statistical analysis. Different letters indicate significant differences among treatments. Explanation of treatment names see Table 1.

Treatment	Water Capacity at −10 hPa	Air Capacity at −10 hPa	Water Capacity at −50 hPa [Vol. %]	Air Capacity at −50 hPa [Vol. %]	Water Capacity at −100 hPa	Air Capacity at −100 hPa
Peat 100	75 ± 4 a	19 ± 4 a	41 ± 1 a	53 ± 1 a	40 ± 1 a	55 ± 1 a
Cal 100	64 ± 3 b	27 ± 3 b	49 ± 2 b	42 ± 2 b	33 ± 1 b	58 ± 1 b
Cal 50	79 ± 1 c	14 ± 1 c	50 ± 5 b	42 ± 5 b	41 ± 1 a	51 ± 1 c
Al 100	31 ± 1 d	60 ± 1d	29 ± 1 c	63 ± 1 c	25 ± 1 c	66 ± 1 d
Al 50	52 ± 2 e	41 ± 2 e	37 ± 2 a	56 ± 5 a	32 ± 1 b	61 ± 1 e
Ty 100	13 ± 1 f	85 ± 1 f	13 ± 1 d	85 ± 1 d	16 ± 1 (n.d.)	82 ± 1 (n.d.)
Ty 50	38 ± 1 g	58 ± 1 d	24 ± 1 e	72 ± 1 e	22 (n.d.)	74 (n.d.)
Ph 100	15 ± 1 f	78 ± 1 g	15 ± 0 d	78 ±0 f	15 ± 1d	78 ± 1 f
Ph 50	40 ± 2 g	54 ± 2 h	26 ± 2 c.e	68 ± 2 g	22 ± 1 e	72 ± 1 g

Table A2. Particle size distribution of the different treatments in stage 1 determined according to DIN EN 11540 [23] (n = 3). Explanation of treatment names see Table 1.

Treatment	<0.2 mm	0.2–0.5 mm	0.5–1 mm	1–2 mm	2–4 mm	4–10 mm	10–16 mm	16–31.5 mm	>31.5 mm
	[% of Total Mass]								
Peat 100	7 ± 1	21 ± 1	21 ± 1	15 ± 2	14 ± 1	14 ± 2	7 ±1	1 ± 1	-
Cal 100	20 ± 6	32 ± 3	23 ± 3	15 ± 3	7 ± 2	3 ± 2	0.4 ± 1	-	-
Cal 50	12 ± 5	28 ± 4	27 ± 1	18 ± 3	10 ± 2	5 ± 1	1 ± 1	-	-
Al 100	9 ± 6	16 ± 5	19 ± 0.2	27 ± 4	24 ± 5	4 ± 1	-	-	-
Al 50	5 ± 1	17 ± 1	22 ± 1	23 ± 2	23 ± 1	9 ± 2	2 ± 1	0.3 ± 1	-
Ty 100	0.4 ± 0.1	0.5 ± 0.1	1 ± 0.3	11 ± 1	55 ± 8	32 ± 8	-	-	-
Ty 50	6 ± 1	14 ± 3	14 ± 3	16 ± 1	31 ± 3	17 ± 3	2 ± 1	-	-
Ph 100	0.1 ± 0	0.3 ± 0	3 ± 0.3	30 ± 2	66 ± 2	1 ± 0.2	-	-	-
Ph 50	5 ± 1	11 ± 3	11 ± 3	35 ± 1	25 ± 3	10 ± 3	2 ± 1	2 ± 0	-

Table A3. General setup of the experiments under practical conditions with the potential new substrate components [13]. Explanation of treatment names see Table 1.

	Vegetables	Ornamentals	Trees
Plant culture	*Occimum basilicum*	*Cyclamen persicum*	*Taxus baccata*
Cultivation period	6 weeks	4 months	5.5 months
Irrigation	ebb and flow system	channel system	irrigation cart
Treatments	Peat 100, Cal 100, Cal 50, Al 50, Al 25, in house growing media		
Fertilization (start)	No N compensation	N compensation for Al 50 and Al 25	
Complementary fertilization recommended	According to the results of the test procedure N compensation recommended for Al 50 and Al 25.		
Complementary fertilization applied	No	Yes	Yes

References

1. Gruda, N. Current and future perspective of growing media in Europe. *Acta Hortic.* **2012**, *960*, 37–43. [CrossRef]
2. Schmilewski, G. The role of peat in assuring the quality of growing media. *Mires Peat* **2008**, *3*, 1–8.
3. Myllylä, I. The role and characteristics of peat in horticulture are unique. *Peatl. Int.* **2005**, *1*, 11–14.
4. Blok, C.; Eveleens, B.; van Winkel, A. Growing media for food and quality of life in the period 2020–2050. *Acta Hortic.* **2021**, 341–356. [CrossRef]
5. Prasad, M.; Maher, M.J. Stability of peat alternatives and use of moderately decomposed peat as a structure builder in growing media. *Acta Hortic.* **2004**, *648*, 145–151. [CrossRef]
6. Noguera, P.; Abad, M.; Noguera, V. Coconut coir waste, a new and viable ecologically friendly peat substitute. *Acta Hortic.* **2000**, *517*, 279–286. [CrossRef]
7. Abad, M.; Noguera, P.; Puchades, R.; Maquieira, A.; Noguera, V. Physico-chemical and chemical properties of some coconut coir dusts for use as peat substitute for containerised ornamental plants. *Bioresoure Technol.* **2002**, *82*, 241–245. [CrossRef]
8. la Bella, S.; Virga, G.; Iacuzzi, N.; Licata, M.; Sabatino, L.; Consentino, B.B.; Leto, C.; Tuttolomondo, T. Effects of irrigation, peat-alternative substrate and plant habitus on the morphological and production characteristics of sicilian rosemary (Rosmarinus officinalis L.) biotypes grown in pot. *Agriculture* **2021**, *11*, 13. [CrossRef]
9. Benito, M.; Masaguer, A.; Moliner, A.; De Antonio, R. Chemical and physical properties of pruning waste compost and their seasonal variability. *Bioresoure Technol.* **2006**, *97*, 2071–2076. [CrossRef] [PubMed]
10. Machado, R.M.A.; Alves-pereira, I.; Ferreira, R. Coir, an Alternative to Peat—Effects on Plant Growth, Phytochemical Accumulation, and Antioxidant Power of Spinach. *Horticulturae* **2021**, *7*, 127. [CrossRef]
11. Gruda, N.S. Increasing sustainability of growing media constituents and stand-alone substrates in soilless culture systems. *Agronomy* **2019**, *9*, 1–24. [CrossRef]
12. Barrett, G.E.; Alexander, P.D.; Robinson, J.S.; Bragg, N.C. Achieving environmentally sustainable growing media for soilless plant cultivation systems—A review. *Sci. Hortic.* **2016**, *212*, 220–234. [CrossRef]
13. Leiber-Sauheitl, K.; Bohne, H.; Böttcher, J. Peat substitutes from renewable resources and land-scape conservation materials. *Acta Hortic.* **2021**, *1305*, 545–552. [CrossRef]
14. VDLUFA. Bestimmung der Rohdichte (Volumengewicht) von gärtnerischen Substraten mit sperrigen Komponenten (Determination of bulk density (volume weight) of horticultural substrates with bulky components). *Methodenb. I* **1997**, *A 13.2.2*, 1–5.
15. VDLUFA. Bestimmung des pH-Wertes (Determination of the pH value). *Methodenb. I* **2016**, *A 5.1.1*, 1–7.
16. VDLUFA. Bestimmung des Salzgehaltes in Böden, gärtnerischen Erden und Substraten (Determination of salt content in soils, horticultural soils and substrates). *Methodenb. I* **1991**, *A 10.1.1*, 1–8.
17. DIN; ISO. Soil quality—Determination of organic and total carbon after dry combustion (elementary analysis). *DIN ISO 10694* **1995**, 1–5.
18. BGK. *Rottegrad im Selbsterhitzungsversuch (Degree of Rotting in Self-Heating Test). Methodenb. zur Anal. Org. Düngemittel, Bodenverbesserungsm. und Substrate*; Kapitel IV; Bundesgütegemeinschaft Kompost e.V.: Köln-Gremberghoven, Germany, 2006.
19. DIN; EN. Soil improvers and growing media—Determination of the aerobic biological activity—Part 2: Self heating test for compost. *DIN EN 16087-2* **2011**, 1–10.
20. VDLUFA. Bestimmung von Haupt- und Spurennährstoffen in Kultursubstraten im Calciumchlorid/ DTPA-Auszug—CAT-Methode (Determination of major and trace nutrients in growing media in calcium chloride/ DTPA extract—CAT method). *Methodenb. I* **1997**, *A 13.1.1*, 1–56.
21. VDLUFA. Bestimmung der Wasserkapazität (WK) von Substraten und Komposten ohne sperrige Komponenten (Determination of the water capacity (WC) of substrates and composts without bulky components). *Methodenb. I* **2002**, *A 13.2.3*, 1–4.
22. DIN; EN. Soil improvers and growing media—Determination of physical properties—Dry bulk density, air volume, water volume, shrinkage value and total pore space. *DIN EN 13041* **2011**, 1–30.
23. DIN; EN. Peats for horticulture and landscape gardening—Test methods, properties, specifications. *DIN EN 11540* **2005**, 1–27.
24. VDLUFA. Bestimmung der Stabilität des Stickstoffhaushaltes organischer Materialien (Determination of the stability of the nitrogen balance of organic materials). *Methodenb. I* **2007**, *A 13.5.1*, 1–7.
25. VDLUFA. Keimpflanzentest zum Nachweis von pflanzenschädigenden Stoffen in Kultursubstraten und Substratausgangsstoffen (Seedling test for the detection of substances harmful to plants in growing media and components for growing media). *Methodenb. I* **2016**, *A 10.2.1*, 1–22.
26. Alef, K. Bodenatmung—Die Isermeyer-Methode (Soil respiration—The method of Isermeyer). In *Methodenhandbuch Bodenmikrobiologie*; Ecomed: Landsberg/Lech, Germany, 1991; pp. 87–90.
27. DeLuca, T.H.; Keeney, D.R. Soluble Anthrone-Reactive Carbon in Soils: Effect of Carbon and Nitrogen Amendments. *Soil Sci. Soc. Am. J.* **1993**, *57*, 1296–1300. [CrossRef]
28. Joergensen, R.G.; Mueller, T.; Wolters, V. Total carbohydrates of the soil microbial biomass in 0.5 M K2SO4 soil extracts. *Soil Biol. Biochem.* **1996**, *28*, 1147–1153. [CrossRef]
29. Amha Amde, Y. Water- and salt-extractable organic carbon from peats: Their relations with long-term CO2 evolution, microbial biomass-C, and the degree of decomposition. In *Microbial Activity and Biomass of Peats in Relation to the Intrinsic Organic Matter Composition, pH, Moisture, and C and N Inputs*; Leibniz University Hannover: Hannover, Germany, 2011; pp. 26–43.

30. Hedges, J.I.; Ertel, J.R. Characterization of Lignin by Gas Capillary Chromatography of Cupric Oxide Oxidation Products. *Anal. Chem.* **1982**, *54*, 174–178. [CrossRef]
31. Dao, T.T.; Gentsch, N.; Mikutta, R.; Sauheitl, L.; Shibistova, O.; Wild, B.; Schnecker, J.; Bárta, J.; Čapek, P.; Gittel, A.; et al. Fate of carbohydrates and lignin in north-east Siberian permafrost soils. *Soil Biol. Biochem.* **2018**, *116*, 311–322. [CrossRef]
32. VDLUFA. Bestimmung der Säure-Detergenzien-Faser (ADF) und der Säure-Detergenzien-Faser nach Veraschung (ADFom) (Determination of acid detergent fiber (ADF) and acid detergent fiber after ashing (ADFom)). *Methodenb. III* **2012**, *6.5.2*, 1–8.
33. VDLUFA. Bestimmung der Neutral-Detergenzien-Faser nach Amylasebehandlung (aNDF) sowie nach Amylasebehandlung und Veraschung (aNDFom) (Determination of neutral detergent fiber after amylase treatment (aNDF) and after amylase treatment and ashing (aNDFom)). *Methodenb. III* **2012**, *6.5.1*, 1–8.
34. VDLUFA. Bestimmung des Säure-Detergenzien-Lignins (ADL) (Determination of acid-detergent lignin (ADL)). *Methodenb. III* **2012**, *6.5.1*, 1–4.
35. RAL. *Substrate für Pflanzen—Gütesicherung RAL-GZ 250 (Substrates for plants—Quality assurance system RAL-GZ 250)*; RAL, Ed.; Beuth GmbH: Berlin, Germany, 2015.
36. Amha Amde, Y. Microbial Activity and Biomass of Peats in Relation to the Intrinsic Organic Matter Composition, pH, Moisture, and C and N Inputs. Ph.D. Thesis, Gottfried Wilhelm Leibniz Universität Hannover, Hannover, Germany, 2011.
37. Campbell, M.M.; Sederoff, R.R. Variation in lignin content and composition. *Plant Physiol.* **1996**, *110*, 3–13. [CrossRef] [PubMed]
38. BGK; RAL. Gütesicherung Kompost—Qualitätskriterien für Substratkompost (RAL quality assurance system for compost—quality criteria for substrate compost). *DOK 251-006-3* **2018**, 1–2.
39. Gruda, N. Sustainable Peat Alternative Growing Media. *Acta Hortic.* **2012**, *927*, 973–979. [CrossRef]
40. Sollins, P.; Homann, P.; Caldwell, B.A. Stabilization and destabilization of soil organic matter—Mechanisms and controls. *Geoderma* **1996**, *74*, 65–105. [CrossRef]
41. Tuomela, M.; Vikman, M.; Hatakka, A.; Itävaara, M. Biodegradation of lignin in a compost environment: A review. *Bioresoure Technol.* **2000**, *72*, 169–183. [CrossRef]
42. Riviere, L.-M.; Caron, J. Research on substrates: State of the art and need for the coming 10 years. *Acta Hortic.* **2001**, *548*, 29–41. [CrossRef]
43. Aendekerk, T.G.L. Decomposition of peat substrates in relation to physical properties and growth of Chamaecyparis. *Acta Hortic.* **1997**, *450*, 191–198. [CrossRef]
44. Verhagen, J.B.G.M. Stability of growing media from a physical, chemical and biological perspective. *Acta Hortic.* **2009**, *819*, 135–142. [CrossRef]
45. Amha, Y.; Bohne, H.; Schmilewski, G.; Picken, P.; Reinikainen, O. Physical, chemical and botanical characteristics of peats used in the horticultural industry. *Eur. J. Hortic. Sci.* **2010**, *75*, 177–183.
46. Handreck, K.A. Composts in the Production and Performance of Growing Media for Containers. *ISHS Acta Hortic. 1018* **2014**, 505–512. [CrossRef]
47. Raviv, M. Composts in growing media: What's new and what's next? *Acta Hortic.* **2013**, *982*, 39–52. [CrossRef]
48. Stucki, M.; Wettstein, S.; Mathis, A. *Erweiterung der Studie Torf und Torfersatzprodukte im Vergleich*; Institut für Umwelt und Natürliche Ressourcen, Zürcher Hochschule für Angewandte Wissenschaften: Wädenswil, Schweiz, 2019.
49. Frangi, P.; Amoroso, G.; Piatti, R. Alternative growing media to peat obtained from two fast growing species of poaceae. *Acta Hortic.* **2012**, *927*, 967–972. [CrossRef]
50. Altland, J.E.; Locke, J.C. Use of Ground Miscanthus Straw in Container Nursery Substrates. *J. Environ. Hortic.* **2011**, *29*, 114–118. [CrossRef]
51. Wichtmann, W.; Schröder, C.; Joosten, H. *Paludiculture—Productive Use of Wet Peatlands. Climate Protection—Biodiversity—Regional Economic Benefits*, 1st ed.; Schweizerbart: Stuttgart, Germany, 2016.

horticulturae

MDPI

Article

Performance of Greenhouse-Grown Beit Alpha Cucumber in Pine Bark and Perlite Substrates Fertigated with Biofloc Aquaculture Effluent

Emmanuel Ayipio [1,2,*], Daniel E. Wells [1], Mollie Smith [3] and Caroline Blanchard [1]

[1] Department of Horticulture, Auburn University, 101 Funchess Hall, Auburn, AL 36849, USA; wellsda@auburn.edu (D.E.W.); ceb0116@auburn.edu (C.B.)
[2] CSIR-Savanna Agricultural Research Institute, P.O. Box TL 52, Nyankpala-Tamale, Ghana
[3] School of Fisheries, Auburn University, 203 Swingle Hall, Auburn, AL 36849, USA; mrs0018@auburn.edu
* Correspondence: eza0035@auburn.edu

Abstract: Using aquaculture effluent (AE) to fertigate plants is gaining popularity worldwide. However, in substrate-based systems, the choice of substrate is essential due to their effects on crop productivity. Differences in the retention of nutrients by substrates makes it necessary to assess suitability for use in AE. This study was conducted from January to July in 2016 and September to October in 2019 to evaluate greenhouse-grown Beit Alpha cucumber (*Cucumis sativus* L. 'Socrates') performance fertigated with AE in pine bark or perlite substrates, grown either as one plant or two plants per pot. A 2 × 2 factorial arrangement in a randomized complete block design with four replications for each season was used. The substrate effect on yield in 2016 depended on the density and season. The pooled yield over seasons in 2016 showed pine bark had a significantly higher yield than perlite by 11% in one plant per pot but lowered by the same amount in two plants per pot. In 2019, pine bark significantly reduced the leachate pH in both plant densities and reduced the leachate EC by about 15% in two plants per pot. The foliar boron was occasionally below sufficiency whilst manganese was above sufficiency in pine bark due to its inherently low pH. We conclude that the effect of the substrates on cucumber yield fertigated with AE is dependent on the season and the number of plants per pot. Therefore, due to the local availability of pine bark, it could be a potential substitute for perlite especially when using one plant per pot for AE. In addition, pine bark could be used as an intermediate substrate to reduce the pH in AE for downstream use.

Keywords: Aquaponics; soilless cucumber; leachate pH; cucumber yield

Citation: Ayipio, E.; Wells, D.E.; Smith, M.; Blanchard, C. Performance of Greenhouse-Grown Beit Alpha Cucumber in Pine Bark and Perlite Substrates Fertigated with Biofloc Aquaculture Effluent. *Horticulturae* **2021**, *7*, 144. https://doi.org/10.3390/horticulturae7060144

Academic Editors: Nazim Gruda and Juan A. Fernández

Received: 2 May 2021
Accepted: 2 June 2021
Published: 9 June 2021

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

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

The use of aquaculture effluent (AE) as a nutrient source for plant production is gaining popularity worldwide with an exponential growth from 2004 to 2012 [1]. Aquaponics is a term used to describe a plant production technique in which at least 50% of a plant's essential nutrients are obtained from an aquaculture system (RAS) [2] and can be coupled with different hydroponic systems. Biofloc technology is used to distinguish the RAS technique in which biofiltration, i.e., the conversion of total ammonium nitrogen into nitrates by nitrifying bacteria, and aquaculture co-habit in the same unit. Therefore, biofloc technology is different from a typical RAS or "clear water" systems in which biofiltration is separated from the aquaculture unit. Biofloc technology shows promising benefits for crop productivity with better growth and quality in lettuce [3].

Substrates differ greatly in their physical and chemical properties leading to differential effects on plant productivity. Substrates of an inorganic or mineral origin such as perlite predominate in hydroponic systems due to their consistent composition and predictable performance. However, perlite substrates tend to have a neutral or near neutral pH, which may not be a good combination with the already high pH of AE. Aged or composted pine bark is an organic substrate that has been used predominantly in containerized

ornamental production [4]. However, pine bark substrate has a higher air-filled porosity resulting in a lower water holding capacity than perlite [5]. In a pour through experiment, pine bark substrate had less available water and retained less nitrogen, i.e., NO_3^--N and NH_4^+-N, implying more N would be drained out [6,7] when used. Although perlite is also porous, due to its smaller particle size it has a higher amount of plant available water [8]. On the other hand, pine bark substrate has a low pH [5], which may offer a better combination with AE than perlite.

An assessment of the substrate effect shows that the marketable yield, fruit count and plant height of cucumbers were the highest in a peat substrate that had a significantly higher water holding capacity, cation exchange capacity and organic matter content than perlite and other substrates with a lower water holding capacity [9]. However, in the same study, when perlite was compared with bark mixed with peat of 50% v/v it resulted in a similar performance of the cucumber crop [9]. Pine bark and perlite substrates also had similar effects on Beit Alpha cucumbers when fertigated with a conventional hydroponic nutrient solution [5]. However, differences in yields exist between conventional hydroponics and aquaponics [10] mostly due to low nutrients, the presence of solids and the high pH of AE. Thus, substrates that work well when fertigated with a hydroponic solution might not adapt well with AE. Therefore, there is a need to explore substrate suitability and performance in AE systems. We hypothesize that the type of substrate used would affect the availability of nutrients and, thus, cucumber productivity. Experiments were conducted to explore if pine bark and perlite substrates would influence Beit Alpha cucumber cv. 'Socrates' differently when fertigated with AE. The study also assessed the effect of the plant number per pot and its interaction with the substrate on cucumber productivity.

2. Materials and Methods

2.1. Plant Material, Growth Conditions and Experimental Design

All trials were conducted at the Auburn University aquaponic project facility located at the E.W. Shell Fisheries Center research station (lat. 32.648935° N, long. 85.486828° W).

The plant production for the three seasons was done in a 9 m × 29 m double-layered plastic covered greenhouse. Three-week-old cucumber (*Cucumis sativus* L. 'Socrates') seedlings were transplanted from 70-cell trays to 11-L rectangular Dutch buckets (Crop King, Lodi, OH, USA) filled with either 100% horticultural grade perlite or aged pine bark based on the treatment. Over the course of the experiment, plants were trellised upwards to a height of approximately 2.2 m then allowed to drape.

The production in 2016 ran from 6 January to 31 July in two rounds of trials covering winter to spring seasons. The first round of 2016 ran between winter and early spring while the second round covered the rest of the spring months. The production in 2019 ran from 3 September to 28 October (late summer–fall), with a total of 55 days from transplanting. Plant spacing was 0.46 m × 1.83 m or 0.84 m^2/pot. During the 2019 trial, the greenhouse temperature and the relative humidity were measured using pendant temperature data and a temperature/RH logger (HOBO, Onset Computer corp. Bourne, MA, USA) placed 2.2 m from the ground at the draping point. Data were logged every 10 min and averaged over a 12 h period. The greenhouse microclimate was considered important to assess the condition of growth of the plants. Although cooling of the greenhouse was done using exhaust fans and a cooling pad controlled by night and day temperature set points, temperatures and the relative humidity still fluctuated throughout the production in 2019. The mean day and night air temperatures over the trial period for 2019 were 28.3 °C and 20.8 °C, respectively. The relative humidity was generally high. The mean day and night relative humidity values were 64% and 92%, respectively.

Water was delivered to the cucumbers via an irrigation pump, with the corresponding foot valve submerged 0.35 m below the surface of a passive clarifier system attached to biofloc tilapia aquaculture unit, as described below, so that the settleable solids further clarified in the bottom of the second clarifier were undisturbed. The irrigation pump was wired to a timer that was scheduled to water for 3 min on the hour, nine times per day.

Iron chelate (13% EDTA Fe) was added at a rate of 2 mg L^{-1} to the second clarifier at monthly intervals.

The aquaculture unit used to irrigate the plants consisted of a 100 m^3 rectangular tank contained in 9 m × 29 m plastic greenhouse and a water clarifier unit consisting of two cylindro-conical tanks of 0.5 m^3 each located just outside the greenhouse. The fish tank was aerated by a 1-hp blower (SweetWater, Aquatic Eco-systems, Apopka, FL, USA) fixed with diffuser tubing. The blower was also used to create an airlift that circulated the water from the tank to the clarifier and back. Using normal operating procedures, effluent and solids from the fish rearing tank flowed into the first clarifier, from which settleable solids were removed 2 to 3 times daily by opening a clarifier drain. The AE then flowed by gravity to a second clarifier where further settleable solids were again removed and clarified effluent either flowed back into the fish tank or pumped into the vegetable greenhouse for irrigation. No other filtration devices were used with this system. Water pH was maintained in the range of 6 to 6.5 by adding $Ca(OH)_2$ directly to the fish tank. Potassium chloride was added to the fish tank to maintain a concentration of 120–150 ppm when measured for chloride. Water into the fish tanks came from a rainwater fed reservoir and flowed by gravity to the fish tank as make-up water to account for plant use and water loss through evaporation and disposal of fish sludge.

Prior to starting the first experiment in 2016, the fish rearing tanks were in continuous operation, producing Nile tilapia (*Oreochromis niloticus* L.). Fish were cultured for 11 weeks and then graded, sorted, and stocked by size into three separate netted structures called hapas, from which 50–75 kg of fish were harvested weekly. To jumpstart fish production, 750 tilapia of 200 g each were stocked into a 6 m^3 hapa to be harvested first during the production cycle. Next 2500 tilapia of 100 g each and 7000 tilapia of 50 g each were stocked into separate 18 m^3 hapas to be cultured and eventually divided into an additional 100 m^3 tank. The fish were fed twice daily at 1.5% of their body weight with a complete diet of floating pellets containing between 40% and 36% protein (Cargill, Franklinton, LA, USA). Thus, the fish culture unit was a mix of different ages and weights that required different feed types and feeding rates.

A 2 × 2 factorial treatment arrangement in randomized complete block design with 4 replications per treatment was used leading to 16 experimental units in each season. The treatment combinations were as follows: Treatment 1: Perlite substrate with two plants per pot; Treatment 2: Perlite substrate with one plant per pot; Treatment 3: Pine bark substrate with two plants per pot; Treatment 4: Pine bark substrate with one plant per pot.

2.2. Measurements and Sampling for Laboratory Analysis (Mineral Composition)

Once harvesting was started in 2016, cucumber fruits were picked daily. Cucumber fruit count and fresh weights were recorded daily from five middle individual pots, for each experimental unit. In treatments with two plants per bucket, fruit numbers and weights were added together to represent count or weight per pot. In the 2016 trial, leaf samples were taken for foliar analysis at day 50 from transplanting. In total, 15 recently matured leaves from each experimental unit were sampled. Leaf tissues were digested in sulfuric acid and analyzed for macro- and micronutrient concentrations, using the ICP-MS approach (Waters Agricultural Laboratories, Inc., Camilla, GA, USA).

In addition to yield recorded in both 2019 seasons, measurements were taken on plant height measured at each destructive sampling for biomass, from just below the cotyledons to the apical meristems using a meter rule. Total nodes per plant were counted and divided by plant height to obtain average internode length. Leaf area was measured using LI 3100 (LICOR, Lincoln, Nebraska, USA). Leaf samples after area measurements were dried in an oven for minimum of 48 h at 77 °C. Specific leaf area (cm^2 g^{-1}) per pot was calculated by dividing leaf area (cm^2) over leaf dry weight (g). Leaf SPAD index was measured with a portable SPAD meter (SPAD-502 plus, Spectrum technologies, Aurora, IL, USA) at five points on newly fully expanded leaves and averaged. Leaf stomatal conductance was measured on the same leaves used for SPAD measurements using a handheld leaf

porometer (Decagon SC-1, Meter Group, Inc. Pullman, WA, USA). Plants were placed on a raised platform constructed using cinder blocks and a fiberglass frame. Containers (4.7-L) were placed below plants to collect leachate daily from which pH and EC were measured using a HI9813-6 Portable pH/EC/TDS/temperature meter (Hanna Instruments, Smithfield, RI, USA) and NO_3^- using a L-AQUA twin handheld meters (Horiba, Kyoto, Japan) and multiplied by 0.22 to obtain NO_3^- -N.

Nitrogen use efficiency was calculated based on the measured nitrate of the AE. Daily nitrate measurements were average over the period, and together with the irrigation schedule (7:00 a.m. to 6:00 p.m. CDT), discharge rate of 3.785 L h^{-1} [11] the amount fertigated over the period was estimated as;

$$A_f = D/60 \times r \times E \times T_p \times N_c \quad (1)$$

where:

A_f = Amount fertigated;
D = duration (minutes) per irrigation event;
r = discharge rate;
E = number of events per day;
T_p = duration of trial;
Nc = N concentration.

The nitrogen use efficiency was then estimated by dividing total yield (kg) over amount of NO_3^- N fertigated (kg)

2.3. Data Analysis

Data were subjected to analysis of variance (ANOVA) using the GLIMMIX procedure in SAS (SAS Institute, Cary, NC, USA). Block and individual sampling units were considered as random variables. For yield and foliar data across seasons in 2016, a three-way ANOVA including substrate, density, and season was used. However, for measurements that were taken in 2019, a two-way ANOVA of substrate by density was used. Post-hoc mean comparison was done using Tukey's HSD at $\alpha = 0.05$.

3. Results and Discussion

3.1. Aquaculture Effluent and the Substrate Leachate Nitrate Concentration, pH and EC

The weekly averages of nitrate-N, pH, EC over the experimental period for 2019 are shown in Table 1. Overall, nitrate-N fluctuated the most, ranging from 59.4 ppm to 77.3 ppm in the AE. The highest average weekly EC was 1.24 mS cm^{-1}. The lowest weekly average pH was 6.17, and reached a maximum at 6.7. Measurements of leachate pH, nitrate, and EC allowed the determination of effect of each substrate and planting density on these parameters. In the first configuration, leachate was collected in a non-replicated manner, which was difficult to determine statistical effects of the substrate or density on leachate parameters. However, the setup in 2019 allowed leachate collection from individual experimental units and a test of treatment effect (Table 2).

Leachate pH was higher in perlite than pine bark by about 9% irrespective of plant density but was not statistically significant. However, difference in leachate EC between the substrates depended on plant density such that for one plant per pot, no significant difference existed between the two substrates whereas for two plants per pot, perlite recorded significantly higher leachate EC (12.9%) than pine bark (Table 2). There was no main effect of substrate, and density or their interaction on leachate nitrate-N concentration. Generally, the EC of leachate collected from the pots was averagely lower than the effluent EC from the fish tanks, indicating a possible effect of plant nutrient uptake and substrate, especially for pine bark, on leachate EC.

Table 1. Weekly AE NO_3-N, pH, and EC supplied from the aquaculture unit. Daily measurement for 2019 trial from the emitter and averaged over a 7-day period.

Week After Transplanting	NO_3-N (ppm)	pH	EC (mS cm^{-1})
Week1			
Mean	61.05 ± 3.3	6.4 ± 0.15	1.08 ± 0.00
N	4	4	4
Week2			
Mean	62.54 ± 4.9	6.2 ± 0.14	1.09 ± 0.09
N	7	7	7
Week3			
Mean	77.31 ± 3.7	6.3 ± 0.21	1.24 ± 0.15
N	7	7	7
Week4			
Mean	69.14 ± 7.6	6.5 ± 0.22	1.01 ± 0.14
N	7	7	7
Week5			
Mean	61.60 ± 11.9	6.7 ± 0.27	1.18 ± 0.39
N	7	7	7
Week6			
Mean	75.43 ± 17.0	6.5 ± 0.34	0.98 ± 0.25
N	7	7	7
Week7			
Mean	61.6	6.7 ± 0.27	1.13 ± 0.20
N	6	6	6
Week8			
Mean	59.4 ± 4.4	6.6 ± 0.10	1.12 ± 16
N	3	3	3

Table 2. Simple effects of substrate for each planting density level on leachate NO_3-N, pH, and EC. Data collected from Dutch bucket drainage in 2019.

Density [z]	Substrate	NO_3-N (ppm)	pH	EC (mS cm^{-1})
1×				
	Pine bark	68.91 a	6.07 b	0.81 a
	Perlite	77.11 a	6.66 a	0.87 a
	p-value	*0.4351*	*<0.0001*	*0.1942*
2×				
	Pine bark	59.29 a	6.13 b	0.74 b
	Perlite	74.17 a	6.61 a	0.85 a
	p-value	*0.1683*	*<0.0001*	*0.0169*

[z] 1× = one plant per pot; 2× = two plants per pot; pot = 11-L Dutch bucket.

3.2. Foliar Nutrient Analysis of Cucumbers Affected by the Substrate and Density

The results showed that foliar nutrient concentration of the plants grown in either pine bark or perlite substrates did not differ significantly ($p > 0.05$). In addition, number of plants per pot did not significantly affect foliar nutrient composition of the leaves (Tables 3 and 4). However, plants grown in winter–spring 2016 had higher N, P, K, and Mg values than those in spring except for Ca and S. Foliar nutrient concentration was higher than sufficiency range for N, P, Ca, and S, but not K and Mg which were below the sufficiency ranges (Table 3). Foliar micronutrient concentrations were generally within reported sufficiency ranges except for B which was at or below the low side of the reported sufficiency range across all treatments in 2016 (Table 4). The nutrient levels in our system are far below the recommended levels for cucumber production [12] which corroborates other studies show-

ing that AE is low in plant essential nutrients, especially micronutrients [13], resulting in low yields of aquaponics systems compared to conventional hydroponics system when there is no nutrient supplementation in the AE [10]. However, even when two plants were grown per pot, we observed no signs of nutrient deficiency indicating superior performance amidst the low nutrient load. The interesting observation of sufficient foliar nutrient concentration in this study was also reported by Blanchard et al. [14] where regardless of pH adjustment, cucumber had sufficient foliar nutrient concentration. There needs to be further investigation into what accounts for this performance. We hypothesize that the presence of solids in the AE could play a role in the availability of nutrients through mineralization over time. Additionally, the biological floc which is characteristic of the biofloc system could be a better source of nutrients than clear water systems, as was demonstrated by Pinho et al. [15] which previously led to better growth of lettuce in a biofloc tilapia system [3]. We anticipated that pine bark, due to its organic nature would lead to enhanced mineralization and thus nutrient availability than perlite which is inorganic in such biofloc systems. In addition, we posited that since pine bark generally has lower pH than perlite, it would present a better substrate level pH adjustment to the AE which is usually maintained at higher pH to favor the fish and nitrifying bacteria. However, our observations showed that although there are isolated cases of higher foliar nutrient content in pine bark than perlite, this is not a general case. The effect of pine bark on pH could however be responsible for the observed spikes in foliar Mn content in spring 2016 which was above the upper sufficiency levels. Manganese availability is easily influenced by pH and, therefore, since pine bark has lower pH than perlite, this could have led to a higher competitive advantage of Mn than the other divalent cations, such as iron in the pine bark substrate. However, these spikes could be potential source of phytotoxicity [16]. This is due to an attempt by the plant to balance its ionic charge concentration especially when iron (Fe^{2+}) is limiting. Foliar B concentration was lower than the lower sufficiency limit in almost all cases except for pine bark in spring 2016. Boron availability is also dependent on pH which must be below 6.0, preferably between 4.5 and 5.5 for maximum availability [17]. In this case, B sufficiency was favored under the low pH condition of pine bark which is supported by the leachate measurement taken in 2019 (Table 2).

Table 3. Effect of substrate and planting density on foliar macronutrient concentration (g 100 g^{-1} dry mass) of 'Socrates' cucumber in two trials in 2016.

	N	P	K	Mg	Ca	S
			Winter–Spring 2016			
Substrate						
Pine bark	5.26	0.86	2.63	0.43	2.07	0.55 a
Perlite	5.16	0.8	2.62	0.42	2.04	0.49 b
p-value	*0.4313*	*0.2284*	*0.9796*	*0.8356*	*0.9153*	*0.0197*
Density [z]						
1×	5.25	0.86	2.70	0.44	2.06	0.53
2×	5.18	0.8	2.55	0.42	2.05	0.51
p-value	*0.6028*	*0.195*	*0.2956*	*0.384*	*0.9636*	*0.2322*
Spring 2016						
Substrate						
Pine bark	4.44	0.61	2.11	0.42	4.06	0.66
Perlite	4.45	0.57	2.14	0.43	4.21	0.6
p-value	*0.9697*	*0.5708*	*0.8397*	*0.8091*	*0.5778*	*0.2014*
Density						
1×	4.43	0.6	1.98	0.44	4.23	0.63
2×	4.47	0.6	2.27	0.41	4.04	0.63
p-value	*0.8694*	*0.9954*	*0.075*	*0.1828*	*0.4921*	*0.9772*
Sufficiency level [y]	4.3	0.3	3.1	0.35	2.4	0.32

[z] 1× = one plant per pot; 2× = two plants per pot; pot = 11-L Dutch bucket. [y] Lower sufficiency level from Mills and Jones Jr [12].

Table 4. Effect of substrate and planting density on foliar micronutrient concentration (mg kg^{-1} dry mass) of 'Socrates' cucumber in two trials in 2016.

	B	Fe	Mn	Cu	Zn
			Winter–Spring 2016		
Substrate					
Pine bark	19.55	69.32	99.37 a	9.75 a	67.3 a
Perlite	22.02	67.13	71.25 b	8.67 b	58 b
p-value	*0.0893*	*0.5552*	*0.0205*	*0.0413*	*0.0246*
Density [z]					
1×	21.43	69.23	88.53	9.23	61.95
2×	20.13	67.22	82.08	9.18	63.35
p-value	*0.327*	*0.5849*	*0.5004*	*0.9088*	*0.6695*
			Spring 2016		
Substrate					
Pine bark	30.66	79.49	215.59	7.68	79.75
Perlite	27.2	74.68	193.25	8.088	80.36
p-value	*0.1214*	*0.6239*	*0.2067*	*0.3942*	*0.9221*
Density					
1×	28.83	79.20 a	213.5	7.73	84.43
2×	29.04	74.96 a	195.34	8.038	75.69
p-value	*0.9187*	*0.6654*	*0.2972*	*0.5149*	*0.1851*
Sufficiency levels [y]	30	50	50	8	25

[z] 1× = one plant per pot; 2× = two plants per pot; pot = 11-L Dutch bucket. [y] lower sufficiency level from Mills and Jones Jr [12].

3.3. Yield and Yield Components of a Cucumber Due to the Substrate and Density Effect

Total yield in 2019 was low due to an early termination of the trial. Maximum yields in 2019 were 3.7 kg m^{-2} and 5.5 kg m^{-2} for one plant per pot and 6.7 kg m^{-2} and 7.1 kg m^{-2} for two plants per pot, respectively, recorded by pine bark and perlite (data not shown). Differences in fruit yield in 2019 was not significantly affected by substrate but plant density (Table 5). The yield advantage of two plants per pot over one plant per pot in 2019 was 63% on a square meter basis. Yield in 2016 were higher with maximum values ranging from 16.5 kg m^{-2} in one plant per pot to 24.3 kg m^{-2} in two plants per pot. Analysis of variance conducted on only 2016 yields showed that season had no significant main effect on cucumber yield ($p > 0.05$). However, there was a significant three-way interaction among season, planting density, and substrate. In the winter–spring season of 2016, plants grown in perlite substrate recorded 2 kg m^{-2} ($\pm$0.917; SE) less yield than those grown in pine bark for one plant per pot, although the effect was not statistically significant ($p = 0.15$). However, in spring 2016, perlite recorded statistically significant (adjusted $p = 0.040$) more yield (2.29 kg m^{-2}) than pine bark for two plants per pot. The average yields across seasons are shown in Figure 1.

Effect of the substrates on yield difference is not direct but due to effect on nutrient availability and uptake because of substrates physical, chemical, and biological properties which affect the root environment. On the other hand, number of plants per pot would influence aboveground parameters which relate to light interception for photosynthesis [18]. The interaction between nutrient and water availability due to the substrate effect and aboveground factors due to effect of number of plants per pot, was anticipated to translate into effect on yield. In terms of productivity of the crop, our results showed that both substrates had similar influence on cucumber yield which was similar to observations made by Shaw et al. [5]. In our case, pine bark only showed superior yield performance over perlite in one plant per pot. This means that the increased above and below ground mass due to the additional plant number did not offer benefit for pine bark substrate in the inherently low nutrient AE. Pine bark is known to be high in potassium [19], which is an essential nutrient for fruit development. In cucumber, potassium is especially required

in increased concentrations at the heavy fruiting stage. Therefore, the high potassium contained in pine bark, coupled with its higher than perlite cation exchange capacity of 10 cmol L^{-1} [20] was expected to confer superior yield performance in both plant densities. It is not known why there was a reduction in fruit yield for pine bark in two plants per pot. It is most likely that high bulk density, which is characteristic of pine bark, had a restricted growth effect on two plants per pot. Few studies examine the performance of cucumbers in different substrates fertigated with AE, making it difficult to examine the performance of the two substrates in respect to other studies. However, Ayipio et al. [10] showed that substrate-based systems resulted in poor yield comparison between aquaponics and conventional hydroponics crop yield; very few studies used substrates, indicating that more research on substrate use with AE is required. For cucumber fertigated with hydroponic nutrient solution, performance in different substrates is affected by the substrate's ability to retain water and was demonstrated by improvement in marketable yield by wood bark when combined with peat [9].

Table 5. Interaction effect of planting density and substrate with season on cucumber fruit yield.

	Yield (kg m^{-2})		
	Winter–Spring 2016	**Spring 2016**	**Spring–Summer 2019**
Substrate			
Pine bark	13.26Aa [z]	11.17Aa	3.37Ba
Perlite	12.52Aa	12.03Aa	3.90Ba
Density [y]			
1×	10.39Ab	9.73Ab	2.91Ba
2×	15.38Aa	13.46Aa	4.37Ba

[z] Means in the same column followed by the same lower-case letter are not statistically different ($p \geq 0.05$); means in the same row followed by the same upper-case letter are not statistically different. Means under 'Substrate' are not compared with means under 'Density'. [y] 1× = one plant per pot; 2× = two plants per pot; pot = 11-L Dutch bucket.

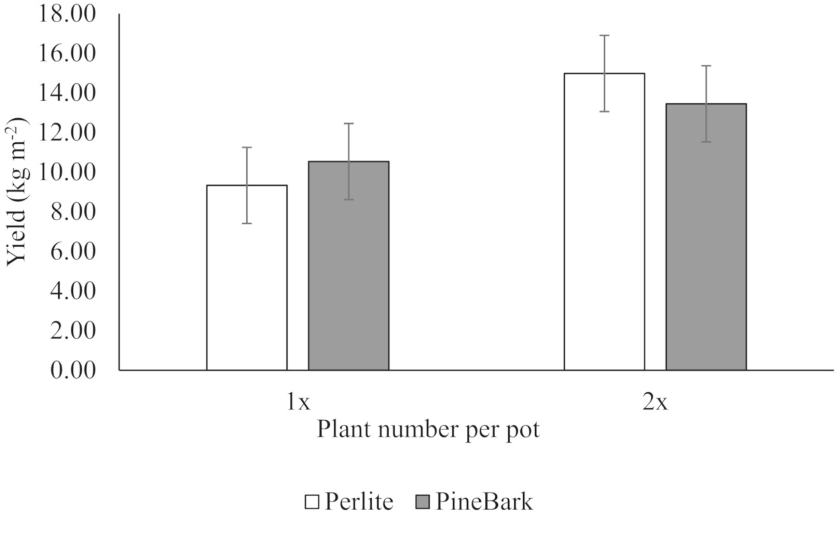

Figure 1. Yield per square meters of cucumber in 2016 trial across seasons of spring and winter–spring. Error bars are ± standard error. 1× = one plant per pot; 2× = two plants per pot.

Although our data show that two plants have, overall, greater yield per square meter than one plant per pot, these data are not sufficient to make conclusions on the economic

productivity of two plants per pot when fertigated with AE. Other economic factors, such as added labor and seed cost, must be considered. We realized that, on a per plant basis, there was no significant effect of number of plants per pot on yield, indicating a lack of mutual benefit of the added leaf foliage to improve yield. Yields obtained in 2019 were generally low for cucumbers grown for 35 days from transplanting due to an early termination of the experiment resulting from observed foliar damage from disease spores. Even the low yield results, obtained in 2019, compare well with an earlier study in the same system [11] where cucumber plants were grown for 44 days from transplanting.

3.4. Morphological Measurements

In the 2019 trial, SPAD value was used as a proxy assessment of the overall health of the plants since there was no foliar nutrient content analysis. Mean SPAD values were 23.27 and 25.77 in one plant per pot, whereas for two plants per pot, SPAD values were 24.73 and 25.98 for perlite and pine bark, respectively. Generally, plants grown in pine bark had significantly higher SPAD values than those in perlite by about 1 SPAD unit which is considered low in terms of horticultural importance. A value of 45.2 SPAD units is considered sufficient to predict yields for cucumber [21]. Therefore, the low SPAD values recorded in 2019 could also explain the low yields recorded in that year. Leaf area and dry weights were used to estimate specific leaf area (SLA) which is usually an essential input for leaf area index conversion when modeling light interception. The SLA of cucumber plants grown in the system ranged from 249.69 cm^2 g^{-1} to 430.35 cm^2 g^{-1} which was similar to that found in fruiting cumber plants for restricted and non-restricted roots at 60 days after sowing [22]. Low SLA values are an indication of high leaf dry matter content as a result high light level. It was expected that SLA be high in two plants per pot due to competition for light. However, our results showed no significant effect of number of plants per pot on SLA indicating similarity in light environment for both configurations. Mean Stomata conductance values were 712.4 and 696.1 mmol [(H_2O)] m^{-2} s^{-1} in perlite but were 674.0 and 729.22 mmol [(H_2O)] m^{-2} s^{-1} in pine bark for one plant and two plants per pot, respectively. However, there was no significant interaction between substrate and number of plants per pot on stomata conductance. The values obtained for stomata conductance are similar to values obtained for cucumber infested with powdery mildew even with full strength nutrient supply [23]. This stomata response was because of the greenhouse growing condition of high humidity and temperature but not due to treatment effects. However, it was evident that in pine bark substrate, growing two plants per pot exacerbated the situation as seen in the reduction in stomata conductance. The low stomata conductance is an additional explanation for the low yield observed in 2019, because stomata opening is necessary for both transpiration and leaf photosynthesis. Leaf area index (LAI) values were also low, with the highest LAI being 3.0 m^2 m^{-2} and the lowest being 1.07 m^2 m^{-2} at 35 days after transplanting with more than 16 leaves. For optimal cucumber productivity, a LAI of greater than 3.5 m^2 m^{-2} is estimated for more than 16 leaves per plant [18]. This means the current LAI estimated from our study is not optimal for cucumber productivity. However, Nikolaou et al. [24] obtained maximum LAI value of 1.84 m^2 m^{-2} at 43 days after transplanting in greenhouse soilless cucumber grown with cooling indicating our results are not an isolated case.

4. Conclusions

We can conclude that generally, although the biofloc AE was low in dissolved ions, it was successful for growing the Beit Alpha cucumbers and had comparable yields between the two substrates assessed. Foliar nutrient concentrations were generally within sufficiency ranges, except foliar B which was lower. Pine bark showed effect on reducing leachate pH and could be used as a pH downward regulator in AE for downstream. Effect of the substrates on yield was dependent on season and number of plants per pot. Use of pine bark as a substitute substrate for perlite is only justified in one plant per pot, when density is increased to two plants per pot perlite is more preferrable.

Author Contributions: Conceptualization, and design; D.E.W., data curation; E.A., M.S., C.B.; data analysis, initial draft; E.A.; Revision; D.E.W., E.A., M.S., C.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the United States Department of Agriculture National Institute of Food and Agriculture (USDA-NIFA), grant number 2016-70007-25758.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is available upon request to the corresponding author.

Acknowledgments: The authors are also grateful to the Alabama Agricultural Extension Service (AAES) for their support and to all the graduate student workers for help in the greenhouse.

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

References

1. Love, D.C.; Fry, J.P.; Genello, L.; Hill, E.S.; Frederick, J.A.; Li, X.; Semmens, K. An international survey of aquaponics practitioners. *PLoS ONE* **2014**, *9*, e102662. [CrossRef]
2. Palm, H.W.; Knaus, U.; Appelbaum, S.; Goddek, S.; Strauch, S.M.; Vermeulen, T.; Haïssam Jijakli, M.; Kotzen, B. Towards commercial aquaponics: A review of systems, designs, scales and nomenclature. *Aquac. Int.* **2018**, *26*, 813–842. [CrossRef]
3. Pinho, S.M.; Molinari, D.; de Mello, G.L.; Fitzsimmons, K.M.; Coelho Emerenciano, M.G. Effluent from a biofloc technology (BFT) tilapia culture on the aquaponics production of different lettuce varieties. *Ecol. Eng.* **2017**, *103*, 146–153. [CrossRef]
4. Papadopoulos, A.P.; Asher, B.-T.; Silber, A.; Uttam, K.S.; Michael, R. Inorganic and Synthetic Organic Components of soilless culture and potting mixes. In *Soilless Culture:Theory and Practice*; Raviv, M., Lieth, H.J., Eds.; Elsevier B.V.: London, UK, 2008; pp. 505–537.
5. Shaw, N.L.; Cantliffe, D.J.; Funes, J.; Shine, C.I.I.I. Successful Beit Alpha cucumber production in the greenhouse using pine bark as an alternative soilless media. *HortTechnology* **2004**, *14*, 289–294. [CrossRef]
6. Jahromi, N.B.; Fulcher, A.; Walker, F.; Altland, J. Optimizing substrate available water and coir amendment rate in pine bark substrates. *Water* **2020**, *12*, 362. [CrossRef]
7. Niemiera, A.X.; Bilderback, T.E.; Leda, C.E. Pine bark physical characteristics influence pour-through nitrogen concentrations. *HortScience* **1994**, *29*, 789–791. [CrossRef]
8. Grillas, S.; Lucas, M.; Bardopoulou, E.; Sarafopoulos, S.; Voulgari, M. Perlite based soilless culture systems: Current commercial application and prospects. *Acta Hortic.* **2001**, *548*, 105–113. [CrossRef]
9. Peyvast, G.; Olfati, J.A.; Roudsari, O.N.; Kharazi, P.R. Effect of substrate on greenhouse cucumber production in soilless culture. *Acta Hortic.* **2010**, *871*, 429–436. [CrossRef]
10. Ayipio, E.; Wells, D.E.; McQuilling, A.; Wilson, A.E. Comparisons between Aquaponic and Conventional Hydroponic Crop Yields: A Meta-Analysis. *Sustainability* **2019**, *11*, 6511. [CrossRef]
11. Pickens, J.M. Integrating Effluent from Recirculating Aquaculture Systems with Greenhouse Cucumber and Tomato Production. Ph.D. Thesis, Auburn University, Auburn, AL, USA, 2015.
12. Mills, H.A.; Jones, J.B., Jr. *Plant Analysis Handbook II: A Practical Sampling, Preparation, Analysis, and Interpretation Guide*; Micro-Macro Publishing, Inc.: Athens, GA, USA, 1996; ISBN 1878148052.
13. Bittsánszky, A.; Uzinger, N.; Gyulai, G.; Mathis, A.; Junge, R.; Villarroel, M.; Kotzen, B.; Kőmíves, T. Nutrient supply of plants in aquaponic systems. *Ecocycles* **2016**, *2*, 17–20. [CrossRef]
14. Blanchard, C.; Wells, D.E.; Pickens, J.M.; Blersch, D.M. Effect of pH on cucumber growth and nutrient availability in a decoupled aquaponic system with minimal solids removal. *Horticulturae* **2020**, *6*, 10. [CrossRef]
15. Pinho, S.M.; de Lima, J.P.; David, L.H.; Oliveira, M.S.; Goddek, S.; Carneiro, D.J.; Keesman, K.J.; Portella, M.C. Decoupled FLOCponics systems as an alternative approach to reduce the protein level of tilapia juveniles' diet in integrated agri-aquaculture production. *Aquaculture* **2021**, *543*, 736932. [CrossRef]
16. Maher, M.J.; Thomson, D. Growth and manganese content of tomato (Lycopersicon esculentum) seedlings grown in Sitka spruce (Picea sitchensis (Bong.) Carr.) bark substrate. *Sci. Hortic.* **1991**, *48*, 223–231. [CrossRef]
17. Maucieri, C.; Nicoletto, C.; van Erik, O.; Anseeuw, D.; Robin, V.H.; Junge, R. Hydroponic Technologies. In *Aquaponics Food Production Systems: Combined Aquaculture and Hydroponic Production Technologies for the Future*; Goddek, S., Joyce, A., Kotzen, B., Burnell, G.M., Eds.; SpringerOpen: Chan, Switzerland, 2019; pp. 76–110. ISBN 978-3-030-15942-9.
18. Xiaolei, S.; Zhifeng, W. The optimal leaf area index for cucumber photosynthesis and production in plastic greenhouse. *Acta Hortic.* **2004**, *633*, 161–165. [CrossRef]
19. Maher, M.; Prasad, M.; Raviv, M. Organic soilless media components. In *Soilless Culture Theory and Practice*; Raviv, M., Lieth, J.H., Eds.; Elsevier: Amsterdam, The Netherlands, 2008; pp. 459–504.
20. Silber, A. Chemical Characteristics of Soilless Media. In *Soilless Culture Theory and Practice*; Raviv, M., Lieth, H.J., Eds.; Elsevier: Amsterdam, The Netherlands, 2008; pp. 210–239.

21. Padilla, F.M.; Peña-Fleitas, M.T.; Gallardo, M.; Giménez, C.; Thompson, R.B. Derivation of sufficiency values of a chlorophyll meter to estimate cucumber nitrogen status and yield. *Comput. Electron. Agric.* **2017**, *141*, 54–64. [CrossRef]
22. Kharkina, T.G.; Ottosen, C.O.; Rosenqvist, E. Effects of root restriction on the growth and physiology of cucumber plants. *Physiol. Plant.* **1999**, *105*, 434–441. [CrossRef]
23. Wang, Y.; Ma, G.; Du, X.; Liu, Y.; Wang, B.; Xu, G.; Mao, H. Effects of Nutrient Solution Irrigation Quantity and Downy Mildew Infection on Growth and Physiological Traits of Greenhouse Cucumber. *Agronomy* **2020**, *10*, 1921. [CrossRef]
24. Nikolaou, G.; Neocleous, D.; Katsoulas, N.; Kittas, C. Modelling transpiration of soilless greenhouse cucumber and its relationship with leaf temperature in a mediterranean climate. *Emir. J. Food Agric.* **2017**, *29*, 911–920. [CrossRef]

horticulturae

MDPI

Article

Soilless Tomato Production: Effects of Hemp Fiber and Rock Wool Growing Media on Yield, Secondary Metabolites, Substrate Characteristics and Greenhouse Gas Emissions

Annika Nerlich [1], Stefan Karlowsky [2], Dietmar Schwarz [2], Nadja Förster [1] and Dennis Dannehl [1,*]

[1] Faculty of Life Sciences, Albrecht Daniel Thaer-Institute of Agricultural and Horticultural Sciences, Division Biosystems Engineering and Division Urban Plan Ecophysiology, Humboldt-Universität zu Berlin, Albrecht-Thaer-Weg 3, 14195 Berlin, Germany; annika.nerlich@hu-berlin.de (A.N.); nadja.foerster@hu-berlin.de (N.F.)

[2] Leibniz Institute of Vegetable and Ornamental Crops (IGZ) e.V., Theodor-Echtermeyer-Weg 1, 14979 Großbeeren, Germany; karlowsky@igzev.de (S.K.); schwarz@igzev.de (D.S.)

* Correspondence: dennis.dannehl@hu-berlin.de

Abstract: Replacement of rock wool by organic substrates is considered to reduce the environmental impact, e.g., through energy savings during production and waste prevention, caused by hydroponically produced crops. A suitable substrate for plant production is characterized by an optimal composition of air- and water-filled pores. In our study, we used hemp fibers as an organic alternative to rock wool in order to cultivate tomato plants in hydroponics for 36 weeks. The leaf area, plant length, and yields, as well as the quality of fruits including soluble solid contents, dry weight content, mineral composition, and contents of phenolic compounds caused by both substrates, were similar. Carotenoids were significantly increased in fruits from plants grown in hemp at some measuring dates. Nevertheless, higher emission rates of greenhouse gases such as N_2O, CO_2, and CH_4 caused by hemp fiber compared to those emitted by rock wool during use are rather disadvantageous for the environment. While hemp proved to be a suitable substrate in terms of some physical properties (total pore volume, bulk density), a lower volume of air and easily available water as well as very rapid microbial decomposition and the associated high nitrogen immobilization must be considered as disadvantages.

Keywords: greenhouse gases; greenhouse; organic substrates; carotenoids; phenolic compounds; carbon dioxide; nitrous oxide; methane; N_2O; CH_4

Citation: Nerlich, A.; Karlowsky, S.; Schwarz, D.; Förster, N.; Dannehl, D. Soilless Tomato Production: Effects of Hemp Fiber and Rock Wool Growing Media on Yield, Secondary Metabolites, Substrate Characteristics and Greenhouse Gas Emissions. *Horticulturae* **2022**, *8*, 272. https://doi.org/10.3390/horticulturae8030272

Academic Editors: Nazim Gruda and Juan A. Fernández

Received: 2 February 2022
Accepted: 14 March 2022
Published: 21 March 2022

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

Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

The world population will grow from 7.3 billion to 9.6 billion people by 2050 [1], which will reduce the amount of land available for growing food on a global scale. This will be accompanied by increasing urbanisation (66% of people will live in large cities [2]). Generally, population growth also increases total anthropogenic greenhouse gas (GHG) emissions, of which 23% currently derive from the agricultural sector [3]. In the future, however, the share of GHG emissions in the agricultural sector will continue to shift towards protected greenhouse production. Precise climate control can reduce plant diseases and improve the quality of products, which has led to an increase in greenhouse area, where greenhouses are mostly defined as permanent structures, excluding tunnels, row covers, etc. These greenhouse structures are common in industrialized countries and cover an area of 497,815 ha to produce vegetables worldwide [4]. Unfortunately, it is not possible to estimate the greenhouse area used in China due to the definition of greenhouses. It was reported that there the total greenhouse area covered with plastic films, including covered open vegetation fields, was 2.5 million hectares in 2004 [5].

From an energy point of view, greenhouse production caused very high CO_2 emissions caused by the heating energy required for plants in many places [6–8]. It is estimated that

a temperature increase by heating of only one degree Celsius raises CO_2 emissions by 100 t ha^{-1} [9]. In addition, there are non-negligible CO_2 emissions caused by energy input during the production of growing media [7]. In intensive hydroponic vegetable production, huge amounts of substrate residue are produced at the end of a production year. Rock wool, for instance, is the preferred growing medium of many horticultural producers [10–13]. Up to 150 m^3 of substrate residue is produced per hectare of tomato production per year [14]. This would amount to 113,700 m^3 of substrate residue from rock wool that must be disposed of in landfills every year if rock wool were to be considered on the entire acreage of tomatoes, cucumbers, and peppers (758 ha) [15] grown under glass or other walkable protective covers in Germany in 2021. In addition, the production of one cubic metre of rock wool requires an average primary energy demand of 275 kWh, with 167 kg of CO_2 being released into the environment [16].

Although rock wool is almost a perfect growing medium for hydroponic systems, alternative environmentally friendly organic substrates must be found to close the circular economy and reduce environmental impact. In this context, organic substrates used for hydroponic systems should have optimal physical properties in terms of total pore space (>85 vol%), air volume (10 to 30 vol%), bulk density (<0.4 g cm^{-3}), easily available water (20 to 30 vol%), and water buffer capacity (4 to 10 vol%) as described by Abad and Noguera [17], Abad, et al. [18], De Boodt and Verdonck [19], Boertje [20], and Jenkins and Jarrell [21]. Maintaining these optimal physical properties when using organic substrates over a very long period up to 330 days, especially for intensive tomato cultivation in hydroponics, is challenging because organic substrates can degrade due to high microbial activity [22]. On one hand, this means that the additional carbon input, in combination with abundant mineral nitrogen applications through the nutrient solution, can promote the growth of denitrifying bacteria [23]. This, in turn, means that high nitrous oxide (N_2O) emissions from denitrification are generated, e.g., when using organic growing bags, because nitrogen is typically supplied in the form of nitrate in hydroponic systems [24]. This effect can vary between different organic substrates because denitrification processes strongly depend on the quality of the C source [25] and can be additionally fueled by reduced oxygen (O_2) concentrations [26]. Under strictly anaerobic conditions, methane (CH_4) emissions from organic substrates are also possible [27], but are typically not relevant in hydroponics [24]. On the other hand, high decomposition processes can also negatively affect water and nutrient supply, as well as plant stability in the root zone, which in turn can have a negative impact on yields, dry matter, and fruit quality characteristics, e.g., soluble solid contents (SSC) [28]. Based on these facts, many organic substrates are tested for their suitability as growing media for hydroponic vegetable production. The use of coconut fiber, bark, or rice husk, for example, did not induce changes in growth, yield and SSC in tomato plants compared to those grown in rock wool [13]. In contrast, SSC in tomatoes could be increased by using almond shells as growing media, while the tomato yields did not differ when this substrate was compared to rock wool [29]. Other research groups combined organic with inorganic or different organic substrates and obtained equal tomato yields when compared to tomato plants exposed to rock wool. Among others, the following mixtures were used: peat and composted bark (66.6%:33.4%, *v*/*v*), sepiolite and leonardite (97%:3%, *v*/*v*), sieved pumice and peat-lite (85%:15%, v/v), sepiolite and perlite (80%:20%, *v*/*v*), as well as perlite and peat (85%:15%, *v*/*v*)] [22,30,31]. Some wood-based substrates, although not all, also seem to be promising alternatives to rockwool. White spruce and fir bark (40%:60%, *v*/*v*) showed high potential for greenhouse tomato production, whereas tomato yields produced with the aid of substrates consisting of fresh white spruce and fir sawdust (40%:60%, *v*/*v*) or white spruce and fir shavings (40%:60%, *v*/*v*) were lower than those achieved with rock wool [22]. This might be caused by phenols located in bark, which can have a phytotoxic effect [32].

Based on this brief overview of possible advantages and disadvantages of using organic substrates in hydroponic systems, it becomes clear that not all organic substrates can be used for intensive vegetable production and therefore further alternatives to rock

wool must be sought. Studies on the effects of different growth media on greenhouse gas emissions hardly exist. Furthermore, there is a deficit in studies on the synthesis of secondary metabolites depending on different substrates used as growth media in hydroponic systems. Therefore, the present study is focused on the evaluation of hemp fiber bags to be used as a substitute substrate for rock wool in intensive tomato production. The main objectives of this research were to analyze the physical properties of the used renewable hemp fiber bags compared to rock wool to discern the differences in water-retention curves and possible disadvantages of using hemp fibers. Since the mineralization of hemp fibers can lead to fixation of nitrogen in microbial biomass that may not be available to the plants, nitrogen immobilization was investigated. Due to our hypothesis that organic substrates produce greenhouse gas emissions during vegetable production through their decomposition, we assessed how the degradation of hemp fibers by bacteria in hydroponic tomato cultivation affects the direct N_2O-, CO_2-, and CH_4-emissions using gas flux measurements. In addition to these study parameters, leaf area and yield development as well as mineral composition in leaves and fruits were investigated. The latter characteristics should provide information on whether nitrogen immobilization in organic substrates leads to nutrient supply bottlenecks in the plants. Based on the knowledge gap mentioned above, SSC, dry matter, carotenoids, flavonoids, and phenolic acids in tomato fruits were analyzed under consideration of the growing media used. We hypothesize that greenhouse gas emissions could cause a change in secondary metabolites in tomatoes.

2. Materials and Methods

2.1. Cultivation of Tomato Plants and Assessment of Crop Growth and Yield

Experiments were conducted in a Venlo-type greenhouse at Humboldt-Universität zu Berlin, Germany (Latitude 52° 46′ 74″, Longitude 13° 31′ 16″) from calendar week (CW) 21–47 in 2020. Hemp fiber was tested for its suitability as growing medium in bags, which were provided by Klasmann-Deilmann GmbH, (Geeste, Germany). Rock wool bags (Cutilene®; Tilburg, The Netherlands) were used as a control since rock wool is an established substrate. Tomato seedlings (*Solanum lycopersicum* L. cv. Avalantino F1) with two shoots were grown in small rock wool cubes (100 mm × 100 mm × 65 mm) and supplied by Jungpflanzen Gernert GbR (Albertshofen, Germany). Tomato transplants in small rock wool cubes were transferred to the growing bags on 13th March 2020, when four leaves were formed. Tomato plants were cultivated on high gullies, each gully equipped with 20 growing bags, and each bag planted with two plants, resulting in a distance between plants of 0.5 m. The plant experiment was conducted with three replicates, randomly selecting three gullies with rock wool bags and three gullies with hemp fiber bags. Two additional outer gutters were planted with tomatoes to ensure equal light conditions of the substrate variants.

A hydroponic system with a recirculating nutrient solution was used for a local drip irrigation that delivered a nutrient solution for 150 s, which started mainly after a light summation of 560 W m^{-2}. To obtain a water overflow of 20% after each irrigation cycle, the light summation for controlling the irrigation was regularly adjusted. Stock solution according to the recipe of Göhler and Molitor [33] was mixed with fresh water up to desired EC values and adjusted to pH 6 using phosphoric acid to obtain the nutrient solution for irrigation. The nutrient solution tank in the closed irrigation system was automatically refilled with the desired nutrient solution several times per day. Energy screens were closed at a global radiation of less than 3 W m^{-2}, in order to save energy. The floor level heating was set at 17 °C for day and night and the ventilation was opened above 23 °C to reduce the temperature inside the greenhouse. These processes were controlled using the application of proportional integral differences. CO_2 enrichment was applied and kept at a level of 800 ppm during daylight hours. When the ventilation opening of the greenhouse exceeded 10%, the CO_2 supply stopped to avoid too much loss of CO_2 into the atmosphere. Set points for cooling, heating, ventilation, and the CO_2 enrichment mentioned before were controlled

by data obtained from different sensors evenly distributed in the canopy. Measurements were forwarded to a central computer and recorded every 30 s.

Leaf number and the leaf area (LA) per plant was documented during the first six weeks after planting from three randomly selected plants per substrate. The number of leaves was noted and the leaf length (L) and width (W) of each leaf was measured with a folding ruler. The measurements were inserted in the commonly used equation A = a + b × (L × W) with a = −61.70 and b = 0.35 to estimate the LA of each individual leaf non-destructively [34]. The calculated values were added up to obtain the LA per plant, which was expressed as m^2 $plant^{-1}$.

Yields were determined by weekly harvesting ripe tomatoes corresponding to ripening stage 10 (according to the Organisation for Economic Co-operation and Development, OECD colour gauge). Yields from every week were summed to calculate the total yield per plant in kg $plant^{-1}$.

At the end of the experiments on 19 November 2020, the plant height of five plants per growing medium was measured after the stem of the plant was cut directly above the small rock wool cubes.

2.2. Analysis of Substrate Characteristics Using Water Retention Curves (pF-Curves)

Different physical parameters such as total pore space (TPS), air volume (AV), bulk density (BD), and easily available water (EAW) were examined for hemp fiber and rock wool. The respective substrate was filled into metal rings with a volume of 100 cm^3 and completely saturated with water. These prepared cylinders were placed on a ceramic pressure plate connected to a manometer. By increasing negative pressure values (pF values), different pore sizes of the previously water-saturated soil sample (pF 0) were drained. Released water volumes extracted at each pressure level (in our case pF 1.0 and pF 1.8) correspond to the pore water volume of a given pore size range. In this way, the water volume fractions (volumetric water content; θ_V (Equation (1))) of different substrate pore sizes, and thus their percentages in the soil could be determined. The density of the water was assumed to be 1 mg cm^{-3}.

$$\theta_V\ [\text{vol\%}] = \theta_g\left[\text{g g}^{-1}\right] \times \text{BD}\left[\text{g cm}^{-3}\right] \times 100 \quad (1)$$

The gravimetric water content (θ_g) is given in g g^{-1} and is the amount of water in gram at each suction point per g substrate (Equation (2)).

$$\theta_g\left[\text{g g}^{-1}\right] = \frac{m_{H_2O}\ [\text{g}]}{m_{\text{substrate}}\ [\text{g}]} \quad (2)$$

Bulk density indicates the dry mass of the substrate per 100 cm^3 (Equation (3)).

$$\text{BD}\left[\text{g cm}^{-3}\right] = \frac{m_{\text{substrate}}\ [\text{g}]}{100\ \text{cm}^{-3}} \quad (3)$$

According to De Boodt and Verdonck [19] moisture content at zero suction (pf 0) is defined as TPS stated in vol% and is the product of gravimetric water content (θ_g) and the BD (Equation (4)).

$$\text{TPS}\ [\text{vol\%}] = \theta_{g\ (\text{pf0})}\left[\text{g g}^{-1}\right] \times \text{BD}\left[\text{g cm}^{-3}\right] \times 100 \quad (4)$$

The air volume is the difference of the gravimetric water content at pF 0 and pF 1 (Equation (5)). The easily available water is the difference of the gravimetric water content at pF 1 and pF 1.8 (Equation (6)).

$$\text{AV}\ [\text{vol\%}] = \theta_{g(\text{pf0})}\left[\text{g g}^{-1}\right] - \theta_{g(\text{pf1})}\left[\text{g g}^{-1}\right] \quad (5)$$

$$\text{EAW } [\text{vol\%}] = \theta_{g(pf1)} \left[\text{g g}^{-1}\right] - \theta_{g(pf1.8)} \left[\text{g g}^{-1}\right] \quad (6)$$

All physical parameters were determined in five replicates per substrate, one time before usage in hydroponic cultivation of tomatoes and one time after the cultivation period (CW 21–47 2020, 26 weeks).

2.3. Determination of Substrate Decomposition during Cultivation Period

Decomposition of the organic hemp material could result in substantial mass loss in the growing bags, thus decreasing stand stability for the plants cultivated. To determine the amount of decomposed material, growing bags were weighed in their unused condition and after usage for 16 weeks in tomato cultivation, including roots grown into the materials. The used growing bags were weighed after drying in a ventilated oven for 10 days. Differences in the weights of used and unused growing bags correspond to the decomposed amount of hemp including root biomass, or in the case of rock wool, to the root biomass grown in the rock wool.

2.4. N-Immobilization

Since it was expected that organic substrates would be mineralized during cultivation and that this could lead to the fixation of nutrients in microbial biomass, nitrogen immobilization was determined. This was necessary so that the nutrient application in the hydroponic system can be adapted to the plants' needs. The determination of the nitrogen immobilization of the substrates was carried out according to VDLUFA [35]. Sample material was mixed with a defined amount of ammonium nitrate and incubated over a period of 20 days at constant temperature and humidity. At the end of the incubation period, the contents of ammonium and nitrate nitrogen were determined separately, thus establishing the quantities in which these N compounds are released or fixed. The results are expressed as mg dm^{-3}.

2.5. Analysis of Greenhouse Gases Released by Growing Media

To evaluate the potential for substrate-related GHG emissions, three growing bags each of rock wool and hemp fiber were incubated on a greenhouse gutter with nutrient solution supplied via drippers starting in September 2020. After six weeks, the first gas flux measurement took place on 15 October 2020 and was followed by two more measurements on 9 November and 1 December 2020. For measuring the gas fluxes, the closed chamber method as described by Karlowsky, et al. [24] was used and modified to determine GHG emissions from unplanted growing bags. Briefly, acrylic glass chambers were fitted around the substrate bags and sealed with foam rubber to obtain a closed headspace on top of the growing bags with a volume of approximately 16 L. Over a period of one hour after closing, four gas samples were taken in 20 min intervals with a syringe through a sampling port on top of the chamber. The gas samples were analyzed on the same day by a gas chromatograph (GC 2010 Plus, Shimadzu Corporation, Kyoto, Japan) equipped with an electron capture detector (ECD) for N_2O, a thermal conductivity detector (TCD) for CO_2, and a flame ionization detector (FID) for CH_4.

2.6. Sample Preparation for Chemical Analyses and Determination of Dry Matter and Soluble Solid Content

Over a period of 24 weeks (11 June to 25 November 2020), fruits from 15 different plants per growing medium were harvested at intervals of three weeks and divided into three pooled samples of five tomatoes each. Only the top two ripe fruits of a panicle and panicles of the same age were considered. Each tomato was quartered. One quarter of five fruits were combined into one sample and immediately frozen in liquid nitrogen and then freeze-dried (Christ Alpha 1–4, Christ; Osterode, Germany) for seven days for analysis of secondary metabolites.

The second quarters of the same five fruits were used to determine dry mass of tomato fruits using a ventilated oven (Heraeus, Hanau, Germany) at 60 °C for seven days. The

fruit's dry matter content was calculated by the ratio of the dry mass to the fresh mass and is expressed as a percentage.

The two remaining quarters per fruit were used fresh to determine the soluble solid content (SSC). Firstly, the quarters of fresh tomatoes were mixed (KenwoodHB856, De'Longhi Deutschland GmbH; Neu-Isenburg, Germany) to obtain a homogenous starting material. Aliquots of the resulting liquid were transferred into centrifuge tubes and centrifuged for five minutes at 5000 rpm to remove coarse components and receive a clear solution for analysis. SSC was analyzed using a digital refractometer (PR101, ATAGO; Karlsruhe, Germany) according to manufacturer's protocol, which detects reducing sugars and other soluble solids. The results obtained for SSC are expressed as grams SSC per 100 g FW.

2.7. Analysis of Phenolic Compounds

To analyze phenolic acids and flavonoids, freeze-dried tomato fruits were ground to a fine powder (MM 30, Retsch GmbH, Haan, Germany) and stored at −80 °C until analysis. Extraction and determination of phenolic acids and flavonoids was performed as described by Förster, et al. [36].For analysis, an HPLC (Ultimate 3000, Thermo Scientific, Dionex Softtron GmbH, Germering, Germany) equipped with a 150 × 2.1 mm C16 column (AcclaimPA, 3 µm, Thermo Scientific, Dionex Softtron GmbH, Germering, Germany) was used. Commercially available standards from Sigma-Aldrich of single compounds were utilized as references.

Peak areas of detected phenolic acids and flavonoids were used for calculating contents of each phenolic acid/flavonoid and further summed to total phenolic acid/flavonoid content in tomato fruits in mg g^{-1} DM.

2.8. Analysis of Carotenoids

Extraction of carotenoids was performed as described by Mageney, et al. [37] with slight adjustments. 10 mg of freeze-dried powdered plant material was weighed and shaken with 500 µL of MeOH-THF solution (1:1, *v*/*v*; extraction solution) for 5 min at 24 °C and 500 rpm. After centrifugation at 20 °C and 4500 rpm for five min, the supernatant was transferred to a glass vial and the pellet was re-extracted two more times with 500 µL of extraction solution. The collected extracts were evaporated under nitrogen flow. The obtained pellet was dissolved in 100 µL dichloromethane and 300 µL isopropyl alcohol and filtered through Corning® Costar® Spin-X® centrifuge tube filters (Merck KGaA, Darmstadt, Germany) by centrifugation at 3000 rpm for five min. At the end, the filtered extracts were transferred into dark HPLC vials with inlay. For analysis, 10 µL were injected and separated at a flow rate of 0.2 mL min^{-1} using an Ultimate 3000 HPLC system (Thermo Scientific, Waltham, MA, USA) equipped with a carotenoid column (YMC-Carotinoid column). Detection was performed at 456 nm. The oven temperature was set to 25 °C. The eluents consisted of a mixture of methanol, methyl tert-butyl ether, and Milli-Q (eluent A: 81/15/4, eluent B: 6/90/4). Separation was performed by the following gradient: 0–10 min: 0% B; 10–40 min: 0–100% B; 40–42 min: 100% B; 42–45 min: 100–0% B; 45–55 min: 0% B.

Commercially available standards from Sigma-Aldrich of single compounds were utilized as references. For each run, 5 µL of lycopene standard solution (1 nmol μL^{-1}) was injected separately, corresponding to 5 nmol. Peak areas of this lycopene standard with known concentration and determined response factors (RF) for β-carotene (RF = 0.65) and Lutein (RF = 0.79) in relation to lycopene were used to calculate the contents of each detected carotenoid. All carotenoids were summed to receive total carotenoid content in tomato fruit in µg g^{-1} DM.

2.9. Analysis of the Mineral Composition of Tomato Fruits and Leaves

Oven dried samples of tomato fruits were ground (MM 30, Retsch GmbH; Haan, Germany) and used for nutrient analysis. Elemental analysis (K, Ca, Mg, P, S) was done after microwave digestion (microwave manufacturer CEM, MARS Xpress, CEM; North

Carolina, USA) according to LUFA protocol Vol. III, 10.8.1.2. In brief, 0.2 g of dried and ground sample was weighed into deionized containers and digested with 5 mL HNO_3 (65%) and 3 mL H_2O_2 (30%) with the following program: Step 1: 20 min to reach 200 °C; step 2: 5 min at 200 °C; step 3: 1 min to reach 210 °C; step 4: 5 min at 210 °C; step 5: 1 min to reach 220 °C; step 6: 5 min at 220 °C; and step 7: 30 min to cool down. The resulting solution was transferred to 50 mL volumetric flasks using distilled water and finally filtrated into plastic flasks. Thereafter, the analysis of the elements in the digestion solution was conducted via ICP-OES with an ICP Emission Spectrometer (iCAP 6300 Duo MFC, Thermo; Waltham, MA, USA). The operating conditions employed for ICP-OES were 1150 W RF power, 0.55 L min^{-1} nebulizer gas flow with argon employed as plasmogen as well as carrier gas. Analysis was performed with a crossflow nebulizer (MIRA Mist, Thermo Scientific; Cambridge, England). For quantification of each element, a single-element calibration curve was used. The elements were analyzed in duplicate at the following wavelengths: K = 766.5 nm; Ca at 317.9 nm; Mg at 279.0 nm; P = 213.6 nm; S = 182.2 nm. Nitrogen and carbon were determined using an elemental analyzer (Vario MAX, Elementar Analysensysteme GmbH; Hanau, Germany) according to DIN-ISO-13878 (1998). An aliquot of 0.3 g of sample material was weighed into crucibles and catalytically combusted at 900 °C with pure oxygen. The combustion products and helium (as the carrier gas) passed through specific adsorption columns at a temperature of 830 °C to separate nitrogen and carbon with a thermal conductivity detector. All results are expressed as g kg^{-1} dry matter (DM) for macroelements and mg kg^{-1} DM for micronutrients.

2.10. Statistical Analyses

Data were statistically analyzed using agricolae package [38] in RStudio Version 1.2.5033 [39]. The data were first tested for normal distribution and variance homogeneity before comparisons were calculated using *t*-tests for all parameters except for greenhouse gases. Significant differences between both substrates with respect to their physical properties and influences on performances of tomato plants in terms of growth, yield, mineral content, SSC, dry matter, and secondary metabolite concentrations were calculated. Significance of statistical analyses in this research was concluded for $p < 0.05$ for a given test.

For the measured gas concentrations, gas fluxes were calculated using the R package "gasfluxes" [version 0.4–4; [40], including automatic selection of the most suitable regression method (linear, robust linear, or non-linear HMR model). Input variables used were gas concentration (µmol m^{-3}, converted from ppm values according to the ideal gas law assuming SATP conditions), chamber volume (m^3), and time after closing the chamber (h). The area was set to 1 in order to obtain gas fluxes (µmol h^{-1}) for each growing bag. Gas fluxes in g ha^{-1} d^{-1} were calculated based on molar masses and assuming a potential plant density of 2 m^{-2} (substrate slab density of 1 m^{-2}). An initial screening of the gas fluxes indicated strong deviations from normal distribution. Therefore, statistical analyses were done using exact two-sample Fisher–Pitman permutation tests from the R package "coin" [version 1.3–1; [41] with the alternative hypothesis that GHG emissions from hemp fiber growing bags are greater than GHG emissions from rock wool growing bags.

3. Results and Discussion

3.1. Substrate Characteristics

3.1.1. Water Retention Curves

A suitable substrate for the cultivation of plants is characterized by an optimal composition of air-filled and water-filled pores (physical properties). This composition can be analyzed by using water retention curves. If an organic substrate is used instead of inorganic rock wool, the degree of mineralization of the organic material may vary depending on the cultivation period and conditions, and the proportions of water- and air-filled pores may change as a result. Thus, in this study, physical properties of rock wool and hemp substrates used during hydroponic cultivation of tomatoes were studied once before their use and once afterwards (Table 1).

The total pore volume (TPV) was 90 vol% for unused rock wool and was thus above the reference value [19] given in Table 1, while unused hemp substrate was in the reference range at 76 vol%. After use in hydroponic tomato cultivation, the TPV increased for both substrates, hemp (83.1 vol%) and rock wool (95.6 vol%). Furthermore, hemp remained within, rock wool outside the reference range. An increase of the TPV can be explained by plant root growth into the substrate [42], which was observed in both substrates used.

Table 1. Physical properties of substrates before and after their use in hydroponic tomato cultivation.

	Rock Wool	**Hemp**	**Rock Wool**	**Hemp**	**Optimum ***
	unused		used		
TPV [vol%]	90.4 ± 2.6 aB	75.9 ± 2.9 bB	95.6 ± 1.6 aA	83.1 ± 2.8 bA	>85
AV [vol%]	18.9 ± 5.1 aA	13.9 ± 3.3 aA	17.7 ± 6.3 aA	10.0 ± 2.9 aA	20 to 30
EAW [vol%]	70.3 ± 5.2 aA	41.4 ± 2.0 bA	63.2 ± 4.1 aA	12.5 ± 1.6 bB	20 to 30
BD [g cm^{-3}]	0.1 ± 0.01 bB	0.1 ± 0.0 aB	0.1 ± 0.02 bA	0.2 ± 0.03 aA	<0.4

Differences between substrates are indicated by different lower-case letters, and differences between unused and used substrates are indicated by different upper-case letters (*t*-test, $p < 0.05$, n = 5, mean ± standard deviation: TPV: total pore volume; AV: air volume; BD: bulk density; EAW: readily available water. * Reference values for evaluation of our results were taken from the publication by Dannehl, et al. [28] and references therein.

The proportion of pores filled with air (air volume, AV) was in the range of values between 10 vol% and 19 vol% and showed no significant difference between both substrates and stayed similar before and after their use. All AV values obtained were not within the optimal range when compared to the reference values (20–30 vol% [43]). The AV for hemp was 10 vol% at the end of the tests and therefore much lower than rock wool. We suspect that this is related to the particle size of the substrate, which is probably smaller for the hemp substrate. The finer the material, the lower the air volume [43].

The proportion of pores with easily available water (EAW) in rock wool was highest with 70 vol% and did not change significantly during use. EAW in hemp was significantly lower (41 vol%) and during cultivation the proportion of EAW dropped drastically to 13 vol% falling below the recommended values of 20–30 vol% [19] at the end of the tests. In a study of Islam, et al. [44], where rock wool, carbonated rice husks, and coconut coir were investigated before and after usage, the air-filled pore space of rock wool didn't change over time and showed similar values as observed in our experiment. The organic materials in that study showed increased TPV and water-filled pore spaces after utilization as substrate compared to the unused material. In our study, we found an increase in TPV as well. Contrary to Islam, et al. [44] who documented increased water filled pores, the EAW declined in rock wool and hemp in our experiment after use. Since we did not analyze complete water-filled pore space but only the proportion of EAW, there is the possibility that the proportion of ultra-micropores increased and water within these pores is usually unavailable to plants. With this in mind, it might be that more water-filled pore space is present, but not taken into consideration due to the focus on EAW.

The bulk density (BD) in unused hemp substrates was 0.10 g cm^{-3} and twice that of rock wool 0.05 g cm^{-3} (Table 1). However, BD had doubled by the end of culture in both rock wool and hemp. The higher BD from hemp could result from decomposition during the culture period and the associated reduction in pore size due to degradation processes. Nevertheless, the BD of both growing substrates corresponds to values < 0.4 g cm^{-3} as recommended by Abad, et al. [18].

3.1.2. Stability of Hemp towards Decomposition

Generally, organic substrates are subjected to chemical mineralization accomplished mainly by bacteria and fungi [45]. Therefore, hemp can decompose during use as a substrate and thus lead to unfavorable properties with regard to the standing stability of the plants, as well as to the immobilization of nutrients through their fixation in microbial biomass. However, this is not the case with rock wool, which is an inorganic material that is stable with regards to degradation. Thus, rock wool bags can help to estimate the root mass

formed in the substrate. Figure 1 shows how the weight of the growing bags changed as a result of their utilization. Rock wool bags increased in mass by about 8% (from 529 g to 574 g DM), which can be attributed to the root mass. The weight of the hemp fiber bags decreased by 54% (from 1628 g to 747 g including roots, in 16 weeks). It should be noted that the total cultivation period was from week 11 to 47, i.e., 36 weeks, and not just the 16 weeks shown in Figure 1. It was very clear at the end of the trial that there was hardly any substrate left in the hemp fiber bags. This means that less nutrient solution can be stored in hemp fiber, which quickly reduces the water and nutrient supply for the plants in the event of pump failures. Therefore, when assessing growing media for suitability in hydroponic systems, the weight loss of these is at least as important as the volume loss described by Gruda and Schnitzler [46].

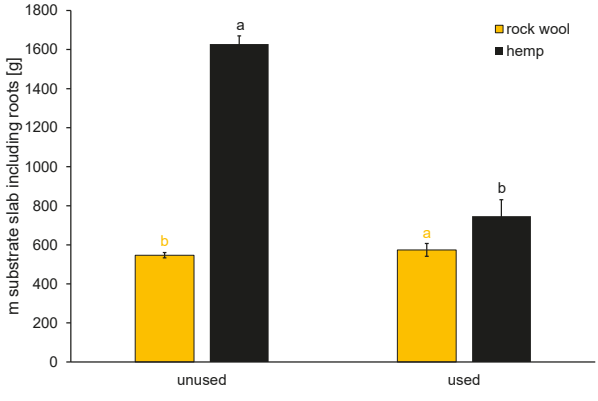

Figure 1. The mass of the substrates used as an indicator of the mineralized content of hemp and the increase in root biomass in rock wool in grams after 16 weeks of cultivation. Differences between unused and used substrates are indicated by different lowercase letters (*t*-test, $p < 0.05$, n = 3 for used substrates, n = 4 for unused hemp, n = 4 for unused rock wool, mean ± standard deviation).

3.1.3. N-Immobilization in Hemp Fiber Bags

The observed rapid degradation of hemp fiber bags (Figure 1) poses the risk that nutrients, especially nitrogen from the nutrient solution, could also be incorporated into the growing microbial biomass involved in the degradation process and thus not be available for the production of plant biomass. Therefore, nitrogen immobilization was determined, which can be triggered by the substrates. This is important in order to adapt the nutrient application in the hydroponic system to the plant. When values of nitrogen immobilization reach 250 mg dm^{-3} and more the material is not recommended to be used as substrate component [35]. It is not stable according to the evaluation of the N-balance (Table 2). According to this investigation, hemp fibers caused a N-immobilization of 601 mg L^{-1} and must therefore be classified as an unsuitable substrate. In comparison, the N-immobilization in wood is only 175 mg L^{-1} and is to be seen as an advantage over hemp fibers [47]. However, hemp fibers are cheaper to procure than wood fibers. In this context, it must be considered that the nitrogen source in hydroponic tomato production is not the substrate itself, but the nutrient solution. A constant supply of necessary nutrients could compensate for the nitrogen immobilization. Therefore, the accumulation of nutrients in the leaves and fruits must also be considered. This will be discussed later.

Table 2. Investigation of nitrogen immobilization of hemp substrates according to the VDLUFA method.

Sample	ΔNO_3-N_{20d}	ΔNH_4-N_{20d}	ΔN_{20d}	Evaluation of N Budget *
		[mg L^{-1}]		
hemp	183	418	601	not stable

* according to VDLUFA [35].

3.1.4. Greenhouse Gas Emissions Released by Hemp and Rock Wool

In general, no traceable N_2O, CO_2, or CH_4 emissions were found from rock wool growing bags incubated with nutrient solution (Table 3). The calculated gas fluxes fluctuated around zero due to background effects from gas chromatographic analyses yielding apparent positive or negative fluxes. The missing GHG emissions from rock wool were probably due to the absence of a C source, which strongly limited microbial activity in the growing bags. In contrast, clear GHG emissions were found from hemp fiber bags incubated with nutrient solution. N_2O emissions were insignificant on 15 October, after 6 weeks of incubation, but increased to a maximum on 11 November before decreasing again on 1 December (Table 3). The highest average value of 4.6 µmol h^{-1} per growing bag on 11 November would correspond to 31 g N_2O-N ha^{-1} d^{-1} (i.e., 49 g N_2O ha^{-1} d^{-1} or 14.7 kg CO_2-eqivalents ha^{-1} d^{-1}) if a plant density of 2 m^{-2} is assumed. This value lies in the upper range of N_2O emission rates reported for rock wool substrates planted with tomato or cucumber [24,48–50], and thus underpins the critical role of organic C sources for N_2O production by denitrifying bacteria. The CO_2 emissions from hemp fiber bags were highest on 15 October and decreased to a similar value at the following two measurements on 11 November and 2 December (Table 3). The average CO_2 emission rate of 3.1 mmol h^{-1} per growing bag on 15 October would correspond to 32 kg CO_2 ha^{-1} d^{-1} if a plant density of 2 m^{-2} is assumed. This value is in the lower range of CO_2 emissions found from tomato and cucumber cultivation on rock wool [24,48,49], possibly due to the missing root respiration and root exudates, which can affect the microbial community through the provision of easily degradable C [51]. In contrast to N_2O, significant CH_4 emissions from hemp fiber bags were found on 15 October (Table 3). Similar emission rates were found on 11 November before they increased to the highest values on 1 December, with on average 6.4 µmol h^{-1} per growing bag. This would correspond to 24.5 g CH_4 ha^{-1} d^{-1} (i.e., 0.7 kg CO_2-eqivalents ha^{-1} d^{-1}) if a plant density of 2 m^{-2} is assumed, which is approximately one tenth of the highest values reported for cucumber cultivation on rock wool by Hashida, et al. [49].

Table 3. Emissions of greenhouse gases from hemp and rock wool.

	Rock Wool	Hemp
	[g-N (N_2O) ha^{-1} d^{-1}]	
15 October 2020	0.17 ± 0.07 a	5.03 ± 4.79 a
9 November 2020	n.d. b	31.02 ± 21.93 a
1 December 2020	0.04 ± 0.05 b	21.97 ± 10.76 a
	[kg-CO_2 ha^{-1} d^{-1}]	
15 October 2020	0.75 ± 0.12 b	32.38 ± 1.36 a
9 November 2020	0.10 ± 0.44 b	16.23 ± 3.85 a
1 December 2020	0.29 ± 0.45 b	17.60 ± 4.01 a
	[g-CH_4 ha^{-1} d^{-1}]	
15 October 2020	n.d. b	8.11 ± 2.91 a
9 November 2020	n.d. b	6.41 ± 4.83 a
1 December 2020	n.d. b	24.49 ± 17.68 a

Measured N_2O, CO_2 and CH_4 emission rates per growing bag (mean ± SEM, n = 3) filled with hemp fiber or rock wool substrates and incubated with tomato nutrient solution. Different small letters indicate significant differences (p = 0.05) for individual measurement days (note that lower p-values are not possible in the used permutation test due to the low number of replicates). N.d.: below detection limit.

On one hand, replacing inert substrates such as rock wool with organic substrates such as hemp fiber offers a compelling opportunity to reduce the climate impact of hydroponic cultivation by lowering the energy demand for substrate production. In detail, if the CO_2 greenhouse gas emissions from the production of rock wool [16] and hemp fibers [52] are compared, they can be reduced by 84% when hemp fibers are used.

On the other hand, it must be considered that the degradation of organic C from organic substrates increases GHG emissions during cultivation. However, if residues are used that would otherwise also be degraded (e.g., in composts), the actual impact might be small, especially for CO_2 and CH_4 [53]. The higher CO_2 emissions from hemp substrates at the beginning of the measurements in October indicate a strong degradation of the hemp fibers, which could have continuously decreased oxygen availability in the substrate slabs. The presence of anoxic conditions in the substrate slabs was furthermore indicated by the perceptible odor of hydrogen sulfide. Denitrification works best under suboxic conditions and decreases again under very anaerobic conditions. However, the latter are necessary for methane formation. This would explain why the nitrous oxide emission was highest in the second measurement, while the methane emission increased again significantly in the third measurement. Thus, it would be desirable to introduce oxygen into the substrate bags to prevent or reduce these anaerobic conditions to prevent emissions of methane and nitrous oxide as suggested by Karlowsky, et al. [24].

3.2. Determination of Plant Growth Parameters

The different physical and chemical properties of both substrates could have an impact on the performance of the plants. In order to be able to make statements on this, the leaf areas during the first six weeks after planting, the plant length achieved at the end of cultivation, and the total yields of the tomato plants were documented as a function of the substrate used (Table 4). Leaf area increased to almost 3 m^2 and the length of the plants reached 9 m. Both parameters did not differ significantly between plants grown on different substrate.

Table 4. Effects of different growing media on tomato plant growth and yield during and at the end of cultivation.

	Rock Wool	**Hemp**
Leaf area per plant [m^2] 6 weeks after planting	2.97 ± 0.19 a	2.82 ± 0.22 a
Plant length [m]	9.51 ± 0.43 a	8.96 ± 0.38 a
Total yield per plant [kg]	9.98 ± 0.72 a	9.27 ± 0.16 a
SSC fruit [g 100 g^{-1} FM] CW 25	6.60 ± 0.08 a	7.07 ± 0.18 a
SSC fruit [g 100 g^{-1} FM] CW 32	5.36 ± 0.04 a	5.40 ± 0.24 a
DM fruit [%] CW 25	7.73 ± 0.34 a	9.12 ± 0.56 a
DM fruit [%] CW 32	5.64 ± 0.26 a	5.54 ± 0.16 a

Leaf area (n = 3) was measured during first 6 weeks after planting and plant length at the end of the cultivation period (n = 8). Total yield per plant was calculated by adding weekly yields (n = 60). SSC: soluble solid content (n = 3). DM: dry matter content (n = 3). CW: calendar week. Different small letters indicate significant differences between the substrates (*t*-test, $p < 0.05$, mean ± standard deviation).

Tomato fruits were harvested weekly, and yields were summed to calculate the total yield per plant at the end of cultivation. No significant differences in the cumulated yields of plants grown on rock wool and hemp were found during our cultivation period (Table 4). These results are in line with other studies that also used organic (composted white spruce and fir bark in 40%:60%, v/v ratio) rather than inorganic substrates [22]. However, a trend seems to be developing that yields from rock wool bags would be higher than yields caused by hemp fiber bags if the cultivation period were extended. Similar results were found with the use of a mixture of white spruce and fir shavings mixed in a 2:3 (v/v) ratio [22]. The reason for this observed increasing trend is probably that hemp fibers were already well advanced in mineralization and thus the conditions in the root zone, e.g., total pore and air volume, were suboptimal at the end of the cultivation period. This hypothesis is supported by Chérif, et al. [54], who showed that tomato plants are sensitive to hypoxia. In

the present study, an odor of hydrogen sulfide coming from hemp fiber bags throughout the cultivation period might be an indication that hypoxic conditions within hemp substrates existed. The rapid mineralization in which mineralizing microorganisms use most of the available oxygen could explain hypoxic conditions, which, in turn, might affect secondary metabolites or yields.

3.3. Mineral Composition of Leaves and Fruits of Tomato Plants

During the 2020 growing season, leaf samples were taken every three weeks for a period of 12 weeks, and fruit samples were taken every three weeks for a period of 24 weeks to determine the mineral composition in tomato leaves and fruits. In this context, a nutrient deficiency was neither observed visually nor detected in leaves and fruits (Table 5). If the individual nutrients are considered, there are no significant differences, neither for the macro- nor for the micronutrients in leaves and fruits. This means that the observed high nitrogen immobilization caused by the hemp substrate could be compensated by the regularly applied nutrient solution and had no negative influence on the nutrient composition in different plant organs. This also refutes the assumption made by Allaire, et al. [22] that lower yields in hydroponic tomato production using organic growing media is favored by nitrogen immobilization.

Table 5. Nutrient contents in tomato leaves and fruits in relation to different substrates.

	Nutrients in Tomato Leaves		**Nutrients in Tomato Fruits**	
	Rock Wool	**Hemp**	**Rock Wool**	**Hemp**
N [g kg^{-1}]	50.2 ± 6.0 a	50.2 ± 6.1 a	17.2 ± 2.6 a	17.0 ± 2.8 a
P [g kg^{-1}]	4.4 ± 0.6 a	4.0 ± 0.3 a	4.0 ± 0.3 a	3.9 ± 0.4 a
K [g kg^{-1}]	49.2 ± 10.8 a	50.2 ± 9.7 a	44.6 ± 3.6 a	43.4 ± 3.8 a
Ca [g kg^{-1}]	19.2 ± 5.9 a	17.2 ± 4.4 a	0.8 ± 0.1 a	0.7 ± 0.2 a
Mg [g kg^{-1}]	8.2 ± 1.2 a	9.4 ± 1.6 a	1.6 ± 0.3 a	1.6 ± 0.3 a
Cu [mg kg^{-1}]	16.7 ± 1.5 a	14.6 ± 1.8 a	9.3 ± 3.8 a	9.8 ± 4.0 a
Zn [mg kg^{-1}]	37.9 ± 7.2 a	36.9 ± 9.0 a	25.1 ± 4.6 a	22.8 ± 4.8 a
Fe [mg kg^{-1}]	174.7 ± 10.5 a	171.3 ± 35.8 a	41.4 ± 15.3 a	39.6 ± 16.3 a

Significant differences are indicated with small letters (*t*-test, $p < 0.05$, n = 3 per sampling date, 9 sampling dates, mean ± standard deviation).

3.4. Effects of Different Growing Media on Quality Parameters of Tomato Fruits

3.4.1. SSC and Dry Matter Content of Tomato Fruits

In addition to mineral content, the soluble sugar content (SSC) and the dry matter content of tomato fruits were determined. The results are shown in Table 4. For both parameters, SSC and dry matter content, no differences were found in the fruits from the different growing media at all measurement dates. Fruit dry weight consists of up to 60% reducing sugars and organic acids [55], making fruit dry weight an important tomato quality parameter. Dry matter contents in our study were between 4.7 and 9.1% and thus in the range of values already published, e.g., in Bertin, et al. [56] or Moraru, et al. [57]. SSC values ranged from 5.1 to 7.1 g 100 g^{-1} FM, which is within documented values for tomatoes from studies from Johnstone, et al. [58] or Verheul, et al. [59]. SSC and dry matter content seem to be influenced more by the measurement date than by the substrate and tend to decrease over the cultivation period. Dry matter content and SSC are influenced by the amount of sucrose produced during photosynthesis, which is transported to the fruit [60]. Photosynthesis, in turn, is closely related to solar radiation. This explains the decreasing dry matter content and SSC in tomato fruit from mid-year (CW 24, 25) to autumn (CW 31, 32).

3.4.2. Secondary Metabolites—Contents of Carotenoids

For the determination of secondary constituents (carotenoids, phenolic acids, flavonoids), fruit from 15 different plants per growing media were harvested at 3-week intervals over

24 weeks (11 June–25 November 2020). The results for the carotenoid analyses are shown in Figure 2. In tomato fruits produced on hemp fibers, there was significantly increased carotenoid content on several measurement dates compared to the fruits from rock wool (CW 25, CW 27, CW 31 and CW 32). In addition, carotenoid content at each test date varied over the culture period, which may reflect the influence of abiotic factors such as light irradiance [61] and temperature [62] on carotenoid content. Furthermore, two hypotheses are possible for significant differences in carotenoids caused by hemp and rock wool: (i) nutrient supply and/or (ii) ethylene release in the substrate. In this context, Bénard, et al. [63] found no effects of different nitrogen levels on the accumulation of carotenoids in tomatoes, whereas a high proportion of K and Mg in the nutrient solution can increase these secondary plant compounds in tomatoes [64]. Since all macronutrients in leaves and fruits were similar regardless of which substrate was used (Table 5), the first hypothesis is not valid. In terms of the second hypothesis, ethylene in organic substrates originates from decomposition of these by microorganisms, where higher rates of ethylene production were detected under anaerobic conditions [65]. Ethylene plays a central role in the ripening of tomato fruit [66]. A dramatic increase in ethylene production is correlated with the rapid accumulation of carotenoids [67]. Based on our results regarding high CO_2-emissions and weight losses caused by the use of hemp, we assume that high ethylene production existed in these growing bags followed by a higher carotenoid accumulation, especially during the end of the cultivation period (Figure 2). This hypothesis must be investigated in more detail. In particular, ethylene concentration and microbial activity and composition must be determined at close intervals during the experimental period.

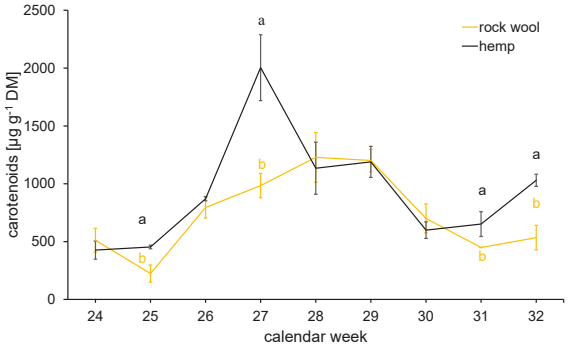

Figure 2. Influence of different substrates on the total carotenoid content in tomatoes. Different small letters indicate significance between variants (*t*-test, $p < 0.05$, n = 3).

3.4.3. Secondary Metabolites—Contents of Phenolic Acids and Flavonoids

In addition to carotenoids, phenolic substances and flavonoids were also investigated as representatives of secondary metabolites. For a first impression, the total phenolic and flavonoid content was determined. In Figure 3 it can be seen that with regard to total phenolic content in tomato fruits, no effect was triggered by the different substrates. The same applied to total flavonoid content. In this context, hypoxia in the root zone can increase phenols in plants [68,69]. In our study, it might be possible that hypoxia was not high enough to increase the phenols in tomatoes.

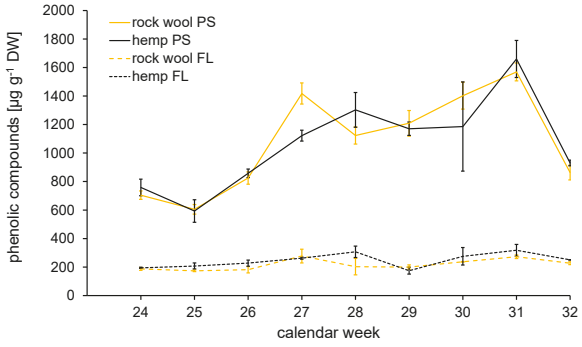

Figure 3. Mean total phenolic acid and flavonoid content in tomato fruit. No significant differences were found (*t*-test, $p < 0.05$, n = 3 over 9 harvest dates 3 weeks apart).

Furthermore, it can be stated that total phenolic content increased during the cultivation period when the last sampling date is not taken into consideration. A positive correlation was found between decreasing temperatures towards the end of the cultivation period and the accumulation of phenolic acid contents in tomato fruits (Figure 4).

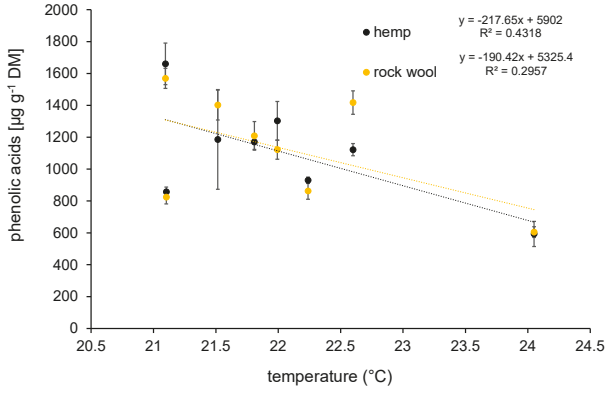

Figure 4. Correlation between phenolic compounds in tomatoes and temperature under consideration of different growing media.

4. Conclusions

The study presented here shows that hemp fibers as an organic substrate in hydroponic cultivation of tomatoes leads to similar yields to the conventionally used rock wool. Likewise, no negative effects on plant growth parameters, nutrient accumulations in leaves and fruits, or phenolic compounds were found. Carotenoids could even be increased by the use of hemp as found in some weeks. Nevertheless, hemp can only be recommended as a substrate for short-term use as the rapid mineralization can be disadvantageous for the root anchoring and thus for the stability of the plants, especially when intensive vegetable production in hydroponics is used.

A higher supply of nitrogen for plants is necessary since mineralization incorporates a significant amount of nitrogen into microbial biomass, making it unavailable to the plants. Although this N-immobilization can be compensated by regularly applied nutrient solution, this increased demand for mineral nitrogen is rather unfavorable from the point of view of sustainability. The observed release of greenhouse gases such as N_2O, CH_4, and CO_2 from hemp fibers also does not correspond to the current goal of making horticulture

more, but environmentally friendly, but could still be lower than CO_2 emissions from rock wool production.

Author Contributions: Investigation: formal analysis, writing—original draft, A.N.; Investigation, formal analysis, writing—original draft, S.K.; Methodology, D.S.; Investigation, N.F.; Project administration, conceptualization, methodology, resources, writing—original draft, formal analysis, supervision, writing—review & editing, D.D. All authors have read and agreed to the published version of the manuscript.

Funding: This project is supported by the Federal Ministry of Food and Agriculture (BMEL) based on the decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support program (funding code 281B203516 for project ´MinTHG´ and funding code 281B204116 for project 'HydroN2O').

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

Acknowledgments: We would like to thank Arlette Harder, Virginia Marten, Wolfgang Pfeifer, and Dirk Behnke as well as Christian Winkelmann from the Division Biosystems Engineering for their technical support during the experiments. We would also like to thank Kirstin Weiss, Gabriele Sommer, Sibylle Noether, and Manuela Alt from the joint laboratory who supported us with their knowledge in the chemical analyses.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. UNFPA. *State of World Population 2011. People and Possibilities in a World of 7 Billion*; Information and External Relations Division of the United Nations Population Fund: New York, NY, USA, 2011.
2. United Nations. *World Urbanization Prospects: The 2014 Revision, Highlights (ST/ESA/SER.A/352)*; Department of Economic and Social Affairs, Population Division: New York, NY, USA, 2014.
3. IPCC. *Climate Change and Land. An IPCC Special Report on Climate Change, Desertification, Land Degradation, Sustainable Land Management, Food Security, and Greenhouse Gas Fluxes in Terrestrial Ecosystems*; Intergovernmental Panel on Climate Change: Geneva, Switzerland, 2019; pp. 1–41.
4. Hickman, G.W. *International Greenhouse Vegetable Production-Statistics (2018 Edition)*; Cuesta Roble Consulting: Mariposa, CA, USA, 2018.
5. Chang, J.; Wu, X.; Liu, A.Q.; Wang, Y.; Xu, B.; Yang, W.; Meyerson, L.A.; Gu, B.J.; Peng, C.H.; Ge, Y. Assessment of net ecosystem services of plastic greenhouse vegetable cultivation in China. *Ecol. Econ.* **2011**, *70*, 740–748. [CrossRef]
6. Cuce, E.; Harjunowibowo, D.; Cuce, P.M. Renewable and sustainable energy saving strategies for greenhouse systems: A comprehensive review. *Renew. Sust. Energ. Rev.* **2016**, *64*, 34–59. [CrossRef]
7. Gruda, N.; Bisbis, M.; Tanny, J. Impacts of protected vegetable cultivation on climate change and adaptation strategies for cleaner production—A review. *J. Clean. Prod.* **2019**, *225*, 324–339. [CrossRef]
8. Gruda, N.; Bisbis, M.; Tanny, J. Influence of climate change on protected cultivation: Impacts and sustainable adaptation strategies—A review. *J. Clean. Prod.* **2019**, *225*, 481–495. [CrossRef]
9. Elings, A.; Kempkes, F.L.K.; Kaarsemaker, R.C.; Ruijs, M.N.A.; Van de Braak, N.J.; Dueck, T.A. The energy balance and energy-saving measures in greenhouse tomato cultivation. *Acta Hortic.* **2004**, *691*, 67–74. [CrossRef]
10. Benoit, F.; Ceustermans, N. Horticultural Aspects of ecological soilless growing methods. *Acta Hortic.* **1995**, *396*, 11–24. [CrossRef]
11. Bussell, W.T.; Mckennie, S. Rockwool in horticulture, and its importance and sustainable use in New Zealand. *N. Z. J. Crop. Hort.* **2004**, *32*, 29–37. [CrossRef]
12. Jeong, B.R.; Hwang, S.J. Use of recycled hydroponic rockwool slabs for hydroponic production of cut roses. *Acta Hortic.* **2000**, *554*, 89–94. [CrossRef]
13. Shinohara, Y.; Hata, T.; Maruo, T.; Hohjo, M.; Ito, T. Chemical and physical properties of the Coconut-Fibre substrate and the growth and productivity of Tomato (*Lycopersicon esculentum* Mill.) plants. *Acta Hortic.* **1999**, *481*, 145–149. [CrossRef]
14. Pieters, J.; Van Assche, B.; Buekens, A. Reducing solid waste streams specific to soilless horticulture. *HortTechnology* **1998**, *8*, 396–401. [CrossRef]
15. Destatis. *Land- und Forstwirtschaft, Fischerei. Gemüseerhebung: Anbau und Ernte von Gemüse und Erdbeeren*; Statistisches Bundesamt: Wiesbaden, Germany, 2019; pp. 2–66.

16. Brandhorst, J.; Spritzendorfer, J.; Gildhorn, K.; Hemp, M. Dämmstoffe aus nachwachsenden Rohstoffen. In *Fachagentur Nachwachsende Rohstoffe e.V.*, 4th ed.; FNR, Ed.; Druckerei Weidner: Rostock, Germany, 2012.
17. Abad, M.; Noguera, P. Los sustratos en los cultivos sin suelo. In *Manual del Cultivo Sin Suelo*; Urrestarazu, M., Ed.; Servicio de Publicaciones Universidad de Almeria, Mundi-Prensa: Madrid, Spain, 2000; pp. 137–183.
18. Abad, M.; Martinez, P.F.; Martinez, M.D.; Martinez, J. Evaluacio´n agrono´mica de los sustratos de cultivo. *Actas Hortic.* **1993**, *11*, 141–154.
19. De Boodt, M.; Verdonck, O. The physical properties of the substrates in horticulture. *Acta Hortic.* **1972**, *26*, 37–44. [CrossRef]
20. Boertje, G.A. Physical laboratory analyses of potting composts. *Acta Hortic.* **1983**, *150*, 47–50. [CrossRef]
21. Jenkins, J.R.; Jarrell, W.M. Predicting physical and chemical properties of container mixtures. *HortScience* **1998**, *24*, 292–295.
22. Allaire, S.E.; Caron, J.; Menard, C.; Dorais, M. Potential replacements for rockwool as growing substrate for greenhouse tomato. *Can. J. Soil Sci.* **2005**, *85*, 67–74. [CrossRef]
23. Firestone, M.K.; Davidson, E.A. Microbiological basis of NO and N_2O production and consumption in soil. In *Exchange of Trace Gases between Terrestrial Ecosystems and the Atmosphere*; Andreae, M.O., Schimel, D.S., Eds.; Wiley: New York, NY, USA, 1989; Volume 47, pp. 7–21.
24. Karlowsky, S.; Gläser, M.; Henschel, K.; Schwarz, D. Seasonal nitrous oxide emissions from hydroponic tomato and cucumber cultivation in a commercial greenhouse company. *Front. Sustain. Food Syst.* **2021**, *5*, 1–13. [CrossRef]
25. Giles, M.E.; Daniell, T.J.; Baggs, E.M. Compound driven differences in N_2 and N_2O emission from soil; the role of substrate use efficiency and the microbial community. *Soil Biol. Biochem.* **2017**, *106*, 90–98. [CrossRef]
26. Morley, N.; Baggs, E.M. Carbon and oxygen controls on N_2O and N_2 production during nitrate reduction. *Soil Biol. Biochem.* **2010**, *42*, 1864–1871. [CrossRef]
27. Le Mer, J.; Roger, P. Production, oxidation, emission and consumption of methane by soils: A review. *Eur. J. Soil Biol.* **2001**, *37*, 25–50. [CrossRef]
28. Dannehl, D.; Suhl, J.; Ulrichs, C.; Schmidt, U. Evaluation of substitutes for rock wool as growing substrate for hydroponic tomato production. *J. Appl. Bot. Food Qual.* **2015**, *88*, 68–77. [CrossRef]
29. Urrestarazu, M.; Martinez, G.A.; Salas, M.D. Almond shell waste: Possible local rockwool substitute in soilless crop culture. *Sci. Hortic.* **2005**, *103*, 453–460. [CrossRef]
30. Manios, V.I.; Papadimitriou, M.D.; Kefakis, M.D. Hydroponic culture of Tomato and Gerbera at different substrates. *Acta Hortic.* **1995**, *408*, 11–15. [CrossRef]
31. Martinez, P.F.; Abad, M. Soilless culture of Tomato in different Mineral Substrates. *Acta Hortic.* **1992**, *323*, 251–259. [CrossRef]
32. Gruda, N.; Rau, B.J.; Wright, R.D. Laboratory bioassay and greenhouse evaluation of a pine tree substrate used as a container substrate. *Eur. J. Hortic. Sci.* **2009**, *74*, 73.
33. Göhler, F.; Molitor, H.D. *Erdelose Kulturverfahren im Gartenbau*; Ulmer: Stuttgart, Germany, 2002.
34. Dannehl, D.; Rocksch, T.; Schmidt, U. Modelling to estimate the specific leaf area of tomato leaves ('Pannovy'). *Acta Hortic.* **2015**, *1099*, 79–86. [CrossRef]
35. VDLUFA. *Methodenbuch Band I-Die Untersuchungen von Böden, Method A 13.5.1, Stabilität N-Haushalt*, 5th ed.; VDLUFA-Verlag: Darmstadt, Germany, 2007.
36. Förster, N.; Ulrichs, C.; Schreiner, M.; Arndt, N.; Schmidt, R.; Mewis, I. Ecotype Variability in Growth and Secondary Metabolite Profile in Moringa oleifera: Impact of Sulfur and Water Availability. *J. Agric. Food Chem.* **2015**, *63*, 2852–2861. [CrossRef]
37. Mageney, V.; Baldermann, S.; Albach, D.C. Intraspecific variation in carotenoids of *Brassica oleracea* var. *sabellica*. *J. Agric. Food Chem.* **2016**, *64*, 3251–3257. [CrossRef]
38. de Mendiburu, F. Agricolae: Statistical Procedures for Agricultural Research. R Package Version 1.3-2. 2020. Available online: https://CRAN.R-project.org/package=agricolae (accessed on 1 February 2022).
39. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2020; Available online: https://www.R-project.org (accessed on 1 February 2022).
40. Fuss, R. Gasfluxes: Greenhouse Gas Flux Calculation from Chamber Measurements, 0.4-4. 2020. Available online: https://cran.r-project.org/web/packages/gasfluxes/gasfluxes.pdf (accessed on 1 February 2022).
41. Hothorn, T.; Hornik, K.; Wiel, M.A.v.d.; Zeileis, A. Implementing a Class of Permutation Tests: The coin Package. *J. Stat. Softw.* **2008**, *28*, 1–23. [CrossRef]
42. Veen, B.; Van Noordwijk, M.; De Willigen, P.; Boone, F.; Kooistra, M. Root-soil contact of maize, as measured by a thin-section technique. *Plant Soil* **1992**, *139*, 131–138. [CrossRef]
43. Verdonck, O. New developments in the use of graded perlite in the horticultural substrates. *Acta Hortic.* **1983**, *150*, 575–581. [CrossRef]
44. Islam, S.; Khan, S.; Ito, T.; Maruo, T.; Shinohara, Y. Characterization of the physico-chemical properties of environmentally friendly organic substrates in relation to rockwool. *J. Hortic. Sci. Biotechnol.* **2002**, *77*, 143–148. [CrossRef]
45. Martius, C. Decomposition of wood. In *The Central Amazon Floodplain*; Junk, W.J., Ed.; Springer: Berlin/Heidelberg, Germany, 1997; Volume 126, pp. 267–276.
46. Gruda, N.; Schnitzler, W.H. Suitability of wood fiber substrate for production of vegetable transplants-I. Physical properties of wood fiber substrates. *Sci. Hortic.* **2004**, *100*, 309–322. [CrossRef]

47. Gruda, N.; von Tucher, S.; Schnitzler, W.H. N-immobilization by wood fibre substrates in the production of tomato transplants (*Lycopersicon lycopersicum* (L.) Karst. ex Farw.). *J. Appl. Bot. Food Qual.* **2000**, *74*, 32–37.
48. Daum, D.; Schenk, M.K. Gaseous nitrogen losses from a soilless culture system in the greenhouse. *Plant Soil* **1996**, *183*, 69–78. [CrossRef]
49. Hashida, S.-n.; Johkan, M.; Kitazaki, K.; Shoji, K.; Goto, F.; Yoshihara, T. Management of nitrogen fertilizer application, rather than functional gene abundance, governs nitrous oxide fluxes in hydroponics with rockwool. *Plant Soil* **2014**, *374*, 715–725. [CrossRef]
50. Yoshihara, T.; Tokura, A.; Hashida, S.-n.; Kitazaki, K.; Asobe, M.; Enbutsu, K.; Takenouchi, H.; Goto, F.; Shoji, K. A Precise/Short-interval Measurement of Nitrous Oxide Emission from a Rockwool Tomato Culture. *Environ. Control Biol.* **2014**, *52*, 137–147. [CrossRef]
51. Dennis, P.G.; Miller, A.J.; Hirsch, P.R. Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities? *FEMS Microbiol. Ecol.* **2010**, *72*, 313–327. [CrossRef] [PubMed]
52. Stucki, M.; Wettstein, S.; Mathis, A.; Amrein, S. *Erweiterung der Studie Torf und Torfersatz-produkte im Vergleich: Eigenschaften, Verfügbarkeit, ökologische Nachhaltigkeit und soziale Auswirkungen*; Institut für Umwelt und Natürliche Ressourcen, Zürcher Hochschule für Angewandte Wissenschaften: Wädenswil, Switzerland, 2019; Volume 3, pp. 3–81.
53. Sánchez, A.; Artola, A.; Font, X.; Gea, T.; Barrena, R.; Gabriel, D.; Sánchez-Monedero, M.Á.; Roig, A.; Cayuela, M.L.; Mondini, C. Greenhouse gas emissions from organic waste composting. *Environ. Chem. Lett.* **2015**, *13*, 223–238. [CrossRef]
54. Chérif, M.; Tirilly, Y.; Bélanger, R.R. Effect of oxygen concentration on plant growth, lipidperoxidation, and receptivity of tomato roots to Pythium F under hydroponic conditions. *Eur. J. Plant Pathol.* **1997**, *103*, 255–264. [CrossRef]
55. Davies, J.N.; Hobson, G.E.; McGlasson, W.B. The constituents of tomato fruit—the influence of environment, nutrition, and genotype. *Crit. Rev. Food Sci.* **1981**, *15*, 205–280. [CrossRef]
56. Bertin, N.; Guichard, S.; Leonardi, C.; Longuenesse, J.J.; Langlois, D.; Navez, B. Seasonal evolution of the quality of fresh glasshouse tomatoes under Mediterranean conditions, as affected by air vapour pressure deficit and plant fruit load. *Ann. Bot.* **2000**, *85*, 741–750. [CrossRef]
57. Moraru, C.; Logendra, L.; Lee, T.C.; Janes, H. Characteristics of 10 processing tomato cultivars grown hydroponically for the NASA Advanced Life Support (ALS) Program. *J. Food Compos. Anal.* **2004**, *17*, 141–154. [CrossRef]
58. Johnstone, P.R.; Hartz, T.K.; LeStrange, M.; Nunez, J.J.; Miyao, E.M. Managing Fruit Soluble Solids with Late-season Deficit Irrigation in Drip-irrigated Processing Tomato Production. *Hortscience* **2005**, *40*, 1857–1861. [CrossRef]
59. Verheul, M.J.; Slimestad, R.; Tjøstheim, I.H. From producer to consumer: Greenhouse tomato quality as affected by variety, maturity stage at harvest, transport conditions, and supermarket storage. *J. Agric. Food Chem.* **2015**, *63*, 5026–5034. [CrossRef] [PubMed]
60. Guichard, S.; Bertin, N.; Leonardi, C.; Gary, C. Tomato fruit quality in relation to water and carbon fluxes. *Agronomie* **2001**, *21*, 385–392. [CrossRef]
61. Llorente, B.; Martinez-Garcia, J.F.; Stange, C.; Rodriguez-Concepcion, M. Illuminating colors: Regulation of carotenoid biosynthesis and accumulation by light. *Curr. Opin. Plant Biol.* **2017**, *37*, 49–55. [CrossRef]
62. Dumas, Y.; Dadomo, M.; Di Lucca, G.; Grolier, P. Effects of environmental factors and agricultural techniques on antioxidant content of tomatoes. *J. Sci. Food Agric.* **2003**, *83*, 369–382. [CrossRef]
63. Bénard, C.; Gautier, H.; Bourgaud, F.; Grasselly, D.; Navez, B.; Caris-Veyrat, C.; Weiss, M.; Génard, M. Effects of low nitrogen supply on tomato (*Solanum lycopersicum*) fruit yield and quality with special emphasis on sugars, acids, ascorbate, carotenoids, and phenolic compounds. *J. Agric. Food Chem.* **2009**, *57*, 4112–4123. [CrossRef]
64. Fanasca, S.; Colla, G.; Maiani, G.; Venneria, E.; Rouphael, Y.; Azzini, E.; Saccardo, F. Changes in antioxidant content of tomato fruits in response to cultivar and nutrient solution composition. *J. Agric. Food Chem.* **2006**, *54*, 4319–4325. [CrossRef]
65. Jäckel, U.; Schnell, S.; Conrad, R. Microbial ethylene production and inhibition of methanotrophic activity in a deciduous forest soil. *Soil Biol. Biochem.* **2004**, *36*, 835–840. [CrossRef]
66. Liu, L.; Shao, Z.; Zhang, M.; Wang, Q. Regulation of carotenoid metabolism in tomato. *Mol. Plant* **2015**, *8*, 28–39. [CrossRef]
67. Marty, I.; Bureau, S.; Sarkissian, G.; Gouble, B.; Audergon, J.; Albagnac, G. Ethylene regulation of carotenoid accumulation and carotenogenic gene expression in colour-contrasted apricot varieties (*Prunus armeniaca*). *J. Exp. Bot.* **2005**, *56*, 1877–1886. [CrossRef] [PubMed]
68. Lara, L.J.; Egea-Gilabert, C.; Niñirola, D.; Conesa, E.; Fernández, J.A. Effect of aeration of the nutrient solution on the growth and quality of purslane (*Portulaca oleracea*). *J. Hortic. Sci. Biotechnol.* **2011**, *86*, 603–610. [CrossRef]
69. Bai, T.; Li, C.; Ma, F.; Feng, F.; Shu, H. Responses of growth and antioxidant system to root-zone hypoxia stress in two *Malus* species. *Plant Soil* **2010**, *327*, 95–105. [CrossRef]

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

Horticulturae Editorial Office
E-mail: horticulturae@mdpi.com
www.mdpi.com/journal/horticulturae

MDPI
St. Alban-Anlage 66
4052 Basel
Switzerland

Tel: +41 61 683 77 34
Fax: +41 61 302 89 18

www.mdpi.com

ISBN 978-3-0365-3902-7