NUTRIENTS RECYCLING IN HYDROPONICS: OPPORTUNITIES AND CHALLENGES TOWARD SUSTAINABLE CROP PRODUCTION UNDER CONTROLLED ENVIRONMENT AGRICULTURE

EDITED BY: Toshiki Asao, Genhua Niu and Md Asaduzzaman

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Topic Editors:
Toshiki Asao, Shimane University, Japan
Genhua Niu, Texas A&M University, United States
Md Asaduzzaman, Bangladesh Agricultural Research Institute, Bangladesh

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Editorial: Nutrients Recycling in Hydroponics: Opportunities and Challenges Toward Sustainable Crop Production Under Controlled Environment Agriculture

Md Asaduzzaman[1]*, *Genhua Niu*[2] *and Toshiki Asao*[3]

[1] Olericulture Division, Horticulture Research Center, Bangladesh Agricultural Research Institute, Gazipur, Bangladesh, [2] Texas A&M AgriLife Research, Texas A&M University, Dallas, TX, United States, [3] Faculty of Life and Environmental Science, Shimane University, Matsue, Japan

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Edited by:
Youssef Rouphael,
University of Naples Federico II, Italy

Reviewed by:
Pradeep Kumar,
Central Arid Zone Research Institute (ICAR), India
Nazim S. Gruda,
University of Bonn, Germany

***Correspondence:**
Md Asaduzzaman
asadcbt@bari.gov.bd

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Editorial on the Research Topic

Nutrients Recycling in Hydroponics: Opportunities and Challenges Toward Sustainable Crop Production Under Controlled Environment Agriculture

NUTRIENT RECYCLING IN HYDROPONICS

Hydroponics is a method of growing plants in soilless media or nutrient solution containing all essential mineral nutrients that potentially lead to yield and quality improvement (Gruda, 2009). In principle, nutrient solutions used in hydroponics can either be reused or discarded (Jensen, 1997; Nederhoff and Stanghellini, 2010). Nowadays, cultivation of horticultural crops including leafy and fruiting vegetables and medicinal herbs with pharmaceutical value are commercially grown in recycled (i.e., recirculating) hydroponics under controlled environments (Resh, 2012; Son et al., 2020).

In recycled hydroponics, nutrient solutions passed through the growing medium are collected into a reservoir and reused repeatedly. In this system, both water and mineral nutrients are used efficiently, therefore minimizing wastage of fertilizer and pollution of the environment. This type of hydroponic system has been widely used in controlled environment agriculture facilities including plant factories using artificial grow lights such as light-emitting diodes. Management of the hydroponic nutrient solution composition, along with the adjustment of environmental conditions may enhance the desired nutritional quality of the produce, regulate bioactive compounds, and increase antioxidants and other phytochemical content in soilless culture (Savvas et al., 2013; Asaduzzaman et al., 2018; Ciriello et al., 2021).

Hydroponics may however be challenged by the accumulation of root exudates that affect plant growth and reduce crop yield and quality. Lower growth and yield performance of several crops including lettuce, strawberry, several leafy vegetables, and ornamentals have been reported in recycled hydroponics (Asao et al., 2004, 2007; Lee et al., 2006). Reduced growth and yield of crops grown in recycled hydroponics because of increased concentration of phytotoxic root exudates have been reported causing allelochemical stress in the strawberry (Kitazawa et al., 2005), tomato (Yu and Matsui, 1993), and cucumber (Yu and Matsui, 1994). Certain phytotoxic chemicals may have a significant impact on plant growth. Moreover, recycled nutrient solutions usually require

sterilization to minimize pathogen loads. In this regard, chemicals and physical treatments such as hypochlorite, ozone, and UV-light, are being used for sterilization.

The present Research Topic collected 12 scientific contributions from the leading research groups throughout the world working on recycled hydroponics, nutrient solution management, the influence of LEDs on crop growth and physiology, salinity impact on growth and nutritional quality, wastewater based nutrient recycling, and also nutrient reclamation or re-mineralization. In addition, this Research Topic complies several aspects of controlled environment agriculture that are useful with for scientific community, and by extension workers and commercial entrepreneurs, for the understanding of nutrient recycling in hydroponics toward sustainable crop production. The original research and reviewed literature also present how the techniques of nutrient recycling, efficient use of available nutrients, quality improvement of crop produce, and nutrient recycling from renewable resources will help the development of sustainable agricultural systems.

NUTRIENT SOLUTION MANAGEMENT IN RECYCLED HYDROPONICS

Recycled hydroponics gained popularity in controlled environment agriculture leading to efficient use of costly fertilizer and a sustainable environment. Rufí-Salís et al. proposed a cascade system with a long-cycle tomato donor crop and five successive cycles of lettuce for a rooftop greenhouse. They quantified the scale between the donor and receiving crops and proposed three major ideas to optimize the nutrient flows while maintaining the yield and quality of the vegetables produced in the receiving crop. The variation of the nutrient content of the leachates produced by the donor crop was a key consideration that determines the number of plants to be planted as the receiving crop. It was found that the early stage of the donor crop could only produce 0.1 lettuces per tomato plant, with N as the limiting nutrient. On the other hand, the late stage of the donor crop was able to leach enough nutrients to feed 9 lettuces per tomato plant. However, attention must be paid to the electrical conductivity (EC) of the water flow to stay within non-harmful values. Nevertheless, the cascade system was shown to be efficient to mitigate the nutrient discharge of open systems, especially in terms of N and P to avoid eutrophication impacts in the early stage of the tomato crop. Considering the nutritional problems at the beginning of the cycle of the donor crop and the harmful salinity that can be reached at the end, future research needs to be designed to test different kinds of horticultural crops to discover possible viable combinations of donor and receiving crops.

Miller et al. reported that nutrient deficiencies in hydroponic production can also be observed due to recycling nutrient solutions. They evaluated the effects of recycling on solution EC changes, tissue nutrient concentration, canopy growth rate, plant water status, and shoot and root weight of lettuce in a greenhouse and suggest the development of optimal strategies for managing recycling nutrient solution in hydroponic production. This study indicates that continuous recycling with tap water containing moderate to high levels of alkalinity can result in an apparent increase in solution EC, nutrient deficiencies in the plants, and reduction in shoot growth, in spite of maintaining the solution EC at a target level. Results of this study also indicated that nutrient recycling significantly decreased N, P, K, and Fe and increased Na and Cu levels in the tissue, in addition to increasing solution EC between adjustments compared to the control. Through image analysis of plants reveals the negative effects of recycling on canopy area started 2 weeks after transplanting. Thus, they hypothesized that certain unwanted compounds (e.g., bicarbonates) and slowly consumed elements (e.g., Ca, Mg) were added to the recycling solution through the alkaline tap water with time.

Ahn et al. designed an EC-based nutrient recycling soilless culture system by theoretical and experimental analyses. An integrated model of solutes such as K^+, Ca^{2+}, and Mg^{2+} and water transport in growing media, automated nutrient solution preparation, and nutrient uptake was designed. In the simulation, the intrinsic characteristics of nutrient changes among open-, semi- closed-, and closed-loop soilless cultures were compared, and stochastic simulations for nutrient control were performed in the closed-loop system. Four automated irrigation modules for comparing nutrient changes among the soilless culture systems were constructed for sweet pepper grown in the greenhouse. Theoretical and experimental analyses exhibited that nutrient variations in these culture systems can be integrated as a function of nutrient supply to the system's boundary areas. Furthermore, stochastic simulation analysis indicated that the nutrient ratio in the soilless culture system reveals the nutrient uptake parameter-based deterministic patterns. Thus, they suggested that the nutrient ratio in the closed-loop soilless culture could be controlled by the long-term feedback of this ratio.

The quality of crop produce can be improved through quantitative management of the hydroponic nutrient solution. The desired mineral content in fruits and vegetables can be either increased or decreased through their elevated or deficient concentration in the culture solution. Zhu et al. studied appropriate NH_4^+/NO_3^- ratio that triggers plant growth and nutrient uptake of flowering Chinese cabbage by optimizing the pH value of the nutrient solution. They analyzed the changes in nutrient solution composition, the content of different N forms in plant tissues and exudates, and expression of plasma membrane H^+-ATPase genes under different NH_4^+/NO_3^- ratios (0/100, 10/90, 25/75, 50/50). Compared with the control, NH_4^+/NO_3^- ratios (0/100, 10/90, and 25/75) significantly reduced the NO_3^- content and increased the NH_4^+, amino acid, and soluble protein contents of flowering Chinese cabbage to varying extents. NH_4^+/NO_3^- ratio (10/90) significantly increased the N use efficiency, whereas NH_4^+/NO_3^- ratio (25/75) significantly decreased it to about 70.25% of that control. Owing to the difference in N absorption and utilization among seedlings, the pH value of the nutrient solution differed under different NH_4^+/NO_3 ratios.

LED LIGHTING, NITROGEN METABOLISM AND ENVIRONMENTAL CONTROLS IN RECYCLED HYDROPONICS

In a plant factory with artificial lighting (PFAL), LEDs have widely been used for economic considerations and also to ensure a lower amount of heat emission inside the controlled room. The influence of LED spectrum along with the application of amino acid as nitrogen source has been studied by Talukder et al. (2018). Li et al. studied the effect of the LED spectrum on the quality and nitrogen metabolism under recycled hydroponics. They found that LED illumination spectra had a significant influence on the growth and nitrogen metabolism of lettuce. Adding green, purple, and far-red light had a negative impact on lettuce growth through decreased photosynthetic photon flux density. Purple LED supplementation was found to be conducive to vitamin-C accumulation in lettuce leaves. Adding purple light inhibited NR (nitrate reductase) and NiR (nitrite reductase) activities and caused a low nitrate, nitrite, and ammonium content while they contributed to amino acid accumulation for nitrogen assimilation. Thus, red, blue, and purple LEDs are recommended for use as supplemental lighting strategy greenhouse production.

He et al. grew purslane (*Portulaca oleracea* L.) in different NaCl salinities in hydroponics under LED lightings. Greater shoot and root dry mass with higher proline and carotene concentration were observed under 100 mM NaCl than fresh water, 200 and 300 mM. However, increasing salinity levels such as 200 and 300 mM NaCl decreases the shoot and root dry mass, ascorbic acid, and total phenolic compounds under lower leaf water content and photosynthetic performance. They concluded that it is feasible to grow purslane under 100 mM NaCl to achieve higher productivity and better quality.

In controlled environment agriculture, environmental factors including VPD fluctuation greatly influence the photosynthesis and yield of a plant. Inoue et al. examined the effects of the vapor pressure deficit (VPD) fluctuation on the photosynthetic and growth parameters in lettuce. In this study, gas exchange, chlorophyll fluorescence, and biomass accumulation were evaluated under drastic (1.63 kPa for 6 min and 0.63 for 3 min) or moderate (1.32 kPa for 7 min and 0.86 kPa for 3 min) VPD fluctuation. The drastic VPD fluctuation induced a gradual decrease in stomatal conductance and thus CO_2 assimilation rate during the measurements, while moderate VPD fluctuation caused no reduction of these parameters. Thus, moderate VPD fluctuation maintained leaf expansion and the efficiency of CO_2 diffusion across the leaf surface, resulting in enhanced plant growth compared with drastic VPD fluctuation.

ORGANIC NUTRIENT SUPPLEMENTATION THROUGH WASTEWATER TREATMENT

Organic nutrients supplemented through wastewater treatment has great advantages in plant growth and yield and environmental sustainability. Researchers are applying a number of methods on the recovery of nutrients from wastewater and agricultural waste for organic greenhouse production (Voogt et al., 2011). Kechasov et al. have designed a closed hydroponic system with an integrated nitrification bioreactor. They compared plant development, fruit yield, and quality of tomatoes grown with the liquid by-product of biogas production from pig manure with greenhouse tomatoes grown with mineral fertilizers. The tomatoes grown with the organic waste-based fertilizer had a similar yield but poorer taste characteristics when compared with tomatoes grown with the high-mineral fertilizer. The plants grown with the organic waste-based treatment accumulated a higher amount of salts, especially tissue Cl^- content. Fertilizers based on organic wastes change plant development toward a generative state and can partially recover the physiological and biochemical responses seen in plants grown under suboptimal fertilization conditions, suggesting that these fertilizers could be favored over mineral fertilizers with similar inorganic compositions. However, the use of organic waste-based fertilizers is less feasible than high-mineral fertilizers because of the lower quality of tomato fruits produced.

Chow et al. verified the viability of nickel electroplating industrial wastewater effluent diluted at different concentrations as a source of nutrient recycling using a hydroponic soilless cultivation system. The significant inhibition of the root and shoot elongation and reduction of photosynthetic pigments were accompanied by the profound morphological distortions in the xylem, phloem, and stomata. It was observed that beyond the maximum tolerable concentration level at 25% of wastewater effluent for hyacinth bean and 5% of wastewater effluent for pak choi. The accumulation of proline and upregulation of POD and APX activities were detected against the nickel electroplating industrial wastewater-induced oxidative stress injury in the plant models.

Celletti et al. investigated the possibility of using innovative fertilizer solutions in hydroponic systems for the growth of agricultural plants. Aqueous hydrothermal carbonized liquid (AHL) derived from cow manure digestate was chemically characterized for pH, electrical conductivity, mineral elements, and organic compounds. The AHL diluted with distilled water (1:30, 1:60, and 1:90, v/v) was used as a source of nutrients instead of standard hydroponic nutrient solution and bio-assayed using maize plants. The results indicated that the dilution ratio 1:30 of the AHL solution showed higher phytotoxicity while the increased dilutions (1:60 and 1:90) had a lower level of toxicity allowing plants to grow. It was clearly evident that higher dilution ratios contain insufficient essential nutrients for the plant, showing pronounced leaf chlorosis. Further studies recommended are identifying appropriate species-specific dilution ratios to supply both low levels of phytotoxins and adequate content of essential nutrients for appropriate plant growth and development.

SUSTAINABLE CROP PRODUCTION THROUGH NUTRIENT RECYCLING IN AQUAPONICS

Cifuentes-Torres et al. mentioned that reclaimed water can, in theory, be used in aquaponics as it has been used as

a water source in agriculture irrigation and aquaculture for many decades. They highlighted that there is an opportunity to use reclaimed water in aquaponics although there are still many questions that arise and more studies are needed to demonstrate that this technology is sustainable. There is the potential that toxic compounds such as certain toxic metals at low concentrations can function as food supplies in fish diets, under strict and controlled conditions. The presence of microalgae in aquaponic systems can be an advantage as it acts as both a food producer and wastewater treatment process. This Research Topic emphasized the studies with aquatic organisms and plants with the ability to metabolize contaminants without the risk to human health.

Lobanov et al. reported challenges of closed environment agriculture for resource-use optimization. The exploitation of readily available, soluble aquaculture effluent would be imperative for nutrient transfer in the hydroponic environment considering the role of microorganisms and the rhizosphere. In this regard, nutrient re-mineralization has to be adopted due to the challenges and carbon reduction and the additional costs associated with existing waste revalorization systems. They investigated micronutrient profiles of the re-mineralized effluent, traditional coupled aquaponics, and commercial hydroponic nutrient solutions were measured. Nutrient concentrations were significantly lower in the aquaculture-derived treatments than the commercial solution, while plant sap analysis did not follow the evidence of higher nutrient content in lettuce grown under excessive nutrient conditions. Lettuce grown in the commercial hydroponic nutrient solution likewise experienced deficiencies in Mg and Ca (young leaves) as well as Na and Si (both young and old leaves). The uptake of certain elements (Cu, Fe, Mg, S, and Zn) was greater across aquaponic treatments than initially predicted, however, Mn was universally absent from aquaponic treatments. B and P were especially low in the standard aquaponics treatment, i.e., fertilization with soluble RAS nutrients only. Together this suggests that the solids treatment system in parallel to RAS soluble effluent may be advantageous for aquaponic facilities seeking to maximize the benefits of the fish solids for plant nutrition. Nonetheless, iron remains the most capricious element to provide for plants. The evidence that neither the commercial solution nor aquaponic treatments were wholly successful in increasing iron uptake suggests a need for future studies to determine minimal "optimal" concentrations for plants and as well the real repercussions of deficiencies on crop yield and nutritional quality.

IMPLICATIONS AND FUTURE CHALLENGES OF RECYCLED HYDROPONICS

Recycled hydroponics has great implications in practice under controlled environment agriculture toward economic considerations and environmental sustainability. It is generally used in greenhouses and indoor farming plant factories for producing a range of high-value crops such as leafy and fruiting vegetables and medicinal plants under artificial light organized vertically. Nutrient recycling is important to achieve high resource use efficiency. Simple EC control of nutrient solution concentration would be sufficient for growing vegetables sustainably under recycled hydroponics (Bamsey et al., 2012; Jung et al., 2015; Chowdhury et al., 2021). As EC indicates the total ionic balance of solution, the specific mineral requirement of the plant is usually overlooked. In this regard, ion-selective electrodes (ISEs) have been used for improving plant growth and quality with efficient use of major nutrients (Rius-Ruiz et al., 2014; Cho et al., 2017, 2018; Chowdhury et al., 2020). Therefore, the profitability of hydroponic farming can be achieved through yield maximization under recycled hydroponics based on either EC- or ISE- control of nutrient solution management.

The main concern of recycled hydroponics is the occurrence of pathogens due to the recirculating nature of the culture solution. Therefore, appropriate sterilization is essential for recycled hydroponics. Another important challenge of recycled hydroponics is the accumulation of inhibitory allelochemicals causing yield and quality reduction due to the autotoxicity phenomenon. Although a number of methods have been suggested to overcome the autotoxicity in several crops more appropriate recovery strategies are the future research need.

AUTHOR CONTRIBUTIONS

MA, GN, and TA have made a substantial, direct, and intellectual contribution to the work, and approved the editorial for publication in Frontiers in Plant Science. MA prepared the original manuscript. GN and TA reviewed and edited revised manuscript.

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ORIGINAL RESEARCH
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Closed-Loop Crop Cascade to Optimize Nutrient Flows and Grow Low-Impact Vegetables in Cities

Martí Rufí-Salís[1,2], Felipe Parada[1], Verónica Arcas-Pilz[1], Anna Petit-Boix[3], Gara Villalba[1,2] and Xavier Gabarrell[1,2]*

[1] Sostenipra Research Group (2017 SGR 1683), María de Maeztu Unit, CEX2019-000940-M, Institut de Ciència i Tecnologia Ambientals (ICTA-UAB), Universitat Autònoma de Barcelona, Barcelona, Spain, [2] Department of Chemical, Biological, and Environmental Engineering, Universitat Autònoma de Barcelona, Barcelona, Spain, [3] Chair of Societal Transition and Circular Economy, University of Freiburg, Freiburg, Germany

Urban agriculture systems can significantly contribute towards mitigating the impacts of inefficient and complex food supply chains and increase urban food sovereignty. Moreover, improving these urban agriculture systems in terms of nutrient management can lead to a better environmental performance. Based on a rooftop greenhouse in the Barcelona region, we propose a cascade system where the leachates of a tomato cycle from January to July (donor crop) are used as the main irrigation source for five successive lettuce cycles (receiving crop). By determining the agronomic performance and the nutrient metabolism of the system, we aimed to define the potential of these systems to avoid nutrient depletion and mitigate eutrophication, while scaling the system in terms of nutrient supply between the donor and the receiving crops. The results showed that low yields (below 130 g per lettuce plant) are obtained if a cascade system is used during the early stage of the donor crop, as the amount of nutrients in donor's leachates, specially N (62.4 mg irrigated per plant in the first cycle), was not enough to feed the lettuce receiving crop. This effect was also observed in the nutrient content of the lettuce, which increased with every test until equaling the control (4.4% of N content) as the leachates got richer, although too high electrical conductivity values (near 3 dS/m) were reached at the end of the donor crop cycle. Findings on the uptake of the residual nutrient flows showed how the cascade system was able to take advantage of the nutrients to produce local lettuce while mitigating the effect of N and P in the freshwater and marine environments. Considering our case study, we finally quantified the scale between the donor and receiving crops and proposed three major ideas to optimize the nutrient flows while maintaining the yield and quality of the vegetables produced in the receiving crop.

Keywords: cascade systems, nutrient recycling, urban agriculture, industrial ecology, urban metabolism

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Md Asaduzzaman, Bangladesh Agricultural Research Institute, Bangladesh

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Niels Holst, Aarhus University, Denmark
Leopoldo Mendoza Espinosa, Universidad Autónoma de Baja California, Mexico

****Correspondence:***
Martí Rufí-Salís
Marti.Rufi@uab.cat

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INTRODUCTION

Cities cover 3% of the Earth's surface area, but host 55% of the world's population (United Nations, 2014; SEDAC, 2016). The global nutritional demand thus concentrates in urban areas, resulting in long and complex supply chains. Urban agriculture (UA) has arisen as a promising solution that brings production closer to consumption points. However, UA is not free of environmental

impacts. Similar to conventional agricultural systems, nutrient discharge resulting from intensive fertilizer use is particularly problematic due its impact on water eutrophication (Muñoz et al., 2008; Torrellas et al., 2012; Romero-Gámez et al., 2014, 2012; Boneta et al., 2019). The application of circular economy strategies in UA systems could help mitigate these impacts. Closing the nutrient cycles in soilless systems maintains the utility and value of scarce resources (Bocken et al., 2017), produces a regenerative effect on the environment (Ellen MacArthur Foundation and McKinsey Center for Business and Environment, 2015), and contributes to a reduction in water and fertilizer consumption (Carmassi et al., 2005; Rufí-Salís et al., 2020).

Two main strategies can be found in the literature to close the nutrient cycles in soilless systems. First, recirculation systems use drained water and nutrients in the same crop. This setup helps to reduce water and nutrient losses by closing resource flows (Ahmed et al., 2000; Agung Putra and Yuliando, 2015). However, it demands additional infrastructure, which requires high investment costs (De Pascale and Maggio, 2005; García-Caparrós et al., 2018) and increases the environmental impacts of the system (Rufí-Salís et al., 2020). Moreover, the high salinity of the drained water can cause yield reduction if it is directly recirculated to the crop without treatment or partial discharge (Magán et al., 2008; Grewal et al., 2011).

Second, cascade systems use drained water and nutrients to irrigate another crop (Incrocci et al., 2003). Since nutrients tend to reach high concentrations in the leachates and thus increase the salinity of this water flow, the salt tolerance of the receiving crop has been the main field of inquiry in the literature. For instance, Shannon et al. (2000) analyzed the salt tolerance of nine leafy vegetables using salty water to simulate drainage water, finding that swiss chard was the most salt tolerant among those evaluated. With a similar method, Grieve and Suarez (1997) focused on potential sulfate and selenium toxicity, concluding that purslane (*Portulaca oleracea*) is an excellent candidate for saline drainage water reuse systems. Real case studies with cascade systems were analyzed by Muñoz et al. (2017), who assessed the performance of beef tomato in a greenhouse as the donor crop and a sequence of lettuce, tomato, and endive as receiving crops. This study underlined the need to further study the observed yield decrease and its causes. Incrocci et al. (2003) assessed a cascade cropping system using tomato as the donor crop and cherry tomato as the receiving crop, highlighting the potential of cascade set-ups to increase the water use efficiency of the system. Nonetheless, little is known about the potential of a cascade system to diminish the nutrient load of drainage flows. García-Caparrós et al. (2016) explored this potential with a pot experiment using ornamental *Juncus acutus*. The authors found that the species used presented a good bioremediation potential based on the uptake of nitrogen (N) and phosphorus (P). García-Caparrós et al. (2018) also explored this potential using horticultural melon as the donor crop and ornamental rosemary as the receiving crop, finding a decrease in yield in the receiving crop, but also a great potential to optimize the water flows and remove the nitrates of the drained water. To our knowledge, there are no references in the literature exploring the potential of horticultural species (both as donor and receiving crops) to produce vegetables while mitigating nutrient depletion through the use of a cascade system and the quantification of the nutrient flows.

The present article aims to tap the full potential of cascade systems to produce local-grown vegetables in the framework of UA while diminishing the nutrient load by closing nutrient cycles in the framework of urban metabolism. Based on the agronomic and nutritional performance of five successive receiving crops of lettuce (*Lactuca sativa, Maravilla*) irrigated by a long-cycle donor crop of tomato (*Lycopersicon esculentum, Arawak*), we determined the feasibility and nutritional implications of cascade systems and provide recommendations to further improve the performance of this setup. This will enable a better implementation of UA systems in cities and inform decision-makers about the main benefits and improvement potential of reusing nutrients in UA by considering the principles of a circular economy.

MATERIALS AND METHODS

System Under Study, Crop Description, and Experimental Design

The study was performed in a rooftop greenhouse (RTG) located on the ICTA-ICP building (41.497681N, 2.108834E) on the campus of the Universitat Autònoma de Barcelona, 15 km west of Barcelona, in the West Mediterranean region of the Iberian Peninsula. The crops grown in the greenhouse used rainwater harvested from a roof surface of 400 m^2 (plus 500 m^2 from the neighboring building). The irrigation system was hydroponic, where the substrate bags are filled with perlite with a pH of 7, an electrical conductivity of 0.09 $dS \cdot m^{-1}$, and a granulometry of [0–6].

The RTG section used for the donor tomato crop was southeasterly facing and occupied an area of 84.3 m^2. 57 substrate bags were used (40L), planting 3 seedlings per bag, totaling 171 plants. The tomato crop was planted on January 14th and uprooted on August 2nd, 2019. The average concentration of fertilizers in kg/m^3 was KPO_4H_2 – 0.283, KNO_3 – 0.138, K_2SO_4 – 0.367, $Ca(NO_3)_2$ – 0.533, $CaCl_2\ H_2O$ – 0.133, $Mg(NO_3)_2$ – 0.178, Hortilon – 0.011, and Sequestrene – 0.011, although the nutrient solution was adapted based on the evolution and phenological stages of the tomato crop. The leachates of the tomato crop were collected through connected pipes from leachate trays and transported to a 300-L tank using a submergible pump.

To maximize the nutrient recycling, these leachates were used to irrigate two crops. First, the same tomato crop using a recirculation system (totaling 7.5 m^3), whose performance was left out of the study to focus only on the cascade system. Second, a receiving lettuce crop located in another section of the RTG southwesterly facing using 2 L/h drippers (totaling 8.8 m^3). **Figure 1** shows the diagram of the water flows of the system.

Five successive lettuce cycles were grown as shown in **Table 1**. The choice of lettuce was based on the lower nutritional demand from this crop compared to tomato. Low nutrient concentrations were expected in the leachates of the tomato crop, and thus lettuce can potentially produce competitive yields with the nutrients

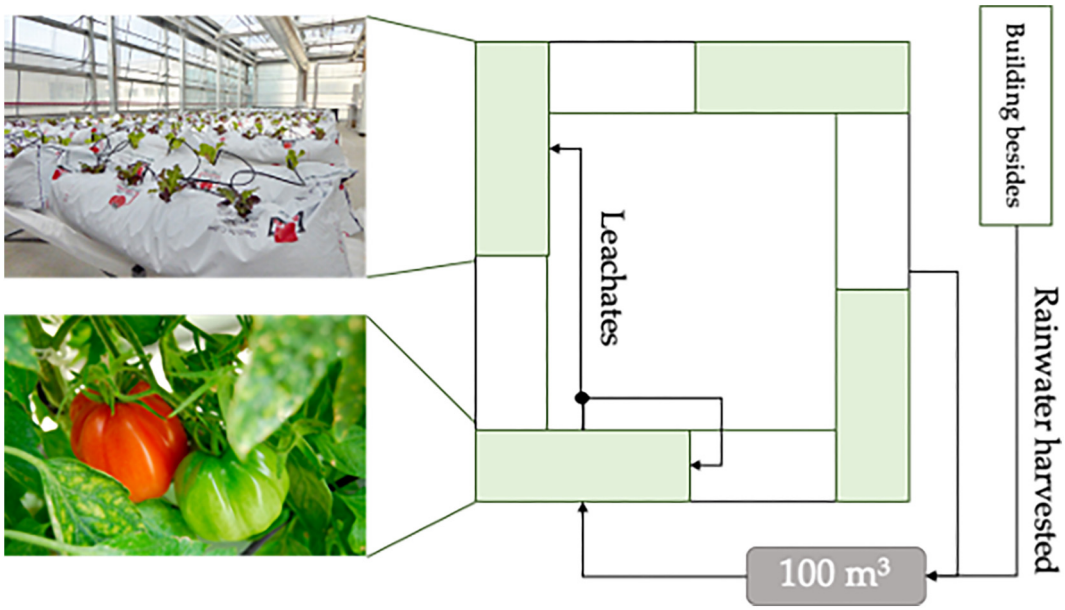

FIGURE 1 | Diagram of the cascade system.

supplied through the cascade system. Average temperatures and relative humidity per test are shown in **Supplementary Figures S1, S2**, respectively.

Plants were distributed in 4 substrate trays, with 8 perlite bags each using a 4 × 2 organization and with a 1.2-m separation between trays. Each substrate bag can allocate 4 plants, totaling 32 plants per tray. All campaigns were planted from nursery plants.

Besides campaigns three to five of the cascade system, we planted mirror control crops using the same species and variety to compare the results in terms of yield and nutrient content during the seasonal period with expected higher water demand and salinity. We used 2 substrate trays for the control, totaling 64 plants.

Water and Nutrient Monitoring

To assess the water flows in the cascade system, analogic flow meters were coupled to the water system. Samples of the irrigation in the receiving crop and the control were collected directly from the drippers placed in the perlite bags. To determine the concentration in the water flows, the respective samples were collected three times per week and analyzed using ion chromatography (ICS-1000 and AS-DV by Dionex) to obtain concentration results for the anions nitrite (NO_2^-) and nitrate (NO_3^{2-}). The same samples were externally analyzed using ICP-OES atomic spectroscopy (Optima 4300DV by Perkin-Elmer) to obtain results for P, potassium (K), magnesium (Mg), calcium (Ca), and sulfur (S). Nutrient content in the lettuce was determined based on three plants randomly selected at the end of the harvest. Samples were sorted into envelopes and dried in a furnace at 65°C for 1 week before analyzing externally the concentration of P, K, Ca, Mg, and S via ICP-OES atomic spectroscopy (Optima 4300 DV by Perkin Elmer) and the concentration of N with elemental analysis (Flash EA 2000 CHNS by Thermo Fisher Scientific). Since the sample for every test and treatment is 3 random plants analyzed twice, a formal statistical analysis was not performed due to the low sample size.

To calculate the accumulated irrigated nutrients per test, the concentration values obtained through the continuous analysis of the water samples for each nutrient were multiplied by the amount of water irrigated between the date a specific sample was taken and the date of the previous sample. The values obtained were then aggregated to obtain the final accumulated irrigated nutrients per test, and divided by the number of plants in a specific test to obtain this parameter per plant.

TABLE 1 | Number of plants and calendar of tests undergone in the present study.

Test	Treatment	Planted	Harvested	Plant number
T1	Cascade	February 8	March 5	128
T2	Cascade	March 5	April 11	128
T3	Cascade	April 24	May 31	128
	Control			64
T4	Cascade	May 31	July 1	128
	Control			64
T5	Cascade	July 1	August 1	128
	Control			64

Statistical Methods

A set of statistical methods and tests were used to verify and strengthen the results obtained in different sections. The tests were performed through the use of different packages developed for R programming software, considering a statistical significance when *p*-values were less than 0.05.

Shapiro Wilk's test (Shapiro and Wilk, 1965) was used as the method to analyze the normality of observations or residuals obtained through linear regressions. Levene's test (Levene, 1961) was used as the method to analyze the homogeneity of variances. Levene's test is an alternative to Bartlett's test with less sensitivity to non-normal data. If the two previous tests (Shapiro-Wilk's and Levene's) presented $p > 0.05$, we applied an analysis of covariance (ANCOVA) to the linear regression to evaluate if the means of a dependent variable were equal under different levels of an independent variable. Oppositely, we applied Kruskal-Wallis test (Kruskal and Wallis, 1952) as the non-parametric analog of ANCOVA, recommended with non-normal distributions and inequality of variances (Feir-Walsh and Toothaker, 1974) to check if samples came from the same distribution.

Three different methods were used to compare the statistical significance between slopes of a linear regression. The first one was based on Meseguer-Lloret (2017b) for homogeneous variances and Meseguer-Lloret (2017a) for non-homogeneous variances, and is based on a *t*-test. This method requires a prior test for normality. If a normal distribution is observed, a F-test can be performed to determine if the variances between treatments were homogeneous, since it is accurate only for normal data. However previous research concluded that the *t*-test is highly robust for equal sample size (Welch, 1938; Milton and Tsokos, 1983), which is the case for most data in this study. Based on the method described by Meseguer-Lloret (2017a) for non-homogeneous variances, a *t*-test should be performed by calculating t_{calc} as expressed in Eq. 1 and t'_{calc} as expressed in Eqs 2–4, being "b" the value of the slope, "s^2" the variance, and "t" the specific value in the *t*-table of probabilities.

$$t_{calc} = \frac{|b_1 - b_2|}{\sqrt{s_{b_1}^2 + s_{b_2}^2}} \quad (1)$$

$$t'_{calc} = \frac{t_1 \cdot s_{b_1}^2 + t_2 \cdot s_{b_2}^2}{s_{b_1}^2 + s_{b_2}^2} \quad (2)$$

$$t_1 = t_{95\%}^{n_1-2} \quad (3)$$

$$t_2 = t_{95\%}^{n_2-2} \quad (4)$$

If $t_{calc} > t'_{calc}$, the slopes analyzed are statistically different. On the other hand, based on the method described by Meseguer-Lloret (2017b) for homogeneous variances, a *t*-test should be performed to calculate t_{calc} as expressed in Eq. 1 and t_{tab}, which is the value in the *t*-table with a 95% of probability and "$n_1 + n_2$ -2" degrees of freedom, as expressed in Eq. 5.

$$t_{tab\ 95\%} = t_{95\%}^{n_2-2} \quad (5)$$

If $t_{calc} > t_{tab}$, the slopes analyzed are statistically different. Oppositely, the slopes analyzed are statistically similar if $t_{calc} < t_{tab}$.

The second method is based on Andrade and Estévez-Pérez (2014). Although the authors state that the *t*-test is a robust method to compare the statistical difference between slopes, they suggest the use of the ANCOVA as a simpler method.

The third method consists on the analysis of the "Estimated Marginal Means Of Linear Trends" through the "emtrends" function from the "emmeans" R package (Lenth et al., 2020). As stated by Lenth et al. (2020), "*emtrends is useful when a fitted model involves a numerical predictor interacting with another predictor.*"

RESULTS AND DISCUSSION

This section presents and discusses the results divided in different sections: see section "Production," section "Water: Irrigation," section "Water: EC and pH," section "Water: Nutrient Content," section "Biomass: Nutrient Content," section "Decreasing Eutrophication Potential and Nutrient Depletion, and section "Scaling of the System." Values in X ± Y form express average ± standard deviation.

Production

Figure 2 shows the average fresh weight per lettuce in every test. We can see that Tests 1 (February) and 3 (May), produced low and similar yields: 108.66 ± 26.85 and 109.82 ± 22.39 g/plant, respectively. Test 2 (April) produced slightly more yield with a higher variability of data (127.95 ± 34.60 g/plant), while Test 4 (June) and Test 5 (July) exerted the highest yields, with 220.31 ± 38.27 and 134.17 ± 34.84 g/plant, respectively. On the other hand, the control treatments in Test 3, 4, and 5 (254.91 ± 52.66, 232.49 ± 62.25, and 185.11 ± 32.75 g/plant) always had more weight than its respective cascade crops. The low yields obtained in the receiving crop in the cascade system coincide with the findings by Muñoz et al. (2012), who used tomato as both donor and receiving crop and Muñoz et al. (2017), who used tomato as donor crop and lettuce as receiving crop.

We determined that only cascade's Test 1 ($p = 0.78$) and 4 ($p = 0.26$) and control's Test 5 ($p = 0.83$) presented a normal distribution ($p > 0.05$). However, a normal distribution was observed when the test was done including all the control data ($p = 0.22$), but not when analyzing the entire cascade dataset ($p < 0.05$). Both of these results were expected based on the homogenous irrigation in the control and the foreseeable variability in nutrient content of the leachates of the donor crop, analyzed in-depth in section "Water: Nutrient Content". When normality is studied for the residuals obtained through multiple combinations between tests and treatments, a non-normal distribution is observed in all of them ($p < 0.05$). On the other hand, the biggest differences between treatments were found in Test 3 and Test 5, in which the control produced 132 and 37% more yield, respectively, than their simultaneous cascade tests. Oppositely, the differences between treatments in Test 4 were narrowed to 6%. Despite this big variability, none of the comparisons between treatments (i.e., Control vs. Cascade in Test 3, 4, and 5) presented a significant homogeneity of variances ($p < 0.05$). Based on the non-normality detected, we applied Kruskal-Wallis test to compare between treatments. As expected, the results showed that the distribution differences between cascade and control were significant in every test ($p < 0.05$).

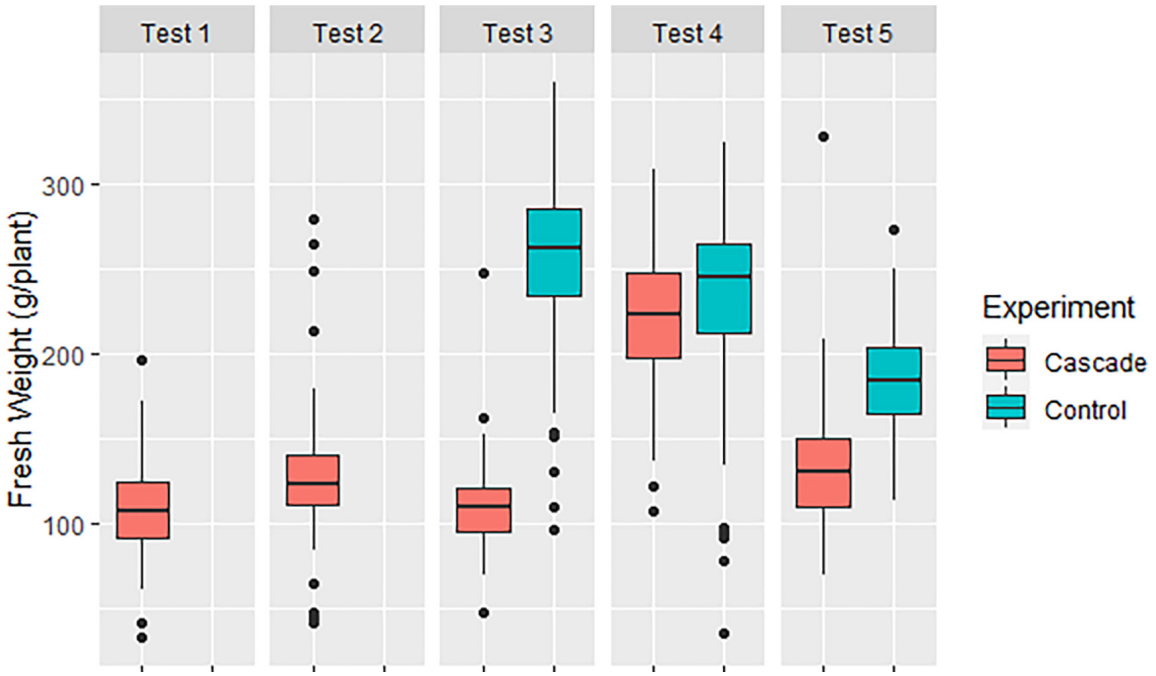

FIGURE 2 | Production of the different tests.

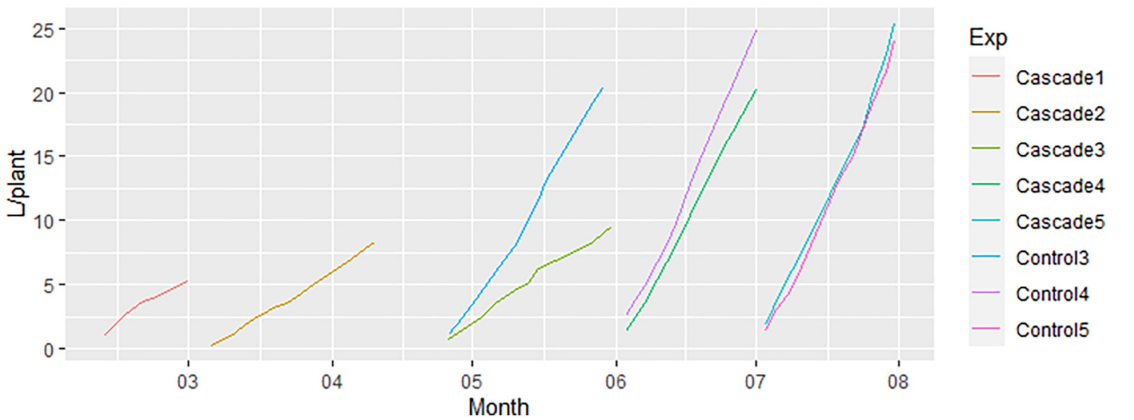

FIGURE 3 | Irrigation per plant of the different tests.

Water: Irrigation

All water irrigated to the receiving crop (8.8 m^3) was supplied through the leachates of the donor crop, thus being a residual flow that the lettuce crop is taking benefit from and avoiding the use of new water. Irrigation was increased in every successive crop based on the climatic conditions inside the greenhouse, irrigating 5 times more water in Test 5 than in Test 1 (**Figure 3**). The slope of the linear regression in Test 1 and 2 of the cascade treatment was similar (0.22 and 0.23 with R^2 of 0.96 and 1.00, respectively). This minimum difference was expected considering the temperatures reached inside the greenhouse during Test 1 and 2 periods, which were really similar (**Supplementary Figure S1**). We observed an anomaly in the water irrigated between the cascade system and the control in Test 3. Control Test 3 irrigated 20.30 L/plant with a slope of 0.60 (R^2 = 1.00), doubling the slope of cascade Test 3 (0.24 with R^2 = 0.99), that irrigated 9.60 L/plant. The statistical differences between the slopes obtained in the different tests and treatments were determined through a multi-step method with a final *t*-test. First, we determined if the individual accumulated irrigation per plant, test and treatment followed a normal distribution, which was confirmed for all of them ($p > 0.05$). A normal distribution was also confirmed for the residuals ($p > 0.05$). In the next step, the variances between treatments were labeled as non-homogeneous in Test 3 ($p < 0.05$) and homogeneous in Test 4 and 5 ($p > 0.05$). Considering the non-homogenous variances in Test 3 we calculated t_{calc} and t'_{calc},

obtaining the values 25.85 and 2.22, respectively. Considering that $t_{calc} > t'_{calc}$, we concluded that the slopes between cascade and control in Test 3 were statistically different. On the other hand, we performed a *t*-test for homogeneous variances for Test 4 and 5. t_{tab} values for Test 4 and 5 were both 1.71. t_{calc} for Test 4 was 6.38, while a value of 0.14 was obtained for Test 5. Given that $t_{calc} > t_{tab}$ for Test 4, the slopes between treatments in this test were observed to be statistically different. Oppositely, the slopes between treatments in Test 5 were statistically similar since $t_{calc} < t_{tab}$.

The ANCOVA applied to the data in **Figure 3** strengthened the statistical results obtained in the *t*-test since the same conclusions were obtained: differences between slopes in Test 3 and 4 were found to be statistically different, while in Test 5 were observed to be statistically similar.

To double-verify the statistical conclusions, we analyzed the "Estimated Marginal Means Of Linear Trends." The outcome of the analysis was the same as the one obtained through the *t*-test and ANCOVA: statistically differences between slopes in Test 3 and 4 ($p < 0.05$) and statistically similar slopes for Test 5 ($p = 0.89$) between cascade and control treatments.

The difference in irrigation rate in Test 3 was related to a shortening in the amount of leachates generated by the tomato crop, coupled with the parallel irrigation with leachates in the donor crop. To solve this problem, three options were considered.

First, to increase the irrigation in the donor crop, thus leaching more water that would be available for the receiving crop. This option was discarded because increasing the irrigation water use efficiency (assumed to be the volume of irrigated water applied per unit of yield) of the receiving crop while decreasing this parameter for the donor crop seemed contradictory. Moreover, the composition of the leachates will likely vary if more water is used in the donor crop. Second, to add water to the cascade system to meet the demand of the receiving crop. This option was also discarded because it would imply a modification in the composition of the irrigation of the receiving crop, thus blurring the effect of a cascade system. More important, both of these strategies were contradictory with the general aim of cascade systems: to take advantage of the residual flows of water and nutrients. If changes in the predefined system are needed, the value of the implementation of cascade systems decrease drastically. What could be seen as the best strategy is based on the practitioner or farmer priorities. When there is shortage of water, the farmer should prioritize in which system the residual flows are used. With a cascade set-up, the receiving crop can be prioritized during water shortage periods, minimizing the recirculation of water in the donor crop itself. However, if the donor crop is already not recirculating its own water and there is still water shortage, the first and second options outlined in this section can be applied considering the mentioned limitations.

The differences between treatments in Test 4 and 5 were narrower than the ones observed in Test 3 since there were not episodes of water shortening. Control Test 4 presented a slope of 0.82 ($R^2 = 0.99$), 18% higher than the slope for cascade Test 4 (0.69; $R^2 = 1.00$). Slope for Test 5 was the same for both treatments (0.81; $R^2 = 0.99$).

Water: EC and pH

Figure 4 shows the electrical conductivity (EC) values for the entire period under study. Since the control irrigation was manually controlled through the addition of fertilizers based on crop requirements, the EC values showed a great stability. On the other hand, the EC of the water used to irrigate the cascade crops increased over time, as the leachates of the tomato crop got richer in nutrients. The highest value was reached in the final cascade cycle, with 2.78 dS/m, much higher than the irrigation of the control (1.46 dS/m). Abou-Hadid et al. (1996) states that the perfect EC value for growing lettuce falls within the 1.0–1.5 dS/m range. Other literature specific for hydroponic lettuce mention that slightly higher values (1.2–1.8 dS/m) could be beneficial (Singh and Dunn, 2016). Most experiments found in the literature report greater yields within these values (Serio and Elia, 2001; Miceli et al., 2003; Samarakoon et al., 2019). Among the cascade test performed in our study, Test 1 (0.92 dS/m), Test 2 (1.25 dS/m) and Test 3 (1.68 dS/m) fell within the optimal range reported by Singh and Dunn (2016) (**Figure 4** – highlighted area). EC readings for drainage water in cascade systems are higher than the ones observed in **Figure 1**, such as 3.62 dS/m reported by García-Caparrós et al. (2016) in drained water of *Ruscus aculeatus*. The authors also reported that a dilution 1:2 with fresh water was able to decrease the EC to values <3 dS/m. In this sense, to compensate the high EC reached in the final cascade cycle, we added fresh water in the leachates, thus triggering the unstable values observed in **Figure 3** for Test 5. However, EC values obtained in the present study weren't as unsuitable as expected considering the focus on salt tolerance from the literature around cascade systems. Oppositely to EC, pH values decreased overtime for the cascade cycle, as shown in **Supplementary Figure S3**. A pH optimal range to grow hydroponic lettuce of 6.0–7.0 is reported by Singh and Dunn (2016), and of 5.6–6.0 by Brechner and Both (2013). Considering a broad range, all cascade tests presented optimal pH mean values except Test 1, that had a mean pH value slightly higher than 7.

Water: Nutrient Content

Figure 5 shows the accumulated irrigated nutrients per plant per test. As expected, the nutrients irrigated in the cascade treatment increased over time, coinciding with the amount of nutrients leached by the tomato crop. K was the nutrient with the highest quantities in the cascade irrigation (reaching 10.7 g/plant in Test 5 of the cascade system), followed by Ca and N, reaching 5.0 and 3.6 g/plant in Test 5, respectively. On the other hand, nutrients irrigated in the control treatment presented a similar behavior among tests.

Although the linear regressions observed in section "Water: Irrigation" regarding the irrigated water presented R^2 values around 0.99, the evolution of the accumulated irrigated nutrients per plant (**Figure 5**) could also be affected by the concentration of nutrients in the water flows. As expected, the nutrient in the control irrigation followed a completely linear regression in all nutrients ($R^2 \approx 0.99$) since the nutrient solution supplied to the crop is completely homogenous. On the other hand, the linear

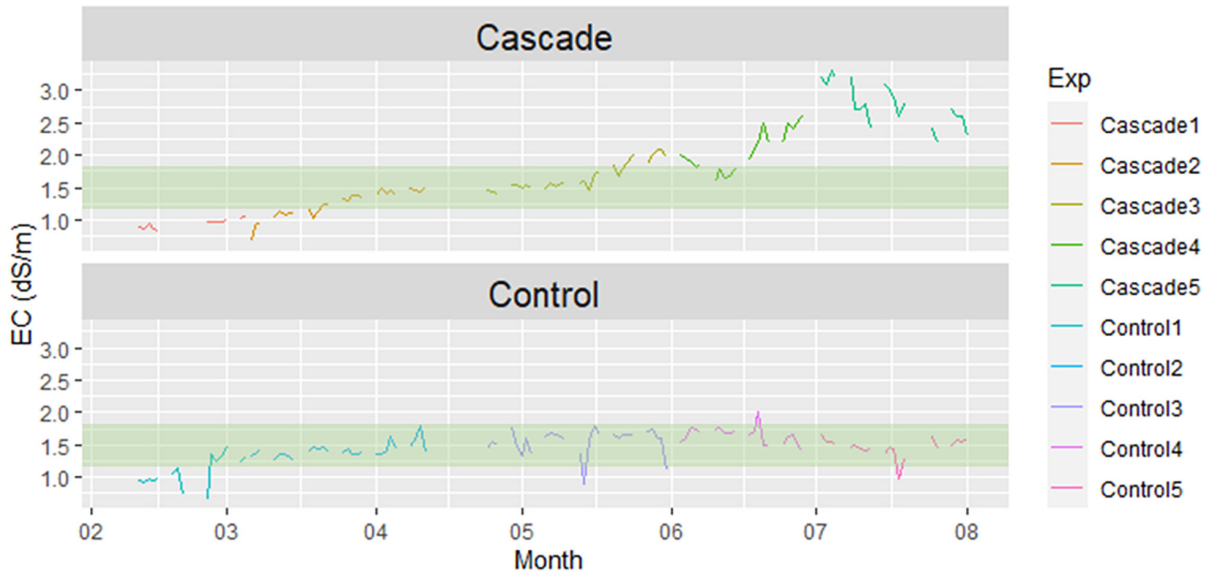

FIGURE 4 | Electrical conductivity (EC). Highlighted area represents the suitable EC range to grow hydroponic lettuce stated by Singh and Dunn (2016).

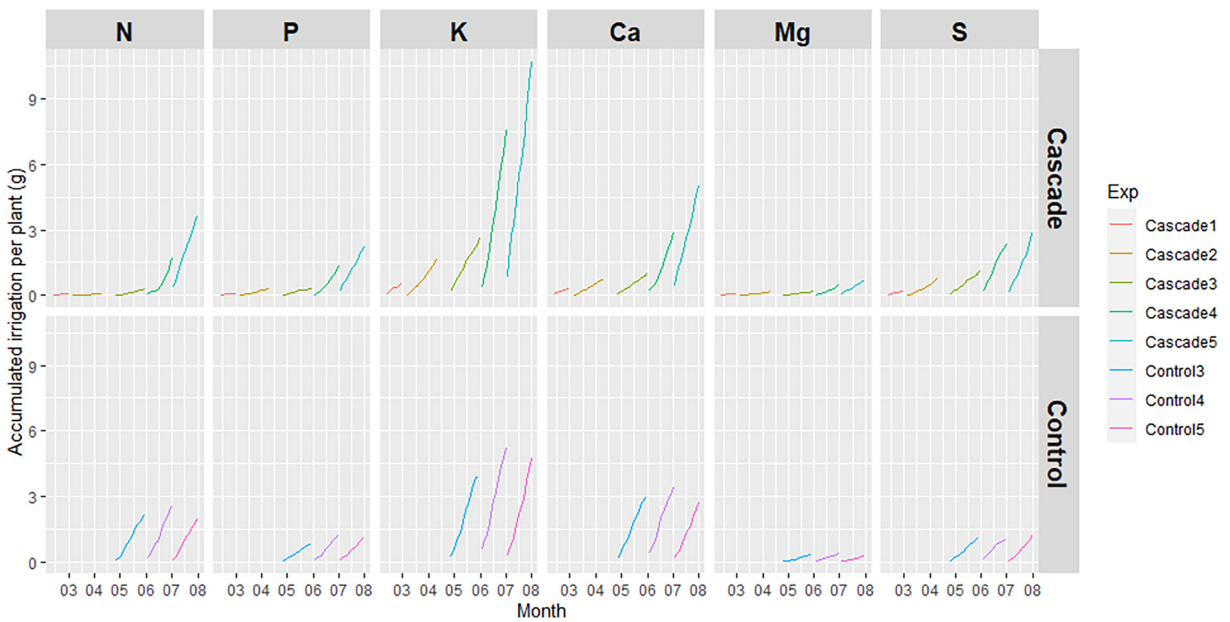

FIGURE 5 | Accumulated irrigated nutrients per plant per test.

regression of the accumulated irrigated nutrients in the cascade treatment highly fitted with the observations, with R^2 values higher than 0.97. The only exception was N in Test 4, which presented a $R^2 = 0.85$. The reason behind this behavior was the low quantities of this nutrient available through the leachates of the donor crop in Tests 1, 2, and 3. In this sense, we can see that this N limitation is overcome in the middle of Test 4 (concentration jumps from 35 to 111 mg/L), probably related to a N increase in the nutrient solution supplied to the donor crop. In this sense, the nutrient supply to the donor crop and its stability can be highlighted as a relevant parameter to also stabilize the nutrient content of the leachates and thus, provide a balanced and reliable source of nutrients to the receiving crop in a cascade system.

The slope of the accumulated irrigated nutrient per plant per test was compared between treatments for every specific nutrient through ANCOVA (Andrade and Estévez-Pérez, 2014). A prior step was done to assess the normality of the residuals, confirming the normality for all nutrients and tests apart from N in Test 3 with a $p < 0.05$. An analysis to determine the homogeneity of

variances was applied to all combinations of nutrients and tests. As expected, Test 3 for N also presented statistically different variances ($p < 0.05$). Nonetheless, other cases were detected: P, K Ca and Mg for Test 3, S for Test 4 and K, Mg and S for Test 5. In this sense, ANCOVA was applied to the remaining cases with normal distribution and homogeneity of variances: Test 3 for S, Test 4 for N, P, K, Ca and Mg and Test 5 for N, P, and Ca, observing statistically different slopes between treatments in all of them ($p < 0.05$). The same outcome was obtained when analyzing the "Estimated Marginal Means Of Linear Trends" for these cases, although one exception was detected. P in Test 4 presented statistical different slopes through ANCOVA ($p < 0.05$), but statistical similarity was observed through "emtrends."

Biomass: Nutrient Content

Figure 6 shows the nutrient content in the lettuce for cascade and control tests. Every nutrient had a different behavior among tests, especially in the cascade treatment. N increased over time, especially between the 2nd and the 3rd test, and the 3rd and the 4th test. The 5th test presented the highest concentration of this nutrient. This tendency was expected based on the findings of the previous section, where we observed the increasing concentration of N in the leachates of the donor crop. Although the tendency related to the concentration in the water flow was observed for most nutrients, the 5th test was not the one with the highest nutrient content in the biomass for other nutrients analyzed. The 4th test was the one with the highest concentrations for P, K, Mg, and S with 0.97, 11.43, 0.34, and 0.35%, respectively. For Ca, the concentration in the 1st test was higher than the one observed for the 4th test (1.65%), which doesn't correlate with its supply through the cascade system (**Figure 5**), and neither with the concentration of Ca in the leachates flow from the donor crop, which increased overtime. This high absorption of Ca in the first test neither correlates with traditional compatibilities/incompatibilities between nutrients (Mulder, 1956), since absorption of Ca may be depressed by excessive amount of Mg, which was also highly absorbed in Test 1, or favored with excessive nitrate concentrations, which was limitedly supplied through the cascade system in the first tests.

As expected, the control presented less differences between tests, especially for N. Due to differences in irrigated water between the cascade and the control in Test 3, nutrient content in the control was higher than in the cascade treatment for this test for all nutrients. When the irrigated water differences between the control and the cascade tests was normalized again in Tests 4 and 5, differences between treatments decreased. The average N content for the control (4.4%) was really similar to the one presented in tests 4 and 5 of the cascade system (4.4%). We could also find really similar concentrations between treatments in Test 4 for K (≈11.02%) and S (≈0.35%), and in Test 5 for P (≈0.78%), K (≈9.9%) and Ca (≈1.3%).

Decreasing Eutrophication Potential and Nutrient Depletion

Table 2 shows the amount of nutrients that were taken up by the lettuce plants (**Supplementary Table S1**) with respect to the irrigated through the cascade system (**Supplementary Table S2**). **Supplementary Table S3** shows the amount of nutrients leached by the lettuce plants. The ratio between nutrients taken up and irrigated decreased over time given the fact that the amount of nutrients being irrigated through the cascade system increased as the leachates from the tomato crop got richer. We can see that all N irrigated through the cascade system was taken up by the lettuce crop in tests 1 and 2, with two major consequences. First, a possible nitrogen deficiency that made Test 1 the one with the lowest yield, making the cascade set-up inefficient in terms of production. Second and with a bioremediation perspective, the removal of all N from the residual flow used to irrigate the receiving crop triggered the complete mitigation of the marine eutrophication impacts caused by the leachates of the donor crop. This mitigation decreased over time, getting down to 43.1% of removal in Test 3, and being only 6.5% in Test 5. The removal of P was already below one third in Test 1, thus just mitigating part of the freshwater eutrophication impacts. The removal rate of P got below 10% in Test 2, and it reached its lowest level in Test 5 with only 1.9%. Given the non-renewable nature of P, the reuse of P is not only relevant to avoid freshwater eutrophication, but also in terms of mitigating its depletion. K was the nutrient irrigated with the most quantity through the cascade system (**Figure 4**). However, high removal rates were only reached in Test 1 (63.4%). Removal rate in Test 2 already got below 25% and kept decreasing over time.

Since the irrigation in the control was more controlled both in terms of water and nutrients, the removal rates among tests were more stable. This was specially the case of N, Ca, and Mg, while P slightly decreased in control tests 4 and 5, probably due to a combination of changes in the nutrient solution and an increase in P consumption of the donor crop in the production stage.

Scaling the System

Other studies that considered the use of a the leachates of a donor crop to irrigate horticultural products did not account for the variability of nutrients along the cycle of the donor crop. For example, Choi et al. (2011) first collected the waste nutrient solution from a hydroponic tomato crop and then applied it to a cabbage crop instead of connecting a donor and a receiving crop simultaneously in a cascade system. Therefore, the nutrient concentration supplied by the authors was stable, avoiding problems related to variability but still subjected to possible nutrient limitations. However, NO_3^- concentration in Choi et al. (2011) study (1285 mg/L) was always above all the NO_3^- values determined in our study. Considering the higher nutrient content of lettuce compared to cabbage (Weber, 2016), it is not surprising that Choi et al. (2011) obtained higher yields than the control. In a similar experiment, Zhang et al. (2006) also obtained higher yields using tomato leachates in muskmelon and cucumber. By using the same donor crop, Incrocci et al. (2003) irrigated a cherry tomato cycle. Since the focus of the author was on the effect of salinity, the waste nutrient solution was adjusted in terms of nutrient content to meet the same amount as the control: 161 mg/L of N. This concentration was lower than the one used by Choi et al. (2011). However, this value was not reached in the cascade system of

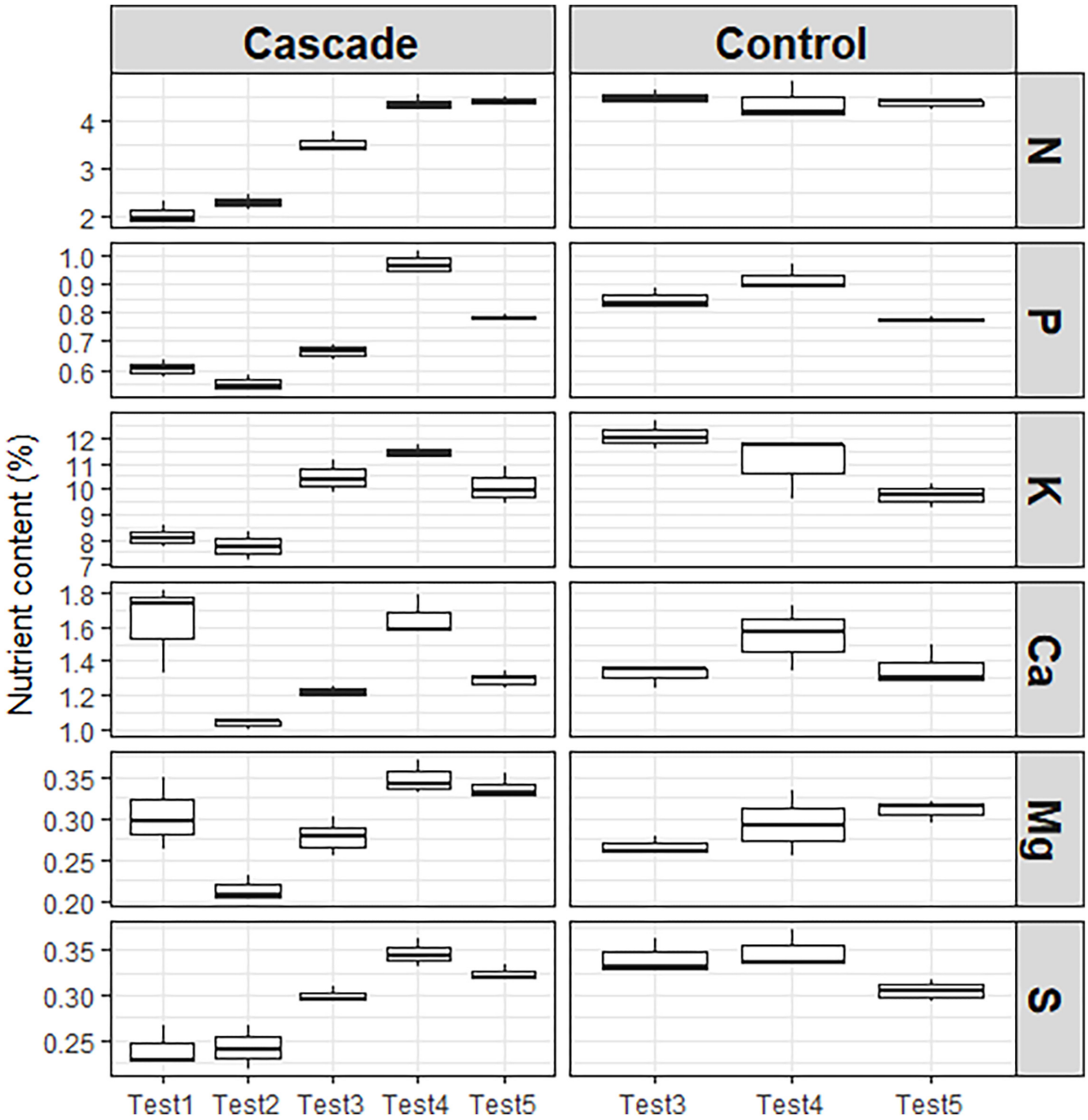

FIGURE 6 | Nutrient context per plant (%).

the present study until Test 4, in which the yield differences between the control and the cascade plants was the narrowest (**Figure 2**). With a cascade set-up using tomato as both donor and receiving crops, Muñoz et al. (2012) converge with the present study since the authors observed yield reductions in the receiving crop compared to the control [as also found by Muñoz et al. (2017)], irrigated with N concentrations of 198 and 310 mg/L, respectively. The N concentration in the tomato leachates in Muñoz et al. (2012) (198 mg/L) is more than two times the average concentration leached by the tomato donor crop of the present study (72.5 mg/L). This probably indicates excessive N in the irrigation of the donor crop, which could be adjusted without yield reductions as suggested and quantified by the same authors.

However, none of these authors considered the variability of nutrient content as a relevant parameter in cascade systems. Taking into account the need for a suitable dimensioning between donor and receiving crops in cascade systems to define best practices (Muñoz et al., 2017), we scaled the system in terms of nutritional demand of the receiving crop.

TABLE 2 | Uptake (%) of irrigated nutrient per plant in cascade treatments.

Nutrients	T1 – Cas (%)	T2 – Cas (%)	T3 – Cas (%)	T3 – Con (%)	T4 – Cas (%)	T4 – Con (%)	T5 – Cas (%)	T5 – Con (%)
N	143.8*	115.9*	43.1	17.1	23.3	16.4	6.5	16.7
P	27.8	9.0	8.7	10.1	6.4	6.9	1.9	5.0
K	63.4	24.4	17.4	31.8	13.4	19.8	5.1	16.0
Ca	23.7	6.9	5.4	4.6	5.1	4.2	1.4	4.0
Mg	15.2	6.4	6.3	8.1	6.3	7.3	2.4	8.1
S	4.5	1.6	1.1	3.2	1.3	3.0	0.6	2.0

*Cas, cascade system; Con, control. *Values above 100% mean complete uptake. Due to the small amount of nutrients leached by the donor crop during T1 and T2 the experimental and analytical error is increased.*

TABLE 3 | Amount of lettuce that could be produced in the cascade system under analysis considering the application of all tomato leachates.

	Units of lettuce plants/tomato plant					Units of lettuce plants/171 tomato plants				
Nutrients	T1	T2	T3	T4	T5	T1	T2	T3	T4	T5
N	0.1	0.6	1.7	6.3	9.1	17.1	101.8	286.4	1073.5	1554.2
P	0.9	8.5	7.1	29.6	30.0	145.6	1460.9	1216.5	5065.1	5131.1
K	0.4	3.4	4.5	12.8	11.0	63.9	585.3	769.7	2188.8	1874.6
Ca	1.6	12.2	13.6	36.8	40.3	267.6	2091.5	2329.4	6288.4	6888.7
Mg	2.1	13.3	12.8	29.5	28.7	366.6	2275.7	2181.9	5036.6	4912.8
S	5.2	52.9	65.3	139.1	99.4	897.6	9042.9	11161.7	23779.4	17005.9

To quantify the scaling between donor and receiving crops in the system under study, we considered the average uptake of the control for all nutrients [N (4.4%), P (0.84%), K (11.0%), Ca (1.4%), Mg (0.29%), and S (0.33%)] and the average yield of the control (224 ± 58 g/plant). **Table 3** shows the quantity of lettuce that could be grown with the maximum quantity of nutrients that could be supplied through the cascade system, considering that all leachates of the donor crop are applied to the receiving crop through the cascade system.

As we can see in **Table 3**, the limiting nutrient was always N, allowing to grow a 1:10 and a 6:10 system for Test 1 and 2, respectively. P (9:10) and K (4:10) in Test 1 were the other nutrients with less than a 1:1 ratio between the tomato donor crop and the lettuce receiving crop. Considering N as the limiting nutrient, we can state that in our system with 171 tomato plants, we could grow 17 lettuce plants in Test 1, while being able to grow 1554 lettuce plants in Test 5, thus giving the practitioners three main options in terms of crop management, considering an ideal situation where we have an area that can allocate 1554 lettuce plants, which is the number of lettuces that we could grow in Test 5 considering the N content in the tomato leachates.

First, to grow 17 lettuce plants in all tests. This option will ensure the production of the same amount of plants in every test but will present huge inefficiencies in terms of space (if the rest of the area is not occupied by other crops) and nutrient flows, discharging a great quantity of nutrients that will increase over time as the leachates of the donor crop become nutrient-richer. This nutrient inefficiency could be mitigated by applying recirculation in the donor crop itself. Second, to grow 17 (Test 1), 101 (Test 2), 286 (Test 3), 1073 (Test 4), and 1554 (Test 5) lettuce plants connected to the cascade system, while growing 1537 (Test 1), 1453 (Test 2), 1268 (Test 3), and 481 (Test 4) lettuce plants irrigated using a conventional nutrient solution. With this option, yield would be maximized, while adapting the nutrient demand of the lettuce cycles to the nutrient supplied through the cascade system without requiring further nutrient management. Third and finally, start with 1554 lettuce plants connected to the cascade system. To avoid nutrient deficiencies as the ones observed in the present study, the nutrient content in the leachates tank used to irrigate the receiving crop must be adjusted. A specific amount of all nutrients should be added in Test 1, while N, P and K should be added in Test 2 and 3, and only N should be added in Test 4. This option would be the most efficient to produce the same amount of lettuce over time while minimizing the nutrient input. However, it would require a detailed control of nutrient flows to avoid nutrient deficiencies. It is important to understand that, despite the strategies mentioned above, the list of management practices is endless: mix and store the leachates during Test 1 to increase the nutrient supply to Test 2 (and omit Test 1 of lettuce), increase the water irrigated to the donor crop, etc. However, the value of all possible strategies rely on their performance in real crops. Therefore, we highlight the need to evaluate and quantify the potential yield that could be reached through the application of these strategies. In addition, the analysis of the nutritional and water flows in pilot or full-scale experiments should be included in the list of upcoming challenges to verify that the plant nutrition is optimal and that the cascade system is well scaled to mitigate eutrophication impacts derived from the depletion of nutrients in the donor crop.

CONCLUSION

The present paper has presented an evaluation of a cascade system with a long-cycle tomato donor crop and five

successive cycles of lettuce. The assessment of the agronomic performance and the nutrient flows have shed light on the potential of these systems to mitigate nutrient depletion in cities while producing food in the framework of urban agriculture.

The variation of nutrient content of the leachates produced by the donor crop is a key parameter to plan the amount of plants that can be planted of the receiving crop. The early stage of the donor crop could only produce 0.1 lettuces per tomato plant, with N as the limiting nutrient. On the other hand, the late stage of the donor crop was able to leach enough nutrients to feed 9 lettuces per tomato plant. However, attention must be paid on the electrical conductivity of the water flow to stay within non-harmful values. Nevertheless, the cascade system was shown to be efficient to mitigate the nutrient discharge of open systems, especially in terms of N and P to avoid eutrophication impacts in the early stage of the tomato crop. To this end, a good scaling between the two crops of the system is vital to tap the full potential of the cascade set-up, while having different options in terms of system management.

Given the findings of this study, we encourage future researchers to test different kind of horticultural crops. Considering the nutritional problems in the beginning of the cycle of the donor crop and the harmful salinity that can be reached at the end, further research should test possible combinations of donor and receiving crops that minimize these two problems. Reporting the limitations of these kind of systems is key to a transparent process of decision-making in the implementation of optimization strategies in urban agricultural systems. In terms of experimental design, further research assessing the nutritional flows of cascade systems should increase the number of plants that will be analyzed in terms of nutrients to precisely determine the variability of concentrations within the same treatment and test.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MR-S, AP-B, GV, and XG conceived the original idea for the study. MR-S, VA-P, and FP set up, supervised, and acquired the data for the experimental tests. MR-S processed and analyzed the data and took the lead in writing the manuscript. All authors were responsible for the conception and design of the study, critically revised the draft for important intellectual content, and gave their final approval to the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.596550/full#supplementary-material

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Recycling Nutrient Solution Can Reduce Growth Due to Nutrient Deficiencies in Hydroponic Production

*Alexander Miller, Ranjeeta Adhikari and Krishna Nemali**

Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN, United States

It is common in hydroponics to supply nutrients to crops by maintaining electrical conductivity (EC) of the recycling solution at a target level. Levels of individual nutrients in the solution are generally not assessed as their regular measurement and adjustment can be both expensive and technically challenging. However, the approach of growing crops at a target EC can potentially result in nutrient imbalances in the solution and reduced growth. We quantified the effects of recycling on solution EC changes, tissue nutrient concentration, canopy growth rate, plant water status, and shoot and root weight of lettuce (*Lactuca sativa*) in a greenhouse. The tap water quality was moderately alkaline and similar to that commonly observed in many commercial greenhouses. In our research, recycling solution maintained at a target EC (1.8 $dS{\cdot}m^{-1}$) significantly reduced shoot fresh (22–36%) and dry weight compared to the control supplied regularly with freshly prepared solution at the target EC. Further, recycling significantly decreased N, P, K, and Fe and increased Na and Cu levels in the tissue, in addition to increasing solution EC between adjustments compared to the control. Using image analysis of groups of plants, we identified that the negative effects of recycling on canopy area started 2 weeks after transplanting. Based on these results, we hypothesized that certain unwanted compounds (e.g., bicarbonates) and slowly consumed elements (e.g., Ca, Mg) were added to the recycling solution through the alkaline tap water with time. Their accumulation "artificially" increased solution EC and "masked" the lower than optimal levels of major nutrients in the solution, leading to the reductions in the concentration of nutrients in the tissue and plant growth. Supporting this, the negative effects of recycling were not observed when the recycling solution was either discarded after 2 weeks of use or made using reverse osmosis water and continuously used. Our findings aid in proper management of recycling solution in hydroponic lettuce production.

Keywords: fertilizer solution, macronutrients, micronutrients, segmentation, water quality

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Toshiki Asao,
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Reviewed by:
Maria Giordano,
University of Naples Federico II, Italy
Amir Khoshgoftarmanesh,
Isfahan University of Technology, Iran

***Correspondence:**
Krishna Nemali
knemali@purdue.edu

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INTRODUCTION

Hydroponics industry is becoming popular in the United States with nearly 2500 enterprises and overall revenue of $ 0.83 billion in 2019 (Us Specialized industry report, 2019). The increased demand for fresh, locally grown, and safe food is driving the growth of the industry in the United States Lettuce accounts for nearly 7% of overall share of the industry in the United States

(Us Specialized industry report, 2019). Hydroponic lettuce is mostly grown in a solution enriched with nutrients and oxygen using different production systems including nutrient film, deep flow, floating bed, and ebb and flow systems (Resh, 2012; Son et al., 2020).

Nutrient solution is usually recycled during hydroponic production (Jensen, 1997; Nederhoff and Stanghellini, 2010) to reduce wastage. Electrical conductivity (EC) of the recycling solution is measured to determine its nutrient status (Brun et al., 2000; Christie, 2014; Jones, 2016). The EC measurement is an indirect and quick-way to measure the total concentration of ions, including nutrients, dissolved in a solution (Graves, 1983; Nemali, 2018). A common practice in hydroponics is to maintain a target EC level in the recycling solution by frequently measuring and adjusting EC using water and nutrients. It is assumed that adequate amount of nutrients can be made available to plants by maintaining the recycling solution EC at the target level. However, maintaining solution EC at a target level may not necessarily result in optimal concentration of individual plant nutrients in the recycling solution. It is not possible to assess the levels of individual ions dissolved in the solution based on EC measurements (Nemali, 2018). Without knowing the levels of individual plant nutrients, it is difficult to ensure their optimal levels in the solution. Therefore, despite maintaining solution EC at a target level, it is possible that certain plant nutrients can become excess or deficient in the recycling solution with time.

Good quality irrigation water can be a limiting factor in many commercial hydroponic greenhouses (Allende and Monaghan, 2015). Irrigation water quality is alkaline in many parts of the United States Midwest (Kaushal et al., 2018), due to the presence of bicarbonates (HCO_3) of calcium (Ca) and magnesium (Mg) (Kaushal et al., 2013, 2018; Guo et al., 2015). When irrigation water with high alkalinity is used in production, Ca, Mg, and HCO_3 can accumulate in the recycling solution (Baars, 1992; Carmassi et al., 2003; Trejo-Téllez and Gómez-Merino, 2012; Sambo et al., 2019). This is because HCO_3 can't be transported through the plant roots (Poschenrieder et al., 2018) while Ca and Mg are slowly consumed by plants (Bugbee, 2004). A direct effect of their accumulation is an "apparent" increase in the recycling solution EC (Zekki et al., 1996). It is possible for solution EC to be close to target EC even when the concentration of major plant nutrients is sub-optimal, due to increased concentration of Ca, Mg, and HCO_3 in the recycling solution. In addition, sodium (Na) in irrigation water can accumulate in the recycling solution (Fernandez, 2017) and cause osmotic stress to plants (Hopkins et al., 2007). There is limited research that quantified the negative effects of using recycling nutrient solution during production and identified physiological reasons for the observed effects of recycling on plant growth in hydroponics.

In addition, research that aimed at developing practical remedies to minimize recycling effects on plant growth is minimal. In some large commercial operations, recycling solution is regularly analyzed in a laboratory and the levels of individual nutrients are adjusted using complicated worksheets on computers. In addition to being expensive, adjusting individual nutrients regularly can be technically challenging to growers. Thus, many growers prefer to discard the recycling solution (Zekki et al., 1996; Lykas et al., 2006; Samarakoon et al., 2006) instead of managing the concentration of individual plant nutrients. However, there are no established guidelines on when to discard the recycling solution. Regardless of species and growth environment, the recycled solution should be discard when the negative effects on plant growth just start to appear. This approach can minimize fertilizer wastage and reduce environmental pollution resulting from frequent discarding. For this, continuous plant growth monitoring is needed to determine the correct stage for discarding. Shoot growth of leafy greens can be monitored by destructively harvesting and weighing plants (Li et al., 2020). However, regular destructive harvests may not be popular due to plant loss. In addition, regular destructive harvests increase labor costs. Currently, there are limited choices for non-destructive crop growth assessments in hydroponic production. Image analysis can be used to non-destructively assess lettuce growth (Li et al., 2020). It is also possible to use image-based measurements for continuous plant growth monitoring on easy-to-use devices like smartphones (Li et al., 2020). However, the efficacy of image analysis technique for timely detection of the negative effects of recycling on plant growth was never tested in hydroponic production.

The aims of the study were to evaluate the effects of continuous recycling on solution EC, tissue nutrient concentration and productivity of lettuce, and develop optimal strategies for managing recycling nutrient solution in hydroponic production. Specifically, our objectives were to (i) quantify the effects of recycling solution on lettuce (*Lactuca sativa*) growth, (ii) identify the stage when recycling effects are observed on plants using image analysis, (iii) relate observed effects of recycling on plant growth to measured physiological responses, and (iv) develop practical remedies to minimize the effects of recycling on lettuce growth.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

We conducted three separate experiments in the study. Experiment 1 was intended to quantify the effects of recycling on plant growth. We designed experiment 2 to understand physiological reasons for the observed effects of recycling in experiment 1 and identify the stage when recycling effects are observed on plants using image-based assessments. Experiment 3 was conducted to validate a hypothesis developed in experiment 2 and identify remedies for minimizing recycling effects in commercial production. We grew leaf lettuce (cv. Black Seeded Simpson) in experiments 1 and 2 because of its fast growth rate, therefore increased probability to detect growth differences. In experiment 3, we used cultivars of lettuce belonging romaine (cv. Amadeus), leaf (cv. Black Seeded Simpson), butterhead (cv. Rex), and oakleaf (cv. Cedar) groups.

Plant materials, seedling production, and growth environment were similar in all experiments. Seeds (Paramount Seeds Co., Stuart, FL, United States) were sown in sheets of rock wool cubes (200 per sheet, 2.5 cm diameter each, Grodan, Roermond, Netherlands) that were placed on watertight trays

(54 cm × 27 cm × 3 cm; Greenhouse Megastore, Danville, IL, United States) for sub-irrigation. The trays were filled every day with approximately one liter of tap water to keep the rock wool cubes moist. After emergence, we thinned the seedlings to one per cube and sub-irrigated them with a dilute nutrient solution containing nitrogen at a concentration of 50 mg·L^{-1}. A water-soluble fertilizer containing 20 N-4.4 P-16.6 K (20-10-20, The Scotts Co., Marysville, OH, United States) mixed with tap water was used to prepare the nutrient solution. Seedlings were transplanted into hydroponic production systems (see "Hydroponic Systems" section below) after 10 days from the sowing. Plants were grown in a glass greenhouse located at Purdue University, West Lafayette, IN, United States. The daily average (standard deviation) air temperature, light integral, and relative humidity in the greenhouse during the experiments were 23.7 ± 1.81°C, 10.5 ± 3.82 mol·m^{-2}, and 80.2 ± 8.76%, respectively.

Hydroponic Systems

A custom-built hydroponic production system similar to commercial flood tables was used in experiment 1 (**Figure 1A**). It was built using black plastic trays (91 cm × 91 cm × 10 cm, 82.8 L volume; Botanicare, Vancouver, WA, United States), nutrient solution reservoirs (76 L; Active Aqua Premium White Reservoir, Petaluma, CA, United States), submersible pumps (9.5 L min^{-1}; TotalPond, West Palm Beach, FL, United States), and vinyl tubing (0.013 m internal diameter; CropKing Inc., Lodi, OH, United States). The trays with covered lids (1.3 cm Styrofoam, U-Line, Pleasant Prairie, WI, United States) were arranged on a greenhouse bench (7.6 m × 1.5 m × 1.1 m). The lids contained holes for inserting net pots (5.1 cm diameter; General Hydroponics, Chico, CA, United States). Reservoirs stored approximately 20 L of the nutrient solution, which was continuously recycled during production. An extension fitting was inserted in the outlet end to enable the nutrient solution to accumulate in the tray before draining back to the reservoirs. The depth to which nutrient solution accumulated in the tray was approximately 1.0 cm. At steady state, approximately 8 and 12 L of recycled solution was present in the flood tables and reservoirs, respectively. Each tray housed 15 net pots in five rows of three each. Each net pot contained one rock wool cube with a germinated seedling. The net pots were spaced 23 cm apart within a row and 15 cm apart between the rows. The base of the net pot rested on the bottom of the tray after inserting through the hole, thereby exposing the lower portion of rock wool cube to the nutrient solution.

Experiment 2 required frequent plant movement and measurements of solution volume (see "Measurements" section below). To facilitate this, we grew plants in hydroponic seeding inserts (72 cell, Hydrofarm, Greenhouse Megastore, Danville, IL, United States; **Figure 1B**) placed on watertight trays (9.4 L; 1020 tray; 54.5 cm × 27.8 cm × 6.2 cm, Greenhouse Megastore). The

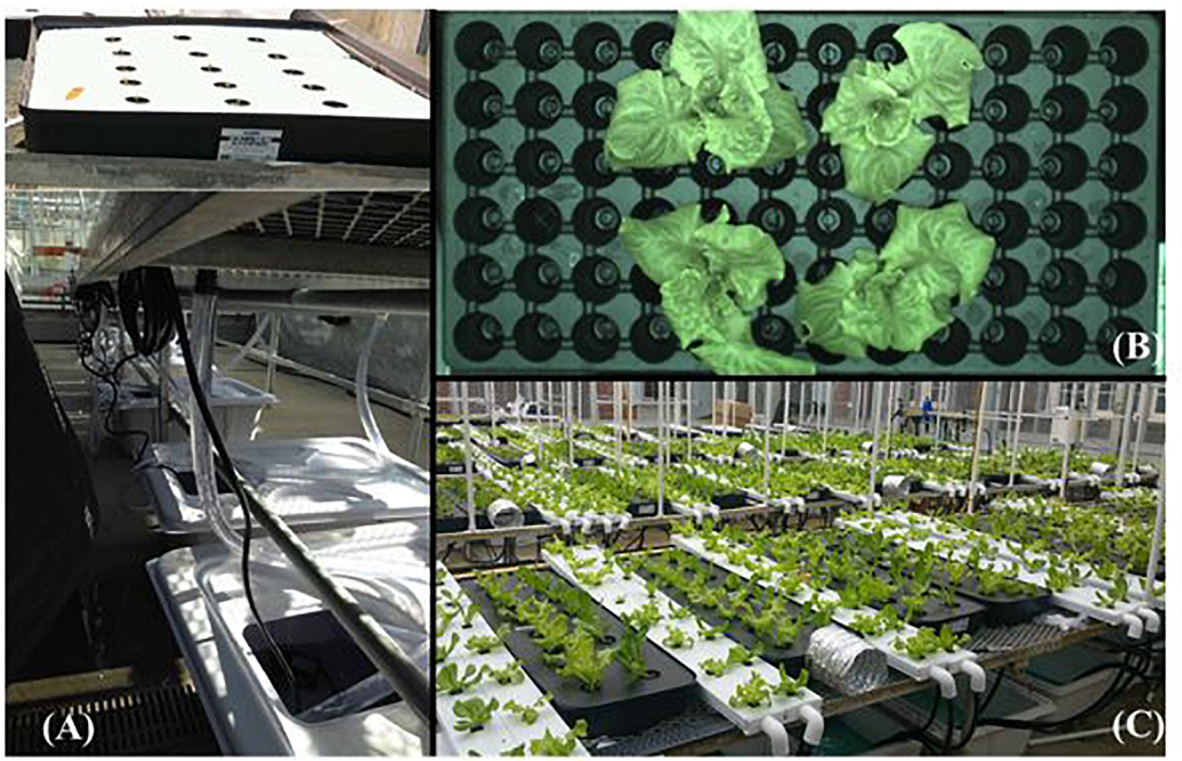

FIGURE 1 | Custom-built hydroponic production systems used in experiments 1 **(A)**, 2 **(B)**, and 3 **(C)**. See "Materials and Methods" section for a description about production systems.

trays were arranged on the greenhouse bench. Each tray stored 2 L of nutrient solution to a depth of approximately 1.5 cm. Although plants were grown in a "passive" hydroponic system, a significant volume of nutrient solution was changed daily (see "Treatments" section below) to ensure that oxygen levels were not compromised. The rock wool cubes with seedlings were directly placed in the cells of the seeding inserts. In each tray, four rock wool cubes with seedlings were arranged in two rows of two each. The spacing between plants was 17 cm within a row and 13 cm between the rows. The bottom of the insert cells was cut open to ensure that the base of the rock wool cubes were in direct contact with the nutrient solution.

Plants in experiment 3 were grown in custom-built constant flood table (CFT) and nutrient film technique (NFT) systems (**Figure 1C**). We built the CFT and NFT systems using reservoirs and submersible pumps similar to those described for experiment I. In addition, black flood tables (122 cm × 31 cm × 10 cm; Botanicare, Vancouver, WA, United States) and lids for the CFT and white channels (150 cm × 12 cm × 4 cm; CropKing Inc., Lodi, OH, United States) and covers for the NFT were purchased from the respective vendors to build the hydroponic systems. The reservoirs stored approximately 20 L of nutrient solution. Black vinyl tubing (1.3 cm internal diameter; Crop King Inc.) was used to connect the pumps to the two production systems and drain the nutrient solution back to the reservoirs. To allow for uniform flow from the inlet to outlet end, the NFT channels were raised by 0.15 m (9.8% slope). An extension fitting (5 cm; Botanicare) was inserted into the outlet of the CFT tray for solution to accumulate (approximately 4 cm depth) in the tray before draining. Flow valves (1.3 cm; Green Back in-line valve, Botanicare) connected to the inlet tubing were used to control the rate of nutrient solution delivery to the CFT and NFT systems. The measured flowrate on the outlet end of the CFT and NFT systems was approximately 6.0 and 1.0 $L{\cdot}min^{-1}$, respectively. Two NFT channels (one unit) and one CFT tray (one unit) were connected to a reservoir. Each production system unit housed 16 plants belonging to four cultivars with four plants each. The rock wool cubes with seedlings were transplanted in the NFT system by inserting through the square holes (3 cm) on the channel covers and making them contact with the base and solution flowing through the channel. The holes were spaced 18 cm within a channel and 13 cm between the channels. In the CFT system, rock wool cubes with seedlings were placed in net pots (General Hydroponics), which were inserted into the holes (6 cm) made on the CFT lids. During the steady flow state, nearly three-fourth of the rock wool cube was immersed in the nutrient solution. The holes on the CFT lids were spaced 17 cm apart within a row and 13 cm apart between the rows.

Treatments

Plants were exposed to two solution treatments ("recycle" and "control") in experiments 1 and 2. Nutrient solution was prepared by mixing the same water-soluble fertilizer used during the seedling stage in both experiments. The target nutrient solution EC in both the treatments was 1.8 $dS{\cdot}m^{-1}$ (pH between 6.2 and 7.1). The EC measurement included the contribution of dissolved salts in the tap water (approximately 0.7 $dS{\cdot}m^{-1}$). In the control treatment, old solution in the trays and reservoirs was discarded and either 20 (experiment 1) or 2 L (experiment 2) of freshly prepared nutrient solution with an EC of 1.8 $dS{\cdot}m^{-1}$ (corresponding to 166 mg $N{\cdot}L^{-1}$) was added to the reservoirs three times a week (experiment 1) or daily (experiment 2). In the recycle treatment, the leftover nutrient solution in the trays and reservoirs was retained. However, solution volume (20 L in experiment 1 and 2 L in experiment 2) and target EC (1.8 $dS{\cdot}m^{-1}$) were maintained, either three times a week (experiment 1) or daily (experiment 2). For this, the volume of leftover solution in the reservoirs (experiment 1) and trays (experiment 2) was measured and adjusted to the target volume (20 or 2 L, respectively) using the tap water. The solution EC was measured after adjusting for the volume by adding a fertilizer stock solution to increase the solution EC to the target level of 1.8 $dS{\cdot}m^{-1}$.

In experiment 3, plants were exposed to three solution treatments [control, 2 week discard (or 2 WkD), and recycling with reverse osmosis (RO) water (or Rec_RO)], two production system (CFT and NFT) treatments, and four cultivar treatments (Amadeus, Black Seeded Simpson, Cedar, and Rex). The control treatment was similar to that described above for experiments 1 and 2. The 2 WkD treatment was similar to the recycle treatment described above for experiment 1, except that the old nutrient solution was discarded 2 weeks after use and reservoirs were refilled with 20 L of freshly prepared solution at target EC. Recycling continued with periodic EC adjustment to target level. The Rec_RO treatment was similar to recycle treatment described above for experiment 1, except that RO water was used instead of tap water to grow plants. The target EC of Rec_RO treatment was 1.1 $dS{\cdot}m^{-1}$, a value equivalent to the EC solely due to dissolved fertilizer ions in other treatments. The EC of the solution was regularly adjusted to the target EC as described above for experiment 1. The differences between the two production systems were previously described in the "Hydroponics Systems" section. The cultivars belonging to different groups varied in their growth rates.

Measurements

Light intensity was measured using quantum sensors (SQ110, Apogee Instruments, Logan, UT, United States) placed at different locations on the bench. Air temperature was measured using aspirated temperature sensors (ST 110, Apogee Instruments) placed in the proximity of the quantum sensors. Both sensors were connected to a datalogger (CR1000X, Campbell Scientific, Logan, UT, United States) for continuous measurements. Daily light integral and daily average temperature were calculated from the instantaneous light intensity and temperature measurements, respectively. Additional temperature sensors were connected to the datalogger in experiment 3 to measure solution temperature in the CFT and NFT systems. Relative humidity measurements were obtained from the environmental control system (Priva, Canada) in the greenhouse. The EC and pH of the nutrient solution were measured using pH/TDS/EC meter (Model #HI9811, Hanna Instruments, Ann Arbor, MI, United States).

In experiment 1, we measured shoot fresh weight (SFW), shoot dry weight (SDW), and root dry weight (RDW) of plants. Plants were harvested on the 22nd day after transplanting. Shoots and roots from a flood table were separated from the rock wool cubes and SFW of all plants was recorded along with the number of plants. From this data, SFW per plant ($g \cdot plant^{-1}$) was calculated by dividing total fresh weight of all plants by the number of plants in a tray. The shoot and root materials from each tray were placed in separate paper bags and dried in a forced air oven maintained at 80°C for 1 week. The dried material was weighed to determine SDW and RDW. From these, SDW and RDW per plant ($g \cdot plant^{-1}$) were calculated by dividing the total dry weight by the number of plants. Root weight ratio (RWR) (dimensionless) was calculated by dividing RDW by the sum of RDW and SDW. Shoot water content (SWC), (%) was calculated by dividing water weight (SFW minus SDW) by SFW and multiplying the result by 100.

In experiment 2, we measured canopy area (CA, cm^2), relative canopy growth rate (RCGR) (d^{-1}), EC of the nutrient solution prior to adjustment (EC_{adj}, $dS \cdot m^{-1}$), end-of-day solution volume (V, L), SFW, SDW, and concentration of different nutrients in the tissue. We used an imaging station (TopView, Aris, Eindhoven, Netherlands) to non-destructively measure CA of plants on different days. Whole-trays with plants were placed inside the image station on each measurement day for capturing images. The height of the tray from the base of the image station was adjusted to ensure that the distance between the camera and top of the plants was similar during each measurement. The images of shoots were captured as the roots were completely covered by the insert. The image processing software automatically separated plants from the background and calculated total pixel area belonging to the plants in each image. The CA (mm^2) was automatically estimated by multiplying the total plant pixel area with a constant (or "magnification factor" of 100) specific to the image station and converting to cm^2. We measured CA on the 10th, 13th, 15th, 18th, 20th, 21st, and 22nd day of the experiment. RCGR was measured as the slope of the linear relationship between ln (CA) and time. Left over solution in each tray was collected into a beaker to measure V around 4.00 pm each day. The evapotranspiration rate (ET, $L \cdot d^{-1}$) was calculated by subtracting V from 2 L. The EC of the solution in the beakers was used to measure EC_{adj}. Plants were harvested on the 22nd day after transplanting. Both SFW ($g \cdot plant^{-1}$) and SDW ($g \cdot plant^{-1}$) were measured as described above. The dried shoot material was grinded, and a representative sample was extracted from each tray. The samples were sent to a commercial laboratory (A&L Great Lakes, Fort Wayne, IN, United States) for complete elemental analysis.

In experiment 3, we measured SFW ($g \cdot plant^{-1}$), SDW ($g \cdot plant^{-1}$), RDW ($g \cdot plant^{-1}$), and EC_{adj} ($dS \cdot m^{-1}$) as described above. We measured the temperature of the solution inside three randomly selected CFT and NFT systems. The measurements of solution temperature were made continuously for seven consecutive days prior to the harvest. From this, hourly average temperature and standard deviation were calculated. In addition, samples of tap water and RO water used in the experiments were sent to the same commercial laboratory described above for water quality analyses.

Experimental Design and Statistical Analyses

Experiment 1 was laid-out in a randomized complete block design with two treatments and six replications. In each replication, solution treatments were represented by separate reservoirs. An experimental unit comprised of fifteen plants on a flood table belonging to a solution treatment and replication. Experiment 2 was also laid-out in a randomized complete block design with two treatments and nine replications. An experimental unit comprised of four plants in a tray belonging to a solution treatment and replication. Experiment 3 was laid-out in a split-plot design with seven replications of main-plot. The solution treatment was as the main-plot, production system was the sub-plot, and cultivar was the second sub-plot. A reservoir belonging to a solution treatment in a replication was connected to one CFT and one NFT unit. There were four plants each of four cultivars in one production system unit. An experimental unit comprised of four plants belonging to a cultivar within a production system and solution treatment in a replication. In all experiments, the treatments were randomly allotted to experimental units. Data were analyzed using a linear-mixed model ("Proc Mixed" procedure) with repeated measures and linear/non-linear regression (Proc "Reg" and Proc "Nlin") using statistical analysis software (SAS, version 9.1, SAS Institute, Cary, NC, United States). Least square means were separated using Tukey's honestly significant difference (HSD) procedure with $P \leq 0.05$ considered statistically significant. Graphs were plotted using SigmaPlot (version 14, Systat Software Inc., San Jose, CA, United States).

RESULTS

Experiment 1

A significant reduction in SFW of lettuce was observed in the recycle compared to control treatment (**Table 1**). SDW of lettuce in the recycle treatment was numerically lower than that of control treatment. However, there were no differences in RDW and RWR between the recycle and control treatments. SWC was significantly higher in the control compared to recycle treatment.

Experiment 2

Similar to experiment 1, a significantly lower SFW was observed in the recycle compared to control treatment (**Table 2**). Decrease in SDW of lettuce was small but significantly lower in the recycle compared to the control treatment. When EC_{adj} was compared, a significant increase was observed in the recycle compared to control treatment. However, there were no significant differences in ET between the recycle and control treatments. Concentration of several nutrients in the tissue including nitrogen (N), phosphorus (P), potassium (K), and iron (Fe), were significantly lower in the recycle compared

TABLE 1 | Shoot fresh weight (SFW), shoot dry weight (SDW), root dry weight (RDW), root weight ratio (RWR), and shoot water content (SWC) of leaf lettuce in experiment 1.

Treatment	SFW	SDW	RDW	RWR	SWC
	g·plant^{-1}	g·plant^{-1}	g·plant^{-1}		%
Control	31.0 (4.35) a	3.5 (0.61) a	3.0 (0.84) a	0.42 (0.038) a	88.8 (1.01) a
Recycle	20.9 (3.61) b	3.3 (0.57) a	2.9 (0.78) a	0.43 (0.033) a	83.9 (1.40) b

Means followed by the same letter are not statistically different ($P \leq 0.05$). Standard error of mean is shown in parenthesis.

TABLE 2 | Shoot fresh weight (SFW), shoot dry weight (SDW), electrical conductivity of nutrient solution before adjustment to the target level (EC_{adj}), evapotranspiration rate (ET), and relative canopy growth rate (RCGR) of leaf lettuce in experiment 2.

Treatment	SFW	SDW	EC_{adj}	ET	RCGR
	g·plant^{-1}	g·plant^{-1}	dS·m^{-1}	L·d^{-1}	d^{-1}
Control	35.3 (0.89) a	1.3 (0.05) a	2.3 (0.05) b	0.73 (0.041) a	0.191 (0.0064) a
Recycle	27.6 (0.61) b	1.2 (0.05) b	2.6 (0.07) a	0.74 (0.042) a	0.164 (0.0038) b

Means followed by the same letter are not statistically different ($P \leq 0.05$). Standard error of mean is shown in parenthesis.

to the control treatment (**Table 3**). Tissue analysis indicated significantly higher levels of copper (Cu) and sodium (Na) in the recycle compared to control treatment.

The image analysis method effectively separated plant area from the background (**Figure 2**). Canopy area assessments indicated that the differences between the control and recycle treatments, although not significant, started earlier by the second week after transplanting. However, CA of plants in the control treatment was significantly higher than that of the recycle treatment starting from the 18th day after imposing treatments (**Figures 2**, **3**). The smaller differences in canopy area became significantly larger with time. By day 22, canopy area of plants in the recycle treatment was approximately 33% smaller compared to that of plants in the control treatment (**Figure 3**). There was a linear relationship between ln (CA) and time in both the control and recycle treatments (**Figure 4**). The overall r^2 for fitted models ranged between 0.93 and 0.94. RCGR of plants was significantly higher in the control than recycle treatment (**Table 2**, also see slope of the fitted models in **Figure 4**).

TABLE 3 | Concentration of nutrients in the tissue of lettuce in experiment 2.

Nutrient	Units	Treatment	
		Control	Recycle
N	(mg·g^{-1})	37.6 (1.29) a	24.3 (0.70) b
P		5.4 (0.11) a	2.7 (0.20) b
K		42.0 (1.15) a	25.4 (1.01) b
Ca		10.7 (0.40)	11.0 (0.34)
Mg		5.2 (0.20)	5.3 (0.27)
S		3.2 (0.17)	3.0 (0.12)
Na		1.9 (0.08) b	3.2 (0.20) a
B	(mg·kg^{-1})	38.8 (2.37)	33.4 (1.79)
Zn		43.1 (2.72)	39.8 (3.16)
Mn		91.8 (5.24)	77.7 (8.65)
Fe		74.2 (8.41) a	46.3 (5.73) b
Cu		13.2 (1.56) b	20.0 (2.68) a
Al		47.4 (9.01)	53.6 (15.44)

Mean values for nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), sodium (Na), boron (B), zinc (Zn), manganese (Mn), iron (Fe), copper (Cu) and aluminum (Al) are shown.Means followed by the same letter within a measurement are not statistically different ($P \leq 0.05$). Values in parenthesis indicate standard error of mean.

Experiment 3

There were no significant differences in SFW and SDW of lettuce among control, 2 WkD, and Rec_RO treatments (**Figures 5A,B**). However, significant differences were observed for RDW among treatments (**Figure 5C**). It was significantly higher in the control than 2 WkD and Rec_RO treatments. Further, RDW was significantly higher in the 2 WkD compared to the Rec_RO treatment. There were no differences in EC_{adj} between the control and 2 WkD treatments (**Figure 5D**). As expected, EC_{adj} was significantly lower in the Rec_RO compared to other treatments. Water quality analyses indicated higher EC and alkalinity in the tap water compared to RO water (**Table 4**). The concentration of Ca, Mg, S, Cl, Fe, Si, and HCO_3 were higher in the tap water than RO water. There were no differences in pH between the tap water and RO water samples.

There was a significant effect of production system on lettuce growth. While SFW, SDW, and RDW were significantly higher, RWR was significantly lower in the NFT compared to CFT system (**Supplementary Table 1**). The difference between the solution and air temperature was consistently higher in the CFT than NFT system, especially during the daytime (**Supplementary Figure 1**). Among the four cultivars, SFW and SDW were significantly higher while RWR was significantly lower in Black Seeded Simpson than other cultivars (**Supplementary Table 2**). No differences were observed among Amadeus, Cedar, and Rex. In addition, there were no differences in RDW among all four cultivars.

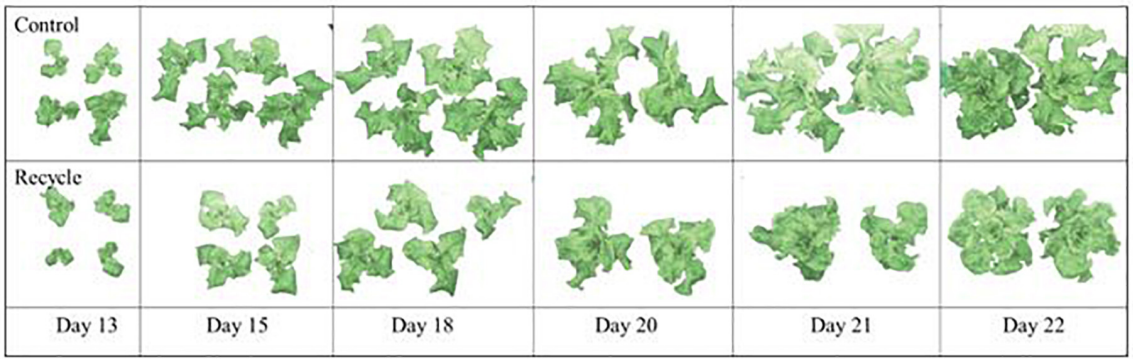

FIGURE 2 | Images of leaf lettuce plants on different days showing segmentation (i.e., background removal) results in experiment 2. Plants were grown in the control and recycle solution treatments. See "Materials and Methods" section for a description of treatments. Images were captured using a TopView image station. Due to large plant size, images of two plants are included in the panels for days 20, 21, and 22. Note visual differences between the two solution treatments start to appear by day 13 and progressively become larger by day 22.

DISCUSSION

Effects of Recycling Nutrient Solution on Plant Growth

We compared plant responses in the recycle treatment with those in the control treatment because maximum plant growth is expected in the control treatment as freshly prepared nutrient solution at target EC was regularly supplied to plants. Therefore, the observed differences between the control and recycle treatment should reflect maximum loss in yield due to recycling nutrient solution. We observed significant reduction in SFW of lettuce due to recycling (**Tables 1**, **2**) in spite of regularly maintaining the solution EC close to the target level in the recycle treatment. This indicates that maintaining target EC of the recycling solution does not necessarily result in optimal plant growth. Our results indicate that negative effects canopy area can start to appear as early as 2 weeks of recycling based on image analysis measurements (**Figure 2**). Decreased canopy area likely resulted in decreased SFW in the recycling treatment as canopy area can affect light interception and biomass production in plants (Niinemets, 2010; Li et al., 2020).

Tissue nutrient levels can potentially indicate reasons for the observed differences in canopy area and SFW between treatments. Our results indicated that tissue N, P, K, and Fe levels were not only lower but also deficient in the recycle compared to control treatment. A tissue N concentration below 30 $mg \cdot g^{-1}$ is considered as deficient for hydroponically grown lettuce (Campbell, 2000). Optimum range of tissue N, P, and K levels, based on lettuce yield in the field, was 43–56, 4.5–7.5, and

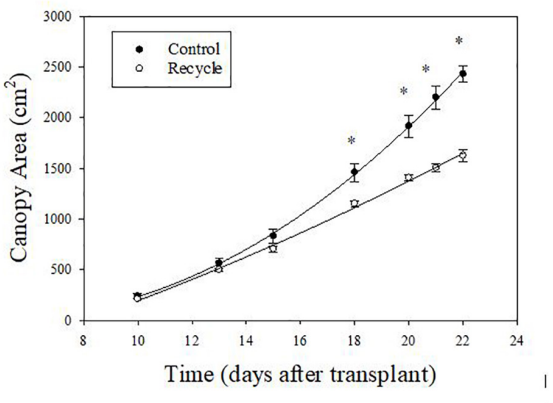

FIGURE 3 | Change in canopy area of plants with time in experiment 2. Canopy area was non-destructively estimated using image analysis. Plants were grown in the control and recycle solution treatments. See "Materials and Methods" section for a description of treatments. Error bars represent standard error of mean. An asterisk (*) indicates statistical difference ($P \leq 0.05$) between the means on a given day.

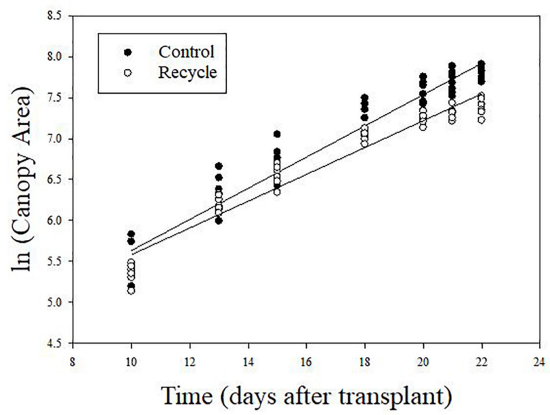

FIGURE 4 | Relationship between natural logarithm of canopy area and time in experiment 2. Plants were grown in the control and recycle treatments. See "Materials and Methods" section for a description of treatments. The fitted equations are ln ($CA_{control}$) = 3.73 + 0.191·time (r^2 = 0.93) and ln ($CA_{recycle}$) = 3.94 + 0.164·time (r^2 = 0.94) for control and recycle treatments, respectively. The slope of the fitted equations represents relative canopy growth rate of plants.

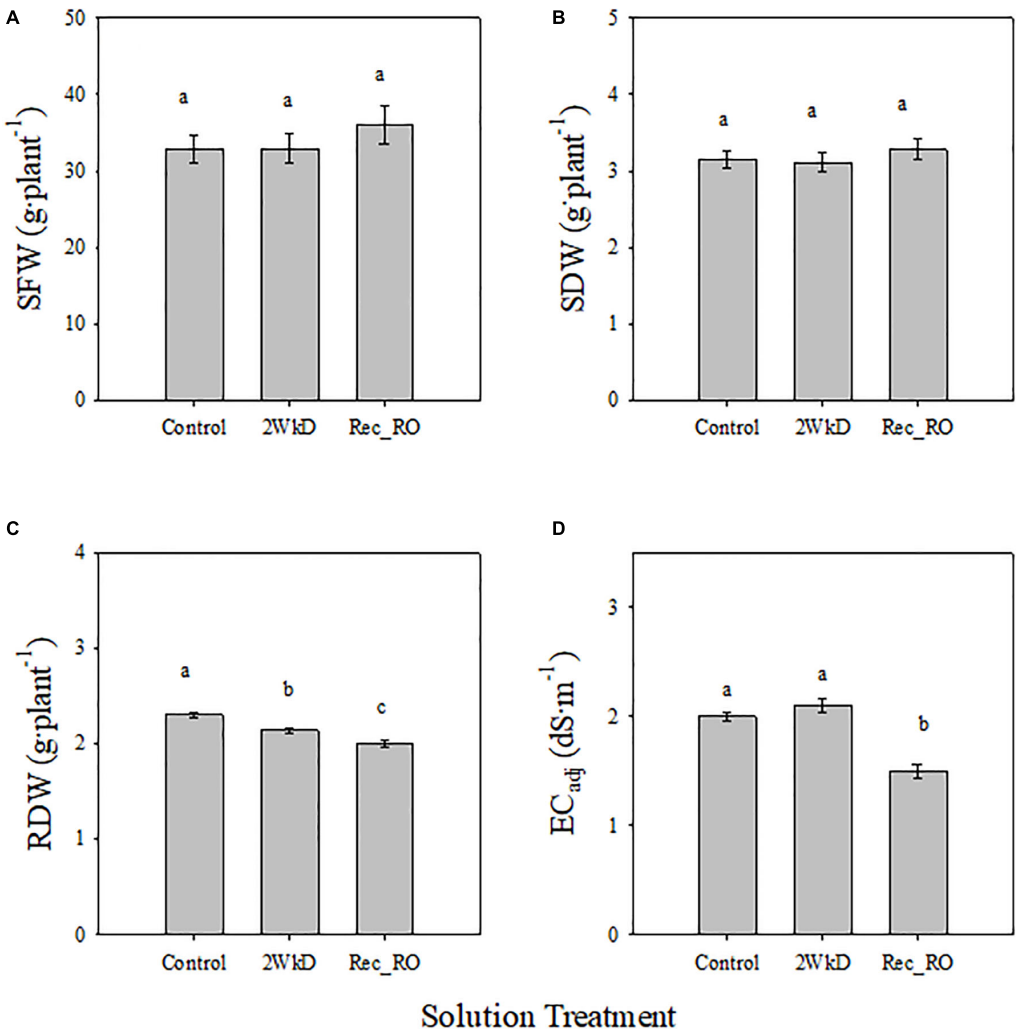

FIGURE 5 | Effect of solution treatments on lettuce shoot fresh weight (SFW) **(A)**, shoot dry weight (SDW) **(B)**, root dry weight (RDW) **(C)**, and electrical conductivity of nutrient solution before adjustment to the target level (EC_{adj}; **D**) in experiment 3. Plants in the control, 2 week discard (2 WkD) and recycling with reverse osmosis water (Rec_RO) treatments. See "Materials and Methods" section for a description of treatments. Different letters above bars indicate statistical significance ($P \leq 0.05$) among treatments. Error bars represent standard error of mean.

33–64 mg·g^{-1}, respectively while optimum range of tissue Fe for lettuce was 86–232 mg·kg^{-1} (Hartz and Johnstone, 2007). Based on these, tissue N, P, and K levels were lower than optimal in the recycling treatment (**Table 3**). While Fe was mildly deficient in the control, it was severely deficient in the recycle treatment (**Table 3**). Although, Cu levels were lower in the control than recycle treatment, the levels were in the sufficiency range (5.6–8.2 mg·kg^{-1}, Hartz and Johnstone, 2007) in both treatments (**Table 3**). Tissue Ca and Mg levels were not different between the recycle and control treatments in spite of their high concentration in the tap water (**Table 4**). Ca uptake is regulated by transpiration rate of plants (Isermann, 1970; Bangerth, 1979). There were no differences in ET between the two solution treatments in experiment 2 (**Table 2**). This is the likely reason for not observing differences in Ca levels in the plant tissue between the two solution treatments. Further Ca can regulate Mg uptake in plants (Tang and Luan, 2017; Liang and Zhang, 2018), which may have likely resulted in no differences in the levels of Mg in the plant tissue. Therefore, growth reduction in the recycle treatment is mostly due to nutrient deficiencies in the tissue. Plants in the control treatment regularly received freshly prepared nutrient solution with balanced levels of individual nutrients, thereby nutrient deficiencies were not observed in this treatment.

We also observed a significant reduction in SWC, a major component of SFW of lettuce, in the recycle treatment (**Table 1**). This is likely associated with increased osmotic stress experienced by plants in the recycle treatment. Tap water contained 14 and 38 mg·L^{-1} of Na and Cl, respectively (**Table 4**). It is possible that NaCl accumulated in the recycle solution with time. Accumulation of NaCl can result in osmotic stress effects on plants (Med-Tek Nutrients, 2016; Ding et al., 2018), which likely decreased water uptake of plants in the recycle treatment. This is

further supported by an increase in tissue Na levels in the recycle than control treatment (**Table 3**). High concentration of Na in the tap water (**Table 4**) likely lead to higher levels of Na in the tissue of plants grown in the recycle treatment. Higher than 5.0 $mg{\cdot}g^{-1}$ of Na in plant tissue can cause injury in plants (WateReuse Foundation, 2007). In spite of being higher than the control treatment, tissue levels of Na were below the injury level in the recycle treatment. Therefore, no visible Na injury symptoms were observed on plants in the recycle treatment. However, an increase in NaCl levels in the solution can potentially reduce water uptake (due to osmotic stress) and subsequently lower SWC and SFW of plants. Collectively, these results indicate that continuously using recycling nutrient solution despite maintaining solution EC at a target level can significantly lower lettuce yield in hydroponic production due to decreased nutrient availability and plant water uptake.

Plant growth differences were observed between the two production systems, when data were pooled from the three solution treatments and four cultivars. The increase in plant growth in the NFT compared to CFT (**Supplementary Table 1**) is likely due to higher solution temperature in the CFT, especially during the daytime (**Supplementary Figure 1**). The average air temperature during the study was 23.7°C. The solution temperature increased above air temperature in both the CFT and NFT systems during the daytime, with higher spikes in the CFT (**Supplementary Figure 1**). It ranged between 0.25 to 1.25 and 0.25 to 0.5°C, respectively in the CFT and NFT systems during different days. The black color of the CFT trays and lids can absorb most of the short wave and infrared radiation from sunlight, which can increase the material temperature. The heat from plastic can be transferred to the solution and increase the temperature of the solution. The optimal temperature for lettuce is between 20 and 24°C (Gent, 2016). Thus, the higher solution temperature in the CFT could have resulted in slower growth compared to the NFT system. However, further research is needed to understand growth differences between the two production systems. Higher SFW and SDW in Black Seeded Simpson than other cultivars is expected as leaf lettuce cultivars can grow at a faster rate than cultivars belonging to other groups (Miller et al., 2020).

Effect of Accumulation of Unwanted Substances on Solution EC and Nutrient Availability

Evapotranspiration can increase and concentrate nutrients in the solution (Bugbee, 2004), thereby increasing EC of the solution. However, this is not the likely reason for higher EC_{adj} in the recycle than control treatment as there were no differences in ET between the treatments in experiment 2 (**Table 2**). Alternatively, high levels of Ca, Mg, and HCO_3 in the tap water (**Table 4**) can potentially increase EC_{adj}. It was reported previously that HCO_3 have difficulty crossing the lipid bilayer of the root cell membrane (Poschenrieder et al., 2018). When HCO_3 are added through the tap water, they can accumulate in the solution (Zekki et al., 1996) and increase solution EC. In addition, plants remove Ca and Mg at a slower rate than other elements (Bugbee, 2004; Med-Tek Nutrients, 2016). Consequently, Ca and Mg can accumulate in the nutrient solution and increase solution EC, especially when their levels are high in the tap water. Although we did not measure the concentration of individual ions in the recycle solution, it is likely that higher EC_{adj} in the recycle than control treatment is due to the accumulation of unwanted compounds and/or slowly removed nutrients in the solution because of their high concentration in the tap water. In the control treatment, the concentration of Ca, Mg and HCO_3 likely was unaffected as old water was discarded regularly. It is important to note that the direct consequence of higher EC_{adj} is a reduction in the volume of concentrated nutrient stock solution added to the recycle solution during EC adjustment. When "apparent" EC of the solution is higher than the target value at the end of each cycle, then a relatively diluted nutrient solution is needed to adjust the EC to the target value. This can reduce the concentration of plant nutrients in the recycling solution compared to control, especially with time. Further, this can reduce the concentration of nutrients available for plant uptake and accumulate in the plant tissue.

Effects of Discarding Old Solution or Using RO Water on Plant Growth

Our results indicate that recycling solution made from tap water likely accumulated unwanted compounds or slowly consumed nutrients over time, which increased solution EC and reduced the quantity of nutrients added during refill. This further likely reduced tissue nutrient levels and decreased growth. Therefore, discarding old solution after 2 weeks (based on image analysis results) or using RO water with minimal levels of Ca, Mg, and HCO_3 (based on water quality analyses) should minimize the negative effects of recycling on plants. Supporting this, there were no differences in SFW and SDW observed among the control, 2 WkD, and Rec_RO treatments (**Figures 5A,B**) regardless of production system and cultivar tested in the experiment. Further, no differences in EC_{adj} between the control and 2 WkD treatments (**Figure 5D**) suggest that discarding old solution after 2 weeks of use can effectively reduce the accumulation of unwanted and slowly consumed elements in the solution. This further suggests that plants in the 2 WkD treatment likely were not limited with nutrient availability as noted in the conventional recycling treatment. This may have resulted in no growth differences between the control and 2 WkD treatments. The EC_{adj} was lower in Rec_RO than other treatments due to lower target EC (1.1 $dS{\cdot}m^{-1}$) maintained in this treatment. In spite of continuous recycling, no negative effects were observed in the Rec_RO treatment (**Figures 5A,B**) as insignificant levels of Ca, Mg, and HCO_3 were measured in the RO water (**Table 4**). Collectively, these results further support that the growth reduction observed in the continuous recycling treatment is partly due to high levels of Ca, Mg, and HCO_3 in tap water and their subsequent accumulation in the recycling solution. Root exudates, especially organic acids like benzoic acid, can reduce lettuce growth in closed hydroponic systems (Lee et al., 2006; Hosseinzadeh et al., 2017). While root exudates are important factors affecting lettuce growth in hydroponics, we do not think that their role was significant in our study. The effect of root exudates will likely be present in both tap and RO water based hydroponics. The fact that continuous recycling with RO

TABLE 4 | Analysis of tap water and reverse osmosis (RO) water used in experiment 3.

Measurement	Units	Irrigation Water	RO Water
pH	n.a.	7.5	7.1
EC	($dS \cdot m^{-1}$)	0.7	0.06
NO_3-N	($mg \cdot L^{-1}$)	0.8	–
NH_4-N		– [$]	–
P		1.1	–
K		3	–
Ca		102	–
Mg		38	–
Na		14	13
S		35	–
Zn		–	–
Mn		0.1	–
Fe		0.47	–
Cu		0.05	0.03
B		0.03	0.03
Al		–	–
Mo		–	–
Si		7	1
Cl		38	3
Alkalinity		250	20
CO_3		–	–
HCO_3		305	24

Values for electrical conductivity (EC), nitrate-nitrogen (NO_3-N), ammonium-nitrogen (NH_4-N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na), sulfur (S), zinc (Zn), manganese (Mn), iron (Fe), copper (Cu), boron (B), aluminum (Al), molybdenum (Mo), silicon (Si), carbonate (CO_3), and bicarbonate (HCO_3) are shown.
[$] Undetected or levels are lower than the sensitivity of equipment.

water did not result in growth reduction suggests that the effect of root exudates may be minimal in our study.

Practical Remedies to Minimize Negative Effects of Recycling

Our results indicate that preparing recycling solution using RO water or discarding recycling solution after 2 weeks of use can minimize the negative effects of recycling in hydroponic lettuce production. Growers should consider increased operational costs associated with producing RO water, especially in large-scale commercial operations requiring large volume of irrigation water. If discarding is more feasible that using RO water, we recommend that growers discard old solution based on plant growth monitoring as in our study. It is possible to make non-invasive CA measurements using easy-to-use devices like smartphones and publicly available software (Li et al., 2020). Interested growers can visit the our website[1] to access free software to estimate CA of plants. Growers can track RCGR (as measured in the present study) on a daily basis using CA measurements from image analysis. In our study, RCGR was 0.191 d^{-1} (or 19.1%) and 0.164 d^{-1} (or 16.4%) in the control and recycle treatments, respectively (**Table 2** and **Figure 4**). Similar to our results, Simko et al. (2016) using six lettuce varieties reported RCGR of 0.201 d^{-1} and Li et al. (2020) reported RCGR of 0.21 d^{-1} for lettuce, using image-based assessments under optimal conditions. These reports, combined with our findings, suggest that a RCGR of 19–21% can be expected in lettuce under optimal conditions. Therefore, growers can discard the recycle solution when RCGR between two consecutive days consistently fall below 19–21%.

[1] https://www.purdue.edu/hla/sites/cea/la-estimated/

CONCLUSION

Our goals for this study were to evaluate the effects of continuous recycling on solution EC, tissue nutrient concentration and productivity of lettuce, and develop optimal strategies for managing recycling nutrient solution in hydroponic production. The research from this study indicates that continuous recycling with tap water containing moderate to high levels alkalinity can result in apparent increase in solution EC, nutrient deficiencies in the plants, and reduction in shoot growth, in spite of maintaining the solution EC at a target level. In our research, discarding old solution after 2 weeks of recycling effectively mitigated negative effects of recycling on growth. We also provide a solution based on imaging technology for plant growth monitoring to accurately determine the stage when recycling solution made with tap water can be discarded. Alternatively, we found that negative effects of recycling were not observed when RO water was used in production. The information from this study can aid in proper management of recycling nutrient solution in hydroponic production.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article is available upon request.

AUTHOR CONTRIBUTIONS

AM contributed to experimental design, experimentation, data collection, and data analyses. RA contributed to manuscript preparation. KN contributed to experimental design, data analyses, and manuscript preparation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.607643/full#supplementary-material

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Integrated Assessment of Nickel Electroplating Industrial Wastewater Effluent as a Renewable Resource of Irrigation Water Using a Hydroponic Cultivation System

*Y. N. Chow[1], L. K. Lee[2], N. A. Zakaria[1] and K. Y. Foo[1]**

[1] River Engineering and Urban Drainage Research Centre (REDAC), Universiti Sains Malaysia, Nibong Tebal, Malaysia,
[2] School of Industrial Technology, Universiti Sains Malaysia, Gelugor, Malaysia

Nickel, a micronutrient essential for plant growth and development, has been recognized as a metallic pollutant in wastewater. The concentration of nickel ions in the water course, exceeding the maximum tolerable limit, has called for an alarming attention, due to the bioaccumulative entry in the water–plant–human food chain, leaving a burden of deteriorative effects on visible characteristics, physiological processes, and oxidative stress response in plants. In this work, the renewable utilization of nickel electroplating industrial wastewater effluent (0, 5, 10, 25, 50, and 100%) as a viable source of irrigation water was evaluated using a hydroponic cultivation system, by adopting *Lablab purpureus* and *Brassica chinensis* as the plant models, in relation to the physical growth, physiological and morphological characteristics, photosynthetic pigments, proline, and oxidative responses. The elongation of roots and shoots in *L. purpureus* and *B. chinensis* was significantly inhibited beyond 25 and 5% of industrial wastewater. The chlorophyll-*a*, chlorophyll-*b*, total chlorophyll, and carotenoid contents, accompanied by alterations in the morphologies of xylem, phloem, and distortion of stomata, were recorded in the industrial wastewater-irrigated groups, with pronounced toxicity effects detected in *B. chinensis*. Excessive proline accumulation was recorded in the treated plant models. Ascorbate peroxidase (APX), guaiacol peroxidase (POD), and catalase (CAT) scavenging activities were drastically altered, with a profound upregulation effect in the POD activity in *L. purpureus* and both POD and APX in *B. chinensis*, predicting the nickel-induced oxidative stress. Conclusively, the diluted industrial wastewater effluent up to the optimum concentrations of 5 and 25%, respectively, could be feasibly reused as a renewable resource for *B. chinensis* and *L. purpureus* irrigation, verified by the minimal or negligible phytotoxic implications in the plant models. The current findings have shed light on the interruption of nickel-contaminated industrial wastewater effluent irrigation practice on the physical and biochemical features of food crops and highlighted the possibility of nutrient recycling via wastewater reuse in a sustainable soilless cultivation.

Keywords: closed hydroponic, food safety, nickel electroplating industry, nutrient recycling, phytotoxicity, wastewater reuse

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Sri Pratap College Srinagar, India
Fabricio Eulalio Leite Carvalho,
Federal University of Ceará, Brazil

****Correspondence:***
K. Y. Foo
k.y.foo@usm.my

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INTRODUCTION

Phytotoxicity of heavy metals, mainly featured by the alterations of numerous physiological processes at the molecular or cellular level, including the inactivation of enzymes, blockage of functional groups at the metabolically important molecules, substitution or displacement of essential elements, and disruption of membrane integrity, has emerged to be a worldwide agenda among the scientific community (Khan et al., 2020; Rizvi et al., 2020; Wakeel et al., 2020). The bio-accumulative entry and indiscriminate discharge of these heavy metals, in particular nickel, lead, and chromium, to the waterways, soils, and eventually to the food chain, specifically from the anthropogenic activities of metal mining, smelting, and electroplating industries; fossil fuel burning; steel manufacture; emissions from vehicle; disposal of household, industrial, and municipal waste; fertilizer and organic manure application; and other miscellaneous sources, constitute a sharp and alarming health risk to the public health and ecosystems (Ceasar et al., 2020; Yan et al., 2020). This pollution becomes more drastic, specifically in urban and agricultural catchments, which receive a variety of organic manures heavily loaded with different toxic metals. In such cases, both the treated and untreated municipal wastewaters in the vicinity of large cities, is a major utility widely applied for the irrigation practice.

The presence of elevated levels of heavy metals in the growing medium of germinating seeds has been reported to suppress the translocation and mobilization of reserve nutrients from the reserve tissues to the growing regions. Similarly, buildup of heavy metals in the rooting medium would significantly retard plant growth; elicit perturbations in cellular metabolism; affect the uptake of potassium, calcium, and magnesium; and decrease the number of flowers, fruits, and crop yield. The most common toxicity symptoms in plants are necrosis, chlorosis, wilting, and disturbance of physiological processes, including photosynthesis, transport of photo-assimilates, mineral nutrition, and plant structure damage (Kasprzak et al., 2003; Rather et al., 2020).

Among all, nickel toxicity has received aesthetic concern, owing to its excessive wide-scale applications in different industries. Under stress conditions, including exposure to the excess concentrations of heavy metals, an imbalanced generation and degradation of reactive oxygen species (ROS) could arise in plant tissues (Georgiadou et al., 2018). This may subsequently result in oxidative injuries of a wide range of important macromolecules including proteins, lipids, and nucleic acids. During this stage, these cells are protected against free oxyradicals by the enzymic and non-enzymic systems that serve as free radical scavengers. The major enzymatic detoxifiers of hydrogen peroxide (H_2O_2) in plants are ascorbate peroxidase (APX, EC 1.11.1.11) and catalase (CAT, EC 1.11.1.6). CAT would catalyze the detoxification of H_2O_2 into water and oxygen molecules; alternatively, H_2O_2 could be eliminated via the ascorbate/glutathione reaction cycle by APX (Kapoor et al., 2019).

Similarly, the rising peroxidase activity is known as a physiological response to abiotic and biotic stressors. Peroxidases are primarily detected in endoplasmic reticulum, vacuoles, cell walls, and Golgi apparatus in plants, and the distribution is presumably associated with different physiological functions. This enzyme has been hypothesized to be a potential bioindicator for sublethal metal toxicity in plants. The accumulation of proline, one of the most widespread proteinogenic metabolites originating from plant tissues against stress conditions, has been well documented (Du et al., 2020). The protective roles of proline have been attributed to its ability to react as an osmoprotectant, source of carbon and nitrogen, membrane stabilizer, protectant of enzymes, and ROS scavenger (Chandrasekhar and Ray, 2017). Meanwhile, an excess of metallic ions has demonstrated destructive effects on the functionality and content of the photosynthetic pigmentations, usually governed by the inhibition of pigment biosynthesis, formation of metal-substituted chlorophylls, or direct oxidative damages on the pigments. This study was conducted to gear toward an in-depth understanding of the concept of renewable utilization of nickel electroplating industrial wastewater effluent, with respect to the physical, physiological, biochemical, and morphological responses of *Lablab purpureus* and *Brassica chinensis* using a closed-hydroponic system. The time course of changes in the (a) photosynthetic pigments, (b) scavenging activities of antioxidative enzymes, and (c) proline accumulation in relation to the wastewater effluent was analyzed. In parallel, the representative growth parameters, including the physical response and the morphological alterations of shoots, leaves, and roots, were elucidated.

MATERIALS AND METHODS

Reagents and Chemicals

The required reagents and chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany): acetone, ascorbic acid, ethylenediaminetetraacetic acid (EDTA), bovine serum albumin, methanol, Coomassie Blue dye G250, aqueous sulfosalicylic acid, H_2O_2, proline, glacial acetic acid, nitric acid, and toluene. On the contrary, sodium hypochlorite, guaiacol, polyvinylpyrrolidone (PVP), ninhydrin acid, and sodium phosphate buffer were supplied by Sigma-Aldrich (St. Louis, MO, United States).

Nickel Electroplating Industrial Wastewater Effluent

The wastewater sample was collected from the effluent discharge point at the multi-electroplating industrial zone of Bukit Minyak, Penang, Malaysia. Water sampling was carried out using 500-ml polyethylene bottles, which were pretreated with concentrated HNO_3 for sample preservation to minimize heavy

Abbreviations: *a*, significance level, probability of rejecting the null hypothesis when it is true; A_{663}, absorbance at 663 nm; A_{645}, absorbance at 645 nm; A_{470}, absorbance at 470 nm; $_{(x+c)}$, total carotenoids (xanthophylls and carotenes); C_a, chlorophyll-*a* concentration; C_b, chlorophyll-*b* concentration; cm, centimeter; *F*, ratio of variation between sample means to variation within the samples; FW, fresh weight; mM, millimolar; *p*, probability value; *r*, Pearson correlation coefficient; *v*, volume.

metals degradation by microorganisms. The physicochemical properties, with respect to the pH, electrical conductivity (EC), total suspended solid (TSS), total dissolved solid (TDS), total phosphorus (TP), ammoniacal nitrogen (AN), dissolved oxygen (DO), 5-day biochemical oxygen demand (BOD_5), and chemical oxygen demand (COD), were determined *in situ* or using a spectrophotometer, by adopting the American Public Health Association (APHA) standard procedures (HACH, DR3900, United States) (American Public Health Association [APHA], 1995). The concentrations of heavy metals were measured with an inductively coupled plasma mass spectrometry (ICP-MS, NexION 300, PerkinElmer, United States). As an indicator of irrigation viability, the pH, EC, TSS, TDS, COD, BOD_5, TP, AN, and DO were analyzed. The pH, TDS, and BOD_5 of the industrial wastewater effluent fell within the suitable range for the applications of wastewater reuse (World Health Organization [WHO], 2006). The raw industrial wastewater effluent, diluted at 0 (control), 5, 10, 25, 50, and 100%, was applied as irrigation water (**Table 1**).

Plant Materials and Nickel Treatment

Healthy, certified seeds of *L. purpureus* (hyacinth bean) and *B. chinensis* (pak choi) provided by the Department of Agriculture, Penang, were applied as plant models. *L. purpureus* is a multifunctional legume, with a rich source of protein and a wide spectrum of therapeutic features. *B. chinensis* not only is a commonly consumed leafy cruciferous vegetables among Asian countries but also demonstrates an important value in Western diet (Sun et al., 2010), with its high resistance against different biotic and abiotic factors and a higher uptake coefficient for heavy metals. These crops have been subjected to the environmental risks of heavy metal-polluted soils and water by environmentalists (Gao et al., 2019; Wang et al., 2020).

The seeds of the plant models were surface sterilized with 75% (v/v) of ethanol, disinfected with sodium hypochlorite solution (2%), and rinsed thoroughly with deionized water. The seeds were cultivated in the hydroponic setups and constantly supplied with the aerated industrial wastewater effluent diluted at 0 (control), 5, 10, 25, 50, and 100%. The setup irrigated with the sole nutrient solution served as the control. The cultivation was conducted using 18 hydroponic setups in a greenhouse, with 16/8 h of photoperiod, 70–90% of relative humidity, and a mean day/night temperature of 30.6°C/23.9°C. The plants were harvested from the hydroponic growth substrate at the completion of the cultivation cycle and cleaned with deionized water to remove impurities from the adhering surface, before being stored for subsequent biochemical analyses.

Bioaccumulative Potential

The plant samples were subjected to oven-drying at 80°C for 3 days before being ground to fine powder using a stainless grinder and digested by concentrated nitric acid in a microwave digester at 100°C. The heavy metal compositions of the plants was determined by ICP-MS.

Growth Parameters

The growth rate was ascertained by the determination of the length of roots and shoots of the plant models. The length between the root–hypocotyl junction and the root tip was recorded as the root elongation, whereas the length from the plumule that emerged from the cotyledon to the farthest end of the leaf was taken as the shoot elongation. The growth rate measurements were conducted on a daily basis in triplicates.

Chlorophyll Content

For the quantification of chlorophyll content, the aliquot (10 mg) of the ground leaves was immersed in acetone (80%, 3 ml) at 4°C, followed by centrifugation for 3 min (Heraeus Megafuge 8, Thermo Fisher Scientific, United States). The absorbance of the resulting extracts was recorded at the optimum wavelengths of 645, 470, and 663 nm (UV-1800, Shimadzu, Japan). The chlorophyll-*a*, chlorophyll-*b*, total chlorophyll, and carotenoid$_{(x+c)}$ content [mg/g fresh weight (FW)] were determined with respect to the given equations, expressed by

$$Chlorophyll - a = 12.21A_{663} - 2.81A_{645} \quad (1)$$

$$Chlorophyll - b = 20.13A_{645} - 5.03A_{663} \quad (2)$$

$$Total\ chlorophyll = Chlorophyll - \mathrm{a} + Chlorophyll - \mathrm{b} \quad (3)$$

$$Carotenoids = \frac{1000\mathrm{A}_{470} - 3.27\mathrm{C}_\mathrm{a} - 104\mathrm{C}_\mathrm{b}}{229} \quad (4)$$

where A_{645}, A_{663}, and A_{470} are referred to as the absorbance at 645, 663, and 470 nm, respectively, and the concentrations of chlorophyll-*a* and chlorophyll-*b* are represented by C_a and C_b (Lichtenhaler and Wellburn, 1983).

Morphological Assessment

The changes of the morphological characteristics for the root, leaf, and shoot of the plant models were analyzed by using a scanning electron microscope (SEM). The plant specimens were prepared according to the procedure recommended by O'Brien and McCully (1981). The fresh root, leaf, and shoot samples were dissected from the same middle portion, and the obtained specimens, at a size of 5 mm^2, were immersed immediately in 99% methanol for 20–40 s, followed by simple air-drying. The plant specimens were sputter-coated with gold, mounted on the aluminum stubs, and analyzed by 15-kV SEM (LEO Electron Microscope Inc., United States) (Pathan et al., 2010).

Proline Level

The standard procedure outlined by Bates et al. (1973) was adopted for the quantitative calculation of free proline concentration. The homogenized filtrate of the fresh leaves was mixed with ninhydrin acid followed by acetic acid and heated at 100°C for 60 min. The resulting red organic layer was mixed vigorously after toluene extraction and applied for spectrophotometric determination of proline at the optimum wavelength of 520 nm, presented as μmol/g FW.

TABLE 1 | Physicochemical characteristics of the nickel electroplating industrial wastewater effluent.

Parameter	Unit	Nickel electroplating industrial wastewater effluent	Recommended maximum level for irrigation (World Health Organization [WHO], 2006)
pH	–	6.5 ± 0.4	6.5–8.0
EC	dS/m	1.50 ± 0.07	0.70–3.00
TSS	mg/L	30 ± 1.43	N.A.
TDS	mg/L	558 ± 20.52	500–2,000
COD	mg/L	260 ± 10.12	N.A.
BOD_5	mg/L	20 ± 0.94	10–30
DO	mg/L	2.20 ± 0.98	N.A.
Turbidity	NTU	14 ± 0.50	N.A.
TP	mg/L	1.03 ± 0.04	N.A.
AN	mg/L	1.120 ± 0.053	N.A.
As	mg/L	N.D.	0.1
Cd	mg/L	0.001 ± 0.000043	0.01
Cr	mg/L	0.001 ± 0.000050	0.1
Cu	mg/L	N.D.	0.2
Fe	mg/L	0.035 ± 0.0017	0.1–1.5
Hg	mg/L	N.D.	N.A.
Mn	mg/L	0.004 ± 0.0002	0.1–1.5
Ni	mg/L	70.40 ± 3.46	0.2
Pb	mg/L	0.003 ± 0.0011	5
Zn	mg/L	0.005 ± 0.0023	2

N.D., not detected; N.A., not available.

Antioxidant Enzymes

The antioxidant enzymes of the plant models were extracted by homogenizing the plant material with a prechilled mortar and pestle in cold sodium phosphate buffer (pH 7.0, 50 mM) that contained ascorbic acid (0.20 mM) and PVP (1% w/v) (Jiang and Huang, 2001). After centrifugation for half an hour at 10,000 rpm (Heraeus Multifuge X1R, Thermo Fisher Scientific, United States), the resulting supernatants were withdrawn for the spectrophotometric analyses of antioxidative enzymes.

Guaiacol Peroxidase Assay

POD assay was initiated with the ambient incubation of the reaction mixture consisting of a supernatant (50 μl), guaiacol (100 μl), sodium phosphate buffer (3.75 ml), distilled water (50 μl), and H_2O_2 (100 μl) for 8 min. The absorbance of the mixture was recorded at 470 nm with reference to the reagent blank (Everse et al., 1994). The specific activity of POD was estimated by the specific extinction coefficient, E (26.6 mM^{-1} cm^{-1}), and expressed as mmol/mg protein/min (Noctor et al., 2016).

Ascorbate Peroxidase Assay

The determination of APX activity was conducted by mixing 150 μl of supernatant with 0.25 ml of sodium phosphate buffer, ascorbic acid, and EDTA. H_2O_2 was added for the immediate initiation of the reaction, with reference to the absorbance value observed at 290 nm. The detoxification capacity of APX was computed, with an E value of 2.8 mM^{-1} cm^{-1} (Nakano and Asada, 1981).

Catalase Assay

The CAT scavenging activity, expressed in the form of required CAT concentration for the liberation of half of the peroxide oxygen, was estimated by mixing 200 μl of H_2O_2, 50 μl of the supernatant, 2.5 ml of potassium phosphate buffer, and 250 μl of distilled water, using an E value of 40 mM^{-1} cm^{-1} (Aebi, 1984).

Protein Content

The Bradford (1976) method was adopted for the quantitative verification of protein content, by referring to the standard curve prepared from bovine serum albumin. The plant sample was subjected to homogenization in the mixture containing potassium phosphate buffer, 1 mM of EDTA, and 1% of PVP, which was subjected to centrifugation for 20 min (11,000 rpm, 4°C) (Heraeus Multifuge X1R, Thermo Fisher Scientific, United States). The resulting supernatant was mixed with 5 ml of Bradford's reagent, and the absorbance was measured at 595 nm with reference to the reagent blank and presented as mg/g FW.

Statistical Analysis

The effects of the industrial wastewater irrigation practice were analyzed using analysis of variance (ANOVA). Analysis was conducted with six biological replicates, and the significant difference between the control and the industrial wastewater effluent-irrigated groups was detected by Duncan's multiple-range test. The correlations among the POD, APX, and CAT were ascertained using Pearson's correlation test. All statistical analyses were performed using IBM SPSS version 24.0 at a significance

level of $p < 0.05$. The significant differences between the tested groups were denoted using superscripted alphabets.

RESULTS AND DISCUSSION

Heavy Metal Composition of Industrial Runoff and Metal Uptake Rate

The compositional characteristics of the nickel electroplating industrial runoff, together with the recommended limit for the irrigation requirement, are given in **Table 1**. The concentrations of the heavy metals in the electroplating industrial wastewater effluent ranged within the respective permissible limits for agricultural irrigation, with an extremely high nickel concentration at 70.4 ± 3.46 mg/L. The heavy metal uptake in the different parts of the plant models is presented in **Supplementary Table 1**. Generally, arsenic and mercury were not detected, while lower concentrations of cadmium, chromium, and lead were recorded, ranging from 0.008 to 0.011, 0.001 to 0.013, and 0.001 to 0.016 μg/g DW, respectively. Meanwhile, the concentrations of copper, manganese, iron, and zinc fell within the range of 0.001–0.025, 0.001– 0.027, 0.002–0.031, and 0.007–0.028 mg/g DW, respectively, with the highest concentrations detected in the roots of the plant models. Specifically, nickel was detected at the highest concentration ranging from 180.15 to 188.26 mg/g DW in roots, at 7.85–9.23 mg/g DW in shoots, and at 23.51–26.28 mg/g DW in leaves. These findings were well corroborated with the heavy metal uptake with wastewater irrigation as reported by Nzediegwu et al. (2019) and Ma et al. (2015).

In particular, the nickel uptake in plants is governed by the root systems via passive diffusion and active transport mechanisms (Seregin and Kozhevnikova, 2006). The ratio of the uptake between the active and passive transport may vary with the plant species, form, and concentration of nickel in the soil or nutrient solution (Dan et al., 2002). The soluble nickel ions could be transferred from the growing surrounding to the plant roots through the cation transport systems for copper, zinc, and magnesium ions and nickel–chelator complex, which involve the high-affinity nickel transport protein, metallothionein, and metallochaperones (Eitinger and Mandrand-Berthelot, 2000; Schor-Fumbarov et al., 2005). The translocation of nickel to the upper plant parts, including the shoots and leaves, is driven by the transpiration stream of the xylem, while the translocation to the meristematic parts of the plants, in particular young leaves, buds, fruits, and seeds, takes place in the phloem, with the regulation of metal–ligand complexes and protein binders (Amari et al., 2016). This uptake and translocation process has been reported to be affected by nickel concentrations, plant metabolism, acidity of soil or solution, or composition of other metals and organic matters (Chen et al., 2009). In the present work, *L. purpureus* and *B. chinensis* demonstrated the accumulation of nickel in the descending order of root > leave > shoot, parallel with the rising concentration of nickel electroplating industrial wastewater (**Supplementary Table 2**). Likewise, Chandrasekhar and Ray (2018) and Rizwan et al. (2017) reported a similar bioaccumulative behavior in *Eclipta prostrata* (L.) L and *Oryza sativa*. Exceedance of 50% of the nickel ions was retained in the roots, mainly due to the sequestration in the cation exchange sites of the xylem parenchyma cell wall and immobilization in the vacuoles of roots. Specifically, majority of these nickel ions were detected in the vascular cylinder, with less than 20% found in the cortex surface, indicating a high mobilization of nickel ions in the xylem and phloem (Sachan and Lal, 2017).

Growth Retardation

Figure 1 illustrates the effect of industrial wastewater effluent irrigation practice on the lengths of roots and shoots of *L. purpureus* and *B. chinensis* as a function of concentration and duration of exposure. Increasing the concentration of wastewater effluent from 0 to 25% showed a gradual reduction in the root length of *L. purpureus*, from 30.7 to 23.1 cm ($F = 132.5$; $p < 0.05$), with the growth inhibition ranging from 10.6 to 24.6%. A dramatic root growth reduction was recorded beyond 25% of wastewater effluent, with an inhibition rate from 35.2 to 45.1% ($F = 66.1$; $p < 0.05$). The same concentration-dependent effect was apparently recorded for shoot length, with a growth reduction from 26.8 to 20.2 cm ($F = 21.8$; $p < 0.05$), while a profound reduction was observed beyond 25% of the industrial wastewater effluent-irrigated group. *B. chinensis* exhibited a minimal root growth reduction of 9.6% at 5% of wastewater effluent. Beyond this concentration, however, a drastic inhibition from 31.9 to 83.3% was observed ($F = 165.2$; $p < 0.05$). A similar but lower degree of reduction was recorded by the shoot of *B. chinensis*, with the percentage ranging at 5.4, 23.2, and 72.6% for 5, 10, and 100% of the wastewater effluent-irrigated groups, respectively ($F = 133.5$; $p < 0.05$).

A similar growth retardation was reported by Khan and Khan (2010), Parlak (2016), and Gupta et al. (2017) under nickel treatment within the concentration range from 0.01 to 40 mg/L, with a reduction from 1.4 to 97.8% for roots and from 0.4 to 89.4% for shoots in chickpea, pea plants, wheat, and millet. The growth impairment may be attributed to the cellular turgor loss, leading to the reduction of cell proliferation and cell division (Gabbrielli et al., 1990). The finding could also be ascribed to the intensification of the cell wall, driven by the lignification and polymerization reactions and regulated by the extracellular peroxidases, which governed the cross-linkage formation between the extension molecules and feruloylated polysaccharides. Additionally, nickel ions may significantly alter the fundamental metabolic pathways in plants, photosynthesis, and translocation processes to inhibit the normal growth of roots and shoots for different plant species (Shahzad et al., 2018).

Alterations of Chlorophyll-*a*, Chlorophyll-*b*, Total Chlorophyll, and Carotenoid Content

The alterations of photosynthetic pigments in response to the percentage of nickel electroplating industrial wastewater effluent are illustrated in **Figure 2**. Chlorosis is considered to be a common toxicity symptom of nickel ions in plants. The rising nickel concentration in the irrigation water has induced a profound inhibition on the photosynthetic pigments

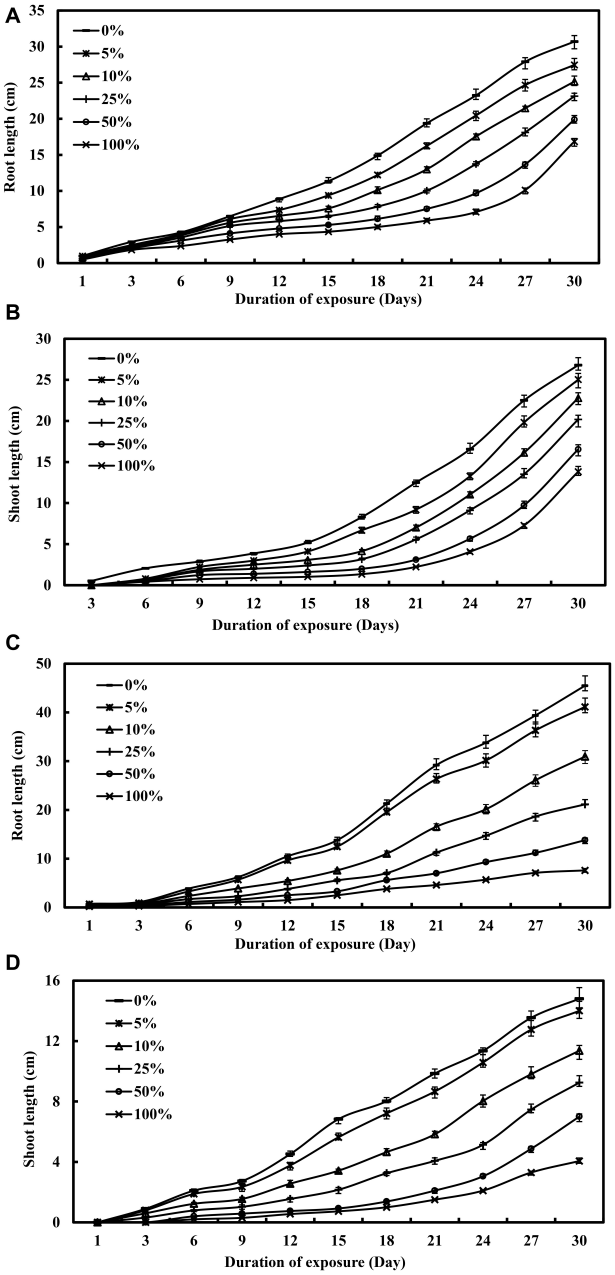

FIGURE 1 | The visual toxicity of nickel electroplating industrial wastewater effluent for *Lablab purpureus* **(A,B)** and *Brassica chinensis* **(C,D)**, respectively, as a function of concentrations and duration of exposure.

of *L. purpureus*, with a statistically significant reduction from 15.2 to 50.4% for chlorophyll-*a* (F = 3,189.3; $p < 0.05$) and from 20.5 to 60.1% for chlorophyll-*b* (F = 1,900.9; $p < 0.05$). Similarly, increasing the percentage of wastewater effluent has caused a significant reduction of carotenoid content from 1.98 to 1.13 mg/g FW (F = 198.1; $p < 0.05$). Specifically, *L. purpureus* showed a drastic reduction in chlorophyll-*a*, chlorophyll-*b*, total chlorophyll, and carotenoid contents beyond 25% of industrial wastewater effluent. Comparatively, *B. chinensis* portrayed a higher vulnerability toward the rising percentage of wastewater effluent, with a dramatic reduction beyond 5% of wastewater effluent, ranging from 31.7 to 71.9%, 23.7 to 77.9%, and 33.1 to 62.2% for chlorophyll-*a*, chlorophyll-*b*, and carotenoid, respectively.

The phenomenon was mainly related to the deficiency of iron and magnesium or the substitutions of these essential ions by nickel ions at the tetrapyrrole ring of the chlorophyll molecules, leading to the denaturation of these chlorophyll structures (Küpper and Andresen, 2016). The chlorophyll reduction may be credited to the nickel-induced oxidative damage on the membranous structure of chloroplast (Fatemeh et al., 2012), with a drastic inhibitory impact on the biosynthesis of chlorophyll and degradation of functional photosynthetic pigments (Fuentes et al., 2014). Similar reductions have been found in the chlorophyll-*a* (46.5%), chlorophyll-*b* (39.5%), and carotenoid (52%) of nickel-treated spinach at the concentrations of 150 and 300 mg/kg of soil (Boostani et al., 2019), and a total chlorophyll reduction, from 34.5 to 43.9% and 39.2%, was detected in nickel-treated soybean at concentrations of 4 and 2 mM, respectively (Sirhindi et al., 2016; Mir et al., 2018). A specific point to be highlighted here is the higher ratio of chlorophyll-*a* to chlorophyll-*b* at 2.03 in *L. purpureus* after wastewater effluent irrigation, indicating a greater sensitivity of chlorophyll-*b* to nickel toxicity compared with chlorophyll-*a*. These acquired results verify the effect of nickel ion-induced oxidative damages on the membranous and molecular structures of chloroplast, with an inhibitory impact on the biosynthesis of chlorophyll, and degradation of functional photosynthetic pigments, to induce a dramatic impact on the Hill reaction, deteriorating the net photosynthesis and transpiration rates in the plant models as demonstrated in this work (Beri and Sharma, 2016).

Morphological Changes

The representative alterations of the industrial wastewater-induced toxicity on the roots, shoots, and leaves of *L. purpureus* and *B. chinensis* are illustrated in **Figure 3**. From the presented images, the transverse section of *L. purpureus* root sample under the control condition demonstrated a normal stellar structure for both xylem and phloem tissues, to support the normal photo-assimilation process (**Figure 3A**). For the 50% of wastewater effluent-irrigated *L. purpureus*, the roots exhibited the structures of crimped xylem and phloem elements, representative of the impairment and disruption of vascular bundles, particularly the xylem vessels (**Figure 3B**). Well-developed structures of root xylem and phloem were portrayed by the control *B. chinensis* (**Figure 3C**), while 10% of wastewater effluent has seriously distorted the root tissue of *B. chinensis* (**Figure 3D**).

Similarly, the SEM findings of the transverse sections of *L. purpureus* and *B. chinensis* shoot samples revealed well-defined hexagonal or pentagonal structures of xylem and phloem (**Figures 3E,G**). However, 10 and 50% of wastewater effluent-induced irrigation resulted in serious deterioration on the orientation of these vascular tissues in *B. chinensis* and *L. purpureus* (**Figures 3F,H**). This suggested that the toxic effect of nickel ions has led to a subdued conduction of water and photosynthates.

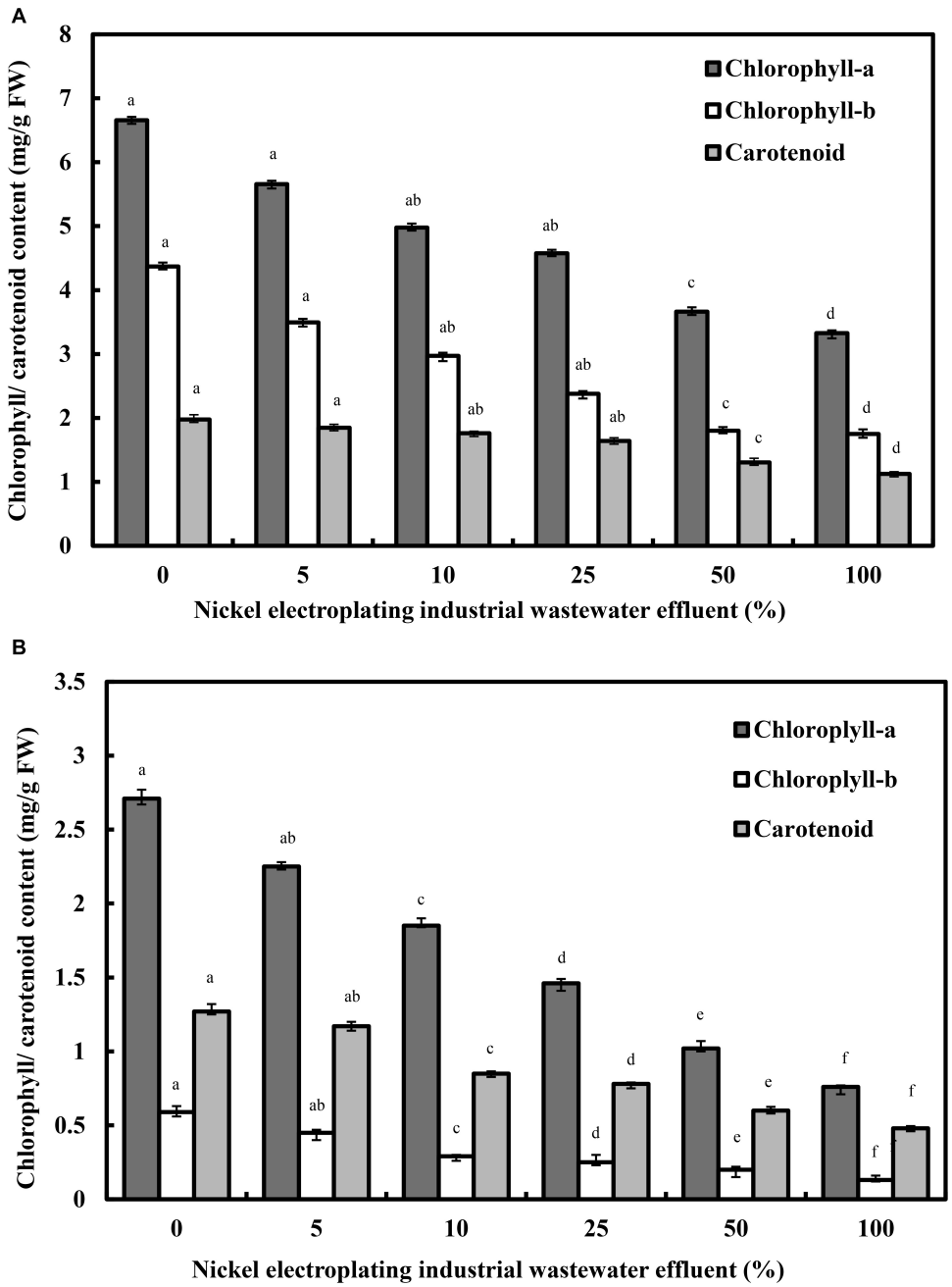

FIGURE 2 | Alterations in photosynthetic pigments of *Lablab purpureus* **(A)** and *Brassica chinensis* **(B)**. The experiment was performed with six replicates, with different alphabets indicating significant difference.

The current findings highlighted the structural deformation of roots, with a noticeable alteration of the cell wall as compared with the control plant models, to induce a dramatic inhibition on the translocation or uptake of nutrient elements and water from the roots to shoots and the aboveground parts of the plant models. These results were in consonance with the findings reported in *Talinum triangulare* and *Vigna radiata* under lead- and mercury-induced toxicity (Kumar et al., 2013; Mondal et al., 2015). The structural malformation may also be attributed to the disintegration of the spongy parenchyma cells, resulting in a significant reduction of intercellular spaces.

From **Figure 3I**, the leaf samples of the control *L. purpureus* exhibited normal stomata with guard cells. Nonetheless, the exposure to 50% of wastewater effluent resulted in a deformed, swelling stomata, with the wide opening of stomatal apertures (**Figure 3J**). Similarly, structurally impaired stomata were

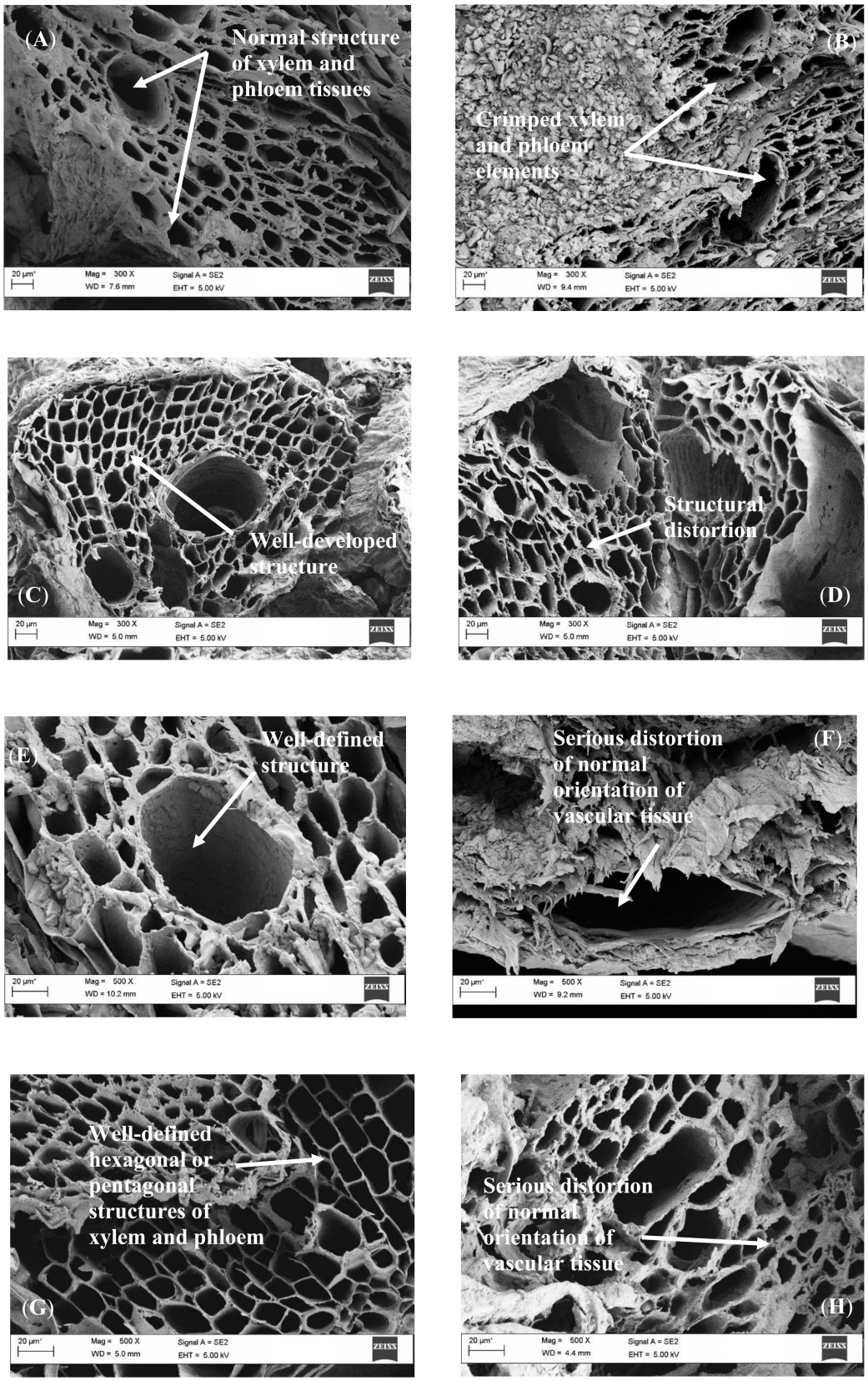

FIGURE 3 | Continued

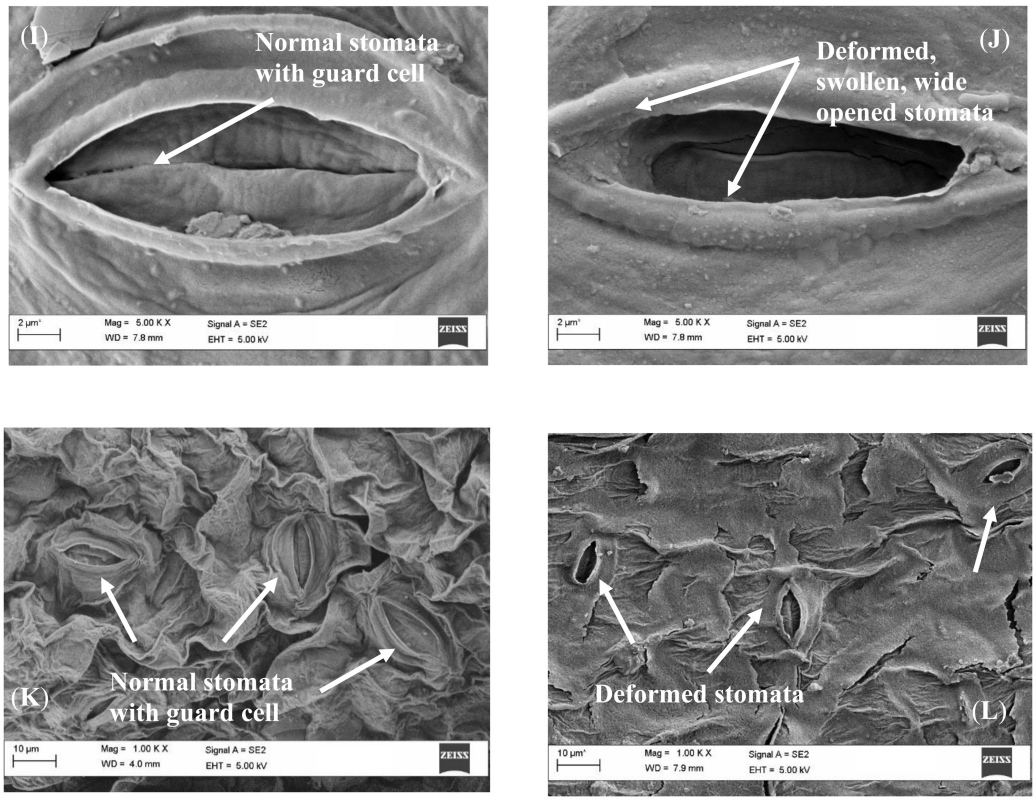

FIGURE 3 | Scanning electron micrographs of the root surface for the control *Lablab purpureus* **(A)**, 50% wastewater effluent-irrigated *L. purpureus* **(B)**, control *Brassica chinensis* **(C)**, and 10% wastewater effluent-irrigated *B. chinensis* **(D)**, the transverse sections of the shoot of control *L. purpureus* **(E)**, 50% wastewater effluent-irrigated *L. purpureus* **(F)**, control *B. chinensis* **(G)**, and 10% wastewater effluent-irrigated *B. chinensis* **(H)**, the leaf of control *L. purpureus* **(I)**, 50% wastewater effluent-irrigated *L. purpureus* **(J)**, control *B. chinensis* **(K)**, and 10% wastewater effluent-irrigated *B. chinensis* **(L)**.

observed in the leaves of *B. chinensis* under the irrigation of 10% wastewater effluent as compared with the normal stomata of the control (**Figures 3K,L**). Significant morphological alterations in the leaves not limited to the wide openings of stomata, with swollen surface, were consistent with the findings reported by Rai and Mehrotra (2008) in the leaves of the chromium-treated medicinal plant *Phyllanthus amarus* and by Mondal et al. (2013) in the leaves of cadmium-treated chickpea. This may be due to the preferential absorption of metal ions by subsidiary cells, and changes in the membrane permeability of the examined plant samples. The opening and closing of stomata were mainly governed by the alterations in the turgor and pressure of the guard cells, which resulted in the widening of the stomatal aperture. With the excessive accumulation of the metal ions, the swelling of intercellular substance between the guard cells took place, leading to the destruction of the connection between the cells and the guard cells, with the wide opening of stomata.

Proline Content

As a multifunctional proteinogenic five-carbon amino acid, proline plays primary roles in protecting the plant system under metal stress, particularly for the stabilization of proteins, membranes, subcellular structures, osmoregulation, and ROS scavenging. Despite the non-fully-elucidated physiological significance of proline, the enhancement of proline content in different plant species has been well documented (Waseem et al., 2019). In this work, a profound concentration-dependent relationship related to the accumulation of proline content has been observed, with a progressive increase of proline content from 1.40 to 2.04 μmol/g FW by increasing the percentage of wastewater effluent concentration from 0 to 25% ($F = 369.3$; $p < 0.05$) (**Figure 4**). However, beyond the concentration of 25%, the proline content of *L. purpureus* exhibited a drastic upsurge from 65.6 to 85.3% ($F = 313.5$; $p < 0.05$). Parallel with the reductive effects in the physical growth and chlorophyll contents, the proline level in *B. chinensis* was increased from 19.8 to 106.6%, with a dramatic peak observed at 10% of the wastewater effluent.

El-Amier et al. (2019) have reported a similar phenomenon in the nickel-treated *Pisum sativum* that recorded 47.6% greater accumulation than the control group at a nickel concentration of 100 μM. The accumulative effect might be driven by *de novo* synthesis, lower degradation and utilization, or breakdown of proteins. The abrupt increase in proline accumulation beyond 5% in *B. chinensis* and 25% in *L. purpureus* could be ascribed to the nickel-induced toxicity, which has exceeded the maximum tolerable threshold level of these plant models and highlighted the higher vulnerability of *B. chinensis* toward nickel ions.

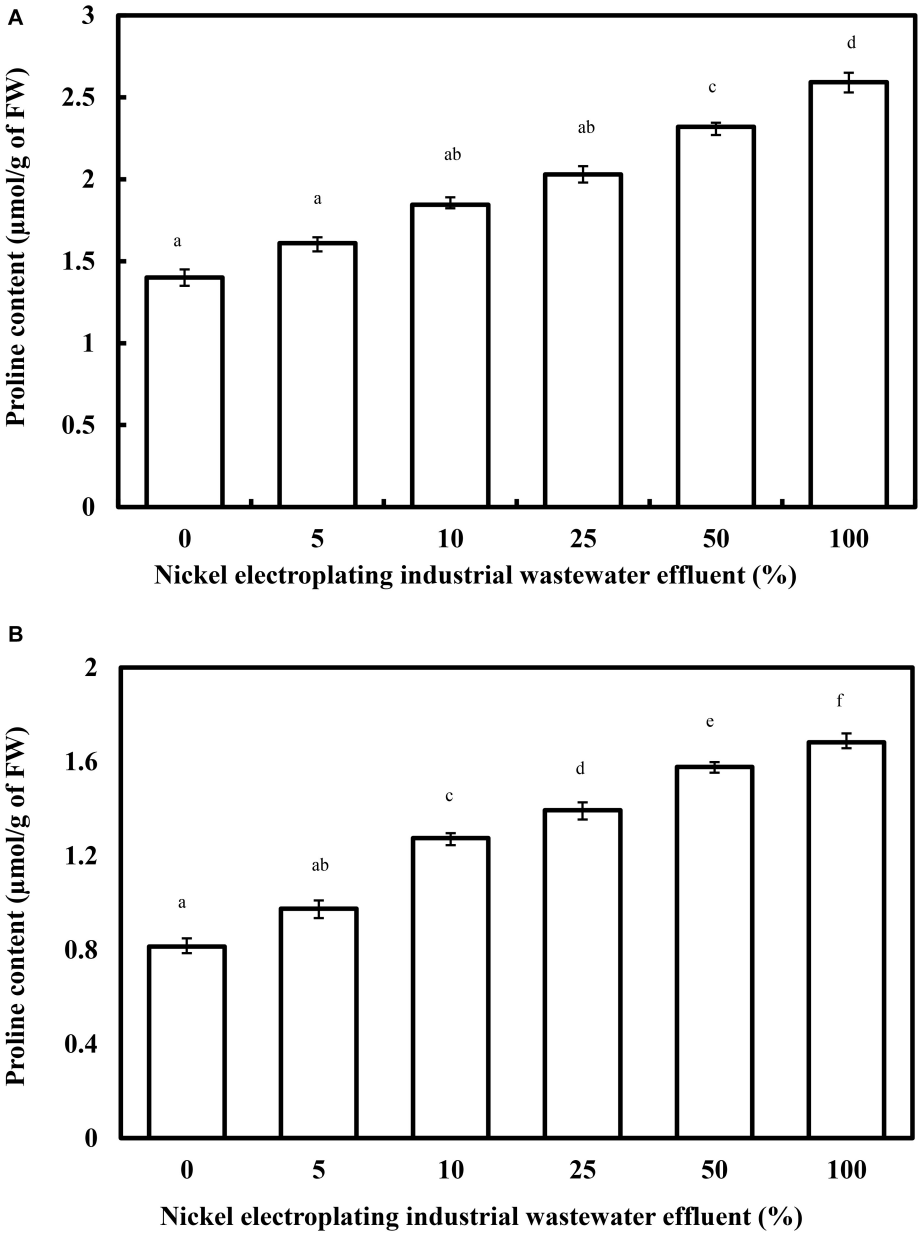

FIGURE 4 | The changing proline content in *Lablab purpureus* **(A)** and *Brassica chinensis* **(B)** with respect to the rising percentage of nickel electroplating industrial wastewater effluent. The experiment was performed with six replicates, and different alphabets indicate significant difference.

Accordingly, a number of metabolic mechanisms in the accumulation of proline against heavy metal-induced toxicity in plants have been elucidated. It is categorized into the adaptation, recovery, and signaling of stress tolerance. The accumulation not only emanated from the heavy metal-induced stress but also resulted from the water deficit syndrome (Kohli et al., 2019). In the present work, proline accumulation was recorded, which could be due to its leading function in osmoregulation or as an osmoprotectant, by adjusting the osmotic balance, altering the expression of specific genes to offset the water deficit syndrome, and controlling the stomatal closure to restrict the uptake or translocation of nickel ions via the suppression of transpiration (Hayat et al., 2012; Szepesi and Szõllõsi, 2018). The unique functional role of proline as a non-enzymatic antioxidant in the plant species under metal stress has been well established using both *in vitro* and *in vivo* studies (Kaul et al., 2008). Under metal stress conditions including nickel-induced stress, proline would prevent the inactivation of enzymes to upregulate the activities of peroxidases, superoxide dismutase, and CAT; induce the formation of phytochelatins; improve the

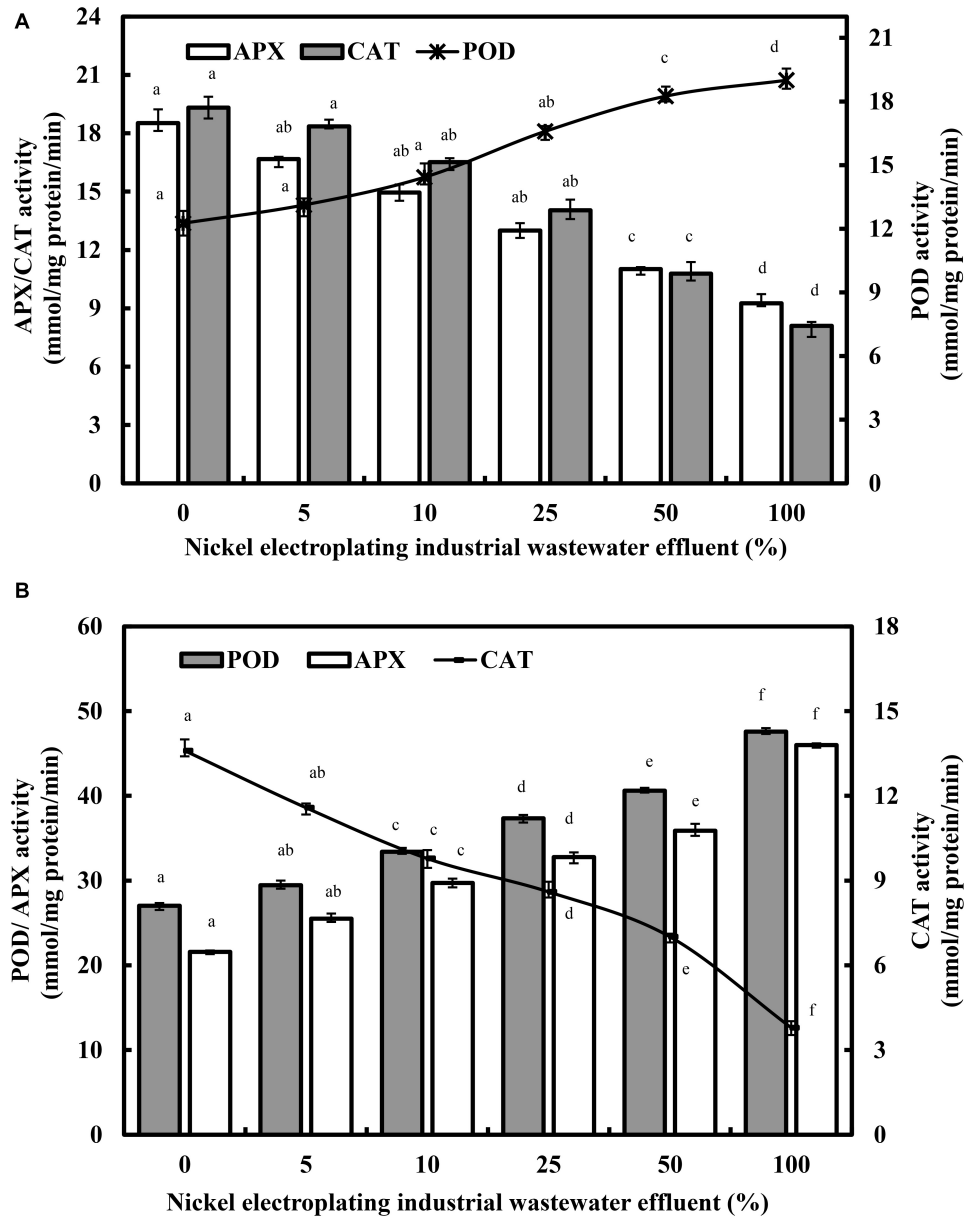

FIGURE 5 | The changing activities of guaiacol peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) in *Lablab purpureus* **(A)** and *Brassica chinensis* **(B)**. The experiment was performed with six replicates, and different alphabets indicate significant difference.

rigidity of the cell wall; inhibit the lipid peroxidation reaction; and govern the potassium ion efflux across the membrane (Kaur and Asthir, 2015).

Antioxidant Systems in Response to Nickel-Induced Toxicity

Figure 5 presents the varietal responses of POD, APX, and CAT enzymes in *L. purpureus* and *B. chinensis* subjected to nickel electroplating industrial wastewater effluent irrigation. Generally, the POD activities in both plant models were stimulated by nickel ions in a concentration-responsive manner. Increasing the industrial wastewater effluent from 0 to 100% promoted the rising POD activity from 12.23 to 18.90 mmol/mg of protein/min ($F = 242.9$; $p < 0.05$) in *L. purpureus* and from 27.02 to 47.60 mmol/mg of protein/min ($F = 313.2$; $p < 0.05$) in *B. chinensis*. Drastic upregulation of POD activities were observed beyond 25% in *L. purpureus* and 5% in *B. chinensis*.

Conversely, the APX activity was significantly inhibited from 18.52 to 9.26 mmol/mg of protein/min in *L. purpureus* ($F = 289.5$; $p < 0.05$), while *B. chinensis* demonstrated a concentration-dependent upregulation of APX activity from 21.58 to 46.01 mmol/mg of protein/min ($F = 359.1$; $p < 0.05$). However, in both plant models, CAT portrayed a similar

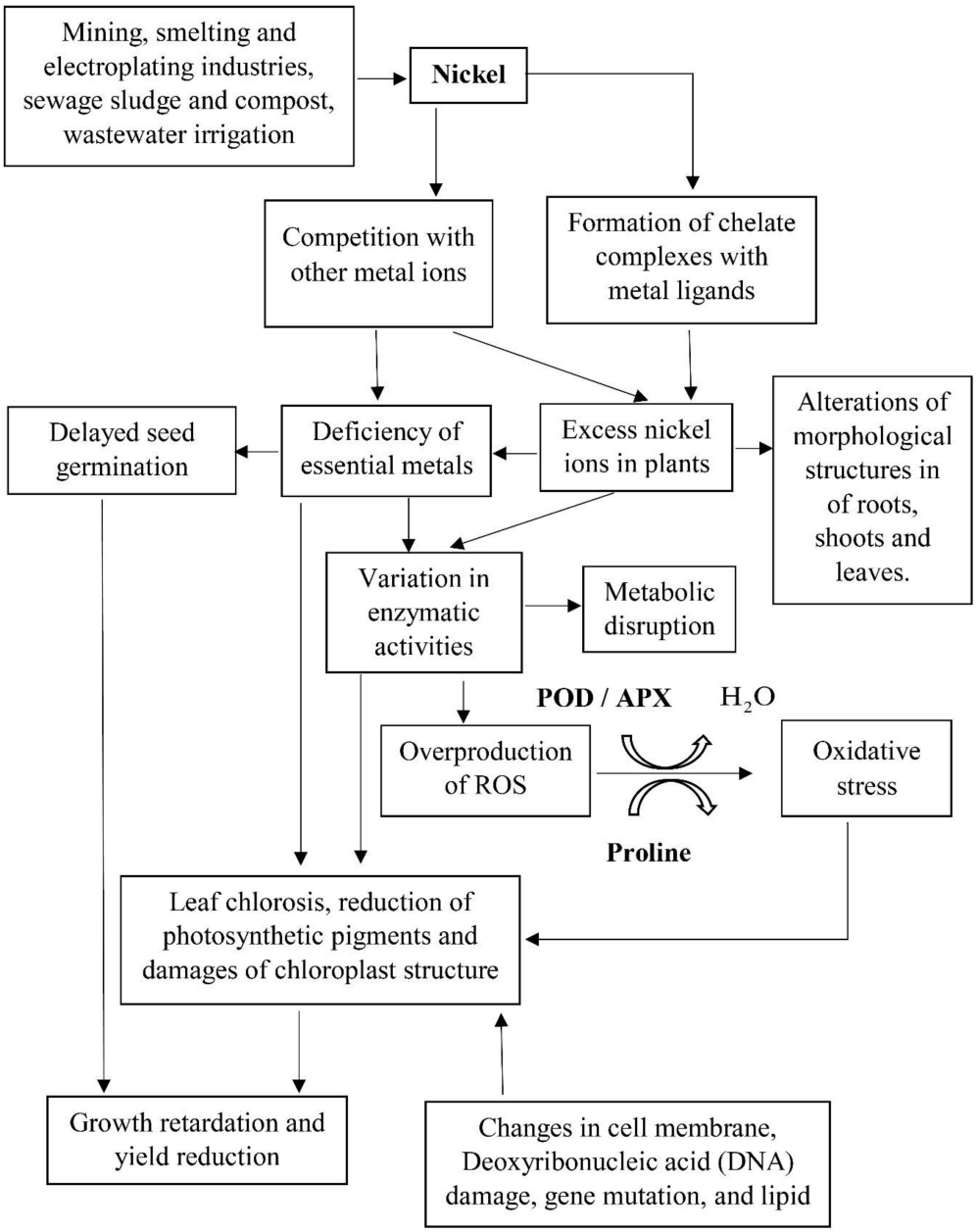

FIGURE 6 | The primary sources of nickel ions, mechanism of nickel-induced phytotoxicity, and the associated antioxidant defense system against the damages on the macromolecules in plant cells.

trend of downregulated activity, from 19.32 to 8.10 mmol/mg of protein/min in *L. purpureus* (F = 436.4; $p < 0.05$) and from 13.60 to 3.79 mmol/mg of protein/min in *B. chinensis* (F = 156.9; $p < 0.05$).

The current findings on the concentration-dependent upregulation of POD agreed satisfactorily with those discovered in the nickel-treated groundnut and soybean (Gopal, 2014; Sirhindi et al., 2016). In the abiotic stress state, the main function of POD is the biosynthesis of lignin, hence the rising POD activity that may, in part, explain the hindrance of root and shoot growth in *L. purpureus* and *B. chinensis* as observed concomitantly in this study. Additionally, POD could be involved as a H_2O_2 scavenger in the lignification reaction in response to the nickel-induced phytotoxicity. Excessive nickel concentration may lead to the overproduction of ROS, with the interaction of different antioxidant enzymes (**Figure 6**). A low concentration of nickel exposure has been shown to enhance the POD activities with the activation of the antioxidant defense system (Gajewska and Sklodowska, 2005; Gajewska et al., 2006; Mir et al., 2018). Conversely, excess nickel ions have been

found to be associated with lower ROS scavenging capability in plants, resulting in ROS accumulation and oxidative stress in plants. The abrupt upsurge of POD activity in *L. purpureus* and *B. chinensis* beyond 25 and 5% of irrigation water illustrated the threshold level of nickel-induced oxidative stress in the plant models, and a significantly higher POD activity is generally upregulated for ROS detoxification to protect plants from macromolecule damage.

On the other hand, the activities of CAT and APX were significantly suppressed in *L. purpureus*. The underlying mechanisms of the findings could be ascribed to the (i) reductive potential of nickel ions to induce iron deficiency of the APX metalloprotein complex in the plant tissues, as APX is a heme-dependent oxidoreductase whose catalytic activity is critically dependent on iron; (ii) binding of nickel ions with the thiol group of cysteine (Cys 32) at the active site of APX, leading to the inhibition of enzyme activity; and (iii) interactive reaction between nickel ions with the functional sulfhydryl groups, which might retard the enzymatic activity of APX in *L. purpureus* (Pandey and Sharma, 2002; Pandey et al., 2017; Karimi and Ghasempour, 2019). The activity of antioxidant enzymes may vary with the plant species, plant tissues, and stress duration, and this could justify the contrasting trends of APX activities in *L. purpureus* and *B. chinensis* (Gajewska and Skłodowska, 2007; Sreekanth et al., 2013). The rising activity of APX in *B. chinensis* could be an indication of the plant defense mechanism against free radical-induced toxicity, and the results were in concordance with those of the nickel-exposed *E. prostrata* (L.) (Chandrasekhar and Ray, 2018). The suppression in CAT activity was reported by Rao and Sresty (2000) and Gupta et al. (2017), which could be ascribed to the nickel-induced essential metal deficiency, resulting in the reduction of the biosynthesis of the iron porphyrin enzyme, as CAT is a metalloenzyme that contains Fe, Cu, Zn, or Mn in its prosthetic groups. Moreover, it has been demonstrated that the CAT activity was negatively related to the concentration of nickel ions in *Lycium barbarum* L. The low scavenging activity of CAT could be in part due to its presence in peroxisomes rather than in the chloroplasts or mitochondria, where majority of the radicals are generated (Pinto et al., 2019).

In the context of *L. purpureus*, the POD was negatively correlated with both APX ($r = -0.942$; $p < 0.01$) and CAT ($r = -0.953$; $p < 0.01$), while APX and CAT were positively correlated ($r = -0.957$; $p < 0.01$), indicating POD was the major antioxidant enzyme in detoxifying the excess-generated ROS under nickel-induced stress. Conversely, for *B. chinensis*, the positive correlation ($r = 0.994$; $p < 0.01$) between POD and APX, further verified by the negative correlations between CAT and POD ($r = -0.977$; $p < 0.01$) and APX ($r = -0.988$; $p < 0.01$), has supported the current findings that both of these antioxidant enzymes concurrently played concerted roles in the detoxification mechanism against nickel-induced oxidative stress. Differential antioxidant enzyme response toward the wastewater effluent irrigation indicated the factor of species variations toward nickel toxicity. The alterations of the POD, APX, and CAT activities under nickel excess in both plant models were in agreement with the literature findings, as presented in **Supplementary Table 3**.

Recent scientific research has highlighted the uptake and redistribution of nickel ions within the plant species via the pathway of cation and metal–ligand complex transport system during the exposure to nickel-contaminated water (Chen et al., 2009). The primary sources of nickel ions, the mechanism of nickel-induced phytotoxicity, and the associated antioxidant defense systems against the damages on the macromolecules in the plant cells are illustrated in **Figure 6**. In the present study, an excess of nickel ions has been depicted to exhibit toxic implications to *L. purpureus* and *B. chinensis*, verified by the inhibition of root and shoot elongation, diminution of chlorophyll content via the disruption of chloroplast structures, and alterations in morphological characteristics of roots, shoots, and leaves, and these perturbations in metabolic pathways could promote the overproduction of ROS. The augmented activities of POD and APX and accumulation of proline have been identified to be the key biomarkers of nickel-induced phytotoxicity to stimulate the defense machinery of *L. purpureus* and *B. chinensis* to accommodate the detoxification reaction.

CONCLUSION

The present research has verified the viability of nickel electroplating industrial wastewater effluent diluted at different concentrations as a source of nutrient recycling using a hydroponic soilless cultivation system. The significant toxicity as evidenced by the inhibition of the root and shoot elongation and reduction of photosynthetic pigments, accompanied by the profound morphological distortions in the xylem, phloem, and stomata, was observed beyond the maximum tolerable concentration level at 25% of wastewater effluent for *L. purpureus* and 5% of wastewater effluent for *B. chinensis*. The accumulation of proline and upregulation of POD and APX activities were detected against the nickel electroplating industrial wastewater-induced oxidative stress injury in the plant models. Highlighting the different resistance of plant species toward nickel electroplating industrial wastewater toxicity, these findings contributed to a better understanding on the possible detrimental impacts resulting from the nickel-contaminated electroplating industrial wastewater irrigation on the food crops, which could be closely associated with the crop yield, plant physiological processes, and the sustainability of ecosystem via wastewater reuse.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

YNC contributed to conceptualization, investigation, methodology, formal analysis, and writing – original draft. NAZ contributed to resources and funding acquisition.

LKL contributed to investigation and supervision. KYF contributed to resources, conceptualization, writing – review and editing, visualization, supervision, funding acquisition, and project administration. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.609396/full#supplementary-material

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High Salinity Reduces Plant Growth and Photosynthetic Performance but Enhances Certain Nutritional Quality of C_4 Halophyte *Portulaca oleracea* L. Grown Hydroponically Under LED Lighting

Jie He*, Xueli You and Lin Qin

National Institute of Education, Nanyang Technological University, Singapore, Singapore

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****Correspondence:***
Jie He
jie.he@nie.edu.sg

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Portulaca oleracea L. (known as purslane) is one of the most nutritious leafy vegetables owing to its high content of antioxidants. In this study, all plants were grown indoors hydroponically with different NaCl salinities. Photosynthetic photo flux density (PPFD) at 200 μmol m^{-2} s^{-1} (12 h) was provided to all plants by LED with red:blue ratio of 2.2. Thirty days after transplanting, plants grown with100 mM NaCl had the highest productivity and the fastest leaf growth followed by those with 0, 200 and 300 mM NaCl. Grown with 300 mM NaCl, purslane had the lowest specific leaf area due to its highest leaf dry matter content and its lowest water content. All plants had similar values of leaf succulence except for those with 300 mM NaCl. Total chlorophyll and carotenoids contents were significantly higher in plants grown with 0 and 100 mM NaCl than with 200, and 300 mM NaCl. All plants had F_v/F_m ratios close to 0.8. However, electron transport rate and $\Delta F/F_m'$ were significantly higher in plants grown with 0 and 100 mM NaCl than with 200 and 300 mM NaCl. CAM-induced purslane with 300 mM NaCl had higher non-photochemical quenching. Maximum net photosynthetic O_2 evolution rate and Cyt b_6f concentration were significantly lower with 300 mM NaCl compared to all other plants while all plants had similar PS II concentration. Proline concentration increased with increasing salinities. All plants had similar levels of total soluble sugars. Plants grown with 0 and 100 mM NaCl had significantly higher concentrations of NO_3^-, total reduced nitrogen, total leaf soluble protein, Rubisco protein, total ascorbic acid, and total phenolic compounds than with 200 and 300 mM NaCl. The highest concentrations of K, Ca, and Mg were found in purslane grown under 0 mM NaCl. Statistically, no significant differences in Fe concentrations were observed among all plants. However, salinity seems to increase Fe concentration. In conclusion, it is feasible to grow purslane under 100 mM NaCl as it is the most optimal condition to achieve higher productivity and better quality. However, the production of antioxidants may depend on not only salinity but also other growth conditions.

Keywords: dietary minerals, hydroponics, LED lighting, photosynthesis, phytochemicals, *Portulaca oleracea* L.

INTRODUCTION

According to the International Water Management Institute, agriculture, which accounts for about 70% of global water withdrawals, is constantly competing with domestic, industrial, and environmental uses for a scarce water supply. Singapore's biggest threat is the water crisis. An integrated approach, therefore, is imperative for increasing food security. To increase food supply locally, the utilization of seawater to produce halophytes as vegetables hydroponically, which uses much less water than traditional farming, could be a strategy to address water scarcity in Singapore. The almost infinite availability of seawater highlights the importance of halophytes as a source of vegetable crops, particularly since they do not compete with glycophytic food crops (Ventura et al., 2015).

Portulaca oleracea L. (known as purslane) is a nutritious leafy vegetable mainly native to the Mediterranean basin and widespread throughout the world (Karkanis and Petropoulos, 2017; Petropoulos et al., 2018). Purslane also has medicinal properties (Uddin et al., 2014; Petropoulos et al., 2016), and it has been listed among the most common used plants for medicinal purposes by WHO (Naeem and Khan, 2013). Interest in cultivating purslane as a vegetable has increased since its bioactive compounds, such as omega-3 fatty acid (Palaniswamy et al., 2001; Teixeira et al., 2010; Bessrour et al., 2018) and ascorbic acid, together with other antioxidants, vitamins, and essential amino acids have been identified (Franco et al., 2011). According to Yazici et al. (2007), purslane is a succulent dicot halophyte, which is drought- and salt-tolerant. However, Kafi and Rahimi (2011) reported that purslane is considered as a moderately salt tolerant species, with a capacity to withstand soil salinity only up to 240 mM. Cultivating purslane under the same NaCl salinity levels hydroponically, Anastaćio and Carvalho (2013) did not observe any toxicity symptoms. Moreover, it was reported that the salinity tolerance of purslane is significantly decreased when plants are grown under low light intensity (Franco et al., 2011). Apart from utilizing the seawater resources to mass produce halophyte vegetables, to compensate for the lack of available land, Singapore also needs to develop vertical farming systems to produce potential edible halophyte vegetables under LED lighting. Our recent studies also showed that an edible halophyte leafy vegetable, *Mesembryanthemum crystallinum* (ice plant) grown indoors was affected by NaCl salinity (He and Qin, 2020a) and induced drought stress (He et al., 2020) as well as LED spectral quality when plants grown with freshwater (He et al., 2017). These studies show that salinity, water supply, and light quality affect productivity, photosynthetic performance, and nutritional quality of *M. crystallinum* grown indoors. However, a very little research has been done on the effects of salinity on growth, photosynthetic performance, and nutritional quality of purslane grown indoors under LED lighting with constant levels of light intensity.

Purslane is the only genus known to have both C_4 and crassulacean acid metabolism (CAM). It is also well-known CAM was elicited if *purslane* was subjected to drought stress (Koch and Kennedy, 1982; Lara et al., 2004; D'Andrea et al., 2014; Winter and Holtum, 2014; Mulry et al., 2015). Lara et al. (2004) reported that the succulent purslane plant shifted its C_4 photosynthetic metabolism to CAM after 23 days of withholding water. In another study with purslane, by measuring the net CO_2 exchange for the above-ground tissues of a young purslane, Winter and Holtum (2014) found that net CO_2 uptake was observed in the dark but CO_2 exchange in the light was limited to a short burst at the beginning of the light period after no watering for 10 days. However, the CO_2 fluxes increased during the light and the dark, reverting to a non-CAM pattern within 24 h after re-watering. Ferrari et al. (2020) evaluated CAM plasticity of 11 subspecies of *P. oleracea* from distant geographical locations, and they concluded that all subspecies expressed CAM in a fully-reversible manner. However, there are different combinations of CAM expression level within the *P. oleracea* complex. When CAM was induced in purslane under drought stress, D'Andrea et al. (2014) found that chlorophyll fluorescence parameters were transitorily affected and non-radiative energy dissipation mechanisms were induced by measuring the effective quantum yield of PSII ($\Delta F/F_m'$), the photochemical quenching coefficient (qP) and non-photochemical quenching (qN or NPQ). In the study of halophyte *M. crystallinum* L., Broetto et al. (2007) found that, when CAM was induced in this species under combination of salinity and high light, the operation of photosystem II (PS II) was affected. The electron transport rate (ETR) and $\Delta F/F_m'$ were reduced while NPQ was increased (Broetto et al., 2007). Our recent studies on *M. crystallinum* L. also showed the similar results (unpublished). These results suggest that CAM induction and its relation to PS II photochemistry make it possible to evaluate photosynthetic performance and the extent of its tolerance to salinity stress. However, to the best of our knowledge, there is very little study on the upregulation of CAM in C_4 purslane under salinity stress.

Accumulations of bioactive compounds have been investigated extensively in purslane (Zhou et al., 2015). When a CAM is induced under drought stress and then reversed back to C_4 upon re-watering in purslane, there was a clear metabolic shift, implying that antioxidants may be involved in photosynthetic machinery protection. Increases in osmolytes, such as proline and total soluble sugar (TSS), could contribute to withstand drought (Rahdari et al., 2012; D'Andrea et al., 2014; Jin et al., 2016). Salt stress also results in high accumulation of proline in purslane (Yazici et al., 2007). Not only proline, but also TSS that can be utilized in functional food, are produced for protection against hyperosmotic stress (Flowers and Colmer, 2008; Agarie et al., 2009; Hsouna et al., 2020). Sdouga et al. (2019) highlighted a correlation between the gene expression varying by population and saline concentration and the level of proline in the leaves of *P. oleracea*. Apart from the accumulation of proline, halophytes are also able to synthesize natural antioxidants such as ascorbic acid and total phenolic compounds under saline and drought conditions (Dat et al., 2000; Ksouri et al., 2007; He et al., 2020). According to Uddin et al. (2012a), the antioxidant potential of purslane was indeed mainly dependent on the total phenolic content. However, Lim and Quah (2007) reported that there was a great variation in the accumulation of total phenolic compounds, which was directly dependent on the season of the year.

Dietary minerals also contribute to the valuable nutritional profile of purslane. Potassium (K), calcium (Ca), magnesium (Mg), and iron (Fe) are abundant in purslane plant (Uddin et al., 2012b). The effects of salinity on mineral composition of purslane leaves have been reported by a number of researchers. For instance, Teixeira and Carvalho (2009) concluded that high salinity resulted in decreases of Ca, K, and Zn contents. Significant differences of the mineral compositions have been also observed among different salinity levels (Uddin et al., 2012b). Chowdhar et al. (2013) reported that Ca was negatively correlated with Na, while there were positive correlations among Na and K, Mg, and Fe.

Impacts of drought stress have been studied frequently in purslane, and the induction of CAM could reduce the negative effects of drought stress. *Portulaca oleracea* engages multiple strategies to cope with the drought and CAM induction is a metabolic strategy of this species to maintain photosynthesis under drought stress (D'Andrea et al., 2014; Ferrari et al., 2020). However, there is little published work exploring the expression of CAM, growth, physiological responses, and nutritional quantity of purslane to salinity when they were grown hydroponically indoors under LED lighting. Thus, this study aimed to address the following objectives: (1) explore the application of saline water to grow purslane hydroponically under LED lighting by studying shoot and root productivity, leaf growth, and its water status; (2) study the impacts of salinity on photosynthetic performance of purslane including photosynthetic light use efficiency, photosynthetic O_2 evolution, CAM acidity, and the concentrations of PS II and Cyt b_6f; and (3) study nutritional quality including both phytochemical and dietary mineral accumulations.

MATERIALS AND METHODS

Plant Materials and Culture Methods

Seeds of purslane (*P. oleracea* L. cv. POR – 2936) produced in Holland were germinated on filter papers before being inserted into polyurethane cubes. All seedlings were incubated under a photosynthetic photon flux density (PPFD) of 100 μmol m^{-2} s^{-1} provided by high-pressure sodium lamps for 5 weeks. Seedlings were then transplanted into an indoor hydroponic systems and were grown under red/blue LED ratios of 2.2 (WR-16 W, Beijing Lighting Valley Technology Co., Ltd., China), and the light spectral distribution is shown in **Figure 1**. All plants were exposed to the same level of PPFD of 200 μmol m^{-2} s^{-1}, 12 h photoperiod and were grown under three NaCl salinities by adding 100, 200, and 300 mM NaCl, respectively, to a full-strength Netherlands Standard Composition with 2.2 ± 0.2 mS cm^{-1} conductivity and pH 6.0 ± 0.2. The room temperature and relative humidity were 24.5/23°C and 56/82% (day/night), respectively.

Measurements of Shoot and Root Productivity, Leaf Growth, and Leaf Water Status

Plants from each treatment were harvested after 30 days of transplanting. Total leaf number was recorded.

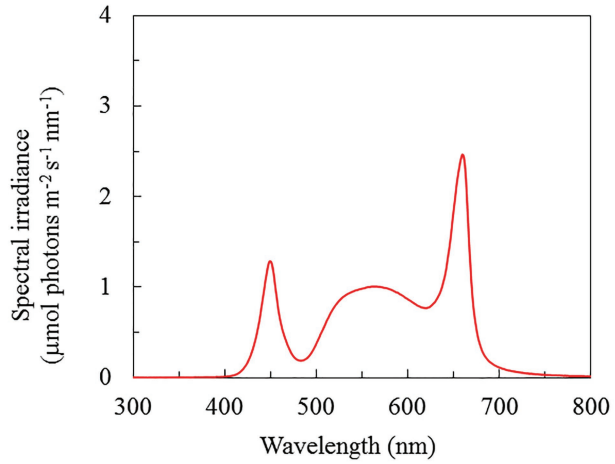

FIGURE 1 | Light spectral distribution of *P. oleracea* grown hydroponically with different NaCl salinities. Spectral scans were recorded every 0.5 nm with a spectroradiometer (PS300, Apogee Instruments, United States).

Shoot (leaves + stem) and root were separated for fresh weight (FW) measurement. Total leaf area (TLA) was measured using a leaf area meter (WinDIAS3 Image Analysis system). Shoot and roots were then dried at 80°C for 4 days, before re-weighing them to obtain dry weight (DW). Specific leaf area (SLA) was determined as L_a/L_{DW} where L_a = leaf area (cm^2) and L_{DW} = leaf dry weight (g; Hunt et al., 2002). Leaf succulence (LS) was estimated as L_{FW}/L_a where L_{FW} = leaf FW (Agarie et al., 2007). Leaf dry matter content (LDMC) was determined by L_{DW}/L_{FW} (Garnier et al., 2001). Leaf water content (LWC) was determined as $(L_{FW}-L_{DW})/L_{FW}$.

Measurements of Total Chlorophyll and Carotenoids Concentration

Twenty-six days after transplanting, leaf discs of fully expanded young leaves were weighed and placed in 5 ml of N, N-dimethylformamide (N,N-DMF, Sigma Chemical Co.) in darkness for 48 h at 4°C. The absorption of pigments was measured using a spectrophotometer (UV-2550 Shimadzu, Japan) at 647, 664, and 480 nm, respectively. Chl a, Chl b and Car concentrations were calculated as described by Wellburn (1994).

Measurement of Chl Fluorescence F_v/F_m Ratio

Maximum quantum yield of PSII was estimated in dark-adapted attached fully expanded young leaves by the F_v/F_m ratio during mid-photoperiod using the Plant Efficiency Analyzer (Hansatech Instruments, UK) according to He et al. (2011).

Measurements of Electron Transport Rate, Effective Quantum Yield of PSII, and Non-photochemical Quenching

The whole young fully expanded leaves were placed on moist filter papers in Petri dishes. They were pre-darkened for 15 min prior to measurements. *Via* the IMAGING PAM MAXI

(Walz, Effeltrich, Germany), the images of fluorescence emission were digitized within the camera and transmitted *via* a Firewire interface (400 megabits/s; Firewire-1394, Austin, TX, United States) to a personal computer for storage and analysis. Measurements and calculations of ETR, $\Delta F/F_m'$, and NPQ were determined according to He et al. (2011).

Determination of CAM Acidity

Based on He and Teo (2007), leaf discs were punched from fully expanded young leaves and then placed in microtitre plate wells before the beginning and the end of photoperiod. The Milli-Q water (1 ml) was added to each well before placing in a 95°C water bath for 15 min. The extracts in the wells were titrated against 0.005 M NaOH, using three drops of phenolphthalein for indicator until end-point was reached. Final volume of NaOH used to reach end-point was used to calculate CAM acidity as both μmol H^+ g^{-1} FW and μmol H^+ g^{-1} DW.

Measurements of Light Response Curves of Net Photosynthetic O_2 Evolution Rate (P_N), PS II, and Cyt b_6f Concentrations

According to He and Qin (2020b), modified from He and Chow (2003), these parameters were measured at 25°C from leaf discs of fully expanded young leaves, which were harvested 16–24 days after transplanting. A whole leaf as placed in a gas-phase oxygen electrode (Hansatech, King's Lynn, UK) chamber contained 1% CO_2 supplied by 1 M $NaHCO_3/Na_2CO_3$ (pH 9). Two illumination regimes were used to obtain photosynthetic O_2 evolution rates: (1) repetitive flash illumination with saturating, single-turnover flashes or (2) continuous white light from light emitting diodes. The measurements of P_N and the calculations of PS II and Cyt b_6f concentrations were carried out as in Zhu et al. (2017) and He and Qin (2020b).

Measurement of NO_3^- Concentration

Dried tissues of 0.01 g were grounded with Mill-Q water, and the details of extraction processes were described in He et al. (2020). The flow injection analyzer (Model Quickchem 800, Lachat Instruments Inc., Milwaukee, United States) was used to determine NO_3^- concentration according to He et al. (2020).

Measurement of Total Reduced Nitrogen

Dried shoot tissues (0.05 g) were digested with a Kjeldahl tablet in concentrated sulfuric acid for 60 min at 350°C (Allen, 1989), and the mixture was allowed to cool before TRN was determined by a Kjeltec 2300 analyzer (Foss Tecator AB, Höganäs, Sweden) through titration.

Measurements of Leaf Total Soluble Protein and Rubisco Protein Contents

Frozen leaf samples of 1 g were ground in liquid nitrogen. The details of extraction processes were described in He et al. (2017). The amount of TSP was determined using the method according to Lowry et al. (1951). Rubisco protein present in the samples was quantified using SDS-PAGE. Solubilized protein was boiled for 5 min and loaded onto the Mini-PROTEAN Precast Gel (TGX gel, any kD, BIO-RAD, United States) to run the gels for 30 min and then stained for 1 h using coomassie brilliant blue according to He et al. (2017). The separated proteins stained were analyzed using a Fluor Chem 8800 gel imagine system under visible light. The amount of TSP loaded were used to calculate Rubisco content based on the band of large subunit (LSU) and small subunit (SSU), and Rubisco content is the sum of LSU and SSU.

Determination of Total Soluble Sugars

After 30 days of transplanting, leaf tissue was dried at 80°C for 4 days. Dry samples of 10 mg were used to extract TSS using hot 80% ethanol. The details of extraction processes were described in He et al. (2020). The concentration of free soluble sugar was determined colorimetrically at 490 nm using a spectrophotometer (UV-2550 Shimadzu, Japan) according to Dubois et al. (1956).

Determination of Proline Concentration

This assay was modified from the protocol by Bates et al. (1973) using frozen leaf tissues of 0.5 g. The details of extraction processes and the measurements of absorbance at 520 nm using a spectrophotometer (UV-2550 Shimadzu, Japan) were described in He et al. (2020).

Measurement of Ascorbic Acids

Total ascorbic acid (Asc) was assayed from 0.5 g of frozen leaves harvested from fully expanded young leaves 33 days after transplanting by the reduction of 2,6-dichlorophenolindophenol (DCPIP) according to Leipner et al. (1997) and modified by He et al. (2020). The Asc concentrations were spectrophotometrically assayed by measuring the absorbance at 524 nm using a spectrophotometer (UV-2550 Shimadzu, Japan). L-ascorbic acid was used as a standard.

Determination of Total Phenolic Compounds

Frozen leaf samples (0.5 g) were ground in liquid nitrogen and 5 ml of 80% methanol (Ragaee et al., 2006). The details of extraction processes and the measurements of absorbances at 765 nm using a spectrophotometer (UV-2550 Shimadzu, Japan) were described in He et al. (2020).

Determination of Dietary Minerals

Dried tissues were digested in 65% nitric acid using an UltraWAVE single reaction chamber microwave digestion system (Milestone, United States). Digested samples were diluted with Milli-Q water. The dietary minerals were measured using Optima 8,300 ICP-OES Spectrometer and WinLab 32 (Perkin Elmer, United States) according to Bocchini et al. (2015).

Statistical Analysis

One-way ANOVA was used to test for significant differences of different variances crossed with the four salinity treatments.

LSD multiple comparison tests were used to discriminate the means (IBM SPSS Statistics 25).

RESULTS

Shoot and Root Productivity

All purslane plants grew well and appeared healthy from transplanting to harvest although plants grown with 200 and 300 mM NaCl were much smaller compared to those grown with 0 and 100 mM NaCl (**Figure 2**). At harvest, the shoot, root FW, and DW of purslane plants were the highest with 100 mM NaCl than with 0, 200, and 300 mM NaCl. Purslane plants with 100 mM had shoot FW almost 10-fold higher than those grown with 300 mM. Statistically, there was no difference in shoot FW between purslane grown with 200 and 300 mM NaCl but they were significantly lower than those grown with 0 mM NaCl (**Figure 3A**). For shoot DW (**Figure 3D**), root FW (**Figure 3B**) and root DW (**Figure 3E**), purslane grown with 100 mM NaCl had the highest values followed by those grown with 0 mM NaCl and then 200 mM NaCl while those grown with 300 mM NaCl had the lowest value. There were no significant differences in shoot/root ratio FW among different treatments (**Figure 3C**). For shoot/root ratio DW, plants grown with 300 mM NaCl had the highest value followed by those with 0 mM NaCl and those with 100 and 200 mM NaCl had similar lowest readings (**Figure 3F**).

FIGURE 2 | Purslane plants grown indoors hydroponically with 0 **(A)**, 100 **(B)**, 200 **(C)**, and 300 mM NaCl **(D)** under LED lighting (R/B ratio of 2.2) for 30 days.

Leaf Growth and Leaf Water Status

Purslane grown with 100 mM NaCl had the highest total leaf number and TLA followed by those grown with 0 and then 200 mM NaCl while those grown with 300 mM NaCl had the lowest value (**Figures 4A,B**). There were no significant difference in SLA between plants grown with 0 and 100 mM NaCl but they were significantly higher than those grown with 200 and 300 mM NaCl. Plants grown with 300 had the lowest SLA (**Figure 4C**). There were no significant differences in LS among plants grown with 0, 100, and 200 mM NaCl, and they were significantly higher than that of plants grown with 300 mM NaCl (**Figure 5A**). The trend for LWC was similar to that of LS except for those grown with 200 mM NaCl had significantly lower LWC than with 0 and 100 mM NaCl (**Figure 5C**). LDMC increased with increasing NaCl concentrations. Plants grown with 300 had the highest LDMC while those with 0 and 100 mM had the lowest values (**Figure 5B**).

Photosynthetic Pigments

There were no significant difference in total Chl concentration between purslane grown with 0 and 100 mM NaCl but they were significantly higher than those grown with 200 and 300 mM NaCl. Purslane plants grown with 200 mM had higher total Chl concentration than with 300 mM NaCl (**Figure 6A**). For total Car concentration, purslane grown with 100 mM NaCl had the highest value followed by those grown with 0 mM NaCl and then 200 mM NaCl while those grown with 300 mM NaCl had the lowest reading (**Figure 6B**). Chl a/b ratios were significantly higher in plants grown with 200 and 300 mM NaCl compared to those grown with 0 and 100 mM NaCl (**Figure 6C**). Purslane grown with 100 mM had the lowest Chl/Car ratio due to its highest Car concentration among the different treatments (**Figure 6D**).

F_v/F_m Ratio, ETR, $\Delta F/F_m'$, and NPQ

Figure 7 shows the maximum quantum yield of PSII, measured by F_v/F_m ratio and photochemical light use efficiency measured by ETR, $\Delta F/F_m'$, and NPQ under an actinic light of 606 μmol m^{-2} s^{-1}, which was the saturating irradiance for ETR. Although there were difference in F_v/F_m ratios statistically, all readings were close or slightly higher than 0.8 (**Figure 7A**). The values of ETR and $\Delta F/F_m'$ were significantly higher in purslane grown with 0 and 100 mM NaCl compared to those grown with 200 and 300 mM NaCl (**Figures 7B,C**). Purslane grown with 300 mM NaCl had the highest NPQ followed by those grown with 200 mM and then 0 and 100 mM conditions (**Figure 7D**).

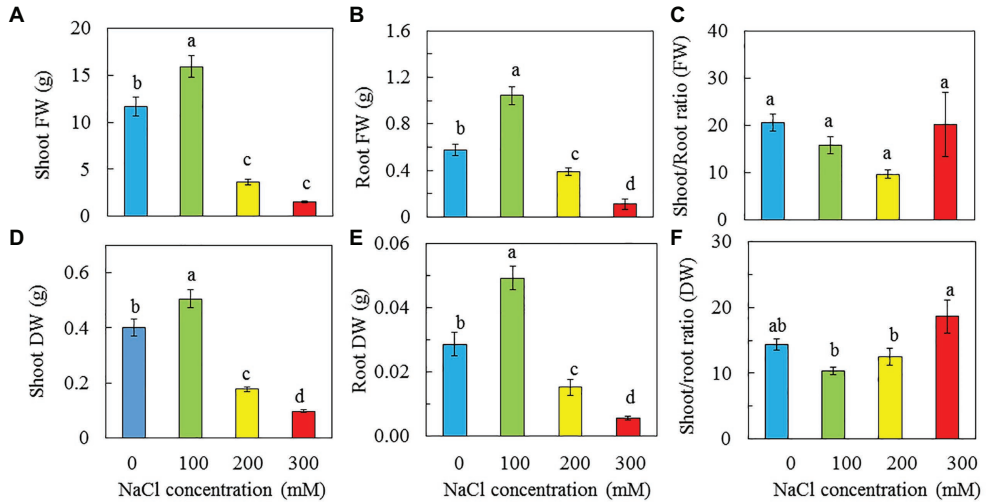

FIGURE 3 | Shoot FW and DW **(A,D)**, root FW and DW **(B,E)**, and shoot/root ratio FW and DW **(C,F)** of *P. oleracea* grown hydroponically with different NaCl salinities for 30 days. Vertical bars represent the standard errors. Means with different letters are statistically different ($p < 0.05$; $n = 5$) as determined by LSD multiple comparison test.

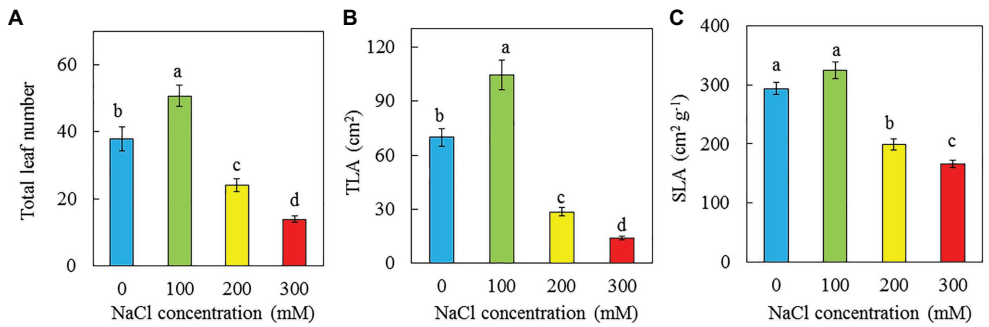

FIGURE 4 | Total leaf number **(A)**, TLA **(B)**, and SLA **(C)** of *P. oleracea* grown hydroponically with different NaCl salinities for 30 days. Vertical bars represent the standard errors. Means with different letters are statistically different ($p < 0.05$; $n = 5$) as determined by LSD multiple comparison test.

Light Response Curves of P_N and CAM Acidity

Light response curves of P_N were determined from all plants grown under different NaCl salinities. The values of P_N for purslane plants grown with 0 and 100 mM NaCl increased with increasing PPFD from 10 to 1,210 μmol m^{-2} s^{-1}. However, P_N of purslane grown with 300 and 200 mM NaCl reached saturated points, respectively, at PPFD of 405 and 808 μmol m^{-2} s^{-1} (**Figure 8A**). Measured under the highest PPFD, P_N of purslane plants grown with 0 mM NaCl was not significantly different from those grown with 100 mM but significantly higher than that of plants grown with 200 and 300 mM NaCl. Although there was no significant difference in P_N between 100 and 200 mM, they were both significantly higher than that of plants grow with 300 mM NaCl (**Figure 8A**). There were no significant differences for CAM acidity among plants grown with 0, 100, and 200 mM NaCl. However, the CAM acidities on the basis of FW and DW for purslane grown with 300 mM were respectively about 4.5- and 2.5-fold higher than the rest plants (**Figures 8B,C**).

PS II and Cyt b_6f Concentrations

PS II concentrations seem to be lower in plants grown with 300 mM NaCl compared to other conditions. However, statistically, there were no significant differences in PS II concentrations among purslane grown with different NaCl salinities (**Figure 9A**). For Cyt b_6f concentrations, they were similar but significantly higher in plants grown with 0 and 100 compared to those with 300 mM NaCl. There was no significant different in Cyt b_6f between purslane grown with 200 and 300 mM NaCl. Cyt b_6f concentrations for plants grown with 0, 100, and 200 mM NaCl were respectively 46, 43, and 29% greater than those of plants grown with 300 mM NaCl (**Figure 9B**).

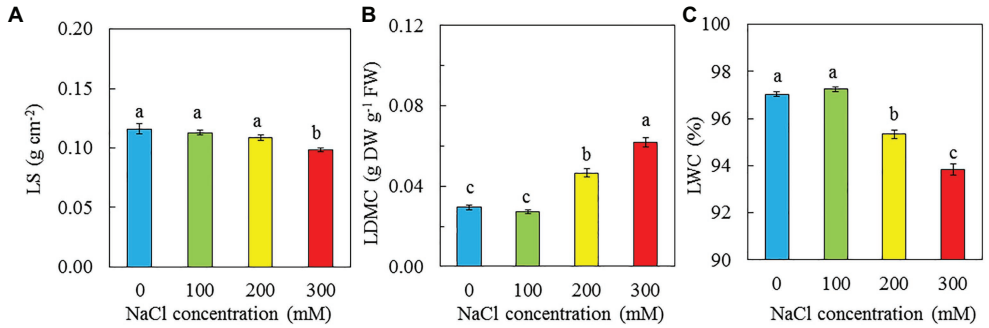

FIGURE 5 | LS **(A)**, LDMC **(B)**, and LWC **(C)** of *P. oleracea* grown hydroponically with different NaCl salinities for 30 days. Vertical bars represent the standard errors. Means with different letters are statistically different ($p < 0.05$; $n = 5$) as determined by LSD multiple comparison test.

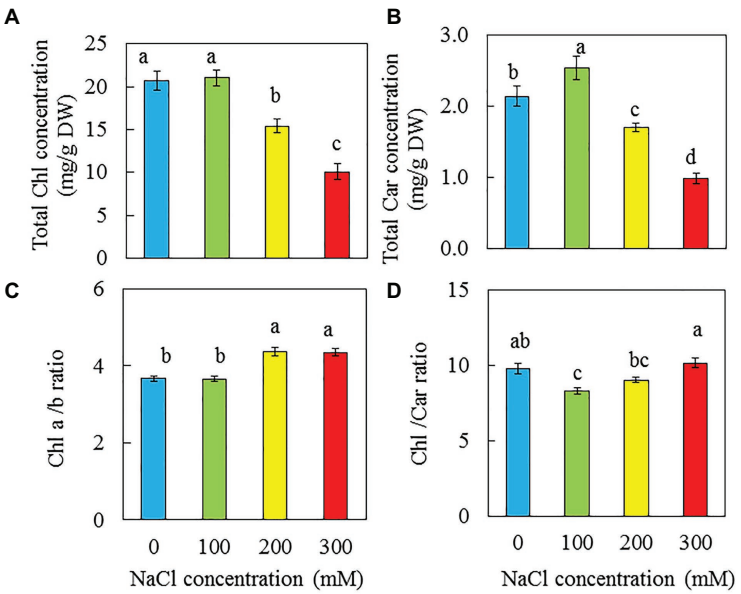

FIGURE 6 | Total Chl concentration **(A)**, total Car concentration **(B)**, Chl a/b ratio **(C)**, and Chl/Car ratio **(D)** of *P. oleracea* grown hydroponically with different NaCl salinities for 26 days. Vertical bars represent the standard errors. Means with different letters are statistically different ($p < 0.05$; $n = 4$) as determined by LSD multiple comparison test.

NO_3^-, TRN, Total Leaf Soluble Protein, and Rubisco Protein

On a DW basis, the concentrations of NO_3^-, TRN, and Rubisco protein exhibit similar trends in response to NaCl salinity, showing that purslane plants grown with 0 and 100 mM NaCl were similar but significantly higher than those grown with 200 and 300 mM NaCl (**Figures 10A,B,D**). For leaf total soluble protein, the plants grown with 0 and 100 mM NaCl had the highest values followed by those grown with 200 mM NaCl while plants grown with 300 mM Nacl had the lowest concentration (**Figure 10C**).

Proline, TSS, Asc, and Total Phenolic Compounds

Purslane grown with 0 mM NaCl had the lowest proline concentration, which increased with increasing NaCl salinity. Plants grown with 300 mM NaCl had the highest proline concentration, which was 2.7-, 3.9-, and 10-fold of those respectively grown with 200, 100, and 0 mM NaCl (**Figure 11A**). However, there were no significant differences in TSS concentration among all plants (**Figure 11B**). Purslane plants grown with 0 and 100 mM NaCl produced much higher total Asc (**Figure 11C**) and total phenolic compounds (**Figure 11D**) compared to those grown with 200 and 300 mM NaCl. Similar highest Asc concentrations were obtained from plants grown with 0 and 100 mM NaCl and they were 98 and 210%, respectively, higher than those grown with 200 and 300 mM NaCl. Purslane grown with 0 and 100 mM NaCl also had similar highest concentration of total phenolic compounds and they were 105 and 160%, respectively, higher than those of plants grown with 200 and 300 mM NaCl (**Figure 11D**).

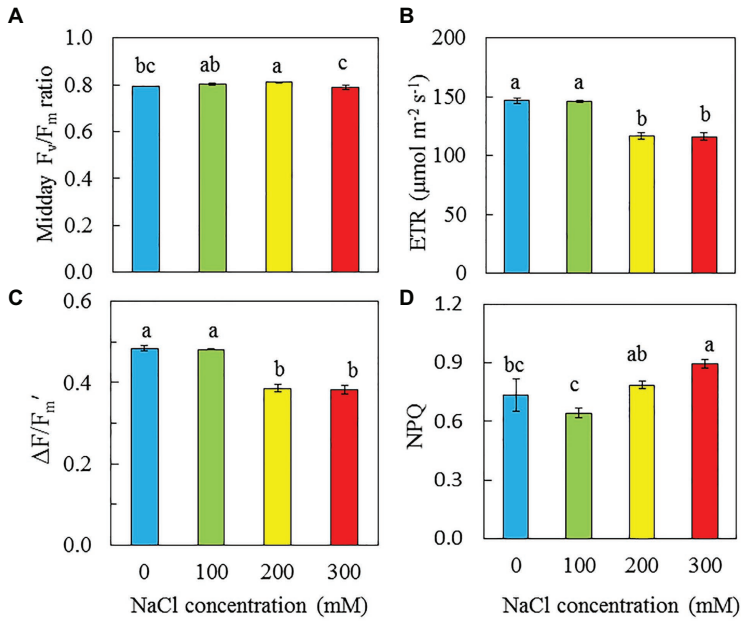

FIGURE 7 | Midday Chl fluorescence F_v/F_m ratio **(A)**, ETR **(B)**, $\Delta F/F_m'$ **(C)**, and NPQ **(D)** of *P. oleracea* grown hydroponically with different NaCl salinities for 26 days. Vertical bars represent the standard errors. Means with different letters are statistically different ($p < 0.05$; $n = 5$ for **A**; $n = 6$ for **B-D**) as determined by LSD multiple comparison test.

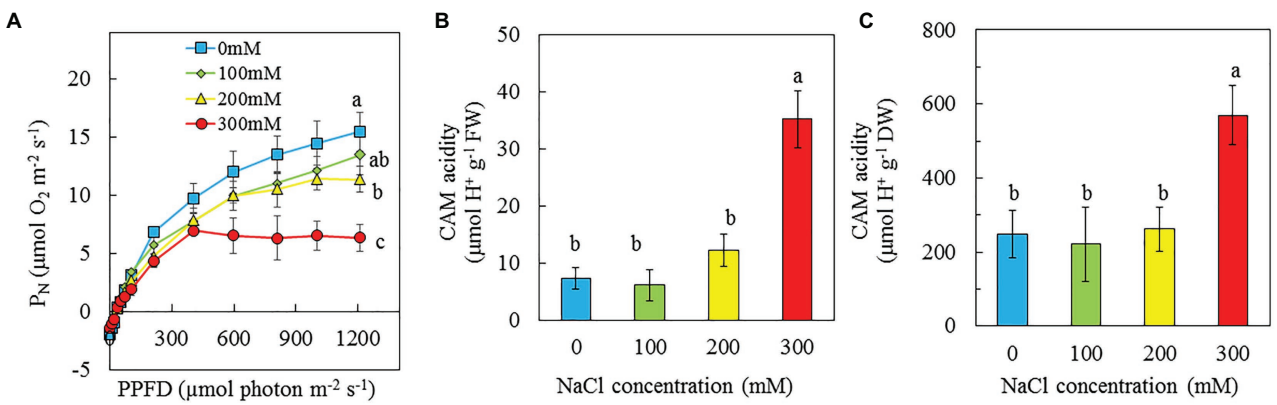

FIGURE 8 | Light response curves of P_N **(A)**, CAM acidity on a FW basis **(B)**, and CAM acidity on a DW basis **(C)** of *P. oleracea* grown hydroponically with different NaCl salinities from 16 to 24 days **(A)** and for 30 days **(B,C)** after transplanting. Vertical bars represent the standard errors. Means with different letters are statistically different ($p < 0.05$; $n = 5$) as determined by LSD multiple comparison test.

Dietary Minerals

High NaCl salinity resulted in decreases of K, Ca, and Mg (**Figures 12A–C**). The concentrations of K, Ca, and Mg were highest in purslane grown with 0 mM NaCl followed by those grown with 100 mM and then 200 mM NaCl while those grown with 300 mM NaCl had the lowest concentration. Statistically, there were no significant differences in Fe concentrations among all treatments. However, salinity seems to increase Fe concentration of purslane (**Figure 12D**).

DISCUSSION

Productivity of Shoot and Root, Leaf Growth, and Leaf Water Status

As common purslane is a drought and salinity tolerant plant (Yazici et al., 2007; Teixeira and Carvalho, 2009), it is an important crop for farming in the Mediterranean basin. According to Alam et al. (2014a), it can also be grown under various soil conditions and types at different locations of West Peninsular, Malaysia. In this study, purslane plants were grown hydroponically

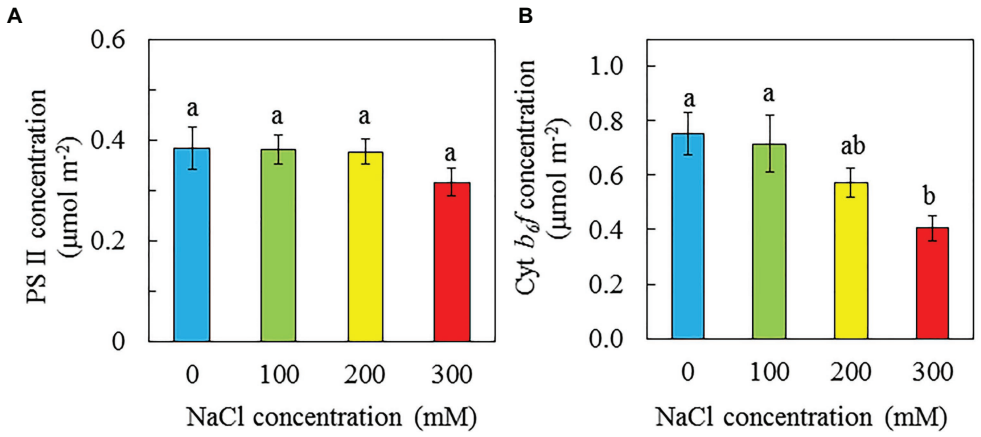

FIGURE 9 | PS II concentration **(A)** and Cyt b_6f concentration **(B)** of *P. oleracea* grown hydroponically with different NaCl salinities from 16 to 24 days after transplanting. Vertical bars represent the standard errors. Means with different letters are statistically different ($p < 0.05$; $n = 4$) as determined by LSD multiple comparison test.

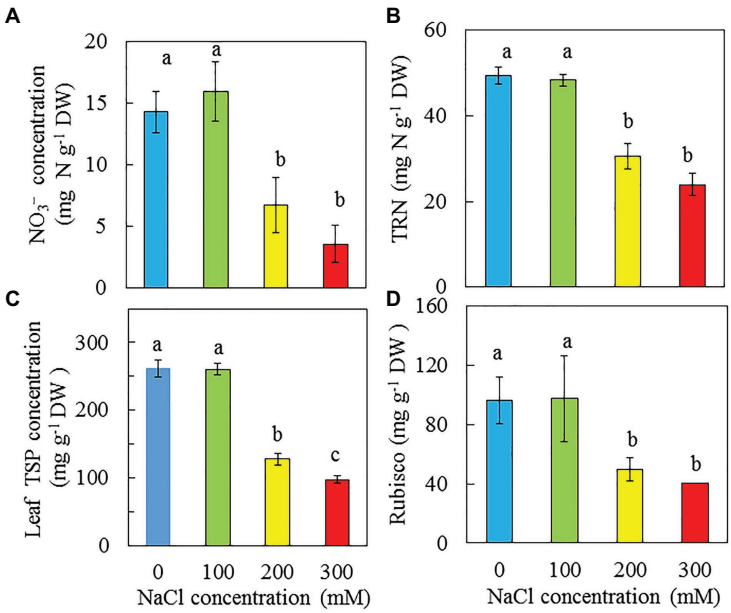

FIGURE 10 | Concentrations of NO_3^- **(A)**, TRN **(B)**, total soluble protein **(C)**, and Rubisco **(D)** in the leaves of *P. oleracea* grown hydroponically with different NaCl salinities for 30 days **(A,B)** and 33 days **(C,D)**, respectively. Vertical bars represent the standard errors. Means with different letters are statistically different ($p < 0.05$; $n = 3$) as determined by LSD multiple comparison test.

indoors with different NaCl salinities under a constant light intensity of 200 μmol m^{-2} s^{-1} provided by LEDs and all plants appeared healthy (**Figure 2**). Plants grown with 100 mM NaCl had the highest shoot and root biomass compared to those with 0, 200, and 300 mM NaCl (**Figure 3**). According to Flowers et al. (1986), most halophytes require saline conditions to attain optimal growth. Our results showed that purslane plants grown with 100 mM NaCl had higher shoot and root productivity compared to those grown with fresh water (0 mM NaCl). Furthermore, according to our observation, purslane plants have the ability to complete their lifecycle on >200 mM NaCl, and thus it is a halophyte crop (Yuan et al., 2019). Grown with different NaCl salinities indoors hydroponically under LED lighting, our study also showed that an editable halophyte *M. crystallinum* L. required 100 mM NaCl to perform better growth compared to those grown with fresh water (He and Qin, 2020a). However, high salt concentration often results in hyperosmotic and oxidative stress, which can hinder the growth and development of plants, and may even lead to death (Xiong et al., 2002; Fan et al., 2011). Although those grown

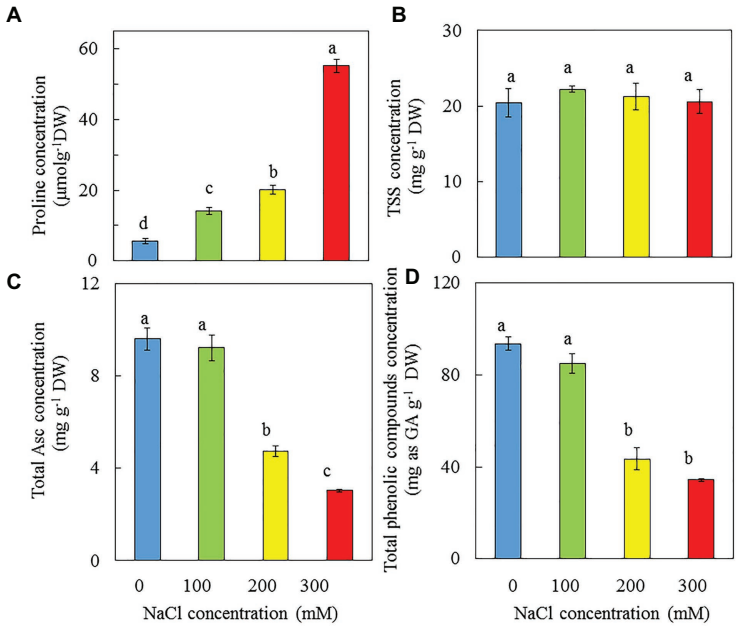

FIGURE 11 | Concentrations of proline **(A)**, TSS **(B)**, total Asc **(C)**, and total phenolic compounds **(D)** in the leaves of *P. oleracea* grown hydroponically with different NaCl salinities for 30 days **(B)** and 33 days **(A,C,D)**, respectively. Vertical bars represent the standard errors. Means with different letters are statistically different ($p < 0.05$; $n = 3$) as determined by LSD multiple comparison test.

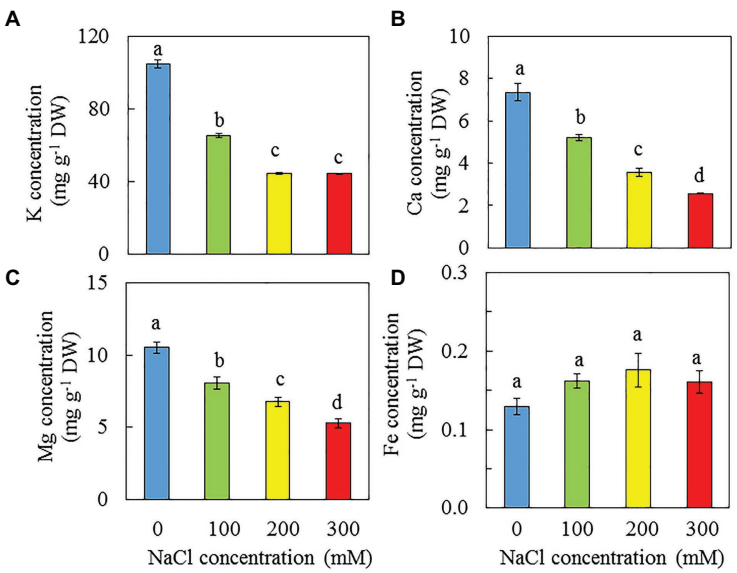

FIGURE 12 | K **(A)**, Ca **(B)**, Mg **(C)**, and Fe **(D)** concentrations *n* in the leaves of *P. oleracea* grown hydroponically with different salinities for 30 days. Vertical bars represent the standard errors. Means with different letters are statistically different ($p < 0.05$; $n = 5$) as determined by LSD multiple comparison test.

with higher salinities, such as 200 and 300 mM NaCl, were much smaller compared to those grown with 0 and 100 mM NaCl (**Figures 2, 3**), none of them showed any visual symptoms of damage, indicating that the purslane plants in this study had a capacity to withstand soil salinity up to 300 mM. Similarly, cultivating purslane under 240 mM NaCl hydroponically, Anastaćio and Carvalho (2013) did not observe any toxicity symptoms. It was reported that the salinity tolerance of purslane and the impact of salinity on purslane growth and development depend on other conditions such as genotypes (Alam et al., 2014b) and light level (Franco et al., 2011). Moreover, Poljakoff-Mayber and Lerner (1999)

reported that shoot growth was frequently inhibited more than root growth under saline conditions. However, with purslane, Franco et al. (2011) found the opposite, with greater reductions in root length than in stem length. In the study with *M. crystallinum* grown with different NaCl salinities aeroponically indoors under LED lighting, we have recently found that shoot/root ratio FW and DW were, respectively, the lowest and the highest in *M. crystallinum* grown with 500 mM NaCl compared to those grown with 100 and 250 mM NaCl. The different responses to salinity between shoot/root ratio FW and DW resulted from the lowest LWC and highest LDMC in *M. crystallinum* grown under the highest salinity of 500 mM NaCl (unpublished). In this study with purslane, all plants had similar shoot/root ratio FW (**Figure 3C**). However, plants grown with 300 mM NaCl had the highest shoot/root DW (**Figure 3F**) due to its highest LDMC (**Figure 5B**) and lowest LWC (**Figure 5C**).

Low productivity of purslane under higher salinity, such as 200 and 300 mM NaCl, could mainly be due to its slow leaf growth and development supported by the lower total number of leaves (**Figure 4A**) and smaller TLA (**Figure 4B**). In the study with two purslane genotypes, Zaman et al. (2019) reported that leaf numbers were significantly decreased in the main stem in both genotypes when grown with high concentration of NaCl due to excess accumulation of salt during the development of leaf. This study also showed that plants grown with 0 and 100 mM NaCl had similar but significantly higher SLA compared to those grown with 200 and 300 mM NaCl (**Figure 4C**). The SLA was the lowest in purslane grown with 300 mM NaCl (**Figure 4C**), resulting from its lowest TLA (**Figure 4B**) and highest LDMC (**Figure 5B**). According to Rodríguez et al. (2005), decreases in leaf area linked to salinity directly affect the SLA of *Asteriscus maritimus* plants as salinity increased. In study with *M. crystallinumm*, we found that plants grown with higher salinity had lower TLA and lower SLA (He and Qin, 2020a). Similar results were observed in this study with purslane plants (**Figures 4A,B**). The decrease of SLA is often associated with the increase in LS of salt tolerant species (Geissler et al., 2009; de Vos et al., 2010, 2013; Rozema et al., 2015). LS is measured as the maximum water content expressed as fresh mass per unit of leaf area (g FW cm^{-2}; Debez et al., 2004). In our study of *M. crystallinumm*, plants with 250 and 500 mM NaCl had much lower SLA compared to those grown with 0 and 100 mM NaCl while salinity did not affect its LS on a leaf area basis (He and Qin, 2020a). In this study, purslane grown with 300 mM NaCl had lower SLA and LS compared to those grown with other lower concentration of NaCl (**Figures 4C, 5A**). The lower LS of purslane grown with higher salinity (**Figure 5A**) could be partially due to its lower LWC (**Figure 5C**). According to Belkheiri and Mulas (2013), LS increased with increasing of salinity. Optimal NaCl concentration for growth has been reported to be the concentration at which LS is highest. A further increase in salinity resulted in decrease of both growth and LS (Khan et al., 2000). However, it appears that the correction between SLA and LS depends on plant species.

Photosynthetic Pigments

Salinity stress could affect the photosynthetic performance due to salt accumulation in leaves (Munns and Tester, 2008) and the deceases in total Chl (Parida et al., 2002; Stepien and Johnson, 2009; Duarte et al., 2013; Zaman et al., 2019). In the study of the glycophyte Arabidopsis and the halophyte Thellungiella, Stepien and Johnson (2009) found that the Chl content of Arabidopsis continuously declined when exposed to salt while there was no significant change in the Chl content of Thellungiella when exposed to same salinity conditions. In our recent study with *M. crystallinumm*, it was found that plants grown with 500 mM NaCl had significantly higher total Chl content than those grown with 0, 100, and 250 mM NaCl (unpublished). Similarly, in the study with two obligate halophytes, *Sesuvium portulacastrum* and *Tecticornia indica*, total Chl contents were significantly enhanced in both species grown under 200 mM and 400 mM NaCl (Rabhi et al., 2012). In the study with two purslane genotypes, Zaman et al. (2019) found that with the increase of NaCl concentrations from 0 to 200 mM, total Chl content decreased. In this study, higher salinity also resulted in lower Chl content in purslane (**Figure 6A**). Apparently, salt-tolerant species show increased or unchanged Chl content under higher salinity conditions, whereas Chl levels decrease in salt-sensitive species. In the study with purslane, Franco et al. (2011) reported that salinity did not affect the Chl content when it was grown in the growth chambers under lower level of light (73.5 μmol m^{-2} s^{-1}). However, in the same study with purslane, it was found that the Chl content decreased with an increase in salinity when they were grown in the greenhouse under higher light intensity (530 μmol m^{-2} s^{-1}). Based on the above discussion, the total Chl content could be considered as a biochemical marker of salt tolerance in plants (Stepien and Johnson, 2009) although the impact of salinity on total Chl content may depend on light intensity (Franco et al., 2011). In the study with halophyte, *Plantago coronopus* (L.), Koyro (2006) reported that salt-induced increase of the Car content, which could function to dissipate the excess energy in the PS I and PS II. In this study, opposite result was obtained for purslane as significantly decreased total Car concentration was observed in purslane grown with 300 mM NaCl while those grown with 100 mM NaCl had the highest value (**Figure 6B**). The Chl a/b ratios of purslane was also affected by salinity, showing a significant increase when grown with 200 and 300 mM NaCl (**Figure 6C**). However, the lowest Chl/Car (or the highest Car/Chl) ratio was observed in plant grown with 100 mM NaCl (**Figure 6D**). Chl b is mainly located in the light harvesting complex, the higher Chl a/b ratio in purslane grown with higher salinity could result in reduced photosynthetic light use efficiency (Koyro, 2006).

Photosynthetic Performance

Chl fluorescence could provide information about the performance of PS II (Maxwell and Johnson, 2000). Maximal efficiency of PS II photochemistry measured by Chl fluorescence F_v/F_m ratio from the dark-adapted leaves is an early indicator of salinity stress (Broetto et al., 2007; Matsuoka et al., 2018; Zaman et al., 2019). In the study with two purslane genotypes,

Zaman et al. (2019) reported that with the increase of NaCl concentrations, F_v/F_m ratio significantly decreased in both genotypes. However, in this study, F_v/F_m ratios in all dark-adapted purslane leaves were close to 0.8 (**Figure 7A**), suggesting that there was no evidence of damage to PS II in any plants (Broetto et al., 2007). The different responses of F_v/F_m ratios of purslane plants to salinity between this study and the study by Zaman et al. (2019) could be due to the different growth conditions. In this study, all plants were grown indoors under a PPFD of 200 μmol m^{-2} s^{-1} while Zaman et al. (2019) cultivated their plants in the greenhouse under a PPFD of 400 μmol m^{-2} s^{-1}. Our recent study with *M. crystallinum* grown indoors under a 200 μmol m^{-2} s^{-1} also showed that salinity stress did not affect the maximal efficiency of PS II photochemistry (He and Qin, 2020a). While the impacts of salinity on maximal efficiency of PS II photochemistry may depend on the light level under which halophytes were grown, general decreases in ETR, qP, and the effective quantum yield of PS II ($\Delta F/F_m'$), but increases in NPQ have been reported under salt stress conditions (Broetto et al., 2007; D'Andrea et al., 2014; Acosta-Motos et al., 2017). Decrease in PS II efficiency and increase in NPQ correlated with the response of plants to salt stress could be a strategy to safely dissipate excess energy (Acosta-Motos et al., 2017). Study with purslane plants, D'Andrea et al. (2014) found that drought stress resulted in decreases of ETR and $\Delta F/F_m'$ and an increase of NPQ. In this study, decreases in ETR and $\Delta F/F_m'$ were observed in purslane grown with 200 and 300 mM NaCl compared to those with 0 and 100 mM NaCl (**Figures 7B,C**). It was reported that PS II and Cyt b_6f may be the sites of the rate-limiting step in the electron transport chain (Eichelmann et al., 2000). Stepien and Johnson (2009) reported that the inhibition of linear electron flow in Arabidopsis under salt stress was accompanied by a downregulation of electron flow through the Cyt b_6f complex. In this study, lower ETR and $\Delta F/F_m'$ observed in purslane grown with high salinity could be due to the tuning of the amount of active PSII reaction centers and regulating the electron transfer by the Cyt b_6f complex (Tikkanen et al., 2012; He and Qin, 2020b). This was supported by the results of lower PS II (**Figure 9A**) and Cyt b_6f (**Figure 9B**) concentrations in purslane grown with 300 mM NaCl compared to other conditions. It was also found that purslane grown with the highest NaCl salinity had the highest NPQ (**Figure 7D**). Increased NPQ implies that purslane grown with the highest salinity such as 300 mM NaCl in this study could safely dissipate excess energy (Acosta-Motos et al., 2017). This also explains why purslane plant grown with high concentration of NaCl was healthy regardless of its slow growth (**Figure 2**).

Acosta-Motos et al. (2015) concluded that the drop in P_N paralleled with a decline in the effective quantum yield of PS II ($\Delta F/F_m'$) and increases in NPQ, acted as a safe mechanism for dissipating excess light energy. In this study, P_N of purslane grown with higher salinity reached saturated points at lower PPFDs. Measured under the highest PPFD of 1,210 μmol m^{-2} s^{-1}, P_N of purslane plants grown with 300 mM was much lower compared to those grown with lower NaCl concentrations (**Figure 8A**). The reduction of P_N could also be due to the decrease of Calvin Cycle enzymes such as Rubisco (Acosta-Motos et al., 2017). It has been reported that salt stress alter Rubisco expression (Dionisio-Sese and Tobita, 2000). In the study with *Desmostachya bipinnata* (L.) Staph, Asrar et al. (2017) found that decreased Rubisco content was observed with increasing salinity treatments. In this study, purslane plants grown with 200 and 300 mM NaCl had much lower soluble protein and Rubisco protein compared to those grown with 0 and 100 mM NaCl (**Figures 10C,D**).

CAM is normally elicited in *purslane* under drought stress (Lara et al., 2004; D'Andrea et al., 2014; Winter and Holtum, 2014; Mulry et al., 2015). Salinity and drought are two often simultaneously occurring stresses. Thus, CAM acidity was also included in this study. In the study with *M. crystallinum*, Borland et al. (2006) concluded that CAM can be induced by abiotic and biotic stresses in responses to changing environmental conditions. Cushman et al. (2008) suggested that CAM acidity levels of *M. crystallinumm*, at least 40 μmol H^+ g^{-1} FW were deemed to be performing CAM under saline conditions. In this study, on the basis of both FW and DW, the CAM acidities for purslane grown with 300 mM were much higher compared to the rest of plants (**Figures 8B,C**). Although CAM acidity of purslane grown with 300 mM was slightly lower than 40 μmol H^+ g^{-1} FW (**Figure 8B**), it was most likely be engaging in CAM-like under high salt stress as it was a 4.5-fold increase compared to those grown with lower concentrations of NaCl. The term "CAM-like" has been used for purslane plants (Lara et al., 2003). *Portulaca* spp. tend to inhabit environments with high light intensities, weak CAM acidity of purslane plants under high salinity in this study could be due to the low level of light used to cultivate this plant species. Furthermore, there are different CAM expression levels within the *P. oleracea* complex (Ferrari et al., 2020). Under drought stress, when CAM was induced in purslane, lower qP, and higher NPQ were observed (D'Andrea et al., 2014). In this study, CAM-like purslane grown under the highest salinity of 300 mM NaCl also had higher NPQ (**Figure 7D**). The regulation of NPQ determines the levels of plant responses under saline conditions. Studies have showed that higher NPQ was observed in CAM-inducible *M. crystalline* under high salt stress (Broetto et al., 2007; Niewiadomska et al., 2011; Matsuoka et al., 2018). Thus, increased NPQ under high salinity condition can also be one of the indicators for CAM induction.

Nutritional Quality

Proline is a metabolite with multiple roles such as antioxidant and osmoprotectant (Szabados and Savouré, 2010). Thus, proline that can be utilized in functional food is produced for protection against hyperosmotic stress caused by high salinity (Flowers and Colmer, 2008; Agarie et al., 2009; Benjamin et al., 2019; Hsouna et al., 2020). It has been reported that the correlation between saline concentration and the level of proline gene expression in purslane varies by population (Sdouga et al., 2019). In this study, the accumulation of proline in purslane plants increased with increasing salinity (**Figure 11A**). Yazici et al. (2007) also reported that salt stress resulted in high accumulation of proline in purslane. TSS is also well-known

osmolyte that enables plants to avoid the consequences of hyperosmotic stress (Hsouna et al., 2020). In our recent study, higher TSS accumulation was observed in *M. crystallinum* grown with 500 mM NaCl compared to those grown with lower concentration of NaCl (unpublished). However, in this study, all purslane plants had similar level of TSS (**Figure 11B**). The natural antioxidants, such as Asc and phenolic compounds, play important roles in various physiological responses to stresses in purslane plants (Lim and Quah, 2007; Uddin et al., 2012a). There are very few studies available on the impact of salinity on the contents of Asc and phenolic compounds in purslane grown under different salinity conditions indoors with hydroponic cultivation. High levels of vitamin C and some vitamins of complex-B have also been identified in purslane (Petropoulos et al., 2016). In this study, Asc concentration of purslane grown with 0 and 100 mM NaCl were 98 and 210%, respectively, higher than those grown with 200 and 300 mM NaCl (**Figure 11C**). These results indicate that increasing NaCl concentration from 100 mM to 200 or 300 mM did not increase the Asc concentration. Our recent studies have shown that drought stress enhanced the concentrations of Asc of *M. crystallinum* grown indoors under combination of red and blue-LED lighting (He et al., 2020). In another study, we also found that Asc concentration of *M. crystallinum* increased with increasing NaCl from 0 to 500 mM NaCl (unpublished data). It was reported that the antioxidant potential of purslane mainly depends on the content of total phenolic compounds (Uddin et al., 2012a). The contents of total phenolic compounds in different plant tissues increased with the increase in salinity have also been reported in a number of plants (Agastian et al., 2000; Muthukumarasamy et al., 2000; Navarro et al., 2006). In the study with halophyte *Cakile maritima*, Ksouri et al. (2007) concluded that plants, such as halophytes tolerant to stress, are potentially interesting systems for production of secondary metabolites, such as phenolic compounds, are useful for food and medicinal applications. In this study, it was unexpected to see that purslane plants grown with 0 and 100 mM NaCl accumulated much higher total phenolic compounds compared to those grown with 200 and 300 mM NaCl (**Figure 11D**). This could be due to the fact that plants vary widely in their concentrations of total phenolic compounds with both genetics and environment affecting the level of phenolic compounds (De Abreu and Mazzafera, 2005). Lim and Quah (2007) reported that there is a great variation in the accumulation of total phenolic compounds, which was directly dependent on the season of the year under natural conditions. Furthermore, lower concentration of Asc and total phenolic compounds for purslane grown under higher salinity could be associated with their lower photosynthetic performance (**Figures 7B,C, 8A**). In the study with *Hypericum perforatum* L. (St. John's wort), a traditional herb, Mosaleeyanon et al. (2005) reported that growing this herb plants under optimal conditions can enhance biomass and secondary metabolite production by increasing net photosynthetic rate. Establishing optimal growth conditions to enhance photosynthetic capacity and secondary metabolites of purslane grown indoors with different saline conditions merit our future study.

According to Corrè and Breimer (1979), purslane has very high NO_3^- content (>2,500 mg kg $^{-1}$ FW). In this study, the NO_3^- concentrations on a DW basis of purslane plants grown with 0 and 100 mM NaCl were similarly and significantly higher (about 2 folds) than those grown with 200 and 300 mM NaCl (**Figure 10A**). Lower NO_3^- concentrations are associated with lower concentrations of TNR, total soluble protein and Rubisco protein (**Figures 10B–D**). Lower total Chl content in the leaves of purslane grown with higher concentrations of NaCl could be partially due to the lower TRN. In this study, after the conversion, the NO_3^- concentrations of purslane grown with 0, 100, 200, and 300 mM NaCl were 1868, 2081, 1,381, and 1,235 mg kg $^{-1}$ FW, respectively, and each of these values was much lower than 2,500 mg kg^{-1} FW as reported by Corrè and Breimer (1979). Purslane is a NO_3^- accumulating plant; the exact contents depend on both cultivars (Egea-Gilabert et al., 2014) and growing conditions (Lara et al., 2011). Although our results showed that the leaf NO_3^- concentrations of purslane grown hydroponically with different NaCl concentrations indoors under LED lighting were much lower than those reported by others, all plants had adequate shoot TRN which was greater than 2% (**Figure 10B**). According to Epstein (1999), an adequate tissue level of N that may be required by plants is around 1.5%. While reduced NO_3^- concentration in the leaves of purslane contributes to good quality of this editable halophyte, concentrations of dietary minerals also contribute to nutritional profile (Uddin et al., 2012b). In the study of purslane, Chowdhar et al. (2013) reported that high salinity resulted in decreases of Ca, and K but increases of Mg and Fe concentration. Teixeira and Carvalho also reported that high salinity slightly increased Mg concentration. However, in this study, high salinity decreased dietary minerals such as K (**Figure 12A**), Ca (**Figure 12B**), and Mg (**Figure 12C**). Lower concentrations of K, Ca, and Mg in purslane grown with high salinity could be attributed to the stunted root architecture (**Figure 2**), which might have limited water and mineral uptake. Although statistically there were no significant differences in Fe among all treatments, salinity seems to increase Fe concentration (**Figure 12D**).

In conclusion, this study reveals that purslane is relatively tolerant to conditions of moderate salinity. It is feasible to grow purslane with 100 mM NaCl indoors hydroponically under LED lightings. Compared to those grown with fresh water (0 mM NaCl), purslane grown with 100 mM NaCl had greater shoot and root productivity and faster leaf growth and development and higher proline and Car concentrations. Increasing salinity from 100 mM to 200 mM and 300 mM resulted in decreases of shoot and root productions could be due to leaf water deficit reflected by lower LWC and reduced photosynthetic performance. Lower concentrations of Asc and total phenolic compounds of purslane grown with higher salinity in this study could mainly be due to its reduced photosynthetic performance. To enhance the nutritional quality of purslane plants without compromising its productivity, it would be feasible to first grow them with low salinity, such as 100 mM NaCl, to enhance photosynthetic performance, to achieve high biomass accumulation, and to increase mineral uptake before transferring to high salinity conditions. Increased shoot and root biomass accumulations and

enhanced photosynthetic performance in purslane plants grown with low salinity may improve the production of phytochemicals after subjecting them to high salinity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JH initiated and funded the expenses for the project and wrote the first draft of the manuscript. JH and LQ planned the experiments, carried out some parts of the experiments, and revised the manuscript. XY carried out most measurements, analyzed the data, and plotted the graphs under supervision of JH and LQ. All authors contributed to the article and approved the submitted version.

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Minimizing VPD Fluctuations Maintains Higher Stomatal Conductance and Photosynthesis, Resulting in Improvement of Plant Growth in Lettuce

Takayasu Inoue[1], *Motoo Sunaga*[1], *Mutsuhiro Ito*[2], *Qu Yuchen*[3], *Yoriko Matsushima*[3], *Kazuma Sakoda*[3] *and Wataru Yamori*[3]*

[1] Fuji Chemical Co., Ltd., Gifu, Japan, [2] Fuji Silysia Chemical Co., Ltd., Gifu, Japan, [3] Institute for Sustainable Agro-Ecosystem Services, The University of Tokyo, Nishitokyo, Japan

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Toshiki Asao,
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Jie He,
Nanyang Technological University, Singapore
Jeremy Harbinson,
Wageningen University and Research, Netherlands

***Correspondence:**
Wataru Yamori
yamori@g.ecc.u-tokyo.ac.jp

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Vapor pressure deficit (VPD) is considered to be one of the major environmental factors influencing stomatal functions and photosynthesis, as well as plant growth in crop and horticultural plants. In the greenhouse cultivation, air temperature and relative air humidity are regulated by switching on/off the evaporative systems and opening/closing the roof windows, which causes VPD fluctuation. However, it remains unclear how VPD fluctuation affects photosynthetic and growth performance in plants. Here, we examined the effects of the VPD fluctuation on the photosynthetic and growth characteristics in lettuce (*Lactuca sativa* L.). The parameters for gas exchange and chlorophyll fluorescence and biomass production were evaluated under the conditions of drastic (1.63 kPa for 6 min and 0.63 for 3 min) or moderate (1.32 kPa for 7 min and 0.86 kPa for 3 min) VPD fluctuation. The drastic VPD fluctuation induced gradual decrease in stomatal conductance and thus CO_2 assimilation rate during the measurements, while moderate VPD fluctuation caused no reduction of these parameters. Furthermore, data showed moderate VPD fluctuation maintained leaf expansion and the efficiency of CO_2 diffusion across leaf surface, resulting in enhanced plant growth compared with drastic VPD fluctuation. Taken together, fine regulation of VPD can be crucial for better plant growth by maintaining the photosynthetic performance in lettuce. The present work demonstrates the importance of VPD control during plant cultivation in plant factories and greenhouses.

Keywords: photosynthesis, VPD, lettuce, rockwool, relative air humidity, stomatal conductance

INTRODUCTION

Natural resource availability has been a limitation of agricultural industry throughout human history; agricultural production has been greatly threatened by water and nutrition shortage and insufficient available land for centuries. In recent years, the development of agricultural technology has enabled a crop cultivation under indoor environments, and the indoor agriculture could protect crops from harmful environments (Kozai et al., 2015).

The current trend in greenhouse cultivation is to extend the crop growing season in order to maximize the equipment operation, elongate the exporting season, and increase the annual yield per unit area, resulting in the profitability improvement.

One major advance for greenhouse and indoor agriculture is water controlling. Plants in greenhouse or similar facilities are less likely to suffer from air water deficit compared with open field. In general, most plants would grow well at vapor pressure deficit (VPD) between 0.5 and 0.8 kPa (Bakker, 1991). The reduction of transpiration rate at high VPD is observed in most crop species (> 2.0 kPa; Gholipoor et al., 2010; Zaman-Allah et al., 2011). Guard cells are vulnerable to turgor loss under high VPD. When the water flux into the stem is too low to meet the high transpiration rate at the leaf, it will eventually cause the closing of stomata. Thus, the stomatal closure decreases the conductance of gas diffusion via stomata, or, stomatal conductance (Chaves, 1991; Ort et al., 1994; Chaves et al., 2003; Flexas et al., 2004), resulting in a decrease in CO_2 assimilation rate (Sinclair et al., 2017). Atmosphere water deficit can suppress the photosynthetic performance also by directly impairing metabolic activities including the enzyme activity of Calvin-Benson cycles (Farquhar et al., 1989) and leads to the loss of biomass production throughout the crop growing period (Tibbitts, 1979; Grange and Hand, 1987; Bakker, 1991; Marsden et al., 1996; Leuschner, 2002; Codarin et al., 2006).

Evaporative systems for cooling and humidifying greenhouses have been developed to provide the ideal growing conditions in a greenhouse (Brandon et al., 2016). Establishment of the appropriate combination of air and water supply would depend on the environmental conditions such as radiation from the sun, ambient temperature and relative air humidity, and it would be essential for maintaining the desired conditions for the plant cultivation in the greenhouse. The main evaporative cooling methods used today are a fan-and-pad system (Davies, 2005) and a fogging system (Sase et al., 2006; Hayashi et al., 2007; Perdigones et al., 2008; Lu et al., 2015). The performance of the fogging system is superior to that of the fan-and-pad system, regarding the uniform distribution of temperature and relative air humidity in the greenhouse (Arbel et al., 2003; Abdel-Ghany and Kozai, 2006; Toida et al., 2006). Research on the plant responses to fogging conditions by the fogging system demonstrated that a fogging system can efficiently improve plant growth (Katsoulas et al., 2001; Leyva et al., 2013).

VPD depends on both temperature and relative air humidity. Evaporative systems mentioned above are used in different situations and in various forms (Brandon et al., 2016; Aljubury and Ridha, 2017), however, most of such instruments are simply controlled by switching on/off, which would cause fluctuation in air humidity due to its binary controlling manner. Therefore, high VPD could be observed during the day even in greenhouse environments (Harmanto et al., 2005; Lu et al., 2015; Zhang et al., 2015). In addition, the temperature in greenhouses often exceed 30°C during midday of sunny winter days in Asian countries. Growers must open the roof windows during this period to lower the temperature inside the greenhouse. The air exchange between the outside and inside of the greenhouse would increase VPD because of the low air humidity in cold seasons (Lu et al., 2015). Thus, VPD can be drastically affected by the on/off switching of evaporative systems and the opening/closing of roof windows. However, to our knowledge, there have been no report studying the effects of a fluctuating VPD condition on the photosynthetic and growth performance in plants. Understanding physiological mechanisms underlying the effect of fluctuating VPD condition on the plant growth would be important for efficient agricultural production in greenhouse with highly controlled environmental conditions. This study was aimed to characterize the effect of the VPD fluctuation on the photosynthetic and growth characteristics in lettuce (*Luctica sativa* L.), the most common vegetable cultivated in greenhouses.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Average, amplitude and cycles of VPD were set according to the values typically monitored in green houses (Garcia et al., 2011; Lu et al., 2015; Zhang et al., 2015). To evaluate the long-term effects of the VPD conditions, romaine lettuce (*Lactuca sativa* L. var. Romana; Takii Seed Co., Kyoto, Japan) were sown in rockwool in an environmentally controlled growth chamber (NK Systems, Japan) at a PPFD of 200 μmol photons m^{-2} s^{-1}, a 16 h photoperiod, a CO_2 concentration of 400 μmol mol^{-1} and two different VPD conditions: moderately fluctuating VPD condition in which a cycle of high VPD (1.32 kPa = relative air humidity of 55%) for 7 min and low VPD (0.86 kPa = relative air humidity of 72%) for 3 min was repeated for 24 h before measurement, and drastically fluctuating VPD condition in which a cycle of high VPD (1.63 kPa = relative air humidity of 42%) for 6 min and low VPD (0.63 kPa = relative air humidity of 80%) for 3 min was repeated for 24 h before measurement. The averages of daily VPD, relative air humidity and temperature were similar between treatments (1.03 ± 0.20 kPa, 65.5 ± 6.9% and 24.0 ± 0.3°C for moderate fluctuating VPD condition, 1.04 ± 0.35 kPa, 64.4 ± 13.7% and 24.0 ± 0.8°C for drastic fluctuating VPD condition). The plants were supplied with sufficient nutrient solution to avoid drought stress. Each rockwool was given 100 ml of nutrient solution at 1/1000 strength (HYPONeX, N:P:K, 6:10:5, Hyponex Japan, Osaka, Japan) everyday.

Analyses of Chlorophyll Fluorescence, P700 and Gas Exchange Measurement

Chlorophyll fluorescence, P700 redox state and gas exchange were measured simultaneously during a 16 h photoperiod using a Dual-PAM-100 and a GFS-3000 measuring systems (Walz, Effeltrich, Germany) in uppermost, fully expanded new leaves of 3-week-old plants grown under moderately fluctuating VPD condition, as described in Yamori et al. (2015, 2016). After leaves were dark-adapted for 30 min, a saturating pulse was applied to obtain the maximum fluorescence and the maximum change in P700. For measurements of photosynthetic parameters, the leaf was firstly allowed to equilibrate at 0.63 kPa or 0.48 kPa of VPD at a CO_2 concentration of 400 μmol mol^{-1} and a PPFD of 200 μmol photons m^{-2} s^{-1} for at least 30 min. A CO_2 assimilation rate and stomatal conductance were measured every 1 min in the environmentally controlled chamber of portable

photosynthesis system under two different VPD conditions: moderately fluctuating VPD, in which a cycle of VPD of 0.63 kPa (=relative air humidity of 80%) for 5 min, VPD of 1.27 kPa (=relative air humidity of 60%) for 4 min and then VPD of 0.95 kPa (=relative air humidity of 70%) for 3 min was repeated for 400 min (maximum amplitude: VPD=0.64kPa, relative air humidity=20%), and drastically fluctuating VPD, in which a cycle of VPD of 0.48 kPa (=relative air humidity of 85%) for 3 min, VPD of 1.74 kPa (=relative air humidity of 45%) for 3 min and then VPD of 0.95 kPa (=relative air humidity of 70%) for 3 min was repeated for 400 min (maximum amplitude: VPD = 1.26, relative air humidity = 40%).

The quantum yield of photosystem I (ΦPS I) was calculated from the complementary PS I quantum yields of non-photochemical energy dissipation, Y(ND) and Y(NA): Y(I) = 1–Y(ND)–Y(NA). The quantum yield of photosystem II [ΦPS II], photochemical quenching [qP] and the fraction of PS II centers in the open state (with plastoquinone oxidized) [qL] were calculated. The electron transport rate (ETR) was calculated as ETR I (or ETR II) = 0.5 × 0.84 × φPS I (or φPS II), where 0.5 is the fraction of absorbed light reaching PS I or PS II, and 0.84 is the leaf absorptance (Genty et al., 1989).

The maximum level of the P700 signal (Pm, full oxidation of P700) and the maximum quantum yield of PS II (Fv/Fm) in the dark was analyzed after the measurements of photosynthesis under moderate or drastic VPD fluctuation as shown in **Figure 1**. The leaves were placed in a temperature-controlled chamber at a CO_2 concentration of 400 μmol mol^{-1} and leaf temperature at 25○C in a Dual-PAM-100 and a GFS-3000 measuring system (Walz, Effeltrich, Germany), and exposed to (1) moderate fluctuating VPD or (2) drastic fluctuating VPD for 5 h at 200 μmol photons m^{-2} s^{-1}. The Pm and Fv/Fm after dark incubation for 15 min were measured before and after the light treatments (Yamori et al., 2016).

Analysis of Gas Exchange Under Different Duration and Frequency of VPD

We also measured a CO_2 assimilation rate, stomatal conductance, transpiration rate, and intercellular CO_2 concentration under different duration and frequency of VPD fluctuation cycles using LI-6800 (Li-Cor, Lincoln, NE, USA) using the 3-week-old lettuce grown under moderately fluctuating VPD condition. For measurements of photosynthesis parameters, the leaf was first allowed to equilibrate at 1.0 kPa of VPD at a PPFD of 200 μmol photons m^{-2} s^{-1} for at least 30 min, and the photosynthetic parameters were recorded every 4 min under various VPD fluctuations, throughout the 400 min period. Three types of VPD condition were generated in the environmentally controlled chamber of portable photosynthesis system: constant VPD of

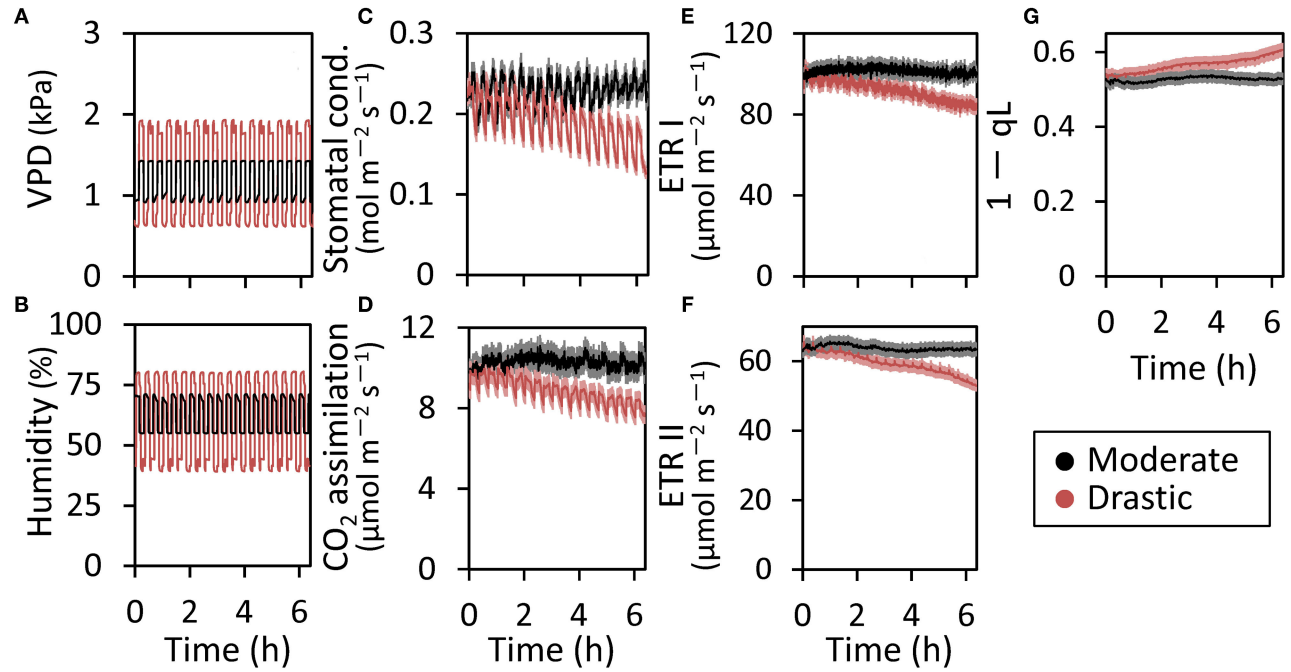

FIGURE 1 | Responses of photosynthetic parameters to moderate and drastic VPD fluctuation in lettuce grown under moderately fluctuating VPD condition. Both VPD **(A)** and relative air humidity **(B)** in the environmentally controlled chamber of portable photosynthesis system were shown. The stomatal conductance **(C)**, CO_2 assimilation rate **(D)**, electron transport rate through PSI (ETR I) **(E)** and through PSII (ETR II) **(F)**, and the redox state of the plastoquinone pool (1-qL) **(G)** were monitored at a CO_2 concentration of 400 μmol mol^{-1} and a PPFD of 200 μmol photons m^{-2} s^{-1} under either moderately or drastically fluctuating VPD conditions for 6 h. The average leaf temperature over the entire period was 25.5 ± 0.32°C for the moderate VPD treatment and 25.8 ± 0.31°C for the drastic VPD treatment. The data are the means ± standard errors of six biological replicates.

0.95 kPa (=relative air humidity of 70%), rapidly fluctuating VPD condition in which a cycle of VPD of 0.48 kPa (=relative air humidity of 85%) for 3 min, VPD of 1.74 kPa (=relative air humidity of 45%) for 3 min and then VPD of 0.95 kPa (=relative air humidity of 70%) for 3 min was repeated for 400 min (maximum amplitude: VPD = 1.26, relative air humidity = 40%) and slowly fluctuating VPD condition in which a cycle of VPD of 0.63 kPa (=relative air humidity of 80%) for 5 min, VPD of 1.27 kPa (=relative air humidity of 60%) for 4 min and then VPD of 0.95 kPa (=relative air humidity of 70%) for 3 min was repeated for 400 min (maximum amplitude: VPD = 0.64 kPa, relative air humidity = 20%). The average of VPD was similar between treatments and showed ∼0.92 kPa (=relative air humidity of 71%). VPD values were calculated according to Buck (1981); VPD = 0.611e [17.502 × Temperature/(Temperature+240.97)] × (1–Relative Air Humidity).

Plant Growth Analysis

The plants cultivated in the environmentally controlled growth chamber were harvested every week after transplanting and shoot fresh weights and total leaf area were measured. The shoot samples were dried under 80°C in an oven for one week and then weighted. Leaf area was measured using a LI-3000 leaf area meter (Li-Cor, Lincoln, NE, USA). Leaf mass per area (LMA) was measured as dry weight per unit leaf area. The experiment of plant growth analysis was repeated twice, switching the treatments between chambers. Number of leaves larger than 0.3 mm were counted by eye on 3rd week.

Chlorophyll and Anthocyanin Contents

The contents of chlorophyll and anthocyanin were quantified at fully expanded leaves of plants grown under two fluctuating VPD conditions for three weeks, using a spectrophotometric method as described in Porra et al. (1989) and ACM-200plus equipment (Opti-Sciences, Inc., USA), respectively.

Statistical Analysis

The data represent the means for three replicate samples of two independent experiment. Data are presented as means ± SE. Analysis of Student *t*-test was performed in the SPSS statistical software (SPSS, Chicago, IL). Differences were considered significant at $P < 0.05$.

RESULTS

Effects of the Amplitude of the VPD Fluctuation on the Photosynthetic Characteristics

The fluctuating VPD condition in the environmentally controlled chamber of portable photosynthesis system induced the fluctuation of a stomatal conductance, CO_2 assimilation rate, ETR I and ETR II, although the amplitude of the fluctuation was much larger in the stomatal conductance and CO_2 assimilation rate than ETR I and ETR II (**Figure 1**). All the photosynthetic parameters were maintained under moderate VPD fluctuation throughout the measurements for 6 h (**Figure 1**). On the other hand, under drastic VPD fluctuation, all the photosynthetic parameters were not affected during the first 1–2 h after the measurements but declined gradually (**Figure 1**). In addition, drastic VPD fluctuation induced the gradual increase in the plastoquinone pool (1-qL), indicating that the electron transport system would accumulate reducing power (**Figure 1G**; Baker, 2008).

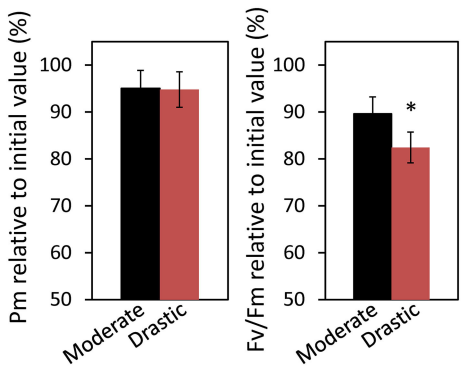

FIGURE 2 | Effect of VPD conditions on alleviation of photoinhibition in lettuce grown under moderately fluctuating VPD condition. The maximum level of the P700 signal of PSI (Pm, full oxidation of P700) and the maximum quantum yield of PSII (Fv/Fm) were measured before and after treatment with moderate VPD or drastic VPD for 6 h, as same in **Figure 1**. Pm and Fv/Fm relative to the initial values before the treatments are shown. The average of Fv/Fm and Pm at the initial values before the treatments was 0.805 ± 0.003 and 1.15 ± 0.01, respectively. The data are the means ± standard errors of six biological replicates. Significant differences between two different VPD conditions are examined by Student's *t*-test (*$P < 0.05$).

At the end of the measurement, we also evaluated the extent of photoinhibition both at PSI and PSII caused by the VPD fluctuation (**Figure 2**). The maximum level of the P700 signal (Pm) under darkness and the maximum quantum yield of PSII (Fv/Fm) were measured before and after moderate and drastic VPD fluctuations. Although the remaining activity of PSI showed no difference between two VPD conditions, that of PSII was significantly lower under drastic VPD fluctuation than moderate VPD fluctuation (**Figure 2**), suggesting that more photodamage at PSII would be accumulated under drastic VPD fluctuation.

Effects of the Duration and Frequency of the VPD Fluctuation on the Photosynthetic Characteristics

Since we have found that the VPD fluctuation differing the amplitude would have different impacts on photosynthesis, we further examined the effects of the duration and the frequency of the VPD fluctuation on photosynthesis in the environmentally controlled chamber of portable photosynthesis system. Under constant VPD condition, transpiration rate, stomatal conductance, CO_2 assimilation rate, and intercellular CO_2 concentration were constant throughout the measurements (**Figure 3**). Both of rapid and slow VPD fluctuations induced the fluctuation of all the parameters.

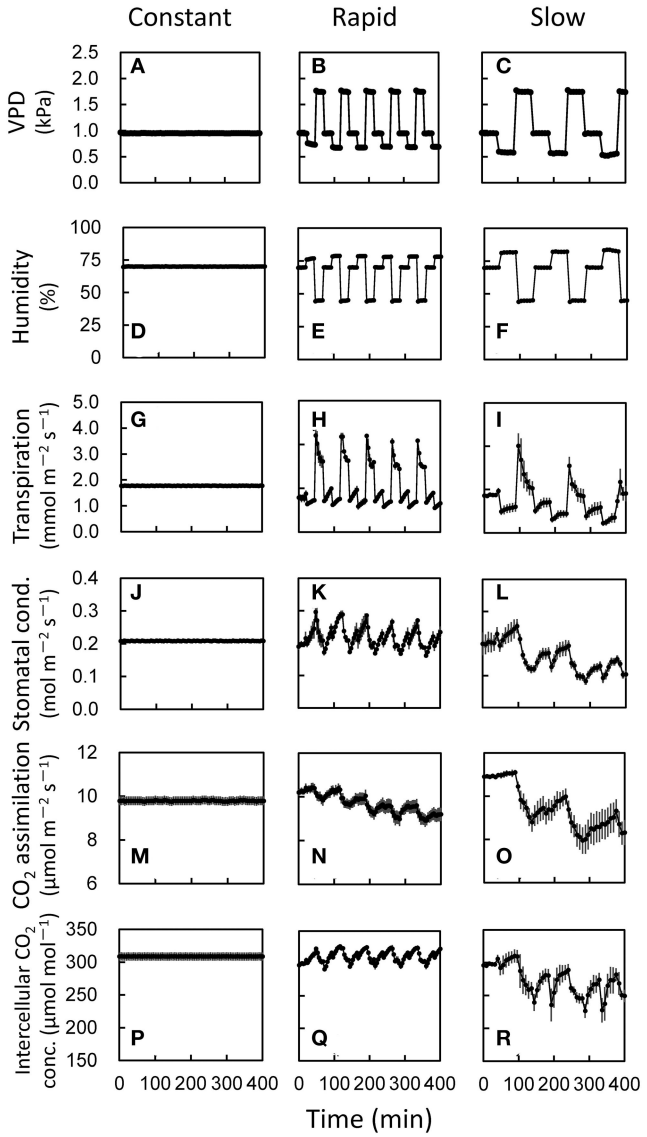

FIGURE 3 | Responses of photosynthetic parameters to several fluctuating VPD conditions in lettuce grown under moderately fluctuating VPD condition. Three different VPD conditions **(A–C)** which corresponds to the relative air humidity **(D–F)** was set in an environmentally controlled growth chamber. The transpiration rate **(G–I)**, stomatal conductance **(J–L)**, CO_2 assimilation rate **(M–O)**, and intercellular CO_2 concentration **(P–R)** were measured at a CO_2 concentration of 400 μmol mol^{-1} and a PPFD of 200 μmol photons m^{-2} s^{-1} under three VPD conditions: (1) constant VPD of 0.95 kPa (=relative air humidity of 70%); (2) rapidly fluctuating VPD, in which VPD of 0.48 kPa (=relative air humidity of 85%) for 3 min, VPD of 1.74 kPa (=relative air humidity of 45%) for 3 min, and VPD of 0.95 kPa (=relative air humidity of 70%) for 3 min; (3) slowly fluctuating VPD, in which VPD of 0.63 kPa (=relative air humidity of 80%) for 5 min, VPD of 1.27 kPa (=relative air humidity of 60%) for 4 min, and VPD of 0.95 kPa (=relative air humidity of 70%) for 3 min. The average leaf temperature over the entire period was 25.2 ± 0.22°C for the constant VPD, 25.4 ± 0.31°C for the rapid VPD and 25.5 ± 0.28°C for the slow VPD treatment. The data are the means ± standard errors of six biological replicates.

Transpiration rate, stomatal, conductance and intercellular CO_2 concentration were maintained, while CO_2 assimilation rate declined gradually under rapid VPD fluctuation. All the parameters declined gradually under slow VPD fluctuation. The larger decrease in CO_2 assimilation rate was shown under slow VPD fluctuation than rapid VPD fluctuation during the measurement.

Drastic VPD Fluctuation Caused Reduction in Plant Growth Compared to Moderate VPD Fluctuation

Plant growth of lettuce was analyzed under moderately and drastically fluctuating VPD conditions (**Figure 4**). There was no clear difference of shoot dry weight, leaf area and LMA between two VPD conditions until two weeks after the beginning of the treatments (**Figures 4C–F**). On the other hand, the plants grown under drastic VPD fluctuation showed less shoot dry weight, leaf area and LMA than those under moderate VPD fluctuation at three weeks after the beginning of the treatments. The shoot dry weight and leaf area in plants grown under drastic VPD fluctuation was 15% and 29% lower than those under moderate VPD fluctuation, respectively. Although there was no difference of the leaf number per plant between two VPD conditions (24.7 ± 0.4 for moderate fluctuating VPD condition, 24.0 ± 0.4 for drastic fluctuating VPD condition; **Figure 5**), the plants grown under drastic VPD fluctuation showed smaller leaves with high LMA. The area of the leaves above the 9th leaf from the bottom were largely different between two VPD conditions at three weeks after the beginning of treatment (**Figures 5D,E**).

Although an air temperature was slightly different between two VPD conditions (**Supplementary Figure 1**), this difference was minimum and had no effect on CO_2 assimilation rate (**Supplementary Figure 2**). There were no significant differences in leaf chlorophyll and anthocyanin content between two different VPD conditions (**Table 1**).

DISCUSSION

Fluctuating VPD Retarded Plant Growth via the Reductions in Leaf Area and Photosynthesis

VPD is considered to be one of the major environmental factors influencing stomatal conductance and photosynthesis (Raschke, 1970; Lange et al., 1971; Grange and Hand, 1987; Xu et al., 1991; Tinoco-Ojanguren and Pearcy, 1993; Bunce, 2006), as well as plant growth and development in crop and horticultural plants (Tibbitts, 1979; Grange and Hand, 1987; Bakker, 1991; Marsden et al., 1996; Leuschner, 2002; Codarin et al., 2006). Most of the previous studies focused on the effects of the averaged or steady-state VPD on plant growth. To our knowledge, there have been no report studying the effects of the VPD fluctuation on photosynthetic and growth performance in plants. The present study clearly showed that drastic VPD fluctuation in the

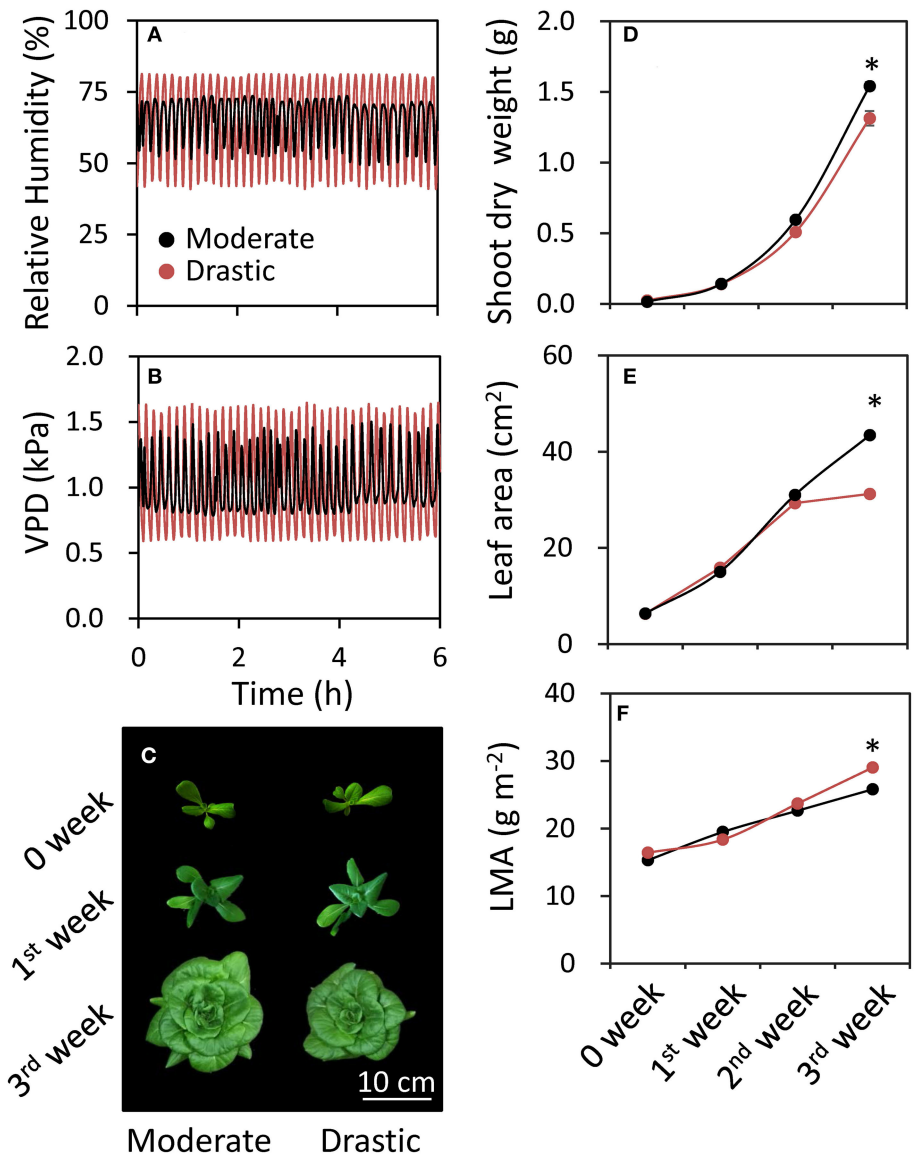

FIGURE 4 | Responses of plant growth parameters to different fluctuating VPD conditions in lettuce. Plants were grown in the controlled growth chamber at a PPFD of 200 μmol photon m^{-2} s^{-1} under two different fluctuating VPD conditions: one is moderately fluctuating VPD condition, in which 7 min high VPD (1.32 kPa = relative air humidity of 55%) and 3 min low VPD (0.86 kPa = relative air humidity of 72%); or drastically fluctuating VPD condition, in which 6 min high VPD (1.63 kPa = relative air humidity of 42%) and 3 min low VPD (0.63 kPa = relative air humidity of 80%). Relative humidity **(A)** and VPD **(B)** during the experiment and a picture of lettuce plants in week 0, 1 and 3 **(C)** are shown. Shoot dry weight **(D)**, leaf area **(E)** and leaf mass per area (LMA) **(F)** of fully expanded leaves were analyzed every week until three weeks after the beginning of VPD treatments. The data are the means ± standard errors of six biological replicates. Significant differences between two different VPD conditions are examined by Student's *t*-test (*$P < 0.05$).

environmentally controlled chamber of portable photosynthesis system declined stomatal conductance and thus CO_2 assimilation rate (**Figure 1**), leading to photoinhibition (**Figure 2**; Yamori, 2016). Moreover, drastic VPD fluctuation for a long-term period resulted in a reduction of biomass production in lettuce (**Figures 4**, **5**).

In general, during atmosphere water deficit, the decrease in stomatal conductance is the primary cause of the reduction of CO_2 assimilation rate (e.g., Bunce, 1997, 2006). In the present study, drastic VPD fluctuation induced the declines in stomatal conductance, CO_2 assimilation rate, ETR I and ETR II (**Figures 1C,E,F**). It would be considered that the drastic VPD fluctuation would cause stomatal closing, leading to simultaneous reductions in the CO_2 assimilation rate and electron transport rate (**Figure 1**). This would result in an over-reduction of the plastoquinone pool (high 1-qL) and

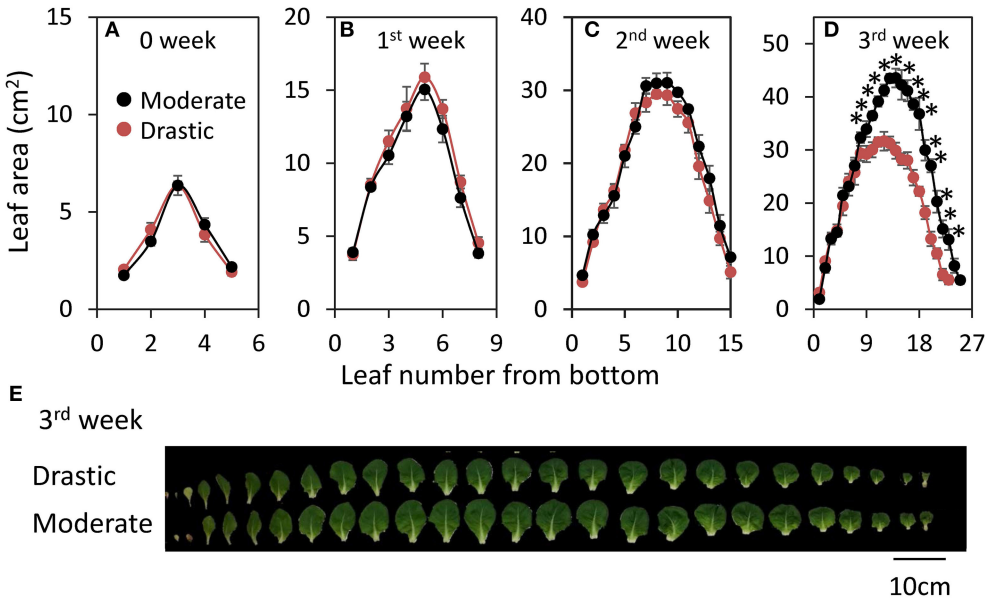

FIGURE 5 | Responses of leaf number and leaf area to different fluctuating VPD conditions in lettuce. Plant growth conditions are similar to **Figure 4**. Leaf area was analyzed in all the leaves every week until three weeks after the beginning of VPD treatments **(A–D)**. Significant differences between two different VPD conditions are examined by Student's *t*-test (*$P < 0.05$). Pictures of each leaf was also summarized **(E)**.

TABLE 1 | Effect of VPD conditions on leaf chlorophyll and anthocyanin contents.

Traits	Condition	
	Moderate	Drastic
Chlorophyll (mmol m^{-2})	0.362 ± 0.009	0.376 ± 0.008
Anthocyanin (mmol m^{-2})	0.107 ± 0.024	0.113 ± 0.026

Significant differences between two different VPD conditions are examined by Student's t-test. There were no significant differences in leaf chlorophyll and anthocyanin content between two different VPD conditions.

the long-term treatment of drastic VPD fluctuation caused severe photoinhibition (**Figure 2**). This was supported by the previous reports that the stomatal response to VPD is actively driven by an abscisic acid, ABA (Bauer et al., 2013) and that, at later stages with increasing severity, drought stress could lead to metabolic impairment including the declines in Rubisco activity (Parry et al., 2002).Thus, the increase in diffusive limitation via stomata and then biochemical limitation would be responsible for the decline in photosynthesis under the fluctuating VPD condition. Further studies would be required to quantitatively partition between stomatal and biochemical limitations with various time course owing to water severity.

Drastic VPD fluctuation declined CO_2 assimilation rate and leaf area with no change in leaf number, resulting in the significant reduction of biomass production in lettuce (**Figures 4**, **5**). There has been reported that VPD affects crop growth through not only a direct impact on CO_2 assimilation rate and stomatal conductance but also on leaf size (Gisleroed and Nelson, 1989; Bakker, 1991). In addition, the reductions of leaf water potential and turgor are, for long, known to have a negative effect on leaf growth (Bradford and Hsiao, 1982; Kramer and Boyer, 1995) since even minimal reductions of leaf water potential or turgor can cause a significant reduction of leaf expansion (Acevedo et al., 1971; Dale, 1988; Hsiao et al., 1998; Alves and Setter, 2004) as well as cell number (Carins Murphy et al., 2014). Taken together, there is a strong relationship between water potential or turgor and leaf sizeas we have observed in leaf area in 3rd week after the biggining of the treatments (**Figure 5**).

Importance of Fine-Regulation of VPD in Plant Growth Conditions

These days, greenhouse operations are moving toward controlling evaporative demand according to VPD from relative air humidity because this approach provides direct information about the driving force of transpiration and evaporation (Katul et al., 2009; Villarreal-Guerrero et al., 2012; du Plessis et al., 2015). The VPD regulation has been demonstrated as an efficient solution to maintain optimal ranges of temperature and relative air humidity simultaneously. Recent work showed that the VPD control via the fogging system improved plant productivity by enhancing the photosynthetic performance during the winter (Lu and Viljanen, 2009; Lu et al., 2015) and summer seasons (Zhang et al., 2015).

Although VPD control is important for the plant cultivation, daily and seasonal changes in VPD and solar radiation are large, and would have significant impacts on stomatal conductance, CO_2 assimilation rate and plant growth (Myers et al., 1997; Prior et al., 1997; Hutley et al., 2000; Yamori, 2016). Even in

greenhouse conditions, VPD fluctuates greatly during the day (Harmanto et al., 2005). In both fogging and fan-and-pad systems which have been commonly used for evaporative systems for cooling and humidifying greenhouses, VPD in greenhouses is commonly controlled by set points for VPD. As the set points are generally lower and upper VPD thresholds, the VPD control in the greenhouse is based on on/off regulation. As shown in the present study, fluctuating VPD consequently retarded plant growth (**Figures 4**, **5**). Thus, VPD in the greenhouse should be controlled not by intermittent regulation but by continuous regulation. The effect of VPD on photosynthesis and plant growth would depend on the extent of fluctuations and the absolute value of VPD as well as other environmental conditions, including growth light intensity, CO_2 concentration and wind velocity. The present study clearly showed that fine-regulation for stable environmental control in greenhouses could maintain the leaf expansion (**Figure 5**) and higher stomatal conductance and photosynthesis during the major part of the day (**Figures 1**–**3**), which would lead to the better plant growth and higher yield with high nutrition values in greenhouses (**Figures 4**, **5**). The effect of VPD fluctuation level might affect processes underlying postharvest quality in lettuce (Chen et al., 2021). Further researches would be needed to optimize the continuous regulation of VPD for plant cultivation, with considering the mean VPD and the fluctuation range, in agricultural production.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

TI, MS, MI, and WY conceived and designed the experiments. TI, MS, and WY performed the experiments and analyzed the data. QY, YM, KS, and WY prepared the manuscript, and all the members contributed extensively to its finalization.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.646144/full#supplementary-material

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Appropriate NH_4^+/NO_3^- Ratio Triggers Plant Growth and Nutrient Uptake of Flowering Chinese Cabbage by Optimizing the pH Value of Nutrient Solution

Yunna Zhu[1,2], Baifu Qi[1], Yanwei Hao[1], Houcheng Liu[1], Guangwen Sun[1], Riyuan Chen[1] and Shiwei Song[1]**

[1] College of Horticulture, South China Agricultural University, Guangzhou, China, [2] Henry Fok College of Biology and Agriculture, Shaoguan University, Shaoguan, China

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Edited by:
Md Asaduzzaman,
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Gonzalez Maria Begoña Moro,
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Zhenhua Zhang,
Hunan Agricultural University, China

***Correspondence:**
Riyuan Chen
rychen@scau.edu.cn
Shiwei Song
swsong@scau.edu.cn

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Compared with sole nitrogen (N), the nutrition mixture of ammonium (NH_4^+) and nitrate (NO_3^-) is known to better improve crop yield and quality. However, the mechanism underlying this improvement remains unclear. In the present study, we analyzed the changes in nutrient solution composition, content of different N forms in plant tissues and exudates, and expression of plasma membrane (PM) H^+-ATPase genes (*HAs*) under different NH_4^+/NO_3^- ratios (0/100, 10/90, 25/75, 50/50 as control, T1, T2, and T3) in flowering Chinese cabbage. We observed that compared with the control, T1 and T2 increased the economical yield of flowering Chinese cabbage by 1.26- and 1.54-fold, respectively, whereas T3 significantly reduced plant yield. Compared with the control, T1–T3 significantly reduced the NO_3^- content and increased the NH_4^+, amino acid, and soluble protein contents of flowering Chinese cabbage to varying extents. T2 significantly increased the N use efficiency (NUE), whereas T3 significantly decreased it to only being 70.25% of that of the control. Owing to the difference in N absorption and utilization among seedlings, the pH value of the nutrient solution differed under different NH_4^+/NO_3^- ratios. At harvest, the pH value of T2 was 5.8; in the control and T1, it was approximately 8.0, and in T3 it was only 3.6. We speculated that appropriate NH_4^+/NO_3^- ratios may improve N absorption and assimilation and thus promote the growth of flowering Chinese cabbage, owing to the suitable pH value. On the contrary, addition of excessive NH_4^+ may induce rhizosphere acidification and ammonia toxicity, causing plant growth inhibition. We further analyzed the transcription of PM H^+-ATPase genes (*HAs*). *HA1* and *HA7* transcription in roots was significantly down-regulated by the addition of the mixture of NH_4^+ and NO_3^-, whereas the transcription of *HA2*, *HA9* in roots and *HA7*, *HA8*, and *HA10* in leaves was sharply up-regulated by the addition of the mixture; the transcription of *HA3* was mainly enhanced by the highest ratio of NH_4^+/NO_3^-. Our results provide valuable information about the effects of treatments with different NH_4^+/NO_3^- ratios on plant growth and N uptake and utilization.

Keywords: ammonium to nitrate ratio, xylem exudate, nitrogen efficiency, plasma membrane H^+-ATPase, flowering Chinese cabbage

INTRODUCTION

Ammonium (NH_4^+) and nitrate (NO_3^-) are two main nitrogen (N) forms that can be absorbed and utilized by plants (Xu et al., 2012), and they have an important effect on crop growth and quality (Hachiya and Sakakibara, 2017). In most aerated soils, the major N form is NO_3^-, whereas NH_4^+ is dominant in acidic and/or anaerobic soils (Zhu et al., 2011). The roots of most plants prefer the uptake of NH_4^+ over NO_3^- in micromolar concentrations (Hachiya and Sakakibara, 2017) owing to the lower energy costs associated with the absorption and assimilation of NH_4^+ than those of NO_3^- (Guo et al., 2007); on the contrary, NH_4^+ often causes ammonium toxicity at millimolar concentrations (Britto and Kronzucker, 2002). Studies have revealed that co-provision of NH_4^+ and NO_3^- nutrition significantly stimulated plant growth in strawberry (Tabatabaei et al., 2008), mini Chinese cabbage (Hu et al., 2015), flowering Chinese cabbage (Song et al., 2017), and Chinese kale (Zhu et al., 2018) in comparison with the addition of NH_4^+ or NO_3^- alone. Moreover, the mixture of NH_4^+ and NO_3^- increased the content of soluble sugars, soluble proteins, and vitamin C in plants (Tabatabaei et al., 2008) and reduced the content of nitrates (Song et al., 2017; Zhu et al., 2018). Therefore, addition of a mixture containing appropriate ratios of NH_4^+ and NO_3^- is beneficial to plant growth and development. Several studies have reported that NH_4^+ and NO_3^- can interact with each other when co-supplying two N forms (Kronzucker et al., 1999; Hachiya and Sakakibara, 2017; Zhu et al., 2020).

NO_3^- and NH_4^+ are absorbed by plant cells from the soil via specific proteins called nitrate transporters (NRTs) and ammonium transporters (AMTs), respectively (Nacry et al., 2013). In *Arabidopsis*, NRTs consist of nitrate transporter 1/peptide transporter family (NRT1/PTR), NRT2, chloride channels (CLC), and slow anion channel-associated 1 homologs (SLAC1/SLAH), which are involved in low or high affinity uptake, xylem loading, and ion efflux of NO_3^- (Krapp et al., 2014). AMTs include two subfamilies, AMT1 and AMT2. Recent research has mainly focused on AMT1, which takes part in the absorption and transport of NH_4^+ (Yuan et al., 2007; Straub et al., 2017). Both NRTs and AMTs are located in the plasma membrane (PM) (Sperandio et al., 2014). PM H^+-ATPase (PM H^+-ATPase, EC 3.6.1.3.) is a proton pump that is necessary to promote cell growth and ion fluxes across the PM (Sperandio et al., 2020). NH_4^+ transmembrane transport is controlled by electrochemical potential inside and outside the cell membrane, and this process does not require energy (Wang et al., 1993). NH_4^+ uptake can lead to depolarization of the cell membrane and can enhance the activity of proton pumps (Schubert et al., 1990); in contrast, NO_3^- transmembrane transport is an active transport process which requires energy and H^+ provided by PM H^+-ATPase (Gaxiola et al., 2007).

Therefore, either NH_4^+ or NO_3^- uptake is related to the activity of proton pumps. H^+-ATPase, which is an important functional protein of the PM, is called the "master enzyme" in plants (Michelet and Boutry, 1995). PM H^+-ATPase is responsible for establishing a proton electrochemical gradient in the membrane energization used for solute transport, and it controls the major transport processes in plants such as root nutrient uptake, cell elongation, xylem and phloem loading, stomatal aperture, and cellular pH regulation (Duby and Boutry, 2009; Zeng et al., 2012). In addition, proton pump in PM is responsible for other important physiological functions, such as stomatal aperture (Janicka-Russak, 2011).

In plants, PM H^+-ATPases (autoinhibited H^+-ATPases), which form one subfamily of P-type ATPases, are encoded by a multi-gene family (Arango et al., 2003). There are 12 members of this family in *Arabidopsis thaliana* (AHA1–AHA12), nine in *Nicotiana plumbaginifolia* (PMA1–PMA9), and ten in *Oryza sativa* (OSA1–OSA10), and they are classified into five subfamilies (Arango et al., 2003; Pii et al., 2014). The members of subfamilies I and II are expressed throughout the plant with different intensity in different organs (Gaxiola et al., 2007). For instance, Ueno et al. (2005) reported that in *Arabidopsis*, *AHA1*, *2*, *3*, *5*, *7*, *8*, *10,* and *11* were expressed in green leaves, and *AHA1*, *2*, *4*, *7*, *8*, *10,* and *11* were expressed in roots. AHA1 is a housekeeping protein found all over the plant (Santi and Schmidt, 2009), whereas AHA2 plays major roles in root metabolism (Gaxiola et al., 2007; Hoffmann et al., 2019). However, subfamilies III, IV, and V are not highly expressed under normal conditions and are expressed in specific tissues (Arango et al., 2003). *AHA6* and *AHA9* are expressed only in the anthers (Gaxiola et al., 2007). The expression of PM H^+-ATPase isoforms is affected by N forms, and *OSA2* and *OSA7* in *O. sativa* are strongly induced in response to N resupply and may be involved in N uptake (Sperandio et al., 2011). The expression and activity of PM H^+-ATPase are affected by other nutrient elements (i.e., P and K^+) (Yuan et al., 2017).

As a variety of Chinese cabbage (*Brassica rapa*), flowering Chinese cabbage (*Brassica campestris* L. ssp. *chinensis* var. *utilis* Tsen et Lee) is an important leaf vegetable in South China whose product organs are leaves and stalks (Song et al., 2012). In previous study, we have shown that a mixture of NH_4^+ and NO_3^- is more beneficial to the growth and quality of flowering Chinese cabbage than a single N source and that it improves plant NUE (Song et al., 2012). Furthermore, ammonium transporter 1.2 (AMT1.2) mediates the interaction of NH_4^+ and NO_3^- under controlled conditions when the pH value is adjusted to 5.8 (Zhu et al., 2020). It is generally known that the absorption of NH_4^+ can induce net H^+ release and acidify the rhizosphere (Schubert and Yan, 1997); on the contrary, the absorption of NO_3^- can increase H^+ uptake through a H^+ co-transport system in PM and alkalize the rhizosphere (Zeng et al., 2012). However, our understanding of the mechanisms underlying

Abbreviations: AMT, ammonium transporter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, H^+-ATPase; N, nitrogen; NH_4^+, ammonium; NO_3^-, nitrate; NRT, nitrate transporter; PM, plasma membrane; qPCR, quantitative real-time polymerase chain reaction; TTC, 2, 3, 5-tetraphenyltetrazolium chloride.

the plant uptake of different forms of N and their transport and assimilation depending on different ratios of NH_4^+ and NO_3^- is limited.

In the present study, we examined the characteristics of different N forms in flowering Chinese cabbage seedlings and nutrient solutions as well as the N form composition of plant exudates. Furthermore, we analyzed the expression of PM H^+-ATPase genes in flowering Chinese cabbage in response to different NH_4^+/NO_3^- ratios using quantitative real-time PCR.

MATERIALS AND METHODS

Plant Material and Treatments

The experiment was carried out in the greenhouse of South China Agricultural University, Guangzhou, Guangdong Province. Flowering Chinese cabbage seeds (cultivar 'Youlv 501') were provided by Guangzhou Academy of Agriculture Science (Guangdong Province). Plug seedlings were used with perlite as the growth medium. Three consistent seedlings with developed third true leaves were selected and transplanted into plastic bucket filled with 5.5 L of nutrient solution. There were 12 replications in each treatment which was arranged in a randomized complete block design. All nutrient solutions were aerated for 15 min per hour using a controlled pump. Hoagland-Snyder formula (3/4 of the dose) was used as basic and contrast nutrient solution, with 3.75 mmol L^{-1} KNO_3, 3.75 mmol L^{-1} $Ca(NO_3)_2$, 0.75 mmol L^{-1} KH_2PO_4, 2.1 mmol L^{-1} $MgSO_4$. In the nutrient solutions with different NH_4^+/NO_3^- ratios nutrient solution, NH_4^+ was supplied by NH_4Cl. KCl or $CaCl_2$ was added to maintain consistent concentrations across the treatments. During the entire growth period, the nutrient solution was not replaced, and it was supplemented with deionized water every 3 days until reaching the original volume. Electrical conductivity and pH were measured during the experiment.

Parameter Measurements

Plant seedlings were harvested randomly when they reached marketable maturity. Their root or shoot weight was measured; the fresh weight of the product organ (flower stalk above the 4th node) was called commercial yield. The growth rate was calculated from the difference in fresh weight before and after sampling divided by the number of days. Roots, stems, and leaves of the seedlings were harvested, immediately frozen in liquid N_2, and stored at −80°C.

The contents of NH_4^+ and NO_3^- were analyzed as described by Ivančič and Degobbis (1984) and Patterson et al. (2010), respectively. Amino acid content was measured as described by Inada et al. (2006), and leucine was used as a standard for amino acid content estimation. Soluble protein content was determined with bovine serum albumin as the standard (Bradford, 1976). Total N concentration was determined with an auto-analyzer (Kjeltec 2300 Analyzer Unit, Foss Tecator, Sweden) as described by Avery and Rhodes (1990), and total N concentration was multiplied by the dry weight of the whole plant to calculate N accumulation. As described by Eckstein and Karlsson (2001), N loss (NL), N loss rate (NLR), mean residence time of N (MRT), N productivity (NP), and NUE were calculated using the following formulas. $NL=[(NS_{applied}-NS_{remain})-(N_{harvest}-N_{transplant})\times n]/n$; $NLR=NL/(NS_{applied}-NS_{remain})$; $MRT=(N_{harvest}-N_{transplant})/[(\ln N_{harvest}-\ln N_{transplant})\times(NL/t)]$; $NP=[(W_{harvest}-W_{transplant})/(T_{harvest}-T_{transplant})]\times[(\ln N_{harvest}-\ln N_{transplant})/(N_{harvest}-N_{transplant})]$; $NUE=NP\times MRT$. In the formulas above, W is the dry weight of the plant; T is the sampling time; N is the amount of N absorbed by seedlings; NS is the amount of N in the nutrient solution; n is the number of seedlings in each hydroponic bucket; and t is the number of days in the whole growth period. Total phosphorus (P) and potassium (K) concentrations were determined through a modified molybdenum blue procedure at 660nm using a spectrophotometer (UV-1800, Shimadzu, Japan) and through atomic absorption spectrophotometry (AA-6800, Shimadzu, Japan) (Westerman, 1990), then their accumulation was calculated by multiplying bythe dry weight.

The Composition of Xylem Exudates of Flowering Chinese Cabbage in Response to Different NH_4^+/NO_3^- Ratios

Flowering Chinese cabbage seedlings were cultured as described in Section "Plant Material and Treatments." After pre-culturing for 5 days, the seedlings were transferred to an N-free Hoagland solution for 2 days. Three consistent seedlings were selected and transplanted in a barrel containing 5.5 L of the nutrient solution. Each treatment had six replications. Seedling exudates were collected after 2 weeks as described by Kehr et al. (2005) to analyze the intensity of xylem exudates and stored at −80°C to measure the content and flux of NO_3^-, NH_4^+, free amino acid, and soluble protein. The activity of roots was measured by using 2, 3, 5-tetraphenyltetrazolium chloride (TTC) as described by Yi et al. (2007).

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Total RNA was extracted from the samples using an Eastep® Super Total RNA Extraction Kit (Promega, Beijing, China), after which it was reverse transcribed using a GoScript™ Reverse Transcription Mix (Promega, Beijing, China). The qPCR was performed in a CFX Connect Real-Time PCR System (Bio-RAD, CA, United States) using SYBR® Premix Ex Taq™ (TaKaRa Bio, Tokyo, Japan). The primer pairs were listed in **Supplementary Table 1**. *Actin2* and *GAPDH* were used as internal controls. Three biological replicates were used to calculate relative gene expression levels by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2002).

Data Analysis

The data were analyzed by one-way analysis of variance (ANOVA) using the software package SPSS 19.0 (SPSS Incorporation, Chicago, IL, United States), and the differences

among treatments were compared using the least-significant difference (LSD) test with a significance level of $P < 0.05$. Figures were made using SigmaPlot v11.1 (Jandel Scientific Software, San Rafael, CA, United States).

RESULTS

Effect of Different NH_4^+/NO_3^- Ratios on the Growth and Biomass of Flowering Chinese Cabbage Seedlings

As shown in **Figure 1**, different NH_4^+/NO_3^- ratios influenced the growth of flowering Chinese cabbage seedlings markedly. T1 (NH_4^+/NO_3^- = 10/90), T2 (NH_4^+/NO_3^- = 25/75), and T3 treatment (NH_4^+/NO_3^- = 50/50) significantly increased the biomass of seedlings during the early growth stage (0–12 days after treatment) compared with the control (NH_4^+/NO_3^- = 0/100) (**Figure 1A**). In contrast to the effect of other treatments, compared to that in the control, T3 significantly decreased the fresh and dry plant weight at the late stage of treatment (15–21 days) (**Figure 1B**). At the harvest stage of flowering Chinese cabbage (21 days), compared with that in the control, the height of flowering Chinese cabbage in T1 and T2 was increased by 1.14, and 1.21 times, respectively, whereas that in T3 was significantly decreased (**Figure 1C**). Similarly, the stem diameter was the highest in T2 and lowest in T3 (**Figure 1D**). Owing to a higher concentration of NH_4^+, T3 significantly inhibited the root growth of flowering Chinese cabbage, and these plants had the lowest root to shoot ratio (**Figure 1E**). Consistent with the changes in biomass, the economic yield was the highest in T2, being 1. 53-, 1. 17-, and 2.41-fold higher than that in the control, T1, and T3, respectively, whereas it was the lowest in T3, being only 69.10% that of the control (**Figure 1F**).

To study the influence of different NH_4^+/NO_3^- ratios on seedling growth, we analyzed the growth rate of flowering Chinese cabbage throughout the experiment. During the period of 0–9 days after treatment, the growth rate of seedlings was the highest in T2, whereas there was no significant difference among the other three treatments (**Table 1**). During the period of 6–15 days, the growth rate was still the highest in T2, followed by T1, and it was the lowest in control and T3, which had no significant difference. Similarly, during the period of 15–21 days, the growth rate was still the highest in T2, with no significant difference between T2 and T1; the growth rate of control plants was significantly higher than that of plants in T3. In summary, at 0–15 days, there was no significant difference between the growth rate of seedlings in the control and T3 group; at 0–21 days, the growth rate was the lowest in T3: it was only 63.16, 48.25, and 41.13% that of the control, T1, and T2, respectively. This indicated that the highest ratio of NH_4^+/NO_3^- (NH_4^+/NO_3^- = 50/50) did not inhibit the growth of flowering Chinese cabbage seedlings at the early stage (at 0–15 days); but it significantly inhibited the growth since 15 days after treatment, its growth rate was only 36.57% of the control at 15–21 days.

Consequently, we concluded that lower ratios of NH_4^+/NO_3^- could promote the growth of flowering Chinese cabbage, among which the effect of T2 (NH_4^+/NO_3^- = 25/75) was the strongest; the highest ratio of NH_4^+/NO_3^- (NH_4^+/NO_3^- = 50/50) could inhibit plant growth, especially at the later stage.

N Content and the Distribution of Different N Forms in Flowering Chinese Cabbage Plants Under Different NH_4^+/NO_3^- Ratios

As shown in **Figure 2A**, the total N content of flowering Chinese cabbage increased at first and then decreased during the growth period. The total N content of plants in T1–T3, among which there was no significant difference, was higher than that of control plants. Regarding the accumulation of total N in plants, it was the highest in plants in T2 throughout the entire growth period, especially at the late stage of growth (i.e., 18–21 days); control plants had the lowest total N in the period from 0 to 18 days of growth, whereas plants in T3 had the lowest total N at 21 days (only 74.56% of that of plants in T2) (**Figure 2B**). Besides the total N content, total N accumulation was also affected by seedling dry weight.

To elucidate the influence of different NH_4^+/NO_3^- ratios on the distribution of N forms, we analyzed the content of NO_3^-, NH_4^+, free amino acids, and soluble protein in different tissues of flowering Chinese cabbage.

As shown in **Figure 3A**, compared with the control (sole NO_3^-), the three treatments (T1–T3) significantly reduced the NO_3^- content of roots, stems, and leaves of flowering Chinese cabbage seedlings; this was particularly visible in T3. In T3, the NO_3^- content of roots, stems, and leaves was only 26.60, 29.88, and 24.76% that of the control, respectively; moreover, there were significant differences in NO_3^- contents among the T1, T2, and T3. Similarly, compared to control, T1–T3 significantly reduced the content of NO_2^- in different tissues; this was particularly visible in T3, in which the NO_2^- content in roots, stems, and leaves was 36.11, 37.04, and 46.00% that of control, respectively (**Supplementary Figure 1**).

Compared with NH_4^+ content of control plants, NH_4^+ content in the roots, stalks, and leaves of plants in the three treatments were significantly increased. Moreover, NH_4^+ content increased with the increase in NH_4^+ concentration of the nutrient solution. NH_4^+ contents of plant roots, stalks, and leaves in T3 were 1.02, 1.15, and 1.20 times higher than those in the control (**Figure 3B**). The root amino acid content in T3 was significantly higher than that in the control and the other treatments; however, there was no significant difference between amino acid content in leaves and stalks among four treatments (**Figure 3C**). Compared to that of the control, soluble protein content in the leaves of flowering Chinese cabbage was significantly increased in T1–T3, whereas the soluble protein content of stalks and roots was significantly increased by T3 (1.34- and 1.56-fold higher than that in the stalks and roots of control plants). Furthermore, the soluble protein content of

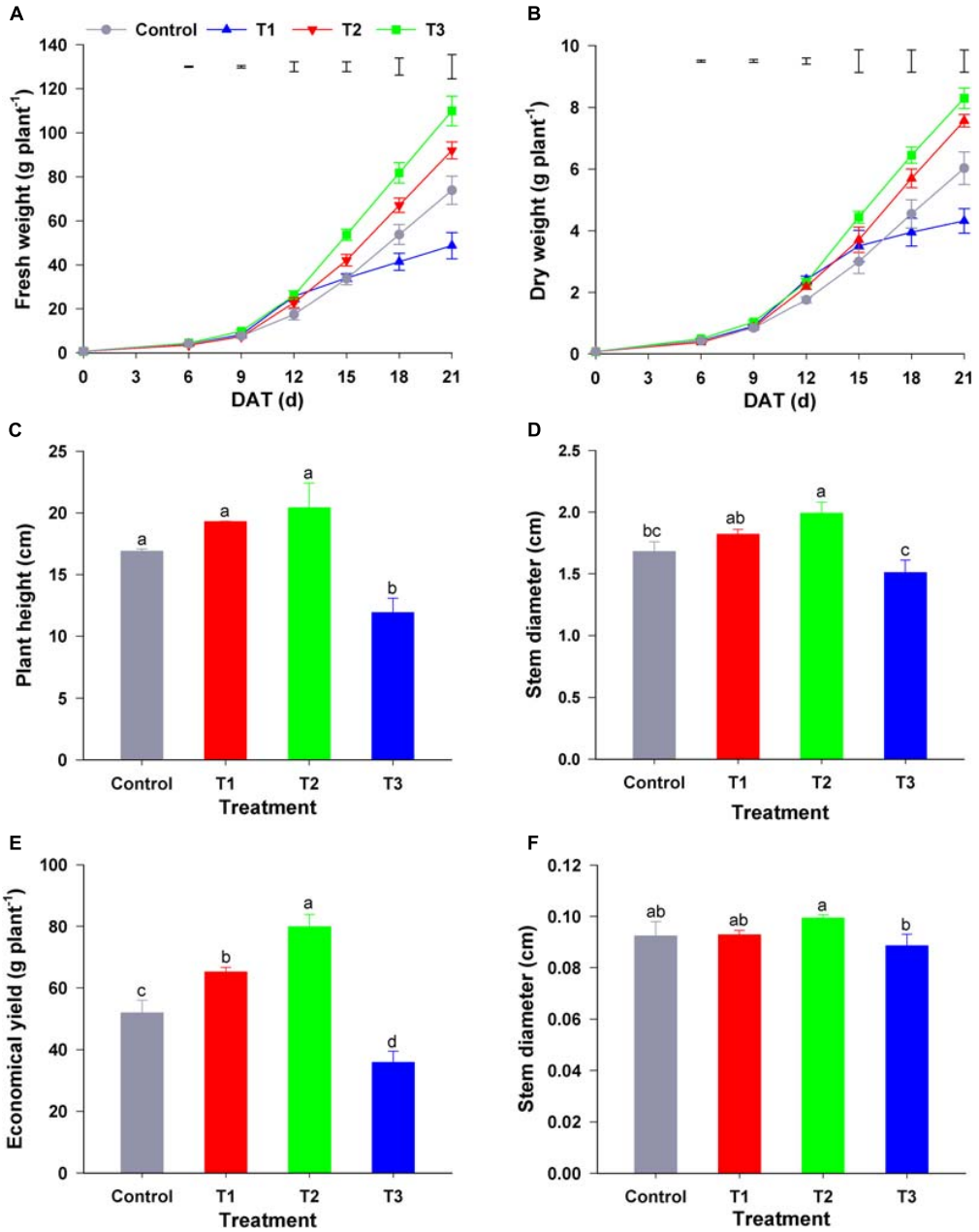

FIGURE 1 | Effect of different NH_4^+/NO_3^- ratios on the growth of flowering Chinese cabbage. **(A)** Dynamic changes in fresh weight; **(B)** dynamic changes in dry weight; **(C)** plant height at the harvest stage (after 21 days of treatment); **(D)** stem diameter at the harvest stage; **(E)** the ratio of root to shoot fresh weight at the harvest stage; and **(F)** economical plant yield at the harvest stage. Control = 0/100, T1 = 10/90, T2 = 25/75, T3 = 50/50. The data represent mean ± SE (n = 6). The vertical ruler in panels **(A,B)** represents the $LSD_{0.05}$ of the average values among the treatments. Different letters in (**C–F**) indicate significant differences at $P < 0.05$.

flowering Chinese cabbage leaves was much higher than that of stalks and roots (**Figure 3D**).

Dynamic Changes in the Nutrient Solution Composition in Response to Different NH_4^+/NO_3^- Ratios

To elucidate the effect of different NH_4^+/NO_3^- ratios on flowering Chinese cabbage, we carried out dynamic monitoring of physicochemical properties of nutrient solution and N content of plants during the experimental period.

With the increase in NH_4^+ concentration of nutrient solutions, their initial EC value started to differ among the four treatments (1.70, 1.78, 1.91, and 2.21 mS cm^{-1} in control, T1, T2, and T3, respectively). This may be due to the increase in the number of ions in the nutrient solution and the interaction between these ions. During the experimental period, the EC value of all nutrient solutions gradually decreased, and the final EC

TABLE 1 | Effects of different NH_4^+/NO_3^- ratios on the growth rate of flowering Chinese cabbage (g d^{-1} $plant^{-1}$).

Treatments	NH_4^+/NO_3^-	0–9 days	6–15 days	15–21 days	0–15 days	0–21 days
Control	0/100	0.77 ± 0.06 b	4.35 ± 0.34 c	6.70 ± 0.64 b	2.20 ± 0.17 c	3.49 ± 0.31 c
T1	10/90	0.74 ± 0.06 b	5.80 ± 0.34 b	8.32 ± 0.22 a	2.76 ± 0.17 b	4.35 ± 0.19 b
T2	25/75	1.01 ± 0.07 a	7.32 ± 0.32 a	9.37 ± 0.69 a	3.53 ± 0.17 a	5.20 ± 0.32 a
T3	50/50	0.84 ± 0.05 b	4.31 ± 0.21 c	2.45 ± 0.70 c	2.23 ± 0.11 c	2.29 ± 0.28 d

Control = 0/100, T1 = 10/90, T2 = 25/75, T3 = 50/50. The data represent mean ± SE (n = 3). Different letters indicate significant differences at P < 0.05.

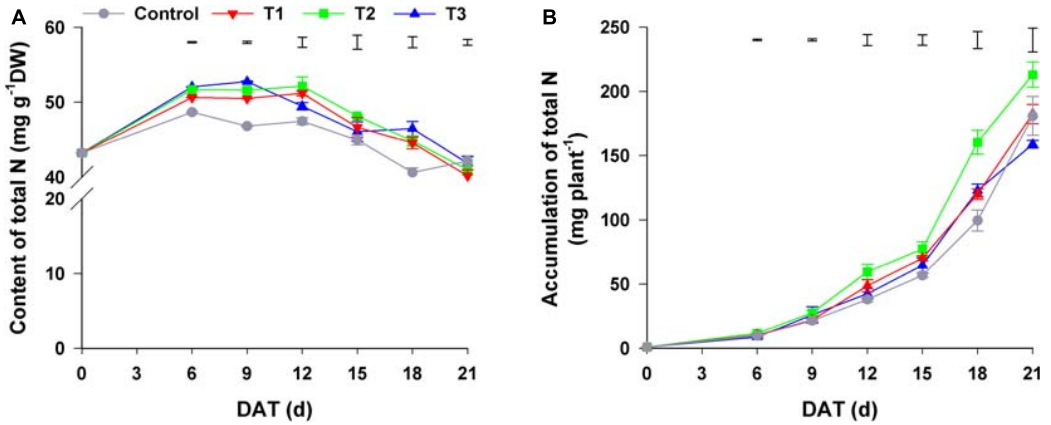

FIGURE 2 | Effect of different NH_4^+/NO_3^- ratios on the content **(A)** and accumulation **(B)** of total N during the growth period of flowering Chinese cabbage. Control = 0/100, T1 = 10/90, T2 = 25/75, T3 = 50/50. DW, dry weight; DAT, days after treatment. The data represent mean ± SE (*n* = 3). The vertical ruler represents the $LSD_{0.05}$ of the average values among the treatments.

values in control, T1, T2, and T3 were 0.44, 0.53, 0.71, and 1.26 mS cm^{-1}, respectively (**Figure 4A**).

Changes in the pH value of the nutrient solutions were completely from the changes in EC value. During the experimental period, the pH values of the nutrient solutions in all four treatments significantly changed. Though the initial pH value of all nutrient solutions was 6.5, the pH values of the control solutions increased gradually until the end of experiment, and the final pH value was 8.14. In T1, the pH value of the nutrient solution decreased slowly (at 0–9 days after the start of the treatment), after which it quickly increased and continued to increase until the end of experiment; its final pH value was 7.86. In contrast, the pH value in T3 declined gradually at 0–9 days, after which it quickly decreased and was only 3.68 at 21 days after the start of the treatment. However, the change in the pH value in T2 nutrient solution was completely different from that in the other three treatments because pH was maintained in the range of 5.64–6.50 throughout the experimental period (**Figure 4B**). Consequently, the changes in the physicochemical properties of these nutrient solutions were distinctly different among the four treatments with different NH_4^+/NO_3^- ratios during the experiment.

As shown in **Figure 4C**, the initial NO_3^- content in the control, T1, T2, and T3 was 160.08, 139.55, 111.33, and 74.09 mg L^{-1}, respectively, decreasing gradually with the decrease in NH_4^+/NO_3^- ratio. During the plant growth period, the content of NO_3^- in all nutrient solutions gradually decreased. The final concentration of NO_3^- in the control, T1, T2, and T3 treatments was 45.56, 30.13, 18.79, and 2.43 mg L^{-1}, respectively, the reduction of NO_3^- with control, T1, T2, and T3 was 114.52, 109.42, 92.54, and 71.75 mg L^{-1}, respectively. Similarly, NH_4^+ content of the nutrient solutions in T1–T3 decreased gradually as time progressed (**Figure 4D**). At harvest, the concentration of NH_4^+ in T1, T2, and T3 was 0.07, 6.73, and 38.85 mg L^{-1}, with the decrease in NH_4^+ being 16.54, 35.00 and 44.41 mg L^{-1}, respectively, compared to that at the beginning of the experiment. The total N content gradually decreased during the experimental period, and the greatest reduction in total N content was observed in T2 (the final content at harvest was 129.75 mg L^{-1}), and the smallest was observed in control (the final content at harvest was 114.52 mg L^{-1}) (**Figure 4E**).

To investigate the change of NO_3^- and NH_4^+ contents in different treatments, we analyzed the ratio of NH_4^+ to NO_3^- in the four treatments. In T1 and T3, during the first 15 d, the NH_4^+/NO_3^- ratio was maintained at 0.10 and 1.10, respectively, after which this ratio in T1 decreased sharply to a stable level of 0.03 at 21 days, whereas in T3, it gradually increased to 16.02 at 21 days. Unlike that in the other three treatments, NH_4^+/NO_3^- ratio in T2 changed little during the experimental period, staying in the range of 0.23–0.41 (**Figure 4F**). However, no significant change was observed in the contents and the ratio of NH_4^+/NO_3^- in the nutrient solution without seedlings during the experimental period (**Supplementary Figure 2**). Therefore, the changes in nutrient solution composition may be related to the cultivation of flowering Chinese cabbage.

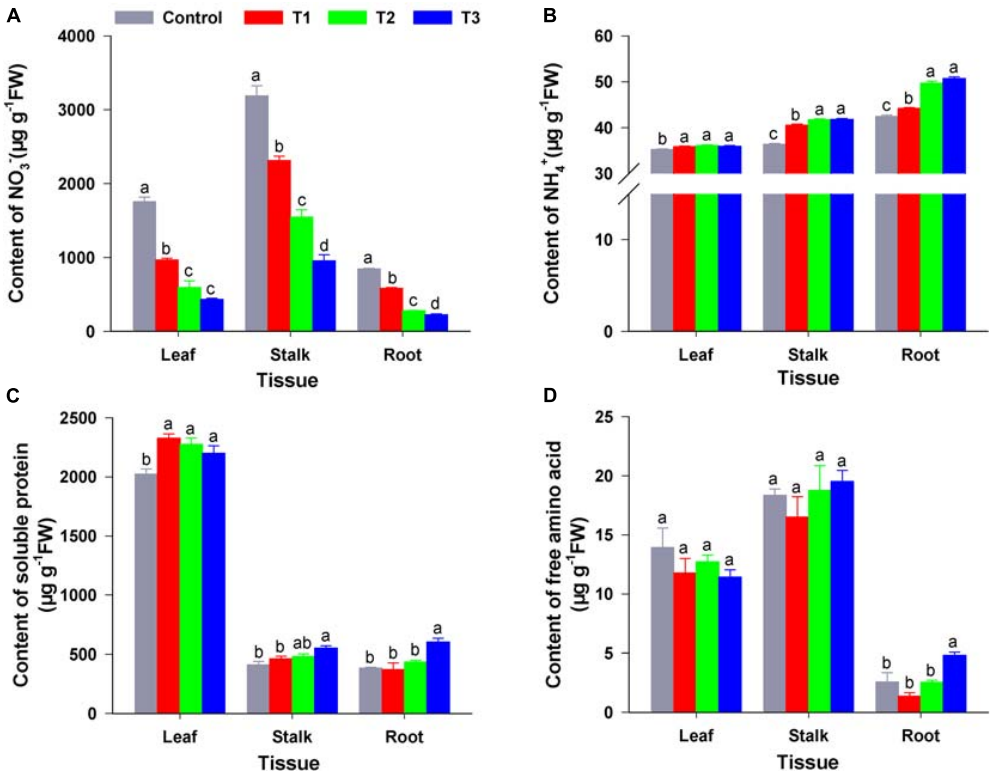

FIGURE 3 | The content of N forms in the roots, stems, and leaves of flowering Chinese cabbage under different NH_4^+/NO_3^- ratios. NO_3^- content **(A)**; NH_4^+ content **(B)**; soluble protein content **(C)**; and free amino acid content **(D)**. Control = 0/100, T1 = 10/90, T2 = 25/75, T3 = 50/50. FW, fresh weight. The data represent mean ± SE (n = 3). Different letters indicate significant differences at $P < 0.05$.

N Loss and N Use Efficiency of Flowering Chinese Cabbage Plants in Response to Different NH_4^+/NO_3^- Ratios

To evaluate the N utilization of flowering Chinese cabbage plants in response to different NH_4^+/NO_3^- ratios, we further analyzed the N loss, N productivity, N residence time, and NUE. We observed that a certain proportion of N loss occurred when flowering Chinese cabbage were grown under hydroponic conditions. Among the four treatments, T3 which had the highest NH_4^+/NO_3^- ratio in the nutrient solution (50/50), had significantly higher N loss than the other three treatments, among which there was no significant difference. Regarding the rate of N loss, it was lower in T2 than in the control, but it was significantly higher in T1 and T3 than in the control owing to the shorter or longer residence time of N remaining in the roots (**Table 2**). The rate of N loss was the highest in T3; it was 1. 44-, 1. 37-, and 1.51-fold that of the control, T1, and T2, respectively. Compared to that in control, N productivity was 1.18, 1.19, and 1.12 times higher in T1, T2, and T3, respectively. Owing to the differences in the N loss rate and N productivity, NUE was significantly different among the four treatments. NUE was the highest in T2, being 1. 22-, 1. 18-, and 1.74-fold that of the control, T1, and T3, respectively; it was the lowest in T3, being only 70.55% that of the control. Therefore, compared to the N loss in the control treatment (sole NO_3^-), the appropriate proportion of NH_4^+/NO_3^- could reduce the rate of N loss, prolong the N residence time, and improve N productivity and NUE, as was the case with T2 (NH_4^+/NO_3^- = 25/75); nevertheless, a higher ratio of NH_4^+/NO_3^- could have the opposite effect.

Xylem Exudate of Flowering Chinese Cabbage Plants in Response to Different NH_4^+/NO_3^- Ratios

To elucidate the influence of different NH_4^+/NO_3^- ratios on N allocation, we analyzed the xylem exudate composition of flowering Chinese cabbage seedlings. To ensure that we could obtain exudates in all treatments, we constantly adjusted the pH values of the nutrient solutions to approximately 6.5 during the experiment. There was no significant difference in the root activity measured by TTC among the four treatments (**Supplementary Figure 4**). However, the exudation intensity in T3 was significantly lower than that in the other three treatments (i.e., 46.90, 55.18, and 53.60% lower than that of control, T1, and T2, respectively) (**Figure 5**).

Compared with NO_3^- flux in the control, the addition of NH_4^+ to the nutrient solution significantly reduced the flux of NO_3^- in the exudates, and this effect was more pronounced with the increase in NH_4^+ concentration, (in T3, NO_3^- flux in the exudates was only 34.11% that of the control). Regarding the NH_4^+ flux in the exudates, compared to the control, T1

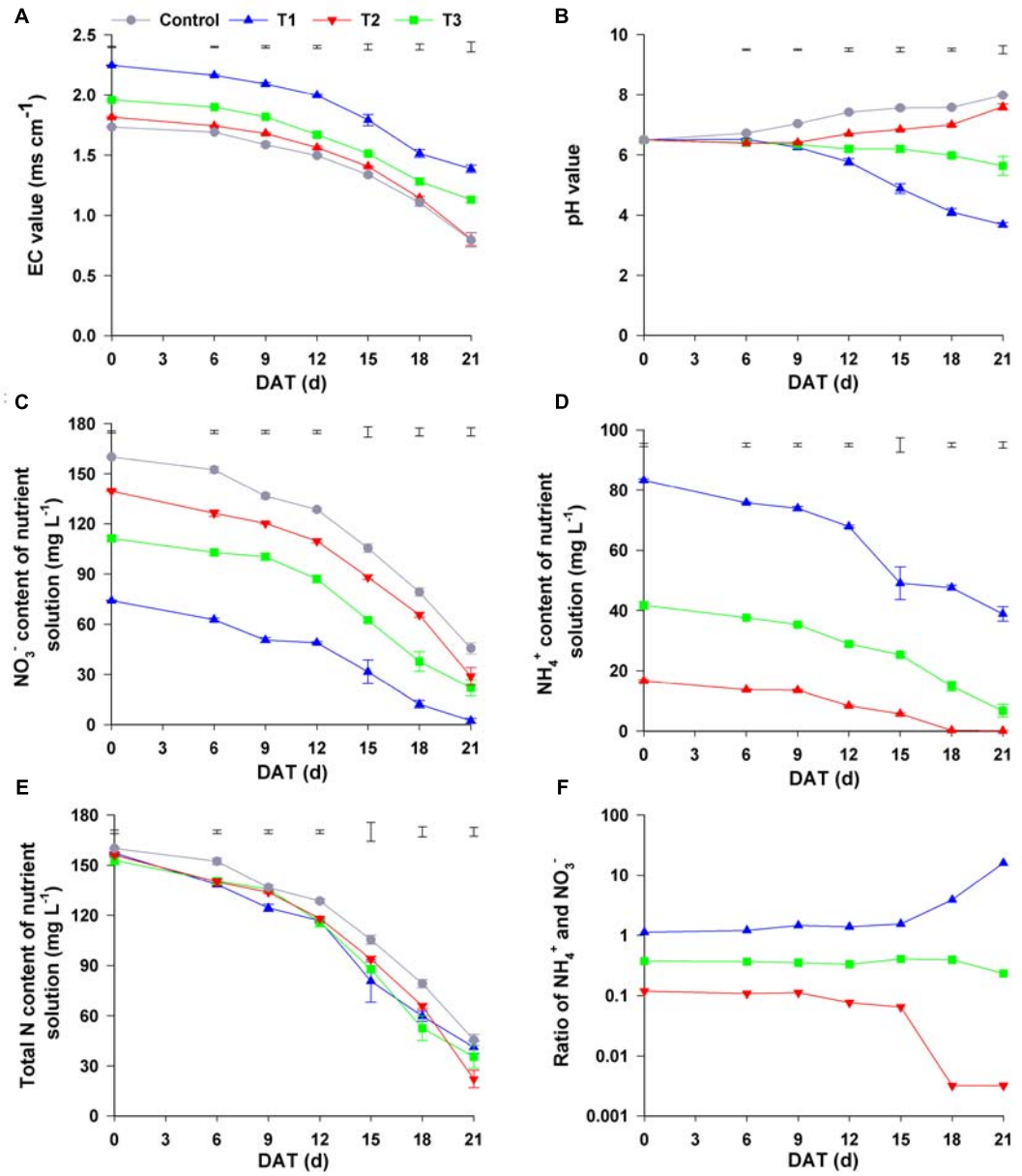

FIGURE 4 | Changes in the nutrient solution composition under different NH_4^+/NO_3^- ratios. **(A)** EC value; **(B)** pH value; **(C)** NO_3^- content; **(D)** NH_4^+ content; **(E)** total N content; **(F)** ratio of NH_4^+ and NO_3^-. Control = 0/100, T1 = 10/90, T2 = 25/75, T3 = 50/50. DAT, days after treatment. The data represent mean ± SE (n = 3). The vertical ruler in **(A–E)** represents the $LSD_{0.05}$ of the average values among the treatments.

TABLE 2 | Rate of N loss, N residence time, N productivity, and N use efficiency in nutrient solutions with different NH_4^+/NO_3^- ratios during the growth period of flowering Chinese cabbage.

Treatment	N loss (mg plant^{-1})	Rate of N loss (%)	N residence time (d)	N productivity (mg mg^{-1} d^{-1})	N use efficiency (mg mg^{-1})
Control	52.59 ± 5.31 b	17.92 ± 1.81 b	18.59 ± 2.26 a	5.36 ± 0.21 b	98.78 ± 4.64 b
T1	54.02 ± 2.81 b	18.87 ± 0.98 ab	16.01 ± 1.18 ab	6.34 ± 0.21 a	101.53 ± 4.55 b
T2	47.87 ± 4.69 b	17.06 ± 1.67 b	19.05 ± 2.71 a	6.39 ± 0.24 a	120.53 ± 7.26 a
T3	74.43 ± 4.64 a	25.80 ± 1.61 a	11.66 ± 0.93 b	5.98 ± 0.22 ab	69.39 ± 1.81 c

Control = 0/100, T1 = 10/90, T2 = 25/75, T3 = 50/50. The data represent mean ± SE (n = 3). Different letters indicate significant differences at P < 0.05.

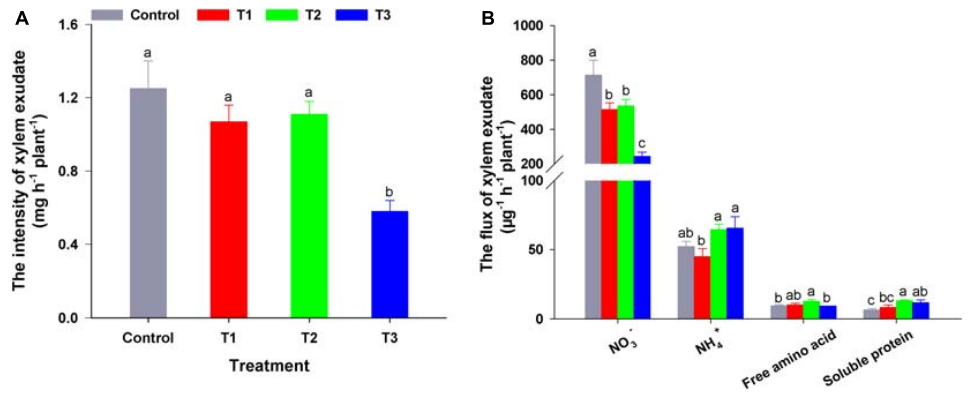

FIGURE 5 | The intensity **(A)** and flux **(B)** of xylem exudate in flowering Chinese cabbage in response to different NH_4^+/NO_3^- ratios. Control = 0/100, T1 = 10/90, T2 = 25/75, T3 = 50/50. The data represent mean ± SE (n = 3). Different letters indicate significant differences at $P < 0.05$.

decreased the NH_4^+ flux, which was lower than that of T2 or T3. Even though the NH_4^+ concentration of T2 nutrient solution was half that of T3 nutrient solution, there was no significant difference in NH_4^+ flux between these two treatments (**Figure 5B**). Compared with the control, the flux of soluble proteins in the exudates of flowering Chinese cabbage was up-regulated by three NH_4^+/NO_3^- ratios treatments, and the soluble protein flux was the highest in T2, being nearly twice the control. Free amino acid flux was similar to that of soluble proteins. Among the exudation fluxes of 4 N-forms, NO_3^- flux was the greatest. The proportion of NO_3^- in total N was 91.27, 89.05, 85.55, and 73.76% in control, T1, T2, and T3, respectively. This indicated that NO_3^- is the main form of N transported from the roots to the shoots in flowering Chinese cabbage. The contents of different N forms in the exudates of flowering Chinese cabbage were similar to the changes of N flux in all treatments (**Supplementary Table 2**). In summary, we confirmed that different NH_4^+/NO_3^- ratios had significantly different effects on plant N flux.

Expression of Plasma Membrane H^+-ATPase Genes (*HAs*) in Flowering Chinese Cabbage Plants in Response to Different NH_4^+/NO_3^-

The pH value of nutrient solutions changed significantly in response to different NH_4^+/NO_3^- ratios (**Figure 4B**). PM H^+-ATPase plays an important role in intracellular pH homeostasis and ensures normal life activities (Duby and Boutry, 2009). Thus, we analyzed the characteristics of *HA* genes encoding PM H^+-ATPase proteins.

As described by Arango et al. (2003), the putative PM H^+-ATPase is distributed into five subfamilies in the phylogenetic tree constructed from *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Nicotiana plumbaginifolia* (Np), and *Brassica rapa* (Br). Seven out of ten putative proteins were clustered in two of the five subfamilies: LOC103855020 (BrHA11), LOC103835963 (BrHA10), and LOC103841882 (BrHA7) were clustered in subfamily I, III, and V, respectively; LOC103828351 (BrHA1), LOC103834686 (BrHA2), LOC103845176 (BrHA3), and LOC10 3842249 (BrAHA5) were clustered in subfamily II; and LOC103842249 (BrHA6), LOC103853056 (BrHA8), and LOC103830447 (BrHA9) were clustered in subfamily IV (**Figure 6**).

HA genes in flowering Chinese cabbage had different response to different NH_4^+/NO_3^- ratios. The expression of *HA1* or *HA7* in plant roots decreased significantly with the increase in NH_4^+/NO_3^- ratios, especially in T3 (their expression was only 39 and 6% that of the control, respectively) (**Figure 7A**). In contrast, *HA3* transcription in T3 was higher than that in the other treatments, being 4. 06-, 5. 17-, and 4.51-fold that of the control, T1, and T2, respectively. The transcription of *HA2* and *HA9* was higher in T2 than in the other treatments. As for the expression of *HA5*, the expression in the control and T3 was higher than that in T1 and T2, whereas the expression of *HA6*, *HA8*, *HA10*, and *HA11* was similar in response to different NH_4^+/NO_3^- ratios, even though it was higher in the control and T2 than in T1 and T3 (**Figure 7A**).

In plant leaves, the expression of *HA3*, *HA5*, *HA9*, and *HA11* significantly increased with the increase in NH_4^+/NO_3^- ratios, especially in T3 (NH_4^+/NO_3^- = 50/50). However, the expression of *HA6*, *HA8*, and *HA10* increased at first and then decreased with the increase in NH_4^+/NO_3^- ratios, especially in T2 (NH_4^+/NO_3^- = 25/75), where their expression was the highest, being 83.64, 17.94, and 85.35 times that of the control. The transcription of *HA1* and *HA2* in plant leaves was little affected by different NH_4^+/NO_3^- ratios, and the expression of *HA7* in plant leaves was not detected in any treatment (**Figure 6**). Overall, the expression of *HAs* was significantly affected by different NH_4^+/NO_3^- ratios.

DISCUSSION

Response of Flowering Chinese Cabbage Plants Growth to Different NH_4^+/NO_3^- Ratios

As the main forms of inorganic N, both NH_4^+ and NO_3^- can be taken up by plants; however, they play different roles

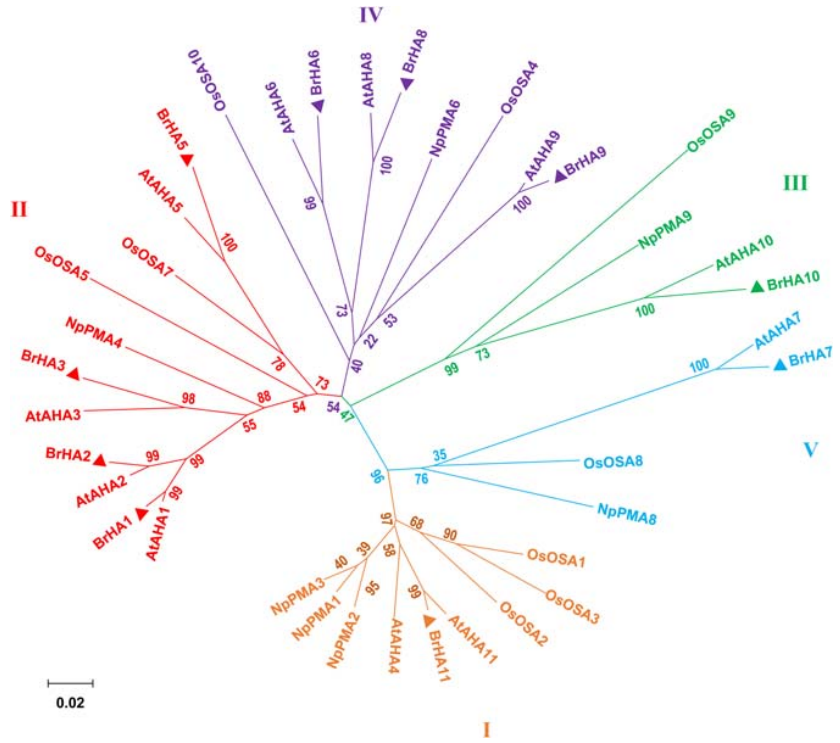

FIGURE 6 | Phylogenetic tree showing the relationships among the plasma membrane H^+-ATPases from *Brassica rapa* (Br), *Arabidopsis thaliana* (At), *Oryza sativa* (Os), and *Nicotiana plumbaginifolia* (Np). Phylogenetic trees were built using the MEGA6.0 (for protein ID codes, see **Supplementary Table 4**). Bootstrap values from 1,000 replicates were used to estimate the confidence limits of the nodes. The scale bar represents a 0.02 estimated amino acid substitutions per residue. Triangles represent the PM H^+-ATPase protein from *Brassica rapa*.

in photosynthesis (Konnerup and Brix, 2010), the absorption of minerals (Zou et al., 2001; Zeng et al., 2012) and water (Ding et al., 2018), and other physiological and biochemical processes. Previous studies have reported the effects of different NH_4^+/NO_3^- ratios on the biomass of several plant species (Tabatabaei et al., 2008; Santos et al., 2013; Hu et al., 2015; Song et al., 2017). In the present study, the results showed that the appropriate NH_4^+ and NO_3^- ratios (i.e., NH_4^+/NO_3^- = 10/90 and 25/75) were beneficial to the growth of flowering Chinese cabbage and resulted in higher fresh weight and economical yield, especially for the treatment of NH_4^+/NO_3^- = 25/75. Santos et al. (2013) reported that supplying N in a nutrient solution in the form of NO_3^- or NH_4^+ exceeding 70% or 50%, respectively, may decrease the yield in *Panicum maximum*. However, in the present study, the high NH_4^+/NO_3^- ratio (50/50) significantly accelerated the growth of seedlings at the early stage (0–12 days), and it inhibited it at the late stage (15–21 days); during this period, plant growth rate was 2.45 mg d^{-1} $plant^{-1}$, which was only 36.57% of plant growth in control with sole NO_3^- (**Figure 1** and **Table 1**). This showed that the effects of different forms of N on plant growth could be also closely related to the duration of treatment, and that the highest concentration of NH_4^+ used in our study would not cause ammonium toxicity when applied for only a short time. In our experiment involving different NH_4^+/NO_3^- ratios, other cations or anions present in the nutrient solution may be involved with supplying together with the N source, and their concentrations were always affected, as indicated by different original EC value of the nutrient solution among four treatments (**Figure 4A**). In this study, 2.25, 5.60, and 11.23 mmol L^{-1} Cl^- was added to the T1, T2, and T3 nutrient solution, respectively, and these concentrations were below the threshold of osmotic salt stress, which is approximately 40 mmol L^{-1} NaCl for most plants (Munns and Tester, 2008). Therefore, the amount of Cl^- in nutrient solution in present study was insufficient to cause salt stress. Furthermore, we analyzed the content and ratio of NH_4^+ and NO_3^- in the nutrient solution. We found that the NH_4^+/NO_3^- ratio in the treatment with NH_4^+/NO_3^- = 50/50 remained at 1.10 at the early stage and increased rapidly at the late stage of plant growth, whereas that in the treatment with NH_4^+/NO_3^- = 27/75 remained unchanged throughout the entire growth period (**Figure 4F**). Thus, we speculated that the appropriate ratios of NH_4^+ and NO_3^- in the rhizosphere environment could be beneficial for the growth of flowering Chinese cabbage.

Response of Plant Absorption and Accumulation of Nutrient Elements to Different NH_4^+/NO_3^- Ratios

Dry matter and nutrient accumulation are necessary for organ differentiation and yield formation, and the absorption of

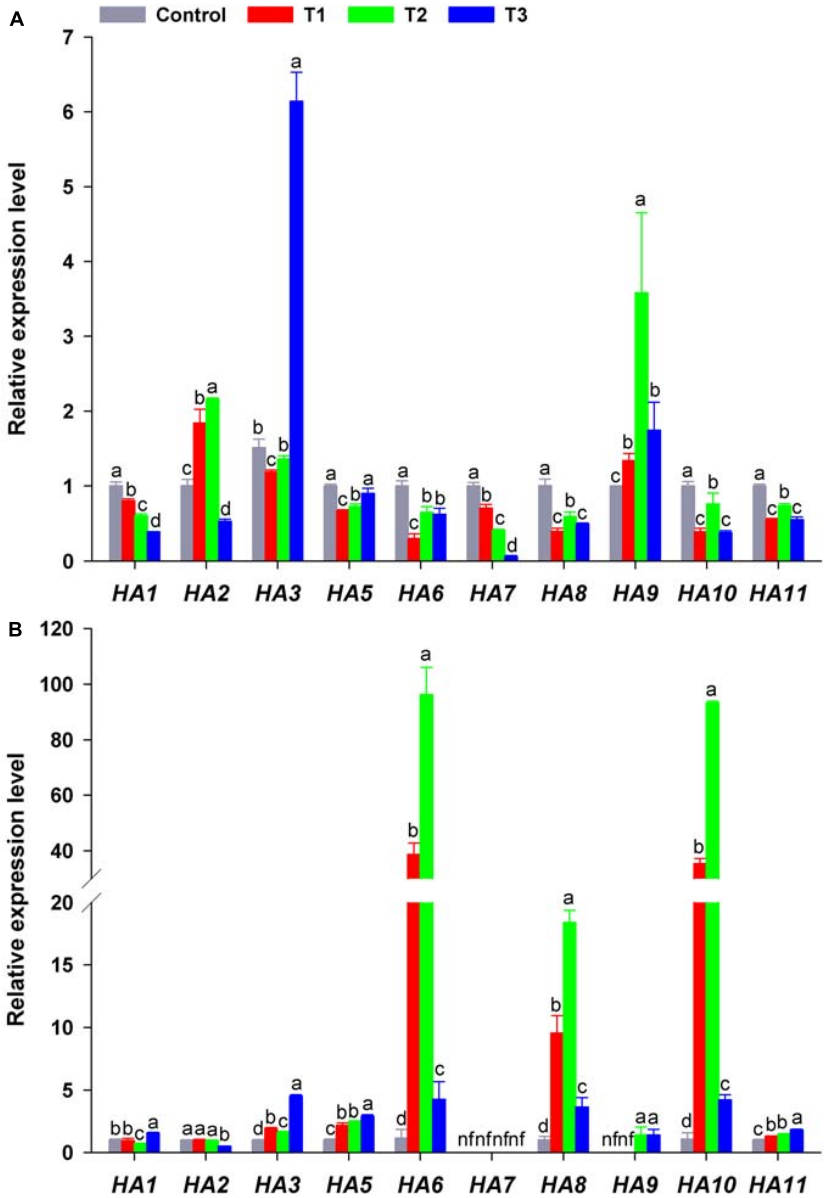

FIGURE 7 | Expression level of plasma membrane H^+-ATPase genes (*HAs*) in the roots **(A)** and leaves **(B)** of flowering Chinese cabbage plants in response to different NH_4^+/NO_3^- ratios during the 21 days of the experiment. Control = 0/100, T1 = 10/90, T2 = 25/75, T3 = 50/50. The data represent mean ± SE (n = 3). Different letters indicate significant differences at $P < 0.05$. nf represents not detected.

nutrients is the basis for dry matter formation and accumulation. Song et al. (2017) reported that different ratios of NH_4^+/NO_3^- had different influence on the content and accumulation of N in plants. Our study showed that the total N content of flowering Chinese cabbage was significantly increased by the three treatments (T1–T3) at 0–18 days after treatment compared with that of the control treatment with sole NO_3^- (**Figure 2A**). Regarding the accumulation of total N, in T2 (NH_4^+/NO_3^- = 25/75), it was significantly increased in contrast to that in the other three treatments over the entire growth period of flowering Chinese cabbage owing to the increase in plant biomass (**Figure 2B**). This finding was similar to previous findings (Song et al., 2012; Yin et al., 2020).

Moreover, the content of different N compounds in the tissues of flowering Chinese cabbage was significantly affected by different ratios of NH_4^+/NO_3^-. At harvest, the NO_3^- and NO_2^- content in leaves, stalks, and roots significantly declined as the NO_3^- content of the nutrient solution decreased, whereas NH_4^+ content exhibited the opposite trend, increasing as the NH_4^+ content in the nutrient solution increased (**Figures 3A,B** and **Supplementary Figure 1**). The changes in free amino acid and soluble protein contents were similar to the changes in

NH_4^+ content (**Figures 3B–D**). This was in accordance with the results reported by Arnold et al. (2015), and we confirmed the hypothesis that higher costs of NO_3^- assimilation, which is used for amino acid synthesis, result in overall lower amino acid synthesis rates, compared with that when a mix of NH_4^+ and NO_3^- is assimilated by plants. Through the analysis of xylem sap, we also confirmed that the flux and content of NO_3^-, NH_4^+, soluble protein, and free amino acid in the exudates showed a similar tendency in response to different NH_4^+/NO_3^- ratios (**Figure 5B** and **Supplementary Table 2**).

The absorption of NO_3^- or NH_4^+ by plants results in rhizosphere alkalization or acidification, respectively, which affects not only N absorption but also the absorption of other mineral nutrients (Sarasketa et al., 2016). In the present study, the pH value of the nutrient solution gradually declined in T3 (NH_4^+/NO_3^- = 50/50), gradually increased in the control (sole NO_3^-) and T1 (NH_4^+/NO_3^- 10/90), and slightly declined in T2 (NH_4^+/NO_3^- = 25/75; pH = 5.64 at harvest) (**Figure 4B**). Response of plant growth and nutrient absorption to the addition of different N forms may vary with changes in external pH, which may in turn modify pH value (Gerendas and Schurr, 1999).

In plants, the transport and assimilation of NO_3^- and NH_4^+ can dominate cellular pH homeostasis, which in turn affects the availability and utilization of other nutrients (Feng et al., 2020). The absorption of K and P was significantly changed by NH_4^+ and NO_3^- (**Supplementary Figures 2A–D**). In the present study, the three treatments significantly increased the content and accumulation of P compared with those in the control treatment (**Supplementary Figures 2A,B**), whereas the high ratio of NH_4^+/NO_3^- significantly inhibited the absorption of K (**Supplementary Figures 2C,D**). The content and flux of P and K in the exudates of flowering Chinese cabbage showed a similar tendency (**Supplementary Table 3**). Previous studies have shown that a low NO_3^-/NH_4^+ ratio inhibits plant growth and changes ion balance, as well as that the absorption of inorganic cations (K^+, Ca^{2+}, and Mg^{2+}) is reduced under high NH_4^+ conditions (Britto and Kronzucker, 2002). Zeng et al. (2012) reported that PM H^+-ATPase is involved in the stimulated uptake of P by rice roots supplemented with NH_4^+. Thus, the changes in rhizosphere pH caused the absorption of NH_4^+ or NO_3^- had important influence on the absorption of other nutrient elements, which may have been caused by the interaction between ions.

Response of NUE and N Loss to Different NH_4^+/NO_3^- Ratios

Previous reports have indicated that nutrient use efficiency may be expressed in different ways (Xu et al., 2012). In the present study, nutrient use efficiency was described as reported by Berendse and Aerts (1987), where nitrogen use efficiency is divided into N productivity and average N retention time, which can reflect a rapid growth strategy of plants and a nutrient retention strategy, respectively. We observed that compared with control and other treatments, treatments with moderate ratios of NH_4^+ and NO_3^- (NH_4^+/NO_3^- = 25/75) improved plant N use efficiency by increasing N productivity and extending the average N retention time, whereas the high ratio of NH_4^+ and NO_3^- (NH_4^+/NO_3^- = 50/50) significantly reduced plant NUE by shortening the average nitrogen retention time (**Table 2**). Thus, N loss in the treatment with high ratio of NH_4^+ and NO_3^- was significantly higher than that in the control treatment with sole NO_3^-.

The major pathways for N losses include leaching to surface- and groundwater, soil erosion, NH_3 volatilization, N_2O and NO_x fluxes to the atmosphere, and N_2 denitrification (Xu et al., 2012). In the present study, the contents of NH_4^+, NO_3^-, and total N in the nutrient solution without plant culture were almost constant during the growth period (**Supplementary Figure 3**). Thus, it was evident that N loss is low in a closed hydroponics system owing to reduced soil leaching and surface runoff. It could be inferred that the loss of N may have been related to the N metabolism of plants or caused by the change in nutrient solution composition during plant cultivation. Among the N loss pathways, NH_3 volatilization is a major pathway for N loss, and up to 23% of applied N can be lost via NH_3 volatilization (Ju and Zhang, 2017), which can be a consequence of photorespiration, NO_3^- reduction, amino acid catabolism, and phenylpropanoid metabolism (Bittsánszky et al., 2015). The unbalanced accumulation and transformation of active N sources (i.e., NH_4^+, NO_3^-, and NO_2^-) in plant leaves is the fundamental source of N volatilization loss in plants (Xu et al., 2012). In the present study, we observed that the ratio of NH_4^+ to NO_3^- in nutrient solution in T3 (NH_4^+/NO_3^- = 50/50) was up to 16.02 in the late stage of the experiment, whereas in T2 (NH_4^+/NO_3^- = 25/75), this ratio was maintained in the range of 0.23–0.41 (**Figure 4D**), which might have contributed to balanced N absorption. In contrast, nutrient solutions with higher NH_4^+/NO_3^- ratio may cause insufficient assimilation of NH_4^+ after its absorption or protein degradation owing to NH_4^+ toxicity, which can increase the loss of N. Therefore, the appropriate ratio of NH_4^+ to NO_3^- (i.e., NH_4^+/NO_3^- = 25/75 for flowering Chinese cabbage) can decrease N loss, improve NUE, and optimize crop performance.

Xylem Exudate Analysis Supported the Results of Nutrient Absorption and Assimilation Analysis Under Different NH_4^+/NO_3^- Ratios

In plants, nutrients are mainly transported from roots to shoots through the xylem, and the intensity and composition of exudates can reflect plant nutrition status (Buhtz et al., 2004). In the present study, medium and high NH_4^+/NO_3^- ratios significantly reduced the NO_3^- content and flux in the xylem exudates compared with that in control treatment with sole NO_3^-, while significantly increasing NH_4^+ content and flux (**Figure 5** and **Supplementary Table 2**). This result was similar to the results in rice (Kronzucker et al., 1999) and *Populus popularis* (Luo et al., 2013). Besides the difference in energy source between sole NO_3^- and the mixture of NH_4^+ and NO_3^- (Hachiya et al., 2012), Arnold et al. (2015) reported that NH_4^+ and NO_3^- might interact with each other when they are both present in the nutrient solution. Our previous results obtained using a scanning ion-selective electrode technique in flowering Chinese cabbage

showed that the absorption of NH_4^+ may be accelerated by the addition of NO_3^- into the nutrient solution, whereas the addition of NO_3^- had the opposite effect (Zhu et al., 2020).

NH_4^+ content in plant exudates can be a consequence of direct NH_4^+ uptake, but also a consequence of the decrease in NO_3^-, amino acid deamination, protein degradation, and photorespiration (Sarasketa et al., 2014). However, we observed that there was a slight difference in the NH_4^+ flux of the exudates between T3 (NH_4^+/NO_3^- = 50/50) and T2 (25/75), although the NH_4^+ content of T3 nutrient solution in was twice of T2 (**Table 2**). To avoid excessive NH_4^+ absorption which would result in ammonium toxicity, plants adopt different strategies, such as the regulation of AMT activity (Bittsánszky et al., 2015) and increase in NH_4^+ assimilation (Setien et al., 2013). In the present study, the flux or content of soluble protein or free amino acid in the xylem exudates increased with the increase in NH_4^+/NO_3^- ratio (**Figure 5B** and **Supplementary Table 2**); this was similar to the results reported by Yin et al. (2020). However, the flux of soluble protein or free amino acid under T3 (NH_4^+/NO_3^- = 50/50) was significantly decreased compared to that under T2 (NH_4^+/NO_3^- = 25/75) (**Figure 5B**). Furthermore, during the later period, the seedlings in the treatment with NH_4^+/NO_3^- = 50/50 showed symptoms of ammonia toxicity, including leaf etiolation, root reddening, and a lower growth rate and less biomass than the seedlings in the other treatments (**Figure 1** and **Table 1**). This implied that the seedlings of flowering Chinese cabbage under T3 might have experienced ammonia toxicity. Esteban et al. (2016) showed that complementation of NH_4^+ with NO_3^- at low doses, which has long been practiced in agriculture, can counterbalance the toxicity of NH_4^+ in laboratory conditions. Furthermore, NO_3^- may accelerate the transport of NH_4^+ or the assimilation product from roots to shoots (Hachiya et al., 2012; Hachiya and Sakakibara, 2017). Consequently, the absorption and transport of NH_4^+, NO_3^-, and their assimilation products may be affected by different NH_4^+/NO_3^- ratios. Therefore, applying appropriate ratios of NH_4^+/NO_3^- is one of the important ways to improve plant NUE (Rouphael et al., 2018).

Response of Plasma Membrane H^+-ATPase to Different NH_4^+/NO_3^- Ratios

Generally, the absorption of NO_3^- or NH_4^+ by plants results in alkalization or acidification of the rhizosphere (Sarasketa et al., 2016), this is related to the H^+ electrochemical gradient, which is controlled by H^+-ATPase in the PM (Haruta et al., 2018). In the present study, the pH values of the nutrient solution with full NO_3^- and that with a low ratio of NH_4^+/NO_3^- (10/90) were significantly increased, whereas the pH of the solution with a high ratio NH_4^+/NO_3^- (50:50) was sharply reduced, being only 3.68 at the time of harvest; the pH of NH_4^+/NO_3^- 25/75 solution was maintained in the range of 5.64–6.50 (**Figure 4B**). Zhu et al. (2009) have reported that the biomass of rice seedlings supplied with NH_4^+ at pH 6.5 is significantly higher than those given NO_3^-, whereas there is no difference between the two N forms at pH 3.0, in which PM H^+-ATPase plays an important role. PM H^+-ATPase pumps create the electrochemical H^+ gradients which energize most transport processes in and out of plant cells through channel proteins and secondary active carriers (Hoffmann et al., 2019).

It is generally believed that the mechanism through which the supply of NH_4^+ acidifies the rhizosphere is related to active PM H^+-ATPase pumping H^+ from intracellular into extracellular space, to maintain H^+ homeostasis in root cells (Palmgren and Harper, 1999; Zhang et al., 2018). The differences in PM H^+-ATPase activity between NH_4^+-supplied and NO_3^--supplied roots could be related to the discrepancy in H^+ generation during the assimilation of NH_4^+ and NO_3^- (Zhang et al., 2018), H^+-ATPase activity shows a narrow pH optimum at pH 6.0 (Zhu et al., 2009). Furthermore, PM H^+-ATPase is involved in the ammonium-nutrition response of plant roots (Zhu et al., 2009; Zhang et al., 2017), the activity of H^+-pumping, H^+-ATPase, and pH gradient across PM were significantly related the pH value of nutrient solution, rather than the N forms (Zhu et al., 2009). Zhu et al. (2009) reported that a high expression of various PM H^+-ATPase genes (*OSA1*, *OSA3*, *OSA7*, *OSA8*, and *OSA9*) is responsible for the adaptation of rice roots to low pH, either under sole NH_4^+ or sole NO_3^-. Similarly, PM H^+-ATPase also participates in NO_3^- uptake in plants (Sperandio et al., 2011; Pii et al., 2014). In rice, *OSA2*, *OSA5*, *OSA7*, and *OSA8* are induced by NO_3^- (Sperandio et al., 2011), which consequently increases N accumulation and rice growth (Sperandio et al., 2014); in winter wheat, *TaHA1* also participates in this process (Jiang et al., 2017); the expression and activity of *VvHA2* and *VvHA4* are induced by NO_3^- in grapevine (Pii et al., 2014).

To elucidate how different NH_4^+/NO_3^- ratios influence N absorption and assimilation, we further analyzed the phylogenetic tree and transcription level of PM H^+-ATPase gene (*HAs*) in flowering Chinese cabbage. A previous phylogenetic analysis based on the homology of sequences showed that 11 genes encoding HA homologs were present in *Brassica rapa* genome and were clustered into five clades, which was similar to the results obtained for *Arabidopsis* and *Nicotiana tabacum* (Arango et al., 2003). The predicted proteins encoded by *BrHA1*, *BrHA2*, *BrHA3*, and *BrHA5* are clustered in clade II; those encoded by *BrHA6*, *BrHA8*, and *BrHA9* are clustered in clade IV; and those encoded by *BrHA7*, *BrHA10*, and *BrHA11* are clustered in clades V, III, and I (**Figure 6**). Most members of subfamilies I and II are usually expressed in all tissues, whereas the expression of the members of other subfamilies is dependent on the tissue or environmental factors (Arango et al., 2003). A similar tendency was observed in the present study: *BrHA1*, *BrHA2*, *BrHA3*, *BrHA5*, and *BrHA11* were expressed in roots and leaves, while *BrHA7* and *BrHA9* were either not expressed or expressed only in roots of flowering Chinese cabbage (**Figures 7A,B**).

Consistent with the results of previous studies (Zhu et al., 2009; Sperandio et al., 2011; Pii et al., 2014), the expression of *BrHAs* in flowering Chinese cabbage was obviously affected by different NH_4^+/NO_3^- ratios. The expression of *BrHA1*, *BrHA5-8*, *BrHA10*, and *BrHA7* in roots and *BrHA2* in leaves was decreased by the addition of the mixture of NH_4^+ and NO_3^-,

whereas the expression of *BrHA3* in roots and *BrHA1*, *BrHA3*, *BrHA5*, *BrHA11* in leaves was increased by high NH_4^+/NO_3^- ratios. In contrast, the expression of *BrHA2* and *BrHA9* in roots and *BrHA6*, *BrHA8*, and *BrHA10* in leaves was significantly up-regulated by low NH_4^+/NO_3^- ratios compared with that in sole NO_3^- or high NH_4^+/NO_3^- ratio treatments (**Figures 7A,B**). The results were similar to those described by Zhang et al. (2018) in barley, the expression of *HvHA3* and *HvHA9* was down-regulated by NO_3^-, but *HvHA7* expression was induced by NO_3^-. In *Arabidopsis*, AHA2 is important for the growth and development of roots regardless of N levels, and this correlation may be related to the control of pH homeostasis (Młodzińska et al., 2015); AHA7 mainly functions when apoplastic pH is above 6.0, and it can make up for the lack of AHA2 (Hoffmann et al., 2019). *OSA7* in rice is induced by NO_3^- (Sperandio et al., 2014), which plays an important role in rice root growth and grain production without affecting N accumulation; this may highlight the importance of other PM H^+-ATPase isoforms in N uptake (Sperandio et al., 2020). Expression studies with *HA3*, *HA6,* and *HA8-10* suggest that they participate in response to different forms of N (**Figures 7A,B**; Pii et al., 2014; Zhang et al., 2018), but again, genetic evidence for this essential function is lacking (Haruta et al., 2018). In the present study, the expression level of *HA7*, along with the expression level of *HA1* in roots, was positively correlated with the pH of nutrient solution, which decreased as the NH_4^+/NO_3^- ratios increased (**Figure 4B**). Whether pH homeostasis is regulated by BrHA1/BrHA7 in flowering Chinese cabbage roots is worth investigating in future studies.

In addition to controlling the nutrient fluxes across the PM, PM H^+-ATPase is involved in several other physiological processes, such as stomatal opening, phloem transport, and cell growth (Arango et al., 2003; Duby and Boutry, 2009). We observed that the stomata of flowering Chinese cabbage leaves in T3 (NH_4^+/NO_3^- = 50/50) were closed, whereas in the other treatments, they were open (**Supplementary Figure 5**). This indicates that the appropriate NH_4^+/NO_3^- ratios might help maintain normal stomata opening and contribute to gas exchange between plants and the atmosphere, thereby benefitting photosynthesis; on the contrary, higher NH_4^+/NO_3^- ratios induce the closure of stomata, thus reducing water absorption, transpiration, and mineral absorption, and especially diminishing NH_4^+ uptake to avoid ammonia toxicity, the symptoms of which were observed during the later period. The activity of PM H^+-ATPase is regulated by abscisic acid, ethylene, and other hormones (Chen et al., 2017). Kinoshita and Hayashi (2011) reported that blue light can induce the activation of H^+-ATPase via phosphorylation of penultimate threonine (Thr) in the C-terminal H^+-ATPase and trigger subsequent binding of the 14-3-3 protein to the phosphorylated H^+-ATPase. The mechanism of H^+-ATPase action in different N conditions should be investigated in the future.

In summary, supplying an appropriate ratio of NH_4^+/NO_3^- (NH_4^+/NO_3^-=25/75) can improve N absorption and assimilation and promote the growth of flowering Chinese cabbage owing to the suitable pH value; on the contrary, the addition of excessive NH_4^+ may induce rhizosphere acidification and ammonia toxicity, resulting in growth inhibition. Our results provide valuable information regarding the influence of different NH_4^+/NO_3^- ratios on plant growth and N uptake and utilization.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

SS and RC conceived and designed the research. BQ carried out the experiments. YZ analyzed the data and wrote the manuscript. YH helped to analyze the data and reviewed the manuscript. GS and HL reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.656144/full#supplementary-material

Supplementary Figure 1 | NO_2^- content in root, stem and leaf of flowering Chinese cabbage under different NH_4^+/NO_3^- ratios.

Supplementary Figure 2 | Effect of different NH_4^+/NO_3^- ratios on the content and accumulation of total P and K in the growth period of flowering Chinese cabbage.

Supplementary Figure 3 | The change of NO_3^-, NH_4^+ and total N content of the nutrient solution without seedlings under different NH_4^+/NO_3^- ratios.

Supplementary Figure 4 | The root activity of flowering Chinese cabbage under different NH_4^+/NO_3^- ratios.

Supplementary Figure 5 | Stomatal conductance of leaf in flowering Chinese cabbage in response to different NH_4^+/NO_3^- ratios.

Supplementary Table 1 | The primers were used in this paper.

Supplementary Table 2 | The content of xylem exudate in flowering Chinese cabbage in response to different ratios of NH_4^+ and NO_3^-.

Supplementary Table 3 | The content and flux of P and K in xylem exudate in flowering Chinese cabbage in response to different ratios of NH_4^+/NO_3^-.

Supplementary Table 4 | The name and accession number of the H^+-ATPase protein used for the generation of the phylogenetic tree.

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Theoretical and Experimental Analyses of Nutrient Control in Electrical Conductivity-Based Nutrient Recycling Soilless Culture System

*Tae In Ahn[1,2], Jong Hwa Shin[3] and Jung Eek Son[1,4]**

[1] Department of Agriculture, Forest and Bioresources (Horticultural Science and Biotechnology), Seoul National University, Seoul, South Korea, [2] Smart Farm Research Center, KIST Gangneung Institute of Natural Products, Gangneung, South Korea, [3] Department of Horticulture and Breeding, Andong National University, Andong, South Korea, [4] Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul, South Korea

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***Correspondence:**
Jung Eek Son
sjeenv@snu.ac.kr

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An electrical conductivity (EC)-based closed-loop soilless culture system is practical for in-field deployment. Literature on the closed-loop soilless culture nutrient management premise the limitations in managing recycled nutrients under dynamic changes in individual nutrient uptake concentrations. However, recent systems analysis studies predicting solutions for nutrient fluctuation stabilization in EC-based closed-loop soilless culture systems suggest that the system may have a deterministic side in nutrient variation. This study aims to derive a nutrient control principle in an EC-based nutrient recycling soilless culture system by theoretical and experimental analyses. An integrated model of solutes such as K^+, Ca^{2+}, and Mg^{2+} and water transport in growing media, automated nutrient solution preparation, and nutrient uptake was designed. In the simulation, the intrinsic characteristics of nutrient changes among open-, semi- closed-, and closed-loop soilless cultures were compared, and stochastic simulations for nutrient control were performed in the closed-loop system. Four automated irrigation modules for comparing nutrient changes among the soilless culture systems were constructed in the greenhouse. Sweet pepper plants were used in the experiment. In the experimental analysis, nutrient concentration conversion to the proportion between nutrients revealed distinctive trends of nutrient changes according to the treatment level of drainage recycling. Theoretical and experimental analyses exhibited that nutrient variations in open-, semi- closed-, and closed-loop soilless culture systems can be integrated as a function of nutrient supply to the system's boundary areas. Furthermore, stochastic simulation analysis indicated that the nutrient ratio in the soilless culture system reveals the nutrient uptake parameter-based deterministic patterns. Thus, the nutrient ratio in the closed-loop soilless culture could be controlled by the long-term feedback of this ratio. We expect that these findings provide theoretical frameworks for systemizing nutrient management techniques in EC-based closed-loop soilless culture systems.

Keywords: Michaelis–Menten equation, sustainability, hydroponics, irrigation, drainage, soilless culture, nutrient recycling

INTRODUCTION

Soilless cropping is attracting attention as one of the principal means for sustainable intensification, which increases yield with minimum adverse environmental impact (Gruda, 2019). The basis for the interest in soilless culture systems is the ease of constructing closed-loop (CL) resource management. However, in practice, the application of CL soilless culture systems is scarce. In South Korea, the proportion of soilless culture occupied by the CL system is only 5% (Lee and Kim, 2019). In Almería, the highest area of soilless production in Spain, only 12% of 3,000 ha soilless cultivation are as uses the drainage reuse system (Massa et al., 2020). On the other hand, the cultivation area of soilless culture continues to increase worldwide (Rodríguez-Delfín, 2012; Walters et al., 2020). For now, the low proportion of the CL system is intertwined with technical constraints. Technological solutions for replacing the open-loop (OL) management practice on the field with the CL system are becoming challenging in soilless culture systems (Massa et al., 2020).

Nutrient management of the recycled nutrient solution is one of the critical barriers in replacing OL nutrient management. Ideally, nutrient management uncertainties can be minimized through the measurement of individual nutrients. Thus, development studies for nutrient management systems using real-time measuring devices, such as ion sensors, have been conducted; however, for a primary nutrient control system replacing OL nutrient management techniques, there were technical constraints (Kläring, 2001; Gieling et al., 2005; Bratov et al., 2010; Lee et al., 2017). The nutrient-sensing technique available at the field level is the electrical conductivity (EC) sensor, representing only the total nutrient concentration. However, nutrient uptake concentration, which complicates nutrient management of the recycled nutrient solution, varies mainly with transpiration and leads to fluctuations in root zone concentrations (van Noordwijk, 1990). Thus, several studies have shown that the CL system could be accompanied by nutrient variation, nutrient imbalance, and subsequent yield loss (Zekki et al., 1996; Kläring, 2001; Hao and Papadopoulos, 2002; Raviv and Lieth, 2008; Miller et al., 2020). Nevertheless, an EC-based system is a promising platform for disseminating nutrient management techniques at the field level. Thus, most of the approaches for managing the recycled nutrients and corresponding adverse effects have been conducted under the EC-based system. These approaches provided various information on nutrient behaviors and crop responses by manipulating the system variables and the parameters, such as the nutrient reuse period (Ehret et al., 2005; Ko et al., 2013), EC control (Rouphael et al., 2016; Signore et al., 2016), semi-closed-loop (SCL) system (level of drainage discharge) (Massa et al., 2011; Rouphael et al., 2016), fertilizer compositions (Hao and Papadopoulos, 2002; Gent and Short, 2012; Neocleous and Savvas, 2015, 2018), and difference in irrigation systems (Zekki et al., 1996; Incrocci et al., 2006; Bouchaaba et al., 2015). In addition to these, Savvas (2002) conducted a study on the control of individual nutrients with 2 weeks intervals of drainage analyses in the laboratory and replenished the adjusted nutrient solutions. Savvas (2002) tested nutrient replenishment methods and discussed their control performance and technical limitations.

However, at the field level, dynamic behaviors in irrigation system variables complicate the understanding and systemizing nutrient control techniques. Transpiration, which impacts nutrient uptake concentration (i.e., the ratio of nutrient to water absorption over time) (van Noordwijk, 1990; Le Bot et al., 1998),subsequently affects drainage volume, leaching fraction, and drainage EC (Ben-Gal et al., 2008; Shin and Son, 2016). Consequently, changes in drainage characteristics influence the recycled nutrient solution (Savvas and Manos, 1999). Understanding nutrient control principles under this condition may require a systems approach. Recently, a theoretical analysis study of a soilless culture system predicted a modified nutrient replenishment method and experimentally verified the theoretical prediction that a modified nutrient replenishment method could stabilize the fluctuations of some individual nutrients in the EC-based CL soilless culture (Ahn and Son, 2019). However, there is still little information on how the individual nutrient could be controlled in the EC-based CL soilless culture so far.

Overall, this study was designed to focus on the nutrient control principle under the irrigation system's dynamic behaviors and corresponding fluctuations in the nutrient uptake concentration, which affects the nutrient variation characteristics in the soilless culture system.

In this study, theoretical and experimental analyses for nutrient control are based on the following theoretical backgrounds:

(1) A plant's nutrient uptake phenomenon can be approximated by the Michaelis–Menten equation (Clarkson, 1985; Le Bot et al., 1998; Cott et al., 2018), and a system that follows this mechanism can have a steady-state solution according to the conditions of input variables and parameters (Golicnik, 2011).
(2) Transpiration produces fluctuations in all nutrients' uptake concentrations in the nutrient solution (van Noordwijk, 1990; Le Bot et al., 1998).

Dynamic changes in nutrient uptake concentration are intrinsically driven by Michaelis–Menten kinetics beneath the transpiration rate variations (Le Bot et al., 1998). On this basis, it can be hypothesized that converting the nutrient concentration to the proportion between nutrients could neutralize the dynamic changes in nutrient uptake concentration and could reveal the nutrient uptake parameter-based deterministic patterns. Consequently, complexity in nutrient control in the EC-based soilless culture system could be expected to be narrowed down to the parameter-based deterministic patterns. This study aims to analyze the nutrient variation patterns according to the degree of nutrient recycling and derive a nutrient control principle in the EC-based CL soilless culture system by constructing an integrated soilless culture system model.

MATERIALS AND METHODS

Automated Irrigation Module Used in This Experiment

The automated irrigation module consisted of a drainage tank, a nutrient solution tank, and a standard nutrient solution tank (**Figure 1**). In the drainage tank, the weight and EC of the collected drainage were measured by a load cell (JSB-20, CAS, South Korea) and an EC sensor (SCF-01A, DIK, South Korea). In the mixing tank, the nutrient solution's mixing process was carried out according to the degree of nutrient recycling. The drainage tank's storage capacity was 11.7 L, and the mixing tank capacity was 19.4 L. Ultrasonic-level sensors (UHA-300, Unics, South Korea) were installed at the top of the mixing tank to monitor the feed amount of drainage, raw water, and standard nutrient solution. Peristaltic pumps (M500, Verderflex, United Kingdom) were used to transfer standard nutrient solutions, drainage, and water from the pre-adjusted standard nutrient solution, drainage, and water tank to the mixing tank. For the irrigation of the mixed nutrient solution, a centrifugal pump (PUN-350M, Wilo Pump, South Korea) was used. A pyranometer (SP-110, Apogee, United States) was used for the integrated solar irradiance for automated irrigation control. A data logger (CR1000, Campbell Scientific, United States) and controller (SDM-CD16AC, Campbell Scientific, United States) were used for measurement, nutrient solution preparation, and irrigation control.

Greenhouse Experiment of the OL, SCL, and CL Soilless Cultures

The cultivation experiment using the four automated irrigation modules was carried out in a Venlo-type greenhouse located at the experimental farm of Seoul National University (Suwon, South Korea, lat. 37.3°N, long. 127.0°E). The four automated irrigation modules consist of one OL, two SCL, and one CL soilless culture systems. Accordingly, the degree of nutrient recycling was manipulated by applying the OL, SCL, and CL soilless cultures, respectively. The OL soilless culture system uses no drainage in the irrigated solution. The SCL soilless culture system partially uses drainage according to the drainage dilution level. In the CL soilless culture system, most of the collected drainage is reused for the nutrient solution. Basically, we referred to the general process of automated nutrient solution preparation (Savvas and Manos, 1999; Savvas, 2002). The OL irrigation controls the mixing tank EC to the target value. Conventionally, the SCL irrigation reuses the drainage by diluting to a lower EC than the supply EC with water, and stock solutions are applied to the supply EC of the irrigation. However, during the experimental period, the target EC for the irrigated nutrient solution was maintained at 2.6 dS m^{-1}. Thus, in the greenhouse experiment, a pre-adjusted standard nutrient solution for the supply EC (2.6 dS m^{-1}) was stored in the standard nutrient solution tank, and this was used to replenish the standard nutrient solution. In the greenhouse experiment, the determination of the mixing ratio of drainage, water, and the standard nutrient solution was based on the calculation procedure described in section "Determination of the Mixing Ratio in the Soilless Culture System," considering the amount of water already added in the pre-adjusted standard nutrient solution.

The four modules of the nutrient recycling degree treatment were composed of OL, CL, and SCL modules with two EC levels for a drainage dilution of 0.65 and 1.3 dS m^{-1} (SCL 0.65 and SCL 1.3). An integrated solar irradiance-based irrigation control was applied. The modules automatically irrigated 150 ml of the nutrient solution prepared in the mixing tank per plant whenever

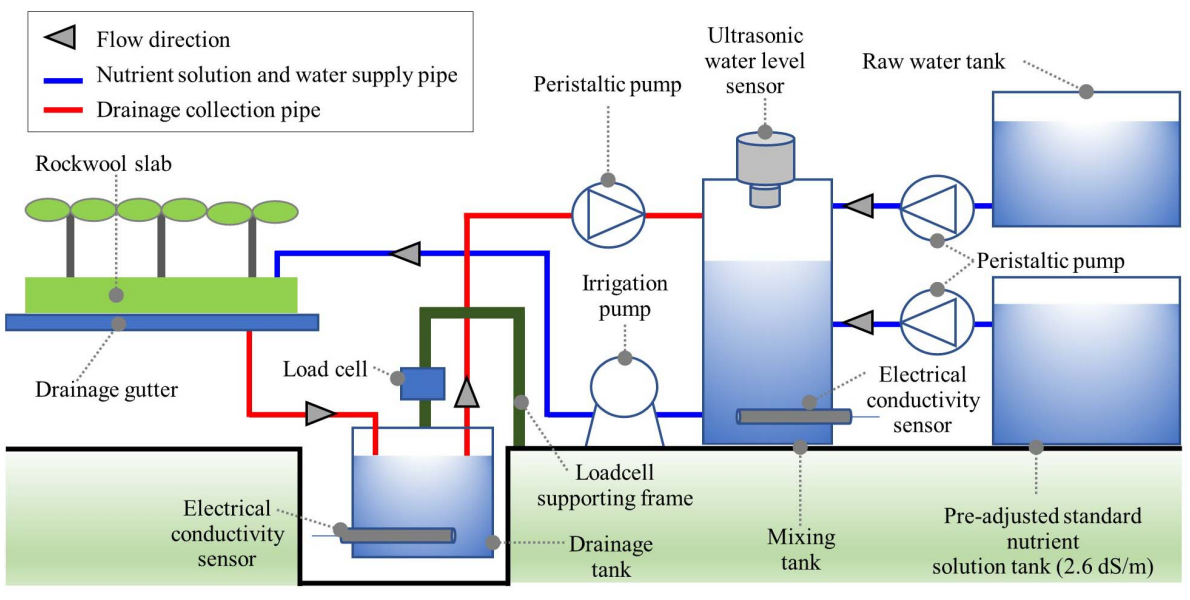

FIGURE 1 | Schematic description of the automated irrigation module for open- (OL), semi-closed (SCL), and closed-loop (CL) soilless culture in the experiment.

the integrated solar irradiance reaches 100 J cm^{-2}. However, the transpiration capacity increases due to plant growth during the cultivation period. Thus, the irrigation frequency or irrigation amount must be adjusted to compensate for the transpiration change. Thus, the supply volume per irrigation event was adjusted by the meteorological condition to compensate for the transpiration capacity change and maintain a drainage ratio of 30%. Each module prepared the nutrient solutions according to the recycling degree before the irrigation event.

Compositions of the standard nutrient solution used in this experiment were 14.56 NO_3^-, 1.18 $H_2PO_4^-$, 3.20 SO_4^{2-},5.96 K^+, 9.56 Ca^{2+}, 3.38 Mg^{2+}, 0.21Na^+, and 0.30Cl^- (in meq L^{-1}) as macro-elements and 18 Fe, 10 Zn, 2 Cu, 10 Mn, and 0.5 Mo (in μM) as micro-elements. The target EC of the nutrient solution mixing module was set to 2.6 dS m^{-1}, and the EC of the raw water was set to 0.15 dS m^{-1}. Daytime temperature, night time temperature, and relative humidity of the greenhouse remained between 25 and 30°C, 15 and 22°C, and 50 and 80%, respectively, by an environmental control system of the greenhouse. Sweet pepper (*Capsicum annuum* L. "Fiesta") plants were used in the experiment and seeded on July 15, 2011. The sweet pepper plants were transplanted into 90 cm (L) × 15 cm (W) × 7 cm (H) rockwool slabs (Cultilene, Netherlands) on September 29, 2011. Three sweet pepper plants were planted per slab, and three slabs were used per experimental treatment. The planting density was 2.8 plants per square meter.

The sweet pepper plants were cultivated by OL nutrient supply until December 15, 2011, and after that, each treatment was applied, and the experiment was finished on March 9, 2012. On the day before the initiation of the treatments, a large amount of a standard nutrient solution was irrigated in an OL nutrient supply in order to make the initial condition of the nutrients balanced in the rockwool slab of all treatments close to the standard composition.

Nutrient Analyses and Statistics

To observe the changes in the ratio between nutrients in the root zone of the soilless culture system, samples of nutrient solution in the rockwool slabs were extracted using a syringe. The collection points of the nutrient solution in the rockwool slab were randomly selected for the collection of representative samples of the overall concentration in the root zone. Then, 10 ml of a root zone nutrient solution was collected for each extraction, and this was performed five times to make a 50 ml sample. Six samples per treatment were collected each time every 2 weeks. K^+, Ca^{2+}, and Mg^{2+} were analyzed using an inductively coupled plasma optical emission spectrometer (ICP-730ES, Varian, Australia). The SAS system (version 9.2, SAS Institute, United States) was used for statistical analysis. In the experimental condition, three rockwool slabs were connected to the single automated module system. Thus, the replicate of the sample could have limited data variability. However, the soilless culture using slab-form substrates has an isolated system boundary of the root zone. Thus, even in the single system (i.e., a single greenhouse with a single irrigation system), each slab condition often shows significant variations in root zone nutrient and water according to the microclimate fluctuations of each slab's location. Therefore, at least, it can be expected that the rockwool slab replicates could statistically rule out these effects in this experimental condition.

The Theoretical Model for Soilless Culture Systems

The models of the soilless culture system used in the simulation consist of water and nutrient transport, nutrient uptake, and nutrient solution mixing (**Figure 2**). This model's basic structures were constructed based on previous models of the soilless culture system (Silberbush and Ben-Asher, 2001; Silberbush et al., 2005). The simulation model reflected the automated nutrient solution mixing process of the EC-based soilless culture system. For nutrients and water transport in the substrate, mass flow and diffusion models in porous media were applied (Shackelford and Daniel, 1991; Corwin et al., 1993; Snape et al., 1995). The nutrient uptake by plants follows the Michaelis–Menten equation. The rate of nutrient uptake is mainly driven by concentration. In this study, a modified Michaelis–Menten equation having an additional variable for the transpiration rate was used to reflect more stochastic changes in absorption parameter variations such as plant growth. This model considers reducing the nutrient depletion zone around the roots due to the accelerated mass flow generated by an increase in transpiration rate with plant growth (Sago et al., 2011; Nomiyama et al., 2013). Since transpiration is a variable that includes parameters for plant growth changes (Baille et al., 1994; Ta et al., 2012), the model was used to reflect the change in the nutrient absorption rate with plants' growth. The definitions, values, units, and sources of the models' parameters are summarized in **Table 1**.

Several assumptions were made to reduce the complexity of the simulation:

(1) Total ion concentration is in a linear relationship with EC in the nutrient solution (Savvas and Adamidis, 1999). Based on this assumption, the EC variable in the automated nutrient solution mixing process was replaced by the total equivalent concentrations in nutrient solution mixing simulation.
(2) The target nutrients selected for the simulation were K^+, Ca^{2+}, and Mg^{2+}, major cations in macronutrients (Marschner and Marschner, 2012).
(3) This simulation aims to analyze the effect of nutrient uptake concentration on nutrient management in the soilless culture system; thus, the interaction between nutrients and changes in the nutrient uptake parameters were not reflected.
(4) The nutrient ratio used in this study indicates the proportion between the milliequivalention concentrations ($C^I/(C^{K+} + C^{Ca2+} + C^{Mg2+})$; $I \equiv K^+, Ca^{2+}, Mg^{2+}$; C is the milliequivalent ion concentration).

Water Transport in the Soilless Culture System

Water transport in a CL soilless culture system occurs between the substrate, the drainage tank, and the mixing tank, and these

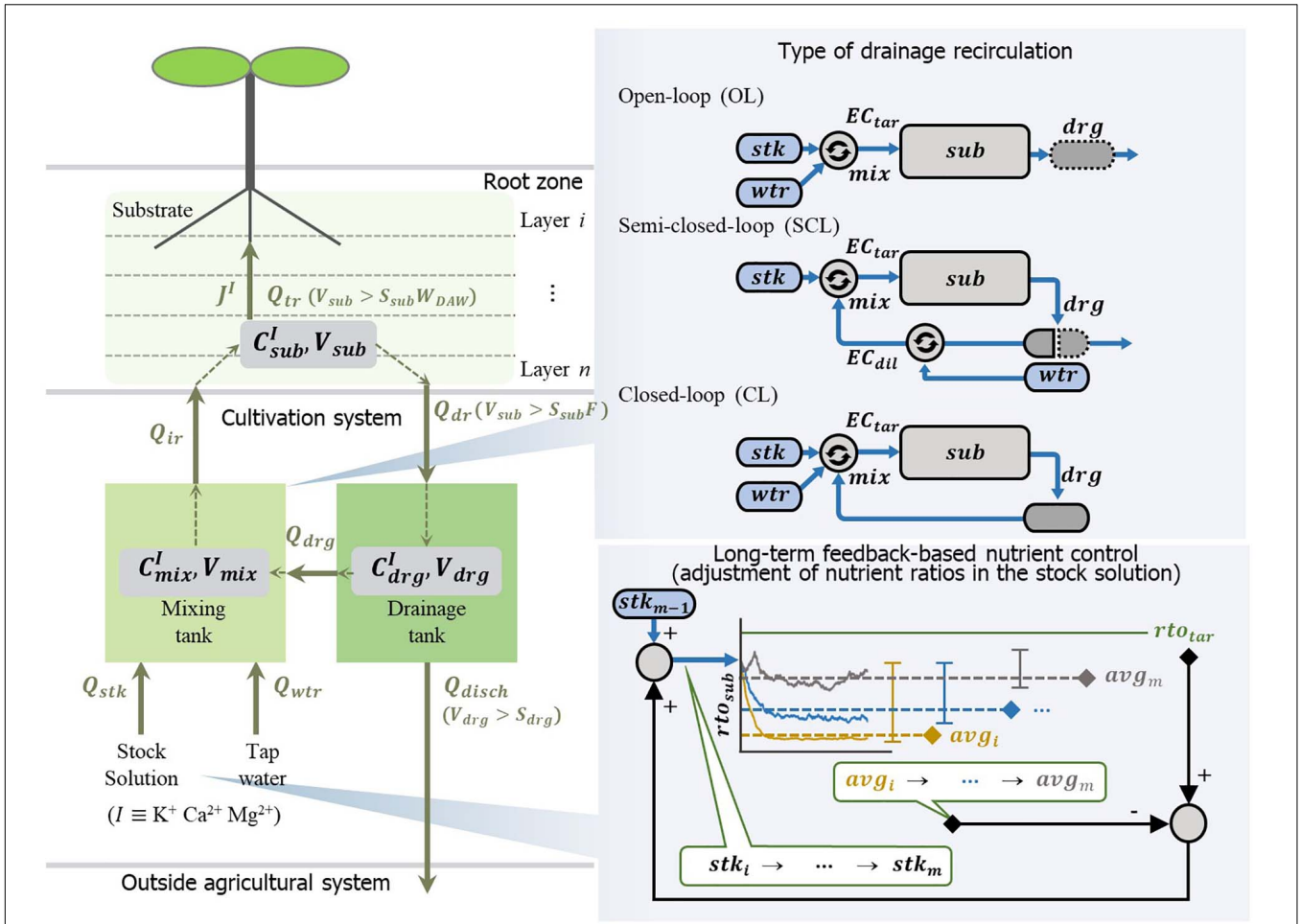

FIGURE 2 | Schematic description of open- (OL), semi-closed- (SCL), and closed-loop (CL) soilless culture systems in the simulation analyses. The water and nutrients are consumed by the transpiration rate ($\mathbf{Q_{tr}}$) and nutrient uptake rate of the plant ($\mathbf{J^I}$), respectively. Nutrients (**I**) from the stock solution ($\mathbf{Q_{stk}}$) and tap water ($\mathbf{Q_{wtr}}$) are circulated through the mixing tank, substrate, and drainage tank with the flow rates of irrigation ($\mathbf{Q_{ir}}$), drainage ($\mathbf{Q_{dr}}$), drainage reuse ($\mathbf{Q_{drg}}$) or discharged ($\mathbf{Q_{disch}}$) outside the system. OL system supplies the nutrient solution by adjusting the mixing ratio of the stock solution (***stk***) and the water (***wtr***) to the target EC ($\mathbf{EC_{tar}}$). SCL system prepare the drainage reuse by adjusting the mixing ratio of the drainage (***drg***) and the water to the dilution EC ($\mathbf{EC_{dil}}$). Subsequently, the SCL system adjusts the diluted drainage to the target EC by the stock solution and supplies it to the substrate (***sub***). CL system reuses all collected drainage (***drg***), and the nutrient solution is prepared by adjusting the mixing ratio of the stock solution (***stk***) and water (***wtr***) to the target EC ($\mathbf{EC_{tar}}$). The long-term feedback-based nutrient control analysis was conducted in the CL system. Whenever the simulation is finished, the difference between the average value ($\mathbf{avg_m}$) of an individual ion ratio in the substrate ($\mathbf{rto_{sub}}$) and target ratio ($\mathbf{rto_{tar}}$) was returned to the previous ion ratio of the stock solution ($\mathbf{stk_{m-1}}$). Subsequently, the modified stock solution ($\mathbf{stk_m}$) was applied to the next simulation.

correspond to Eqs. 1–3, respectively.

$$\frac{dV_{sub,n}}{dt} = \begin{cases} Q_{ir} - Q_{dr,1} - Q_{tr,1}, n\ 1 \\ Q_{dr,n-1} - Q_{dr,n} - Q_{tr,n}, and\ n\ 1 \end{cases} \quad (1)$$

$$\frac{dV_{drg}}{dt} = Q_{dr,n} - Q_{drg} - Q_{disch} \quad (2)$$

$$\frac{dV_{mix}}{dt} = Q_{drg} + Q_{stk} + Q_{wtr} - Q_{ir} \quad (3)$$

where $V(\mathrm{cm}^3)$ is the volume of water, subscript *sub*, *n* is the water content of each layer when the substrate is divided into *n* layers, *drg* is the drainage tank, and *mix* is the mixing tank. $Q(\mathrm{cm}^3\ \mathrm{min}^{-1})$ is the flow rate of the water; subscript *ir* is the irrigation flow rate to the top of the substrate ($n = 1$);subscripts *dr*,*n*-1 and *dr*,*n* are the flow rates of the drain from the *n* - 1 layer and the *n* + 1 layer, respectively; and *tr*,*n* represents the transpiration rate of the substrate layer *n*. Here, $Q_{dr,n}$ is the difference between the irrigation rate to the substrate and the transpiration rate (Q_{ir} – $Q_{tr,1}$or $Q_{dr,n-1}$ – $Q_{tr,n}$) (Shin et al., 2014). However, $Q_{dr,n}$ and $Q_{tr,n}$ are restricted by the field capacity (*F*, dimensionless) and difficult available water (W_{DAW}, dimensionless), respectively, for the volume of the layer of the substrate (S_{sub}, cm^3). And $Q_{dr,n}$ flows only when $V_{sub,n} > S_{sub}F$, and $Q_{tr,n}$ flows only when $V_{sub,n} > S_{sub}W_{DAW}$. Q_{drg}, Q_{stk}, and Q_{wtr} correspond to the drain, stock solution, and raw water flow rates, respectively, introduced into the mixing tank when irrigation to the root zone is required. Q_{disch} is a variable that

is applied to the SCL system, meaning the flow rate of the nutrient solution discharged to the outside when the volume of stored drainage (V_{drg}) exceeds the capacity of the drainage tank (S_{drg}, cm^3).

Nutrient Transport and Uptake Models in the Soilless Culture System

In a soilless culture system, the movement of nutrients follows the same path as water, and the movement of nutrients between the substrate, drainage tank, and mixing tank can be expressed as Eqs. 4–6, respectively.

$$V_{sub,n}\frac{dC^I_{sub,n}}{dt} = \begin{cases} Q_{ir}C^I_{mix} - Q_{dr,1}C^I_{sub,1} - J^I_1 - \frac{D^I A\left(\theta_1 C^I_{sub,1} - \theta_{n+1}C^I_{sub,n+1}\right)}{Z}, & n = 1 \\ Q_{dr,n-1}C^I_{sub,n-1} - Q_{dr,n}C^I_{sub,n} - J^I_n - \frac{D^I A\left(\theta_n C^I_{sub,n} - \theta_{n+1}C^I_{sub,n+1}\right)}{Z} - \frac{D^I A\left(\theta_n C^I_{sub,\ n} - \theta_{n-1}C^I_{sub,n-1}\right)}{Z}, & n > 1 \end{cases} \quad (4)$$

$$V_{drg}\frac{dC^I_{drg}}{dt} = Q_{dr,n}C^I_{sub,n} - Q_{drg}C^I_{drg} - Q_{disch}C^I_{drg} \quad (5)$$

TABLE 1 | Parameters and nomenclature used for the simulations of soilless cultures.

Parameter	Definition	Value	Unit	Source
J^K_{max}	Ion flux parameter	1.89	10^{-3}meq/plant/min	Estimated from the field experiment data
J^{Ca}_{max}	Ion flux parameter	1.60	10^{-4}meq/plant/min	
J^{Mg}_{max}	Ion flux parameter	1.61	10^{-4}meq/plant/min	
K^K_m	Ion flux parameter	9.00	10^{-3}meq/cm^3	
K^{Ca}_m	Ion flux parameter	2.44	10^{-4}meq/cm^3	
K^{Mg}_m	Ion flux parameter	1.33	10^{-3}meq/cm^3	
D^K	Diffusion coefficient (K^+)	117.6	10^{-5} cm^2/min	Lide, 2005
D^{Ca}	Diffusion coefficient (Ca^{2+})	47.4	10^{-5} cm^2/min	Lide, 2005
D^{Mg}	Diffusion coefficient (Mg^{2+})	42.6	10^{-5} cm^2/min	Lide, 2005
W_{DAW}	Difficult available water of the substrate	0.0068	Dimensionless	Dubský and Šrámek, 2009
F	Field capacity of the substrate	0.74	Dimensionless	Dubský and Šrámek, 2009
A	Cross-sectional area of the substrate	630	cm^2	
S_{drg}	Volume of drainage tank	11,700	cm^3	
S_{sub}	Volume of substrate layer n	472.5	cm^3	
C	Miliequivalent ion concentration		meq cm^{-3}	
Q	Water flow rate		cm^3 min^{-1}	
V	Volume of water		cm^3	
θ	Water content		Dimensionless	
J	Nutrient uptake rate		meq/plant/min	
EC	Electrical conductivity		dS m^{-1}	
OL	Open-loop			
SCL	Semi-closed-loop			
CL	Closed-loop			
Subscript				
drg	Relative to drainage			
sub	Relative to substrate			
mix	Relative to mixing tank			
stk	Relative to a stock solution			
wtr	Relative to raw water			
tar	Relative to a target value			
dil	Relative to dilution value			
n	Relative to the substrate layer			
i	Relative to starting number			
m	Relative to the number of simulation			
ir	Relative to irrigation			
tr	Relative to transpiration			
Superscript				
I	Relative to a type of ion			

$$V_{mix}\frac{dC^I_{mix}}{dt} = Q_{drg}C^I_{drg}+Q_{stk}C^I_{stk}-Q_{ir}C^I_{mix}+Q_{wtr}C^I_{wtr} \quad (6)$$

where C(meq cm^{-3}) represents the equivalent concentration of nutrients and superscript I is the type of ions (K^+, Ca^{2+}, and Mg^{2+}). Subscripts *sub*, *drg*, *mix*, *stk*, and *wtr* represent the source of the nutrients and indicate substrate, drainage tank, mixing tank, stock solution, and raw water, respectively. J^I(meq plant^{-1} min^{-1}) means the uptake rate of nutrients. The final term of Eq. 4 refers to the diffusion of solutes in a substrate, where D(10^{-5} cm^2 min^{-1}) is the diffusion coefficient of an ion I, A(cm^2) is the cross-sectional area of the substrate; θ_n is the water content of the n layer ($V_{sub,n}/S_{sub}$, dimensionless); Z(cm) is the height of the n layer. In the last layer, the diffusion term from n + 1 is excluded because the diffusion occurs from the previous layer only.

The Michaelis–Menten equation was used to model the nutrient uptake rate of the plant (Eq. 7).

$$J^I_n = \frac{J^I_{max}C^I_{sub,n}}{K^I_m + C^I_{sub,n}} \quad (7)$$

where J^I_{max}means the maximum absorption rate of the ion and K^I_m is the Michaelis–Menten constant. J^I_n is the absorption rate of individual nutrient I in layer n, and the equation applying the change in the rate of absorption of nutrients to the increase of transpiration rate ($Q_{tr,n}$) is shown in Eq. 8.

$$J^I_n = \frac{J^I_{max}C^I_{sub,n}Q_{tr,n}}{K^I_m+C^I_{sub,n}Q_{tr,n}} \quad (8)$$

Determination of the Mixing Ratio in the Soilless Culture System

In the simulation, the degree of nutrient recycling was manipulated by applying nutrient solution mixing processes of the OL, SCL, and CL soilless cultures. The nutrient solution preparation process for the OL, SCL, and CL soilless cultures of the above section of the greenhouse experiment of the OL, SCL, and CL soilless cultures was converted to this study's soilless culture system model.

Q_{stk}, Q_{drg}, and Q_{wtr} of the SCL soilless culture system are determined by Eqs. 9–11, respectively.

$$Q_{stk} = \begin{cases} Q_{ir}-Q_{wtr}-Q_{drj},\ if\ V_{drg} > 0 \\ \frac{Q_{ir}(C_{tar}-\sum C^I_{wtr})}{\sum C^I_{stk}-\sum C^I_{wtr}},\ if\ V_{drg} \le 0 \end{cases} \quad (9)$$

$$Q_{drg} = \begin{cases} \frac{Q_{ir}\left(C_{dil}\sum C^I_{stk}-\sum C^I_{stk}\sum C^I_{wtr}-C_{dil}C_{tar}+C_{tar}\sum C^I_{wtr}\right)}{\sum C^I_{stk}\sum C^I_{drg}-\sum C^I_{stk}\sum C^I_{wtr}-C_{dil}\sum C^I_{drg}+C_{dil}\sum C^I_{wtr}}, \\ \qquad if\ V_{drg} > 0 \\ 0,\ if\ V_{drg} \le 0 \end{cases} \quad (10)$$

$$Q_{wtr} = \begin{cases} \frac{Q_{drg}\sum C^I_{drg}-Q_{drg}C_{dil}}{C_{dil}-\sum C^I_{wtr}},\ if\ V_{drg} > 0 \\ Q_{ir}-Q_{stk},\ if\ V_{drg} \le 0 \end{cases} \quad (11)$$

where C_{dil}(meq cm^{-3}) is the equivalent target concentration for the dilution of the total nutrients in the drainage and C_{tar} is the target equivalent concentration for the total nutrients in the nutrient solution to be supplied to the plant. The OL system corresponds to the case when $V_{drg} \le 0$ in Eqs. 9–11.

Q_{stk}, Q_{wtr}, and Q_{drg} for the CL system are determined by Eqs. 12–14, respectively.

$$Q_{stk} = \frac{Q_{ir}(C_{tar}-\sum C^I_{wtr})-Q_{drg}(\sum C^I_{drg}-\sum C^I_{wtr})}{\sum C^I_{stk}-\sum C^I_{wtr}} \quad (12)$$

$$Q_{wtr} = Q_{ir}-Q_{drg}-Q_{stk} \quad (13)$$

$$Q_{drg} = Q_{dr,n} \quad (14)$$

Parameter Estimation and Theoretical Analysis of the Nutrient Variations

The CL soilless culture system discharges no drainage. Water replenishment into the system follows the transpiration amount in the system. Nutrients removed from the system correspond to the absorbed nutrients by plants. Thus, the Michaelis–Menten parameters of the nutrient uptake model were estimated by performing a numerical integration-based progress curve analysis using the water and nutrient inputs, transpiration, and nutrient concentration data measured in the CL soilless culture experiment. Simulation and progress curve analysis of these models were performed using Berkeley Madonna 8.3.23 (Berkeley Madonna, Inc., University of California, Berkeley).

The estimated nutrient uptake model parameters were applied to the OL, SCL, and CL soilless culture system models. We performed two simulation analyses as follows:

(1) According to the degree of nutrient recycling, namely, OL, SCL, and CL irrigation models, the nutrient ratio changes were analyzed. The nutrient ratio changes were investigated concerning the degree of nutrient supply into the OL, SCL, and CL systems' boundaries.
(2) This study hypothesizes that converting the nutrient concentration to the nutrient ratio between nutrients could neutralize the dynamic changes in nutrient uptake concentrations and reveal the nutrient uptake parameter-based deterministic patterns. On this basis, a simulation scenario was applied for the derivation of the long-term feedback-based nutrient control technique.

Under the simulation scenario, the transpiration rate, one of the main disturbance factors for nutrient uptake concentration, was manipulated by a random walk. The long-term feedback nutrient control was performed by changing the nutrient ratios in the stock solution for nutrient replenishment. The feedback period was set to 12 weeks, which is the total period of the simulation. After each simulation, the difference between the target nutrient ratio and average nutrient ratio (target average) was fed back to adjust the stock solution's nutrient ratio (**Figure 2**). Thus,

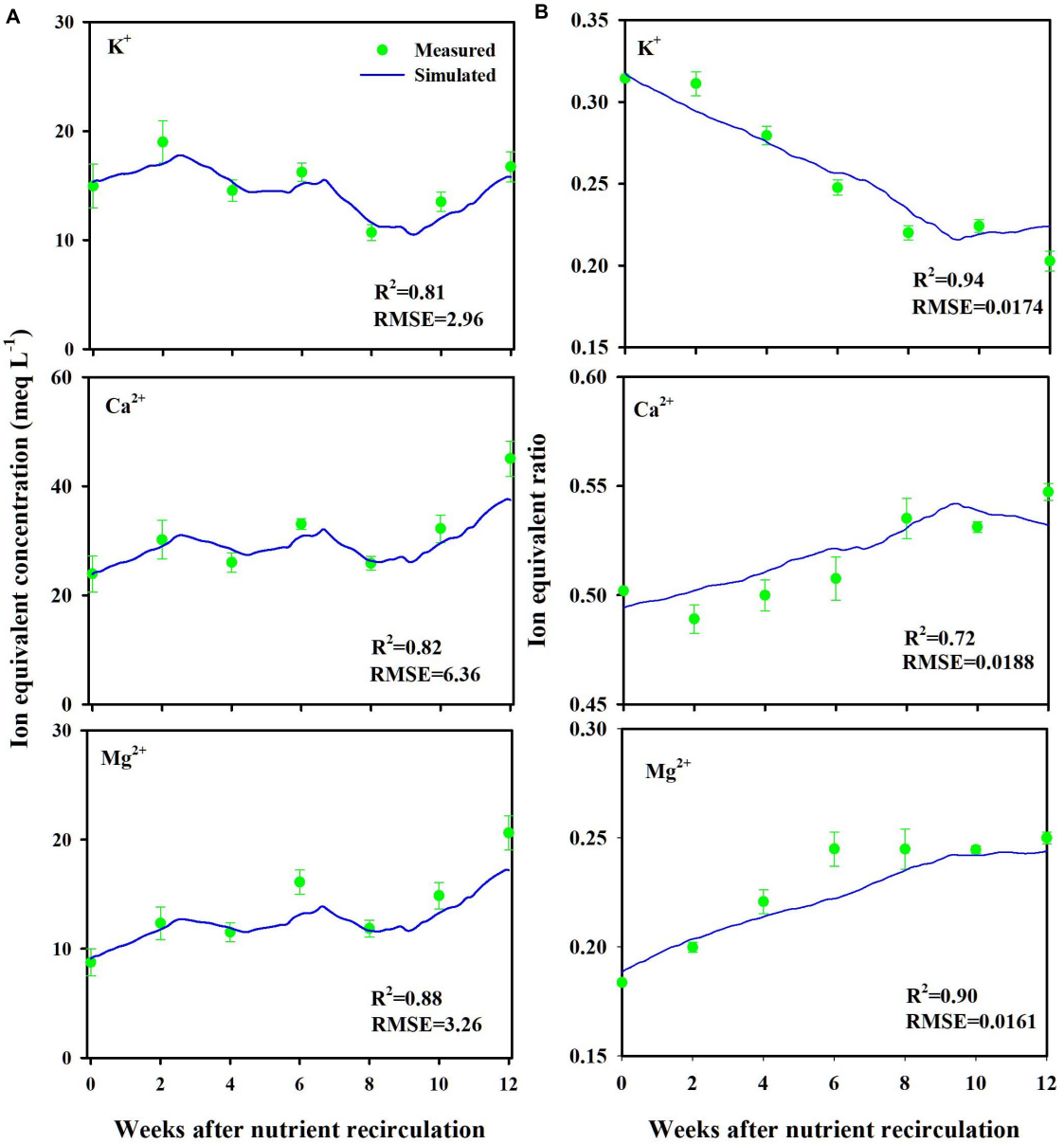

FIGURE 3 | Measured (mean ± SD) and simulated ion equivalent concentrations **(A)** and ion equivalent ratios **(B)** in the substrates of the closed-loop soilless culture system (CL). Ion equivalent concentrations of the substrates represent ion concentration in the whole layers.

as an example, if the average nutrient ratio is higher than the target ratio, negative feedback is returned to the stock solution's nutrient ratio.

RESULTS AND DISCUSSION

Soilless Culture System Model Verification

Measured and simulated nutrient concentrations and ion ratios in the CL substrate showed close agreements with R^2 values of 0.81–0.88 and a root mean square error (RMSE) of 2.96–6.36 meq L^{-1} (**Figure 3A**) and R^2 values of 0.72–0.94 and an RMSE of 0.0161–0.0188 (dimensionless) (**Figure 3B**), respectively. Measured and simulated values in all nutrient concentrations fluctuated with similar patterns. However, the nutrient ratio of the CL showed distinguishable directional trends. The cumulative nutrient supply to the simulated and experimental CL systems showed good agreement with the measured data with an R^2-value of 0.95 (**Figure 4**).

Overall, the soilless culture system model closely simulated the nutrient concentration variations in the CL soilless culture. Furthermore, the nutrient supply into the CL soilless culture system's boundary was simulated with a determination coefficient

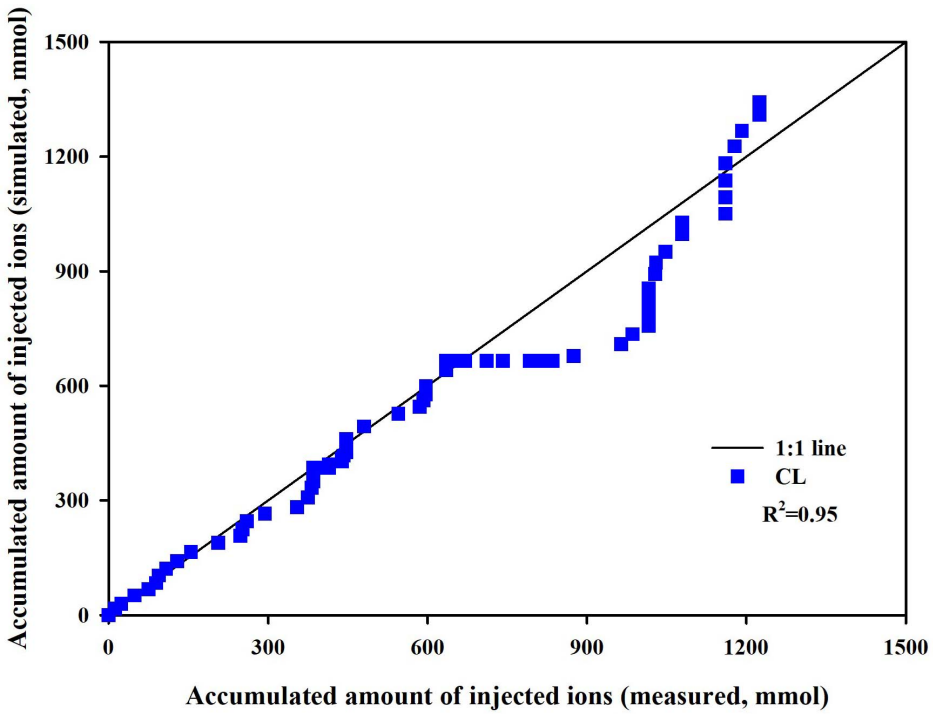

FIGURE 4 | Measured versus simulated amounts of total ions supplied to the closed-loop soilless culture system (CL).

of 0.95. The replenished nutrients in a CL soilless culture system have a relation with the absorbed nutrient and water in the system (Savvas, 2002). Nutrient concentration variations observed in the CL soilless culture system represent the combined effect of nutrient and water absorption, nutrient replenishment, irrigation, and drainage recycling. Thus, the nutrient preparation process and nutrient variation aspects in the soilless culture system were verified here. However, it needs to be noted that the simulation model estimated a relatively high K_m^K. This might be derived from the current model's structural limitations. The modified nutrient uptake model applies transpiration variations to the nutrient uptake parameter variations. Thus, the parameter estimation could be valid in the simulation condition.

Changes in Nutrient Concentration and Ratio in the Experiment

The nutrient concentration in the substrate indicated similar trends throughout the experiment period. The concentration variations were not distinct from the treatment and showed little consistency in the treatments' significant differences (**Figure 5A**). On the other hand, when the nutrients were converted to a ratio between the nutrients, the nutrient variation trends indicated more distinguishable trends in individual nutrients and each treatment (**Figure 5B**). The ratio of K^+ in CL, which has the least nutrient supply, was significantly lower than that of the other treatments and deviated most from the initial value. OL, which has the highest nutrient input, was the closest to the initial ratios. In the CL treatment, Mg^{2+} and Ca^{2+} remained higher than those in other treatments after observing significant differences 4 weeks after treatment. Mg^{2+} and Ca^{2+}were significantly lower in OL and tended to be the closest to the initial ratios (**Figure 5B**). Thus, unlike nutrient concentrations, the nutrient ratio conversion revealed increasing or decreasing individual nutrients under the nutrient concentration's overall fluctuation.

Furthermore, those increasing or decreasing trends in nutrient ratios and K^+ leveled off 6 weeks after treatment. These results suggest that the nutrient ratios have more deterministic aspects than the nutrient concentration. On the other hand, the nutrient concentrations were relatively difficult to distinguish between the nutrients. The concentration trends between the treatments also showed complicated fluctuations. Changes in nutrient concentrations in the root zone in soilless culture systems vary depending on the difference between irrigated nutrient concentration and nutrient uptake concentration (van Noordwijk, 1990). The nutrient uptake concentration represents the ratio of nutrients to water taken up over time (Le Bot et al., 1998). Thus, the change in the uptake concentration results from variations in the transpiration and nutrient uptake rate. The transpiration could affect mass flow near the root system (Marschner and Marschner, 2012). The transpiration could also partially drive the uptake rate of ions like Ca^{2+} (Adams and Ho, 1993; Mengel, 2009). Thus, an ion-specific interaction between transpiration and nutrient uptake could affect the substrate's nutrient concentration variations.

However, in this study, the concentration of the substrate nutrient showed the synchronized fluctuations. As explained above, the nutrient concentration indicated similar trends and showed an overall gradual increase from the 8 weeks onwards.

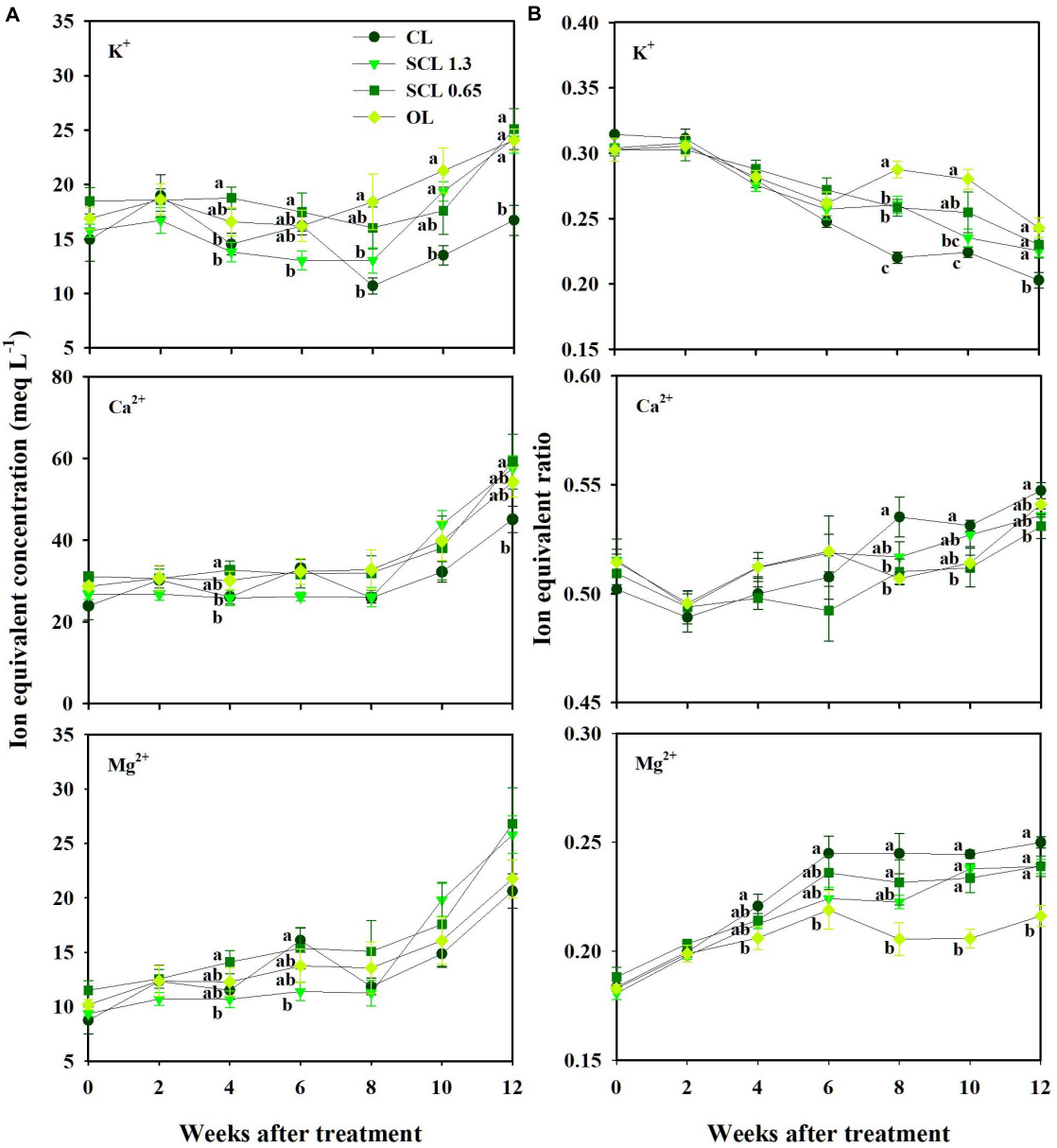

FIGURE 5 | Changes in the ion equivalent concentration **(A)** and ion equivalent ratios **(B)** in the substrate of the closed-loop (CL), semi-closed-loop (SCL 1.3 and SCL 0.65), and open-loop (OL) soilless culture system, respectively. SCL 1.3 means an EC level for a drainage dilution of 1.3 dS m^{-1}. Data represent the mean ± SD from three rockwool slabs of each treatment. Significant differences ($P < 0.05$) between treatments are indicated by different letters.

The drainage EC of CL, SCL, and OL systems followed similar tendencies (**Figure 6**). However, the CL system's drainage EC showed more sensitive variations to the nutrient concentration change. In contrast, in SCL and OL, which have relatively low drainage reuse proportions, less sensitive EC changes were observed. The low drainage reuse leads to the accumulation of residual drainage in the tank, and subsequently, the EC in the drainage tank responds less sensitively to the substrate nutrient concentration changes. As a result, depending on each system's storage conditions, the sensitivity of the EC fluctuation in the drainage tank was different, but the overall trends indicated similarity with variations in the substrate nutrient concentration. The overall increase in the nutrient concentration 8 weeks after treatment could mainly result from the increased transpiration rate as plant growth progresses. In general, a plant's transpiration rate is accelerated as the plant expands its leaf area during growth (Bailey et al., 1993). In this study, the transpiration gradually increased to the end of the greenhouse experiment (**Figure 8A**). This may suggest that the change in transpiration has a more dominant global effect on the nutrient concentration changes in nutrient concentrations.

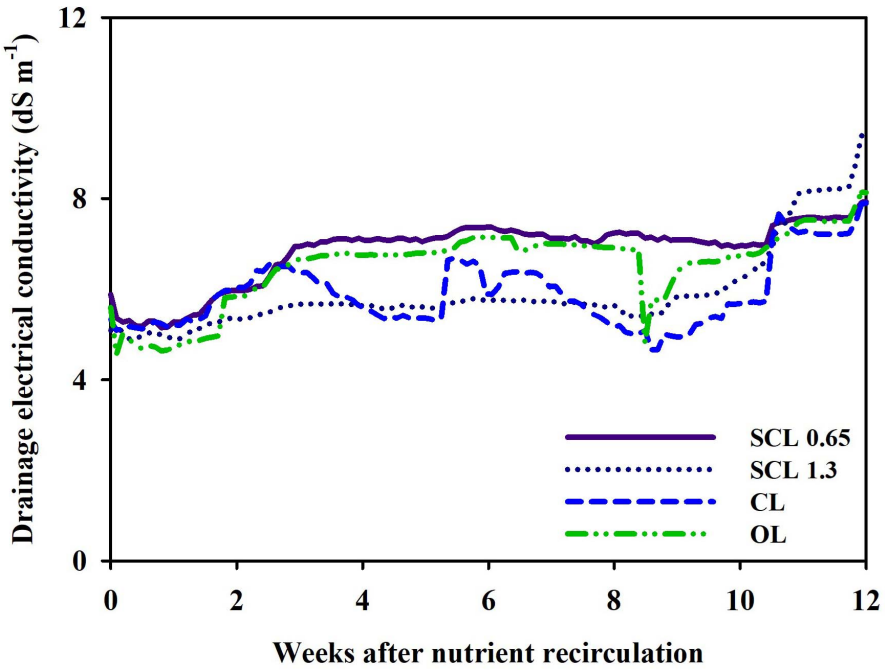

FIGURE 6 | Changes in electrical conductivity (EC) in the drainage tank of the closed-loop (CL), semi-closed-loop (SCL 1.3 and SCL 0.65), and open-loop (OL) soilless culture system, respectively.

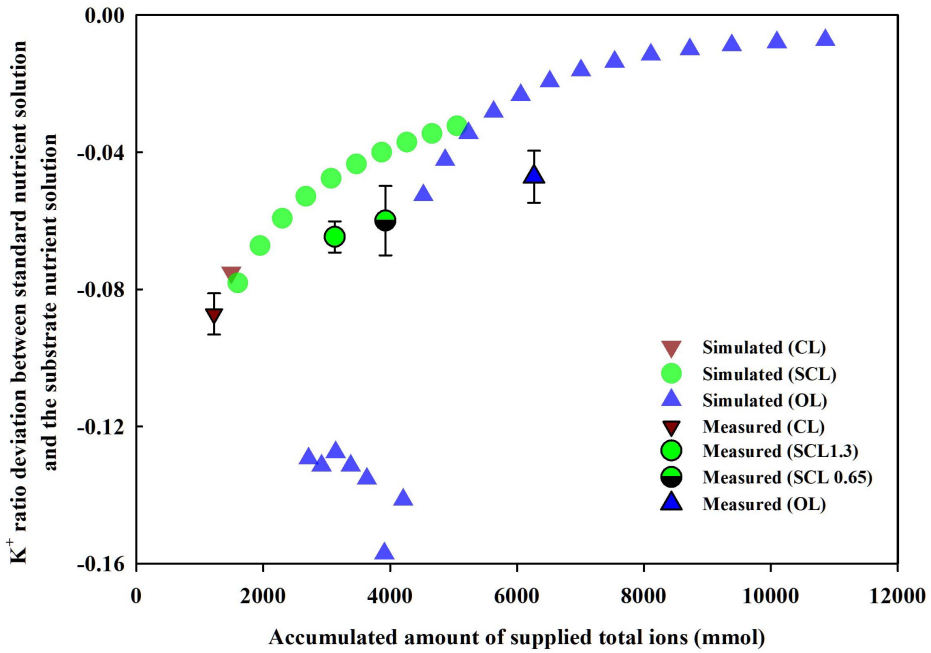

FIGURE 7 | Changes in the measured (mean ± SD) or simulated K^+ ratio deviation between standard nutrient solution and the substrate nutrient solution by supplied amounts of total ions of the soilless culture systems at the end of the experiment and the simulation. CL, SCL, and OL mean the closed-loop, semi-closed-loop, and open-loop soilless culture systems, respectively. SCL 1.3 means an EC level for a drainage dilution of 1.3 dS m^{-1}.

Changes in transpiration and consequent variations in nutrient absorption concentration mainly drove the macroscopic fluctuations in the overall nutrients and EC. On the other hand, nutrient absorption is mainly dominated by transporters' arrangement located on the root surface (Arsova et al., 2020). Thus, converting the nutrient concentrations to nutrient ratios

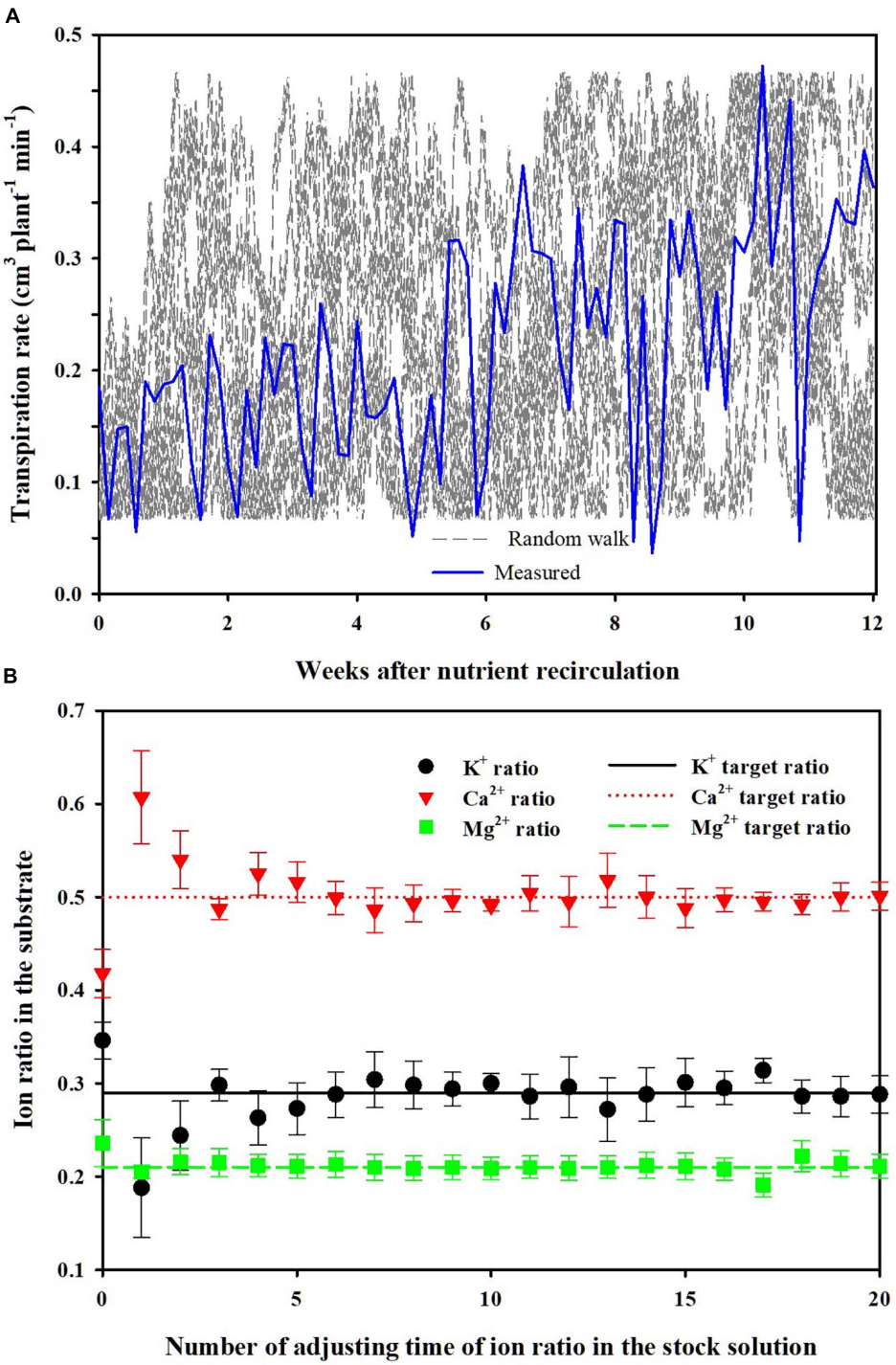

FIGURE 8 | Random walk transpiration rates considering disturbance in the uptake concentrations by simulation and measured transpiration rates in the experiments **(A)** and changes in ion ratios (mean ± SD) in the substrate according to the number of adjusting the time of ion ratios in the stock solution under the random walk disturbance **(B)**.

might have reduced the global effect of transpiration on the nutrient concentrations and revealed the distinguishable directional trends. Subsequently, it can be conjectured that the nutrient ratio changes in a soilless culture system mainly reflect the nutrient uptake phenomenon driven by the nutrient transporter. That is, the nutrient ratio changes could implicate the Michalis–Menten-driven nutrient uptake. Consequently, the nutrient ratio filtered the variations in nutrient uptake

concentrations variations and showed the combined effect of nutrient recycling degree and nutrient uptake parameters.

Theoretical Analysis of Nutrient Ratio in the OL, SCL, and CL Soilless Cultures

Simulation (1), described in section "Parameter Estimation and Theoretical Analysis of the Nutrient Variations," showed the nutrient ratio changes according to the degree of nutrient recycling, namely, OL, SCL, and CL irrigation models. The degree of nutrient recycling is also strongly related to the degree of nutrient supply into the system boundaries of OL, SCL, and CL systems. Thus, the nutrient ratio changes of the soilless culture systems were summarized on the basis of a nutrient supply (**Figure 7**). As a representative, when the K^+ ratios in the soilless culture systems were summarized for nutrient inputs, specific trends were observed. As nutrient supply increases, i.e., as the degree of nutrient recycling decreases, the nutrient ratio's deviation from the standard ratio approached zero. Furthermore, the K^+ ratios in the experiment showed similar trends as the simulation. The deviation between the nutrient ratio measured in the experiment and the standard ratio decreased in the order of CL, SCL 1.3, SCL 0.65, and OL.

The nutrient variations in the CL soilless culture system are often considered a black box in some way for its dynamic behaviors in uptake concentration (van Noordwijk, 1990). Thus, in CL soilless culture, managing the nutrient uptake concentration effect is often approached as empirical methods. The literature on the EC-based CL soilless culture system investigated the appropriate interval or level of nutrient solution renewal (Ehret et al., 2005; Ko et al., 2014), drainage discharge (Massa et al., 2011), and EC control (Rouphael et al., 2016; Signore et al., 2016). The CL soilless culture system was featured due to uncertainties in nutrient management. However, Simulation (1) shows that although the OL, SCL, and CL soilless culture systems have been separated from each other, the systems' nutrient ratios can be summarized continuously based on the systems' nutrient inputs.

In Simulation (1) of section "Parameter Estimation and Theoretical Analysis of the Nutrient Variations," a modified concept on the nutrient variations of OL, SCL, and CL soilless culture systems was presented. The nutrient ratio changes of each system were macroscopically integrated into nutrient inputs to the systems. In this respect, the soilless culture models could be simplified by the inflow and the outflow into the system boundary and the system's uptake rate (Eq. 15). Moreover, with these critical parameters, the theoretical framework for the nutrient variations of OL, SCL, and CL soilless culture systems could be reconstructed.

$$V_{sys}\frac{dC^I_{sys}}{dt} = Q_{stk}C^I_{stk}+Q_{wtr}C^I_{wtr}-\frac{J^I_{max}C^I_{sys}}{K^I_m+C^I_{sys}}-Q_{out}C^I_{sys} \quad (15)$$

where Q_{stk} and Q_{wtr} are the flow rates of the stock nutrient solution and raw water flowing into the boundary of the soilless culture system, respectively, and Q_{out} is the flow rate of the nutrient solution discharged outside the boundary. C^I_{sys} corresponds to the concentration of a nutrient in the system. C^I_{stk} and C^I_{wtr} are concentrations of a nutrient in the stock solution and raw water, respectively. V_{sys} is the volume of nutrient solution in the system. V_{sys} in Eq. 15 cannot exceed the system's capacity, and the consumed water is replenished repeatedly and thus is assumed to be constant.

In the SCL system, the boundary of the system includes the mixing tank and the drainage tank because the drainage is partially circulated or discharged outside the system. Therefore, Q_{stk} and Q_{wtr} correspond to the supply rate of nutrients and water to the soilless culture system, respectively; and Q_{out} in Eq. 15 corresponds to the flow rate of the nutrient solution discharged from the drainage tank (Q_{disch} in Eq. 5). In the case of the CL system, the boundary is the same as that of the SCL system, but Q_{out} does not occur, and the steady-state solution, in this case, can be summarized as in Eq. 16.

$$C^I_{sys} = \frac{K_m C^I_{stk}Q_{stk}+K^I_m C^I_{wtr}Q_{wtr}}{J^I_{max}-C^I_{stk}Q_{stk}-C^I_{wtr}Q_{wtr}} \quad (16) \qquad (16)$$

This indicates that the point of convergence for C^I_{sys} is determined according to the concentrations of the supplied stock solution (C^I_{stk}) and raw water (C^I_{wtr}) and their inflow rates (Q_{stk} and Q_{wtr}, respectively) and means that when the inflow rates exceed the maximum nutrient uptake rate of the plant (J^I_{max}), the system becomes unstable. However, in the EC-based CL soilless culture system, Q_{stk} is determined based on the total equivalent concentration of nutrients in the system; as a result, the feeding rate of the total nutrients follows the uptake rate of total nutrients at a certain level. Thus, too excessive increases or decreases can be controlled by general EC control practice. Under this condition, the nutrient ratio conversion could reveal the parameter-based nutrient variations. Unlike dynamic variations of nutrient concentration, the parameter-based nutrient variations could indicate deterministic changes. Thus, these deterministic changes could be controllable by long-term feedback.

Assuming that the C^I_{sys} is the total equivalent concentration of all nutrients in the system measured by EC and C^I_{sys} is controlled in steady-state through EC feedback, Eq. 16 theoretically predicts that C^I_{sys} converges to a constant value. This means that the individual nutrient variations could also level off to steady-state. Additionally, the terms including Q_{wtr} in Eq. 16 may contribute to the system fluctuation by replenishing the water consumed through transpiration. Thus, its influence is determined by the mineral concentration in the raw water. However, in most CL or SCL soilless culture studies, including the cultivation experiment of this study, and OL soilless culture, irregular fluctuations in the total nutrient concentrations relative to its initial values have been reported often (Hao and Papadopoulos, 2002; Massa et al., 2011; Shin and Son, 2016; Signore et al., 2016; Lee et al., 2017; Moon et al., 2018, 2019).

However, the recent literature theoretically deduced a modified nutrient replenishment method for steady-state management of EC and experimentally demonstrated its effect (Ahn and Son, 2019). Here, this study's theoretical prediction on the effect of steady-state EC control was observed in the greenhouse experiment. When the total ion concentration was

controlled to the initial target value, the individual nutrients of the EC-based CL soilless culture system rapidly converged into the average steady state.

Overall, Simulation (1) defines that the SCL and OL soilless culture systems are mainly driven by the nutrient input and discharge. On the other hand, the nutrient variation in a CL soilless culture system is mainly determined by the relationship between nutrient inputs and nutrient uptake parameters.

Nutrient Ratio Control in the CL Soilless Culture

Simulation (2) shows the average changes of the nutrient ratios and their standard deviations over the simulation period when performing the nutrient balance control described in section "Parameter Estimation and Theoretical Analysis of the Nutrient Variations." For each simulation, randomized transpiration rates were generated by the random walk method (**Figure 8A**). The mean values of K^+, Ca^{2+}, and Mg^{2+} ratios gradually converged to the control target values as the times of stock solution adjustment increased (**Figure 8B**). Also, the standard deviations gradually decreased. Even though the random walk generated arbitrary uptake concentration in every simulation, the nutrient ratios in the CL soilless culture system gradually approached the target ratios. Thus, every soilless culture system simulated in this scenario has experienced different nutrient uptake concentrations.

In this simulation, a different path of the transpiration by random walk was applied every time, within the range of maximum fluctuation during the cultivation period. Furthermore, the theoretical and experimental analyses were conducted under the substrate condition, which has a non-homogeneous root-zone condition. These can be regarded as extreme conditions. However, the average nutrient ratio approached the target value even in a long-term feedback period of 12 weeks under various nutrient uptake concentrations. This means that the discussion about Eq. 16 is also theoretically valid. Moreover, other soilless culture systems using no substrate such as nutrient film and deep-flow technique could have less spatial variations in nutrient and water distribution than a substrate-based system. Thus, this theoretical framework can be expected to be compatible with other soilless culture systems. Consequently, the results of the theoretical and experimental analyses suggest that the nutrient variations in EC-based soilless cultures could be managed even under the weekly based long-term nutrient analysis conditions.

In a previous study, nutrient control with a 2 weeks feedback period was conducted in an EC-based CL soilless culture system (Savvas, 2002). Here, the difference between the target concentration and the current concentration at every nutrient analysis event was applied as the feedback value. This corresponds to the proportional control from the viewpoint of control engineering. The proportional control can generate a steady-state error, and the literature reported similar trends in the nutrient concentrations. However, no discussion in this respect was conducted so far. Although the nutrient uptake concentration fluctuated by transpiration could be a short-term phenomenon, the cumulative difference between the supplied nutrient concentration and nutrient uptake concentration could result in a particular trend of nutrient concentration or nutrient ratio changes on a time series. Neutralizing the transpiration effects from the nutrient management in the recirculating system could reveal the kinetic domain of nutrient uptake. Thus, in this domain, nutrient variation complexity could be narrowed down to nutrient uptake parameter aspects.

CONCLUSION

This study hypothesized that converting the nutrient concentration to the nutrient ratio could neutralize the dynamic changes in nutrient uptake concentration and reveal the nutrient uptake parameter-based deterministic patterns. The greenhouse experiment of the degree of nutrient recycling, namely, OL, SCL, and CL soilless cultures, showed that the nutrient ratio conversion could provide more distinguishable nutrient behaviors. Thus, the complexity of nutrient uptake concentration in the CL soilless culture was narrowed down to the parameter-based level. The simulation analysis confirmed that the nutrient ratio, which represents the parameter-based deterministic changes, could be controlled by long-term feedback control. Although this study analyzed limited nutrients, the three macro-elements K^+, Ca^{2+}, and Mg^{2+} are ions that account for almost half of EC and total equivalent concentrations in a soilless culture system. We expect that subsequent studies based on the theoretical framework established in this study could be advanced to the extended techniques for the rest of the ions, including the micro-elements. In conclusion, our findings provided theoretical frameworks for systemizing nutrient management techniques and suggested a new nutrient control technique in the EC-based CL soilless culture.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

TIA and JES conceived the research and prepared the manuscript. TIA and JHS performed the experiments and analyzed the results. All authors read and approved the final manuscript.

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Can Reclaimed Water Be Used for Sustainable Food Production in Aquaponics?

*Liliana Cifuentes-Torres, Gabriel Correa-Reyes and Leopoldo G. Mendoza-Espinosa**

Oceanographic Research Institute, Autonomous University of Baja California, Ensenada, Mexico

Aquaculture is a technology used for the production of animal protein but produces a great amount of waste that decreases productivity and adversely affects the environment. Sedimentation and filtration have been used for the treatment of the suspended fraction of these wastes although dissolved substances like nutrients can be an asset. Therefore, the management of aquaculture waste remains a challenge. Aquaponics is a technology that can eliminate dissolved N and P from aquaculture systems as they serve as nutrients for plants, which are absorbed through the roots and are incorporated into their tissues. Several reports and studies exist on the benefits of aquaponic systems for the combined production of plants and aquatic organisms and its advantages in terms of economics and environmental protection. The great majority of the studies use the wastewater from the aquatic production tanks as a source of nutrients for plants production. However, domestic or municipal wastewater is a resource that has been used extensively in other production systems such as conventional agriculture and aquaculture, yet its potential as a source of water for aquaponics has not been established. The current analysis hypothesizes that reclaimed water can be used for aquaponics. Despite the extensive use of reclaimed water in agriculture and aquaculture and the low risk to human health when properly managed, there are no academic studies that have tackled this issue. In order to overcome the generalized mistrust of the public in consuming crops irrigated with reclaimed water or fish growing in reclaimed water, it is recommended that only ornamental fish and plants would be cultivated by this method. There is an urgent need for studies to verify the safety and advantages of such cultivation technique. Finally, it is necessary to establish guidelines for the responsible use of reclaimed water in aquaponics.

Keywords: integrated farms, wastewater reuse, sustainability, reclaimed water, aquaculture, ornamental fish

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Mushtaaque Ahmed, Sultan Qaboos University, Oman
Yuli Andriani, Padjadjaran University, Indonesia

***Correspondence:**
Leopoldo G. Mendoza-Espinosa
lmendoza@uabc.edu.mx

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INTRODUCTION

One of the challenges that science currently faces is the optimization of traditional production systems in ecological, economic, and social terms. The production of food in a sustainable and safe manner requires the recycling and improvement of ancient techniques like hydroponics which, since Babylonia times, already produced quality crops and provided urban landscaping (González-Carmona and Torres-Valladares, 2014). Currently, hydroponics is used as a technique that facilitates the growth of crops in cities, thanks to the implementation of urban farms and vertical gardens (Al-Chalabi, 2015). In these places another technique for food production has

been implemented, in which in a synergetic way, hydroponics is integrated with waste generated by aquatic organisms in an aquatic recirculation system (Goddek et al., 2015). This type of production system is known as aquaponics and takes advantage of the symbiotic relationship between aquatic organisms, plants and bacteria (Rakocy, 2012). In aquaponics, two products are obtained that can be either edible or of economic value and that can be commercialized. Therefore, the combination of cultivation systems for fishes and aquatic plants such as duckweed and water hyacinths is not considered aquaponics for the present analysis.

Several studies have highlighted the potential of aquaponic systems (Diver, 2006; Endut et al., 2011; Rakocy, 2012). In all of them, aquaponics has been used as a treatment system for wastewater from aquatic systems. However, there are no studies in which reclaimed water has been used in such systems. The nutritional potential of reclaimed water has been identified in many studies in developed countries (Bixio et al., 2008), and public policy regarding the use of this type of water generally requires advanced treatment and disinfection (United States Environmental Protection Agency, 2012).

In developing countries, the treatment of wastewater is not common. It has been estimated that approximately 80% of the wastewater generated worldwide is discharged without proper treatment (Winpenny et al., 2013). Regardless of this, the FAO has promoted the concept of integrated farming in which all available resources, both agricultural and livestock, are used in a sustainable way, contributing to increase the quality of life of farmers and improving the natural environment. Some of such practices established by the FAO is the recycling of nutrients from livestock in fish cultivation such as tilapia. Therefore, it can be asserted that potentially, reclaimed water can be used after an adequate treatment for the cultivation of plants and fish, therefore, aquaponics.

In the present analysis, wastewater is referred to as crude wastewater that has not received any type of treatment, while reclaimed water is wastewater that has received some sort of treatment, usually secondary treatment. Taking this into account, our hypothesis establishes that it is possible to integrate in a planned and responsible way the use of reclaimed water as a source of nutrients in aquaponics systems, reducing the need to add synthetic nutrients to the system, thus, making the production more environmentally friendly. This last issue is important from a commercial point of view due to the fact that organic products usually have a higher value than non-organic ones, that results in higher income for the producer, and a shorter return in investment. The present article will analyze traditional aquaponics and highlight the potential for the use of reclaimed water in aquaponics and the challenges that lay ahead.

TRADITIONAL AQUAPONICS

Aquaponic systems consist of a unit that cultivates plants without soil and another with a tank cultivating fish. The excreta from the fish are used as nutrients by the plants (Maucieri et al., 2018; Yep and Zheng, 2019). This type of production system was called integrated system (Rakocy, 2012), and has its origin is sustainable agriculture, that has as its goal to achieve the production of plants and livestock by using efficiently the resources, without damaging the environment by integrating the natural cycles in production systems to increase the quality of life of farmers and the society as a whole (The Food Agriculture Conservation and Trade Act of 1990, 1990). Therefore, aquaponics can be considered a sustainable agricultural system.

Some of the advantages of aquaponics systems are the efficient use of water (95–99%), the lower need for the addition of synthetic fertilizers (<50%), the elimination of the need of agrochemicals for pests and diseases control, the non-dependence to soil, the simultaneous production of plants and aquatic organisms and the low discharge of waste to the environment (Al-Fedh et al., 2008; Lampreia, 2016; König et al., 2018; Maucieri et al., 2018).

Historically, the species of fishes that have been commonly used in aquaponics are, in order of its frequency of use: tilapia (*Oreochromis niloticus*), catfish (*Clarias gariepinus*), carp (*Cyprinus carpio*), trout (*Oncorhynchus* mykiss), and pacu (*Piaractus mesopotamicus*) (Rakocy, 2012; Love et al., 2014a). Ru et al. (2017) highlights that due to its high tolerance to suspended solids, levels of nitrite above 44.67 mg L^{-1} and low concentrations of oxygen, *O. niloticus* is the most common species cultivated in commercial systems. With respect to the hydroponic component, the most common type of crops cultivated are leafy vegetables due to their ability to grow at high N concentrations, their shorter growth period, their relatively low nutrients requirements and their high demand (Bailey and Ferrarezi, 2017). Additionally, Love et al. (2015) found that the most common species of crops in aquaponic systems are basil (*Ocimum basilicum*), tomato (*Solanum lycopersicum*), lettuce (*Lactuca sativa*), cabbage (*Brassica oleracea*), beetroot (*Beta vulgaris*), pak choi (*Brassica campestris*), peppers (*Capsicum annuum*), and cucumber (*Cucumis sativus*). Exotic species of plants such as *Salicornia persica* have also been cultivated in aquaponic systems using brackish water. This has opened new windows into the possibility of using aquaponics as an option for the cultivation of marine organisms and plants tolerant to salt. Moreover, *S. persica* has a high concentration of lipids, omega 3 and minerals, making it attractive for cosmopolitan markets such as the European (Turcios and Papenbrock, 2014).

In a study by Hu et al. (2015) the dynamics of the nitrogen compounds in aquaponics systems with tomato (*Lycopersicon esculentum*) – pak choi (*Brassica campestris* L. *subsp. Chinensis*) – tilapia (*Oreochromis niloticus* was tested. Such study demonstrated that the assimilation of N varies with the type of crop, reaching 41.3% for tomato and 34.4% for pak choi. Such a difference is explained by the larger surface area of the roots of the tomato plant, that increases the amount of biofilm of nitrification bacteria, responsible for the oxidation of ammonia (NH_4^+) to nitrates (NO_3^-) (Endut et al., 2016). A lower concentration of ammonia was reached in water from tomato than from pak choi. The importance of the surface area of the roots for the removal of nutrients was mentioned by Endut et al. (2016). They used an aquaponics system with water spinach (*Ipomoea aquatica*) – green mustard (*Brassica juncea*) – catfish (*C. gariepinus*) and found that the removal of N compounds and orthophosphates

were very similar but was more efficient for water spinach than for green mustard. Spinach had roots with larger surface area than green mustard and nutrients removal was 88.76 vs. 78.21% for ammonia, 92.51 vs. 86.67% for nitrite, 90.04 vs. 86.87% for nitrate, and 88.99 vs. 78.72% for orthophosphates. Henfi et al. (2015) evaluated the decrease of nutrients in aquaponic systems of *Cherax quadricarinatus* – *I. aquatica*. The rate of survival of *C. quadricarinatus* was 90% while the removal of nutrients was 84.6, 34.8, and 44.4% for NH_3, NO_3^- and orthophosphates.

Some factors that affect the dynamics of N in aquaponic systems are pH, dissolved oxygen, the hydraulic loading rate and the C:N ratio. The pH affects all organisms that interact in the aquaponic system. In the case of nitrification bacteria, it has been demonstrated that their activity decreases when pH is below 6.4 and greater than 9.0, and NUE (Nitrogen Use Efficiency) is 50.9% at pH 6.4 (Ruiz et al., 2003; Zou et al., 2016). This value is relatively high as it has been demonstrated that one of the problems with aquaponics is its low NUE (40%) (Hu et al., 2012; Wongkiew et al., 2017a) Dissolved oxygen (DO) also has a direct effect on nitrification bacteria. In the case of ammonia oxidizing bacteria (AOB), their transformation efficiency decreases at DO levels below 4.0 mg l^{-1} while at less than 2 mg L^{-1} the activity of nitrite oxidizing bacteria (NOB) is greatly reduced. Therefore, levels between 5 and 6 mg L^{-1} are recommended, which is ideal for the majority of aquatic organisms (Kim et al., 2005; Rakocy, 2012). The hydraulic loading rate (HRL, m^3 day^{-1}), which is the liquid flowrate per unit of cultivation area, is a variable that affects the retention time of nutrients, sediments and microorganisms (Li et al., 2009). A low HRL can cause a decrease of OD, while a high HRL can reduce the retention time of water, that can cause a decrease in the assimilation of nutrients by the crops' roots and the washing of the bacterial biofilm thus, the deterioration of water quality (Endut et al., 2010). Finally, the C:N ratio is related with the population of nitrification bacteria and heterotrophic bacteria that coexist within the system. A high C:N ratio increases the growth rates of heterotrophic bacteria and decreases the level of nitrification bacteria (Ebeling et al., 2006). Also, a high population of heterotrophic bacteria decreases the concentration of DO and causes an inefficient transformation of ammonia to nitrate which can cause problems to the survival of fishes that can be exposed to toxic levels of NAT and nitrites (Wongkiew et al., 2017b).

The majority of the studies have demonstrated successful results in terms of nutrients recycling and assimilation. Buzby and Lin (2014) undertook a study in which the hydroponic element was isolated from the aquatic cultivation pond in order to evaluate nutrients uptake independently. The cultivation tanks with *L. sativa* and *Tropaeolum majus* were spiked with ammonia and water samples were taken every hour for 4 h. It was found that both types of plants were effective in reducing ammonia (81% with *L. sativa* and 89% with *T. majus*).

A study by Buzby and Lin (2014) demonstrated that it is unclear the relation between the amount of nutrients added to the aquatic culture and the removal efficiency by the hydroponic system; aspects like the species used for cultivation, the type of system, cultivation density and others have to be taken into consideration. Rakocy (2012), Al-Fedh et al. (2008), and Endut et al. (2010) studied this relation and obtained values between 14 and 42 g food m^{-2} to 60–100 g food m^{-2}. Additionally, fish cultivation commonly requires the addition of synthetic fertilizers because normal fish food generally lacks nutrients such as K, Ca, and Fe (Graber and Junge, 2009).

Delaide et al. (2016) studied the effect of supplementing water with high-purity mineral salts for fishes in an aquaponics system to simulate a commercial hydroponic solution. They used three types of water: commercial hydroponic solution (HP), water for aquaponics non-supplemented (AP) and supplemented water for aquaponics (CAP). The study consisted in evaluating the nutrient concentration of leaves and the weight of sprouts and roots. Results showed that sprouts had a significantly higher weight with CAP compared to HP and AP. However, the weights obtained in HP and AP suggest that AP can be an alternative for conventional hydroponic systems. Results for roots showed that AP and CAP were larger than HP. The authors attributed this to the fact that water for the fish cultivation contained components like organic matter, rhizobacteria and fungi that stimulated the growth of roots and, in return, increased nutrients intake. Other substances in SRA that promote the growth of sprouts and roots are humic acids and phenols (Spaccini et al., 2009; Hambly et al., 2015).

In this respect, the addition of probiotics to the food for fishes in cultivation has been evaluated and it was found that the addition of microorganisms like *Saccharomyces cerevisiae*, *Lactobacillus acidophilus*, *Bacillus subtilis*, *Aspergillus oryzae*, *Rhodopseudomonas*, *Actinomyces*, and *Nitrobacter* have a significant effect in the feed conversion ratio (FCR), an increase in the production of catfish and the efficiency of organic matter removal in aquaponic systems (Zhou and Wang, 2014; Santoso and Sunadji, 2020). This is caused by the ability of probiotics to facilitate the absorption of nutrients in the digestive system of fishes by increasing the production of digestive enzymes like proteasas. An effect on the immune system of fishes has also been observed, due to the fact that the presence of beneficial bacteria suppresses the population of pathogenic microorganisms. All combine to a better growth of aquatic species in cultivation (Zhou and Wang, 2014).

Zahidah et al. (2018) studied the use of the Red Water System (RWS), a system that uses probiotics from the fermentation of *Lactobacillus casei* and *Saccharomyces cerevisiae,* for the cultivation of catfish. This technique uses probiotics for the decomposition of organic matter and the fact that probiotic microorganisms can reduce ammonia by oxidation in cultivation ponds. In aquaponic systems, the benefit is evident as the decrease of ammonia that cannot be undertaken by plants can be undertaken by these microorganisms. In an experiment by Zahidah et al. (2018) the concentration of ammonia decreased when the microbial activity in the RWS started, which allowed for the transformation of ammonia into nitrates. It was demonstrated that probiotic microorganisms are capable of degrading residual organic matter from food and feces, thus preventing its accumulation in aquaponic systems and improving the water quality of the culture.

Love et al. (2014b) evaluated the worldwide use of ornamental fishes and plants in aquaponic systems. They found that 48% of the cultivation systems had, as a primary organism, an

ornamental fish like koi, goldfish or tropical fish. In contrast, only 20% of plants used in aquaponics were ornamental plants. A study by Mchunu et al. (2018) found that in South Africa, in 45 aquaponic systems located in main cities, 25% cultivated ornamental plants and 16% ornamental fish.

Aquaponic systems could be implemented in urban areas, effectively taking part in what is known as urban agriculture. The definition of urban agriculture includes the production, process and merchandising of foods within urban or peri-urban areas, through techniques like horticulture and aquaculture, optimizing the use of resources and improving the nutritional value of the products and creating jobs (Hernández, 2006; Ribeiro et al., 2015). In most cases, urban agriculture is undertaken in small scale systems and dispersed throughout the urban area. This type of agriculture has grown in popularity to supply food to a growing population established in urban settlements (Lampreia, 2016). This is key to provide a stable access to affordable food and avoid the so called nutritional desserts, that lead to nutritional and public health problems (Tomlinson, 2017). Thus, studies on urban aquaponics have grown in the last decade, and particularly after 2019, according to Wirza and Nazir (2020). Such studies have dealt with the social acceptance of urban aquaponics and its role in urban planification. Pollard et al. (2017) studied focal groups with experience in the production of urban foods, food distribution and business administration, by means of interviews and their opinion on urban aquaponics in the city of Adelaide (Australia). The majority of those interviewed were not familiar with aquaponics and there was a persistent negative opinion about the technology. This was attributed to the lack of knowledge and due overall fear to a more competitive new production technology. To solve this, the authors recommend to plan and create public policies to promote and facilitate the concept of aquaponics to the market, guaranteeing long-term profitability.

Having said that, aquaponics does present challenges. Rakocy (2012), Goddek et al. (2015), and Greenfeld et al. (2019) evaluated the profitability of aquaponic systems and concluded that its feasibility is complex due to several factors like the type of system used, the type of organisms in cultivation, the location and the size of the production. Likewise, Vermeulen and Kamstra (2013) found that aquaponics appears to be a production system less efficient than sustainable practices in organic agriculture, because factors such the recirculation of nutrients, energy efficiency and the optimization of land tend to be more expensive than traditional methods. However, the optimum ratio between the cultivated organisms and the development of less expensive technological systems for water treatment makes the technology more profitable (Goddek et al., 2015; Greenfeld et al., 2019).

In relation to public policies on urban aquaponics, the European Union has begun to plan the integration of policies and strategies of areas such as agriculture, fisheries, food production and environment and recognize the importance of aquaponics in each of these fields. The goal of these policies are to promote innovation, increase competitiveness, increase sustainability, increase the quality of the resources, optimize the use of land, contribute toward the wellness of organisms being cultivated and achieve economies with lower carbon footprint. The EU program is designed to provide financial backing for research projects and commercial assistance to people and businesses interested in establishing a business in aquaponics (Hoevenaars et al., 2018).

It is clear, thus, that aquaponic systems are an alternative for the production of food and the improvement of water quality. The symbiotic relations found in such systems, promotes the growth of bacterial communities and fungi that favors the growth of roots and increases nutrients assimilation. However, to date the optimum relationship between aquatic organisms and plants is not clear, and factors such as species, age and feeding habits influence the system so a "standard" value for its success is not yet possible. More investigations on these factors and the interaction of these factors is needed.

USE OF WASTEWATER IN AGRICULTURE AND HYDROPONICS

Taking into consideration that approximately 57% of the energy used in agriculture is used for the production of nitrogen fertilizers (Yep and Zheng, 2019), and that it has been calculated that the reserves of phosphate will decrease down to half in the following 60 years, meaning that the cost of extraction will increase significantly (Goddek et al., 2015), it is of great importance to have new sources of nutrients. Reclaimed water could be such a source.

The increase in human population has generated an increase in the demand of food and greater pressure on natural resources such as water. This has led to countries such as Australia, United States of America, China, Israel, and Spain to include in their water management policies the reuse of reclaimed water for the irrigation of crops (Pescod, 1992). The success in its implementation with the production of fruits and vegetables has modified the water-food nexus in countries with arid and semiarid climate, and has established the reuse of reclaimed water as a viable water management option (Elgallal et al., 2016). Many studies have been published on the use of wastewater for agriculture (Gupta et al., 2010; Licciardello et al., 2018; Salgot and Folch, 2018) while its use in hydroponics has been demonstrated in lab-scale and pilot-scale experiments but examples of full scale commercial systems are very limited (Cifuentes-Torres et al., 2020).

USE OF WASTEWATER IN AQUACULTURE

Animal protein and other products of aquatic origin can be supplied through aquaculture; this sector has reported an annual growth of 5.3% from 2001 to 2018 (Food and Agriculture Organization of the United, 2018) yet it has been estimated that approximately 75% of the nutrients are not used by the cultivated organisms, which results in environmental pollution and other aquaculture systems downstream (Liu et al., 2021). The use of reclaimed water can be a sustainable and dependable alternative for aquaculture. For example, reclaimed water can reduce the geographical dependency of aquaculture to freshwater, so that it

can be located anywhere near a city with wastewater treatment plants. This would result in a decrease in production costs and the procurement of fresh produce of high protein content to the benefit of local communities (Zaibel et al., 2020).

The first experiences with the cultivation of fish using wastewater were in Germany at the end of the XIX century, in which trout, carp and salmon were cultivated in tanks fed with sewage-field drains (Prein, 1990). In China and India wastewater with high levels of nutrients, are traditionally considered an input for aquaculture because they promote the growth of plankton and other microorganisms that are food for fishes. In India, for example, the yield reached in ponds with reclaimed water cultivating tilapia and carp was 5 t ha^{-1}, which represents 16% of the total sales of carp and tilapia in the municipality of Calcuta (Adhikari et al., 2009). Vo and Edwards (2005) reported a production of 7 t ha^{-1} of Tilapia Mozambique (*Oreochromis mossambicus*), Tilapia nilótica (*O. niloticus*), two Indian major carps (rohu, *Labeo rohita* and mrigal, *Cirrhinus mrigala*) and Chinese silver carp *(Hypophthalmichthys molitrix*) in 330 ha of ponds cultivating fishes in periurban areas in Vietnam.

Nowadays, there are numerous studies dealing with the risks of using reclaimed water for crops irrigation or aquatic organisms and take into account the high probability of microbial pollution, toxic metals accumulation, and emerging contaminants. Terechovs et al. (2019) evaluated the effect of 49 emerging contaminants in reclaimed water in fishes of the species *Bidyanus bidyanus* in the semi-rural region of Shoalhaven in Australia. They detected 20 emerging contaminants in the reclaimed water and 23 and 19 emerging contaminants in flesh and liver of the fishes, respectively. However, the concentration of all contaminants was below the limit established by Australian authorities, with the exception of benzotriazol with a concentration of 675 ng L^{-1} in reclaimed water, well above the 7 ng L^{-1} established by the Australian legislation.

The use of reclaimed water in aquaculture interconnected with the production of crops was evaluated in Bangladesh by a company called "Agricuatics," in the city of Mirzapur, with a population of 3,000–4,000 habitants and a wastewater production of 100 L s^{-1} (Drechsel and Hanjra, 2015). The system consisted of 5 tanks. The first tank received the effluent from the city's wastewater treatment plant and worked as a sedimentation tank; the second tank one was designed to have a high hydraulic retention time and was sown with duckweed. The 3rd, 4th, and 5th tanks were used for the cultivation of fish (carp and tilapia) and its effluent was used for the irrigation of fruit trees. The production of fish was 15 t ha^{-1} y^{-1} and for duckweed was 220–400 t ha^{-1} y^{-1}. All of the products (the fish and the fruits) were sold in the local market so the production system was self-sufficient.

In a study by Terechovs et al. (2019), 17β estradiol, diazepam, verapamil and trimethoprim were found in the liver of fish, which indicates that they can accumulate and be metabolized by this organ. Adhikari et al. (2009) studied the concentration of Pb, Cd, Cr, Cu, and Zn in water, sediments and internal organs of fish cultivated in ponds with wastewater from Calcuta (India). They found that Pb exceeded the maximum level allowed by the Canadian Environmental Quality Guidelines (22 μg l^{-1} vs. 7 μg l^{-1}). The five toxic metals were detected in sediments although only Cd and Pb exceeded the maximum levels established by the EPA (Cd: 10.1 μg g^{-1} dw vs. 1.2 μg g^{-1} dw; Pb: 50.5 μg g^{-1} dw vs. 46.7 μg g^{-1} dw). The only toxic metal that appeared to be bioaccumulated by the aquatic organisms was Zn, mainly in kidneys. The concentrations of all five toxic metals in flesh were many times lower than the safety margins established by the WHO and FAO thus, fish were considered safe for human consumption.

A study by Mark et al. (2019), found that fish of the species *Clarias gariepinus* cultivated in ponds with domestic reclaimed water can have Fe and Cd levels within the maximum levels allowed by the FAO, WHO, and NOAA. The tissues that presented a higher bioaccumulation of toxic metals were the gills and liver with concentrations of 0.1 – 2.0 mg kg^{-1}, which were still below the safety threshold for human consumption (1 – 2 mg kg^{-1}). Although *Escherichia coli* was detected in levels of 104 UFC 100 ml^{-1} in reclaimed water and sediments of the cultivation ponds, the fish tissues did not present *E. coli* nor other pathogens like Salmonella and helminths. However, as with agricultural produce, it is necessary to use adequate hygiene measures such as proper wash and adequate cooking of the fish after harvest, disinfection of hands and appliances to avoid contamination by pathogens.

Sharma and Olah (1986), Sahoo and Singh (2015), and Li et al. (2017) documented the use of waste from a porcine farm for tilapia cultivation obtaining good results in terms of growth without detrimental effects of the cultivated organism nor human health concerns. Thus, it has been put forward that an adequate management of livestock wastes can decrease the probability of contracting diseases by virus and bacteria such as *Escherichia coli* or *Toxoplasma gondii* (FAO/WHO, 2014).

With the goal to prevent infections by bacteria and contamination of fish meat, the WHO established a maximum levels of certain microbiological parameters in water, for example 1,000 fecal coliform bacteria per 100 mL in water. And to avoid the risk of infection by helminths, water has to be free of helminth eggs to prevent diseases such as schistosomiasis, fasciolopsiasis and clonorchiasis (Blumenthal et al., 2000). The same authors also mention the need to monitor the microbiological quality of fish once they have been fished. The handling of the fish is particularly important since the concentration of bacteria can be particularly higher in guts than in muscle, so during the process of evisceration the risk of contamination to other parts of the fish is very high.

As mentioned before, toxic metals such as arsenic, cadmium, lead and mercury can be bioaccumulated in carnivore fish; however, it is highly unlikely that the fish would be harvested at an adult age so the concentration of these metals would be low (WHO, 2006). The plants can also bioaccumulate toxic metals but at a level that is not considered a hazard to human health (WHO, 2008). In relation to chemical contaminants such as pesticides, these are, generally speaking, not a problem in the aquaculture industry. However, in countries with weak regulations and there is widespread use of agrochemicals, the possibility to be exposed to these substances increases substantially. Such is the case of

glyphosate in waters of the Amazon region where it has been found bioaccumulated in tissues of fish for human consumption (Gómez-Ramírez et al., 2012).

The European Union (Unión Europea [UE], 2019) and Codex Alimentarius (Inter-Organization Programme for the Sound Management of Chemicals [IOMC], 2008) have established maximum levels of toxic metals allowed for in fish tissue for human consumption. For example, for Hg the maximum concentration in fish is 0.5 mg kg^{-1} fresh tissue; for Cd, it shouldn't be above 0.050 mg kg^{-1} fresh tissue per day (Unión Europea [UE], 2019). For Pb, concentrations above 0.30 mg kg^{-1} can be considered a health risk and, according to The Joint FAO/WHO Expert Committee on Food Additives (JECFA), the weekly intake of inorganic As is 0.015 mg kg^{-1} (WHO, 2008).

Therefore, in order to avoid the risk for the consumers of fish cultivated with reclaimed water due to the bioaccumulation of toxic metals and microcontaminants, the cultivation of ornamental fishes can be an attractive option. It has been estimated that approximately 4,000 species of freshwater ornamental fish are commercialized worldwide, of which between 700 and 800 are cultivated. In contrast, only 180 species of freshwater fish are used as food (Ramirez et al., 2010). For ornamental fishes, wastewater of lower quality can be used, which means fewer wastewater treatment processes and higher profitability.

As highlighted above, more studies on the bioaccumulation of substances, using different species of fishes and changing cultivation conditions in order to gather further experiences with the cultivation of fish in reclaimed water are needed.

AQUAPONICS WITH RECLAIMED WATER

It was not possible to find an academic study that used wastewater or reclaimed water for aquaponics. The only apparent study that does mention wastewater for aquaponics is Rana et al. (2011) that used domestic wastewater at various dilutions for the growth of tomato (*Lycompersicum esculentum*). Although the title does mention aquaponics, there is no description of the aquatic organism used nor about its growth or survival rates. Having said that, there are a considerable number of studies in which wastewater has been used for the growth of fish, plants or vegetables which might indicate that such activities could be integrated (into aquaponics) and become viable taking into consideration factors such as health standards, concentration of contaminants in water and monitoring of certain contaminants in the flesh of plants and fishes to avoid human health risks.

Some examples of such studies are the following. Siqwepu et al. (2020) demonstrated that the addition of 30 mg kg^{-1} of ferrous sulfate ($FeSO_4$) to the diet of fish (*C. gariepinus*) increased their hematologic profile and produced an effluent adequate in terms of Fe (0.16 mg L^{-1}) for the growth of plants. Luo et al. (2020) analyzed the influence of the use of Selenium (Se) on the growth, ornamental features and health of the Koi carp (*Cyprinus carpio Koi)* and lettuce. In diets using 1.55 – 1.57 mg Se kg^{-1} it was observed a greater weight gain, a larger content of carotenoids and improved immunological capacity of the carp. In the hydroponic system, the lettuce did not show adverse effects with the addition of Se. It was concluded that Se is a microelement essential for animals and a cofactor in glutathione peroxidase (GSH-Px), which is an important antioxidant and eliminator of free radicals. The use of reclaimed water for the cultivation of fishes does not adversely affect their growth rate and survival rate, according to Zaibel et al. (2020). They conducted a study with *Cyprinus carpio* cultivated in solutions of 0, 50, and 100% municipal reclaimed water for 5 months. Similarly to other studies, the concentrations of toxic metals in the flesh of the fish were below the maximum levels established by the FAO. These results can make us conclude that reclaimed water can be safely used for the cultivation in aquaponics for some species of fish.

Although no specific reports on the use of reclaimed water in aquaponics were found, several published studies have dealt with the treatment of wastewater during the cultivation of aquatic organisms, mainly through phytoremediation by aquatic plants such as water spinach (*Ipomoea aquatica*) and duckweed (*Lemna minor*). These plants are added as a remediation component (Effendi et al., 2015) or as a diet supplement of fishes and other organisms (Pinandoyo et al., 2019). Studies by Effendi et al. (2015) demonstrate the nutrient removal capacity of an aquaponic system with crayfish (*Cherax quadricarinatus*) and water spinach, obtaining 85% reduction for NH_4^+, 34% for NO_3^- and 44% for PO_4. Endut et al. (2009) used water spinach to treat wastewater from an aquatic system cultivating African catfish at three hydraulic loading rates (HLR). Results demonstrated the removal of 65% of BOD, 83% of total suspended solids, 78% of ammonia and 89% of nitrites and a positive correlation between removal rates and hydraulic loading rate (HLR). All hydraulic loading rates (HLR) were efficient for the removal of nutrients and to maintain water quality conditions for the growth of fishes.

Other studies have demonstrated that small densities of fishes in reservoirs filled with reclaimed water can help regulate the growth of microalgae and undesirable vectors such as mosquitoes and snails (Terechovs et al., 2019). The growth of microalgae in aquaponic systems has been used to promote the improvement of water quality by increasing its buffer capacity, dissolved oxygen levels and the production of polyunsaturated fatty acids that can be added to the diet of the fish. The latter is very relevant as a common deficiency of aquaponics systems (and indeed aquaculture systems) is the need to add external substances like concentrates, which represent one of the highest costs in aquatic production systems (Addy et al., 2017).

As mentioned earlier, other options for the use of reclaimed water in aquaponic systems is the use of ornamental plants (*Dianthus, Chrysanthemum, Gerbera, Euphorbia, Anthurium, Alstromeria, Lilium, Rose*) and ornamental fishes (**Table 1**). The worldwide market for ornamental plants has been calculated in 60 billion dollars per year. The countries with the larger demand for flowers are Switzerland, Japan and the United States and the main producers are the European Union, United States, Japan, and Colombia (van Uffelen and de Groot, 2005). Reclaimed water could serve as an important source of nutrients for the cultivation of ornamental plants although risks for human health

TABLE 1 | List of ornamental fishes that can be cultivated in captivity and with the potential of being used in aquaponics with reclaimed water.

Specie	Care level	Diet	Max. Size (cm)	Sale price (USD)
Cyprinus carpio	Easy	Omnivore	7.6	$17.99
Carassius auratus	Easy	Omnivore	20.3	$2.69 (3 – 5 pack)
Pterophyllum scalare	Easy	Omnivore	15.2	$4.99
Danio rerio	Easy	Omnivore	6.4	$70 (10 pack)
Poecilia reticulata	Easy	Omnivore	4.5	$14.99 (3 pack)
Puntius tetrazona	Easy	Omnivore	7.6	$11.99
Gymnocorymbus sp.	Moderate	Omnivore	6.4	$11.99
Symphysodon sp.	Moderate	Carnivore	20.3	$479.99 (3 pack)

Source: https://www.liveaquaria.com/. The costs are for organisms in aquariums and presented only as a reference. Costs are subject to change and depend on availability, quality and quantity of organisms required for each system.

need to be taken into consideration (De Bon et al., 2010). The market for ornamental fish in 2010 was calculated at 10 billion dollars. The largest importers of ornamental fish are the United States, the European Union and Japan, while the main exporters are Belgium, The Netherlands, United States, Australia, Brazil, and Colombia. It has been calculated that more than half of the total commerce for wildlife are fishes, and only in the United States there are more than 160 million aquariums (Biondo and Burki, 2014). Therefore, reclaimed water could be used for the production of ornamental fishes and it wouldn't affect its commercialization as these would not be used for human consumption.

In relation to legislation for the use of reclaimed water in aquaponics, there are no current norms. The United States, the European Union and the WHO have guidelines for the use of reclaimed water in agriculture, in order to guarantee low risk to human health (Cifuentes-Torres et al., 2020). With respect to the risks associated with the use of wastewater in aquaculture and, therefore, applicable to aquaponics, the WHO has established that the primary concern is the presence of pathogens, followed by the exposure to chemical substances. The adequate treatment of wastewater can significantly decrease the transmission of illnesses. It has been established that other measures such as adequate cooking of the food and hygiene facilities within the households can decrease the risks of contamination of bacteria like *E. coli*, *Vibrio Cholerae*, *Salmonella* spp. and *Shigella* spp., protozoa like *Giardia intestinalis* and *Entamoeba* spp., and virus like hepatitis A, hepatitis E, adenovirus and rotavirus. The risk of disease by helminths such as *Ascaris*, hookworms and *Taenia* spp. is higher for farmers and consumers of contaminated plants than for aquaculture workers and consumers of contaminated fish. Special considerations have to be taken with nematodes such as *Clonorchis, Opisthorchis* y *Fasciola* which can be transmitted by direct contact with contaminated water or if the infected plant or fish are eaten raw.

In relation to the possible consideration of aquaponic products as organic, according to the guidelines established by the National Organic Standards Boards (NOBS), the addition of synthetic materials and products from an industrial nature, limit the possibility of obtaining an organic certification in products from aquaponic systems (National Organic Standards Board [NOSB], 2016). In these, inorganic substrates are commonly used for the hydroponic component and pelleted food is used for aquatic organisms (Kledal et al., 2020). Additionally, according to the regulations by the EU (834/2007), for the production of organic horticulture, plants have to grow on soil, using the biological interactions generated in this ecosystem and, thus reducing the addition of agrochemicals to the soil. This has created problems due to desertification, monocultures, and the decrease of nutrients in agricultural soils so for traditional agriculture it is a challenge to grow organic products (Altieri, 2002). This has caused the need to implement new technologies, like hydroponics, that partially solves the problem of the lack of nutrients in soil. Nevertheless, organic horticulture prefers to pursue soil management rather than the adoption of a technology that dispenses with soil, causing that the certification of products from aquaponics, hydroponics or aquaculture will probably won't happen in the near future, specifically in the UE (Kledal et al., 2020).

Despite this panorama, some private certification agencies in the United States, approved by the USDA, are generating organic certifications to vegetables produced by aquaponics under the argument established by the National Organic Standards Board [NOBS] in 2002 that defines organic production as "a production system that follows the agreements by law and regulations through the promotion of the natural cycle of the resources, through the integration of cultural, biological and structural components (referring to the assembly of the production system), promoting the ecological equilibrium and conservation of biodiversity" (National Organic Standards Board [NOSB], 2016). Thus, the methods used by hydroponics and aquaponics are legally entitled to be certified for the production of organic products as long as the producer can demonstrate the use of organic products guidelines (National Organic Standards Board [NOSB], 2010).

However, there is still controversy on the use of organic labels in crops produced from soil-less technologies (such as hydroponics and aquaponics) due to the opposition by soil-based farmers that argue that new labeling could cause confusion for consumers (Agricultural Marketing Service [AMS], 2016). The reality is that consumers of products from soil-less systems do not necessarily seek organic-product labels to consider these products more environmentally friendly than those from traditional agriculture (Kledal et al., 2020). Considering the fact that fertilizers in aquaponics originate through reclaimed water with nutrients from other systems, the organic certification would make even more sense, when following all health-safety protocols.

The development of new policies for the use of reclaimed water in aquaponics must include, following the proposal by Alcalde Sanz and Gawlik (2017), the development of operational procedures such as: implementation of a risk management evaluation team, characterization of the reclaimed water, the effluent and receiving waters and processes validation. This management framework is similar to the one used to regulate the reuse of reclaimed water in irrigation and aquifer recharge.

The data included in the present analysis are those found in academic publications. It is evident that, in theory, the potential for the use of reclaimed water in aquaponics is high. However, the potential is only theoretical so there is a need for studies that can translate the potential into reality by means of experimental demonstrations, and pilot-scale studies would be particularly useful.

CONCLUSION

Reclaimed water can, in theory, be used in aquaponics as it has been used as a water source in agriculture irrigation and aquaculture for many decades. The current analysis highlights that there is an opportunity to use reclaimed water in aquaponics although there are still many questions that arise and more studies are needed to demonstrate that this technology is sustainable. There is the potential that toxic compounds such as certain toxic metals at low concentrations can function as food supplies in fish diets, under strict and controlled conditions. The presence of microalgae in aquaponic systems can make it an advantage as it acts as both a food producer and wastewater treatment process. It is necessary to develop guidelines for the use of wastewater in aquaponic systems. To do so, it is necessary to continue studies with aquatic organisms and plants with the ability to metabolize contaminants without the risk to human health. Studies on the effects of water quality and possible bioaccumulation of contaminants in fish and plant tissue would have to be undertaken to prove its eventual safety and facilitate its commercialization.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

LC-T, GC-R, and LM-E contributed to the conception of the manuscript and contributed to manuscript revision, read, and approved the submitted version.

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frontiers in Plant Science

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Improving Plant Health Through Nutrient Remineralization in Aquaponic Systems

*Victor P. Lobanov[1], Doriane Combot[2], Pablo Pelissier[2], Laurent Labbé[2] and Alyssa Joyce[1]**

[1] Department of Marine Sciences, University of Gothenburg, Gothenburg, Sweden, [2] PEIMA-INRAe, UE0937, Fish Farming Systems Experimental Facility, Sizun, France

The exploitation of readily bioavailable fish excreta as a source of plant nutrients lies at the cornerstone of aquaponics farming. Research on nutrient cycling in aquaponic systems has devoted considerable attention to the plant uptake of dissolved nutrients in fish excreta, however, the integration of particulate-bound nutrients into downstream hydroponic farming has remained elusive. The high amount of organic carbon present in fish sludge may lead to biofouling if directly incorporated into hydroponic circulation systems, reducing the utility of incorporating fish solids on a large scale. In this study, we implemented a novel treatment system capable of reducing the carbon and nitrogen load of fish solids to produce a liquid fertilizer for a downstream hydroponics unit. Lettuce (*Lactuca sativa*) fertilized with exclusively a commercial nutrient solution, the biofilter effluent (coupled aquaponic system), effluent from the solids treatment system, or the latter two combined were grown in nutrient flow technique gutters downstream of a recirculating aquaculture system stocked with rainbow trout (*Oncorhynchus mykiss*). While crop yields were lower for the aquaponic treatments compared to lettuce grown in a commercial nutrient solution, plant sap analysis demonstrated a contrasting picture with respect to internal nutrient concentrations. Lettuce grown in the commercial hydroponic solution were deficient in several mineral nutrients (Mg, Ca, Na, and Si) nor did they have higher iron concentrations despite the significantly higher EDTA-chelated aqueous iron (460× greater than other treatments) in the nutrient solution. Nutrient uptake in the rhizosphere was not investigated on a molecular level, although stunted rhizosphere growth in the commercial nutrient solution control suggests a weakened capacity for nutrient uptake in comparison to other treatments. Alongside the remineralization of micronutrients, the solids treatment system addressed the common issue of excess carbon leading to biofouling via a total suspended solids reduction of 87.27% ± 9.95 during the coupled aquaponics cultivation period. Ultimately, these data lead to two important conclusions. Firstly, optimizing nutrient bioavailability is not synonymous to increasing the presence of a nutrient in the water column. Secondly, estimating ideal nutrient solution concentrations involves both preventing nutrient blocking and improving bioavailability.

Keywords: controlled environment agriculture, aquaponics, nutrient remineralization, solid waste treatment, recirculating aquaculture system, liquid fertilizer

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Genhua Niu,
Texas A&M AgriLife Research, Texas A&M University, United States

Reviewed by:
Catello Di Martino,
University of Molise, Italy
Daniele Del Buono,
University of Perugia, Italy

***Correspondence:**
Alyssa Joyce
alyssa.joyce@gu.se

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HIGHLIGHTS

- The implementation of a low-cost solids treatment system for a freshwater recirculating aquaculture system resulted in a liquid fertilizer more bioavailable to plants than a commercial nutrient solution.
- This study establishes a baseline for macro- and micronutrient acquisition from both soluble RAS waste and fish solids in aquaponics.
- Mineral nutrient uptake in the roots was not found to be linearly correlated to the nutrient solution (background) concentration.
- Aquaponic systems are well adapted to provide most, but not all essential plant nutrients at sufficient concentrations.

INTRODUCTION

In terms of land-use, agricultural production currently occupies half of the world's habitable land (Ellis et al., 2010; Ritchie and Roser, 2013). A staggering 70% of the global freshwater consumption is currently devoted to agriculture, reaching up to 90% of local supply in some regions (Meier et al., 2018). The need for high nutrient-use efficiency in existing agricultural systems has also risen in importance due to extreme instances of eutrophication from intensive food production as well as potential phosphorus scarcities (Cordell et al., 2009; Metson et al., 2012; Steffen et al., 2015; Schaum, 2018). These challenges have led to the increase of controlled environment agriculture (CEA), a term that covers protected agriculture (e.g., greenhouse, polytunnels, row covers) and technology-integrated crop management systems (e.g., vertical farming, aquaponics) (Benke and Tomkins, 2017; Shamshiri et al., 2018; Hickman, 2019; Yanes et al., 2020). As of 2019, protected agriculture covers 8.83% of all arable land; a figure up from 3.5% in 2016 (Hickman, 2019; Sambo et al., 2019). While CEA platforms are more efficient cultivation strategies, they must contend with significantly higher infrastructure costs in comparison to traditional soil-based agriculture (Lichtenberg et al., 2013; Savvas and Gruda, 2018).

Aquaponics is a potentially interesting growing method that can help mitigate some of the additional infrastructure costs of CEA by coupling hydroponic crop production to recirculating aquaculture systems (RAS) (López-Mosquera et al., 2011; Carvalho et al., 2018; Goddek et al., 2019). In most aquaponics systems, the main aquaculture contribution to hydroponics cultivation is via the biofilter. Biofilters are essential to RAS stability as they remove ammonia that is highly toxic to fish, but they are also the first major N-removal step in coupled aquaponics, with the second being the uptake of nitrogenous species by the crops themselves. The biofilter is simultaneously responsible for the bioconversion of ammonium to nitrate, as well as the reduction of nitrogenous species to nitrogen gas, and removes a significant portion of nitrogen as nitrogen gas (Holl et al., 2011; Yogev et al., 2017). The remaining N-fraction leaves the biofilter largely as nitrate with a minority concentration present as nitrite (Wongkiew et al., 2017). The soluble effluent from a RAS is insufficient to address all plant needs. However, there is significant controversy around the extent to which the nutritional profile should be supplemented with nutrient solution for maximum crop productivity (Endut et al., 2010; Bittsanszky et al., 2016; Ayipio et al., 2019).

While hydroponic nutrient supplementation is an easy way to address specific deficiencies, there is an underexplored potential for the remineralization of RAS solid waste as a parallel waste-to-nutrition pipeline to manage agricultural yields. The first solid waste treatment systems in aquaponics were based on either aerobic or anaerobic microbial digesters to increase the solubility of matrix-bound nutrients, with attention mainly devoted to phosphorus and a few plant-relevant micronutrients (Goddek et al., 2016a, 2018; Khiari et al., 2019). Hitherto unexplored in aquaponics production system are the wide range of aerobic and anaerobic nutrient remineralization systems currently used in municipal wastewater treatment plants worldwide, such as enhanced biological phosphorus removal (EBPR) (Yuan et al., 2012; Cieślik and Konieczka, 2017; Bunce et al., 2018). EBPR has been shown to cheaply and efficiently remineralize the diverse substrate compositions typical of municipal waste (Barnard, 1976; Greaves et al., 1999; Yuan et al., 2012; Cieślik and Konieczka, 2017; Schaum, 2018; Rahman et al., 2019; Takiguchi et al., 2019). Typical to EBPR systems is the enrichment of phosphate accumulating organisms (PAO), which play a pivotal role in simultaneous denitrification, carbon catabolism, as well as cyclic phosphorus uptake and release (Yuan et al., 2012; Stokholm-Bjerregaard et al., 2017). An alternating aerobic-anaerobic environment, typically carried out in a sequential batch reactor (SBR), is essential to the activity of these systems.

While canonical PAOs consist mainly of *Candidatus* Accumulibacter spp., the past decade has shown PAO lifestyles among members of the Actinobacterial genus *Tetrasphaera*; bacteria capable of metabolizing a diverse range of carbon sources (Kristiansen et al., 2013; Marques et al., 2017). Recent studies have furthermore hinted at a relationship between iron and phosphorus in the PAO lifestyle, although the mechanism of action remains unknown (Braak et al., 2016; Bollyn et al., 2017). Hydrazine reduction, an essential aspect of methanotroph and anammox metabolism, requires considerable amounts of iron and may play a role in the movement of the metal through the EBPR environment (Maalcke et al., 2016; Fernández et al., 2020; Versantvoort et al., 2020). These biomechanical properties render EBPR systems potentially interesting for aquaponics given that alongside the augmentation of the macronutrient phosphorus, there is an unexplored potential for the remineralization of other plant-relevant nutrients.

The reutilization of one industry's waste products (aquaculture) as a beneficial input to another industrial production process (hydroponics) has made aquaponics into a posterchild for circular economies. The size of an aquaculture system determines the potential scaling of fish to plant production volumes based on waste nutrient availability. It also

Abbreviations: BF, biofilter; CEA, controlled environment agriculture; DO, dissolved oxygen; EBPR, enhanced biological phosphorus removal; EC, electric conductivity; HNS, hydroponic nutrient solution; PAO, polyphosphate accumulating organisms; RAS, recirculating aquaculture system; RM, remineralization effluent; SBR, sequential batch reactor.

sets internal limits, without supplementation, on the ability to satisfy plant nutritional needs based on the availability of specific nutrients poorly represented in soluble RAS effluent (e.g., Fe, Mn, Zn, B, Mo, and Cu). While fish nutritional requirements are controlled via the external addition of feed, gauging plant nutritional needs in a coupled aquaponics system is more challenging due to nutrient dynamism across the aquaculture production cycle and across the plant lifespan, not to mention the complex physiochemical influences on nutrient bioavailability (Yogev et al., 2016; Gichana et al., 2018; Goddek and Körner, 2019). Furthermore, the role of the rhizosphere—and its importance in nutrient bioavailability and assimilation—remains poorly understood (Badri and Vivanco, 2009; Richardson et al., 2009; Chaparro et al., 2014; Garcia and Kao-Kniffin, 2018; Guyonnet et al., 2018; Jacoby et al., 2020), especially in the hydroponic context (Lee and Lee, 2015). The multifactorial increase in both diversity and abundance of the microbial ecosystem in aquaponic systems, as compared to hydroponic counterparts, has previously been discussed as an explanative factor for the discrepancy in fertilizer requirements between the two cultivation systems (Wongkiew et al., 2017; Bartelme et al., 2018; Ayipio et al., 2019; Joyce et al., 2019; Melo and Corney, 2019; Yang and Kim, 2020). None of the nutrient streams (commercial solution, soluble effluent, remineralized effluent, and soluble + remineralized effluent) received additional supplementation over the duration of the study. While it was not expected that this would achieve the maximal yield for any of the aquaponics treatments, it does provide an important perspective into the capacity of the RAS waste streams to supply nutrients to the hydroponics component. If this results in comparable *in situ* nutrient concentrations as determined by plant sap analysis, this may suggest that elevated aqueous nutrient concentrations are alone insufficient at improving agricultural quality and yield.

In these experiments, a novel solids treatment system remineralizing nutrients from fish solids into liquid fertilizer was developed capable of reducing C and N while preserving the trace nutrient profile. Lettuce was grown in four parallel circuits, containing an inorganic hydroponic nutrient solution, a traditional coupled aquaponics loop, and two treatments investigating the remineralization capacity of an in-line solids treatment system as an auxiliary source of nutrient to complement standard aquaponics (with and without coupling to a coupled aquaponics loop).

By simultaneously exploring aquaponics as a fertilizer production system as well as a waste treatment system, we examine the applicability of aquaponics as a value addition to freshwater RAS. Besides contributing to more efficient, resource-conscious fish and plant production, this study explores the concept of crop quality with respect to mineral nutrients. We demonstrate that nutrient excess does not necessarily improve nutrient bioavailability and thus may not translate into improved product quality. Nutrient concentrations in the greenhouse water supply were compared to plant sap analysis data, allowing for a detailed characterization of the capacity for each of the four treatments to satisfy their nutritional demands. This study is the first to assess the capacity of an aquaponics system to target mineral nutrient bioavailability in downstream agriculture through an in-line solids treatment system.

MATERIALS AND METHODS

Experimental Design

An experimental aquaponics system was developed at INRAe-PEIMA (Sizun, France) to evaluate the performance of the solids treatment system within a fully functional aquaponics facility. The goal of this experiment was therefore to establish the boundary conditions for the commercial installation of this treatment system. The cultivation system consisted of three separate recirculating aquaculture system loops operating in parallel in three separate rooms (**Figure 1**). Nutrient solutions were diluted to their final concentration in the four wells within the greenhouse and automatically pumped through eight basins of five parallel nutrient film technique (NFT) gutters (Goponics, France) used to grow the lettuce.

Of the three RAS units, RAS1 ran independently, RAS2 was linked to the hydroponics treatment BF, and RAS3 was linked to hydroponic treatments RM and RM+BF. RAS2 was thus a traditional coupled aquaponics system, whereby oxidized water exiting the system's biofilter was pumped through the corresponding hydroponics treatment before returning to the fish (BF). The biofilter effluent from RAS3 likewise circulated through the greenhouse, however, it was combined with the effluent from the solid waste treatment system (RM+BF). Effluent from the solids treatment system not mixed with biofilter effluent was stored in a separate well (RM). A commercially available hydroponics nutrient solution (Flora series; General Hydroponics, United States) was used as a control group (HNS), manually drained and replaced weekly. The Flora series consists of an N, P, and K heavy solution (FloraGro), a trace nutrient heavy solution (FloraMicro), and a third nutrient solution geared toward flowering, fruiting, and seed production (FloraBloom). Due to the variety of influences on the ultimate *in situ* nutrient concentrations in the plants, no additional nutrient supplementation was done apart from the treatment.

In this study, rainbow trout (*Oncorhynchus mykiss*) were raised from fry on site. Biofilters were set up for RAS 2 months prior to the addition of fish. An autochthonous lettuce cultivar well-adapted to the temperature and humidity profile of the region (Brittany, France) was chosen for this study and seedlings purchased from Tecnosem (France). Seedlings were transferred to the NFT gutters 3 months after fish cultivation began. The greenhouse unit, while not actively heated, was equipped with a thermometer and an automatic ventilation system that could keep the interior air temperature between 15 and 25°C throughout the cultivation period, with late-stage temperatures at the lower end of the range. Automatic pumping systems distributed nutrient solutions from the wells to the gutters.

Design of the Solids Treatment System

The solid waste treatment system involved in the study consisted of a settling basin, an anaerobic fermenter, and a sequential batch reactor (**Figure 2**). Fish solids, passing into

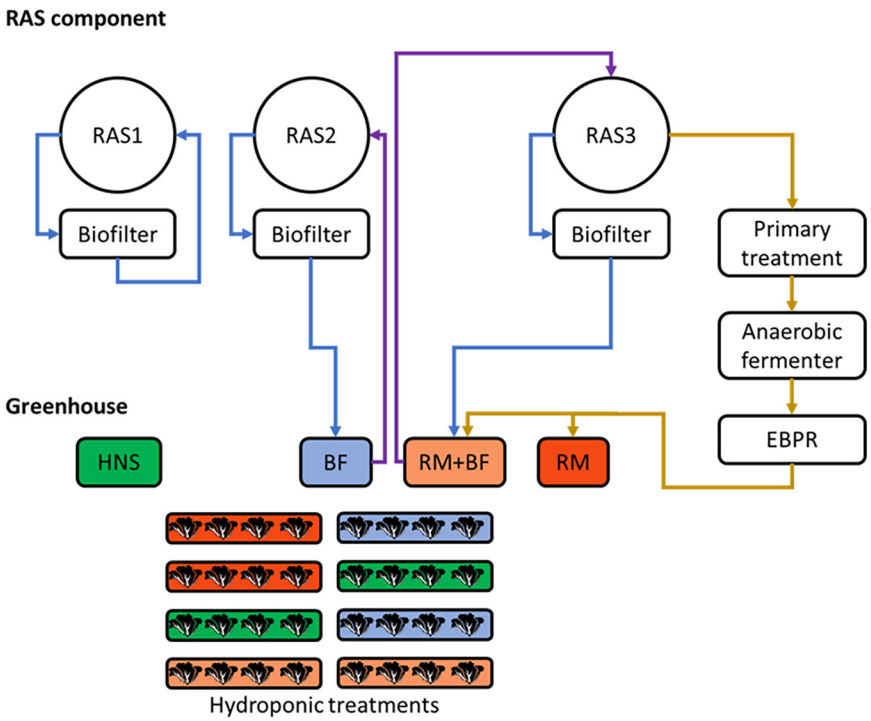

FIGURE 1 | Schematic plan of the three parallel recirculating aquaculture system (RAS) units (left) and greenhouse (right). Blue arrows represent the transfer of wastewater toward the greenhouse, brown arrows represent the transfer of fish solids through the solids treatment pipeline, purple arrows represent the return flows from the greenhouse. Treatments were randomly assigned to their respective gutters.

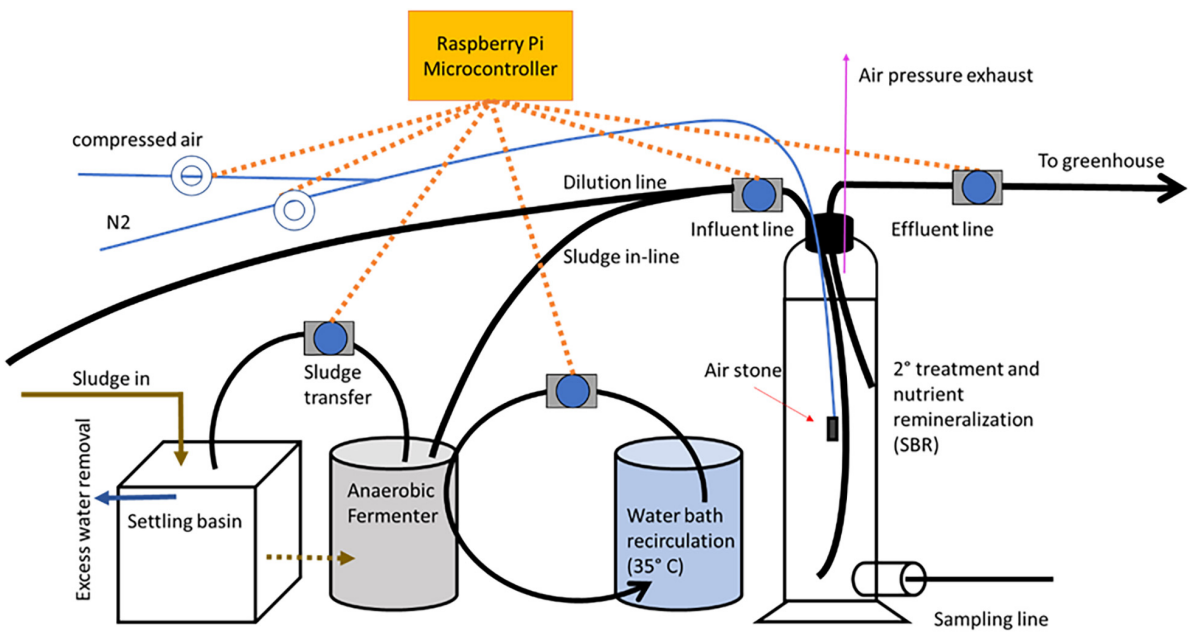

FIGURE 2 | An overview of the solid waste treatment system.

the settling basin directly from the RAS drum filter, were first concentrated in the settling basin with excess water evacuated via a lateral pipe that permitted water, but minimal solids, to pass through. A Raspberry Pi microcontroller was utilized to regulate the pumping of the fish solids from the settling basin into the anaerobic fermenter, then into the SBR, and

finally from the SBR into the RM and RM+BF wells located in the greenhouse. The microcontroller additionally regulated the aeration through an air compressor and a nitrogen delivery system. The anaerobic digester was kept between 25 and 35°C by an adjacent water bath, with a pump recirculating water through tubing from the water bath, through the fermenter, and back into the bath in a closed loop. Prior to entering the SBR, the sludge was diluted 1:2 in water originating from the RAS sampling basin. This water, rich in ammonium, was chosen over sourcing from the aquifer to help balance the C:N ratio within the SBR.

The SBR itself consisted of a 3 L vessel with a main opening at the top, and a secondary lateral opening. That allowed a highly controllable environment where the dissolved oxygen (DO) could be maintained between 0 and 2 mg O_2/L, and that regulated by the duration that either the compressed air or nitrogen lines were open.

To enrich the PAO proportion in the solid waste treatment pipeline, the SBR followed an alternating aerobic (DO = 2 mg/L) and anaerobic (DO = 0 mg/L) cycle. Due to the physical constraints of accessing the interior of the SBR, the DO, ORP, and pH over the course of the SBR cycle were calibrated externally and not monitored in real time. DO was thus set by measuring the shift during aeration with compressed air, or nitrogen gas, using a portable monitor. ORP proved to be a challenging parameter to measure, and thus was estimated through proxy based on the amount of bioavailable carbon entering the SBR. The SBR cycle was carried out as described in **Table 1**.

Sampling

Sampling of aqueous nutrients was done biweekly for each RAS and the hydroponics nutrient solutions across the duration of the respective fish and plant cultivation periods. The pH in each RAS was regulated daily with $NaHCO_3$ to maintain a pH of 7. Similarly, pH in the anaerobic digester was maintained at 7.5. Elsewhere, no modification was carried out as the pH remained stable and within acceptable boundaries.

TABLE 1 | Sequential batch reactor (SBR) cycling regime used in this study.

Phase	Action	Duration (s)	Description
1	Effluent	100	Evacuation of 1.5 L from the SBR
2	Influent	100	Import of anaerobic digester sludge diluted in RAS water totaling 1.5 L
Anoxic phase			
3	N2	60	Establishment of an anoxic environment
Anaerobic phase			
4	Still	1,240	Anaerobic fermentation
Aerobic phase			
5	Air	600	Aeration of the SBR
6	Still	300	Aeration turned off to keep DO from surpassing 2 mg/L
7	Air	900	Aeration of the SBR
8	Still	900	Shift toward starvation regime to promote P-release in PAOs

During sampling, ammonia, nitrite, nitrate, phosphorus (total phosphorus and phosphate), chemical oxygen demand (COD), and biological oxygen demand (BOD) were measured (Hach Lange, Germany).

In addition to *in situ* measurements, samples were assessed for plant relevant nutrients at harvest using commercial technology for greenhouse nutrient monitoring. This allowed a broad survey of nutrients in the RAS, solid waste treatment system, and hydroponics unit wells to be measured using inductively coupled plasma-mass spectroscopy (ICP-MS). Five hundred milliliter of each sample was sent for nutrient analysis with all handling was done in line with the NF EN ISO 5667-3 standard and specific analyses following other standard ISO protocols. Aqueous nutrient analysis was performed by Capinov SAS (France), while plant sap analysis was carried out by NovaCrop Control, Netherlands. Plant sap analysis was conducted on a pooled set of leaves from a specific treatment. Young leaves, old leaves, and roots were packaged separated after being collected 2 h after sunrise as per NovaCrop Control guidelines. All generated bioinformatics data were processed in Microsoft Excel and R.

Lettuce plants were harvested after 8 weeks of hydroponic growth. Fresh and dry shoot and root weights were measured, as well as root length, overall plant health, and total yield. ANOVA and Tukey multiples tests were used to determine whether treatments differed significantly in harvest parameters.

RESULTS

Aqueous Nutrient Concentrations

In situ water quality measurements indicated that the stepwise oxidation of nitrogenous species in the RAS was relatively stable. Total phosphorus and phosphate did not exceed 1 mg/L but did increase following RAS coupling to the HP units, although by the end of the experiment concentrations returned to their original figures (**Figure 3**).

Aqueous nutrient concentrations indicated that many, but not all, essential plant nutrients were available in the water supply (**Figure 4A**). Unsurprisingly, virtually all nutrient concentrations in the output from the solid waste treatments (SBR) were elevated compared to the RAS water alone (**Figures 4A,B**). The notable exception to this rule was Mo. As uneaten feed was directed to the solids treatment system along with excreta, these data imply the nutrient is absent or minimally present in the feed. Charts for P, Fe, NH_4^+, and NO_3^- mirror the shifts expected to occur in the reducing environment. Importantly, as the pH remained slightly alkaline, we can attribute the liberation of P and Fe to microbial activity and not acidic dissociation.

Nutrient composition in the greenhouse wells highlighted the elevated concentrations of virtually all plant-relevant nutrients in the commercial hydroponic nutrient solution (control), with the exceptions being Na, Al, and Si (**Figure 5**). Likewise, the pH across all nutrient solutions remained similar. Due to this high proportion of solutes, the EC of the HNS was proportionally higher. The $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$

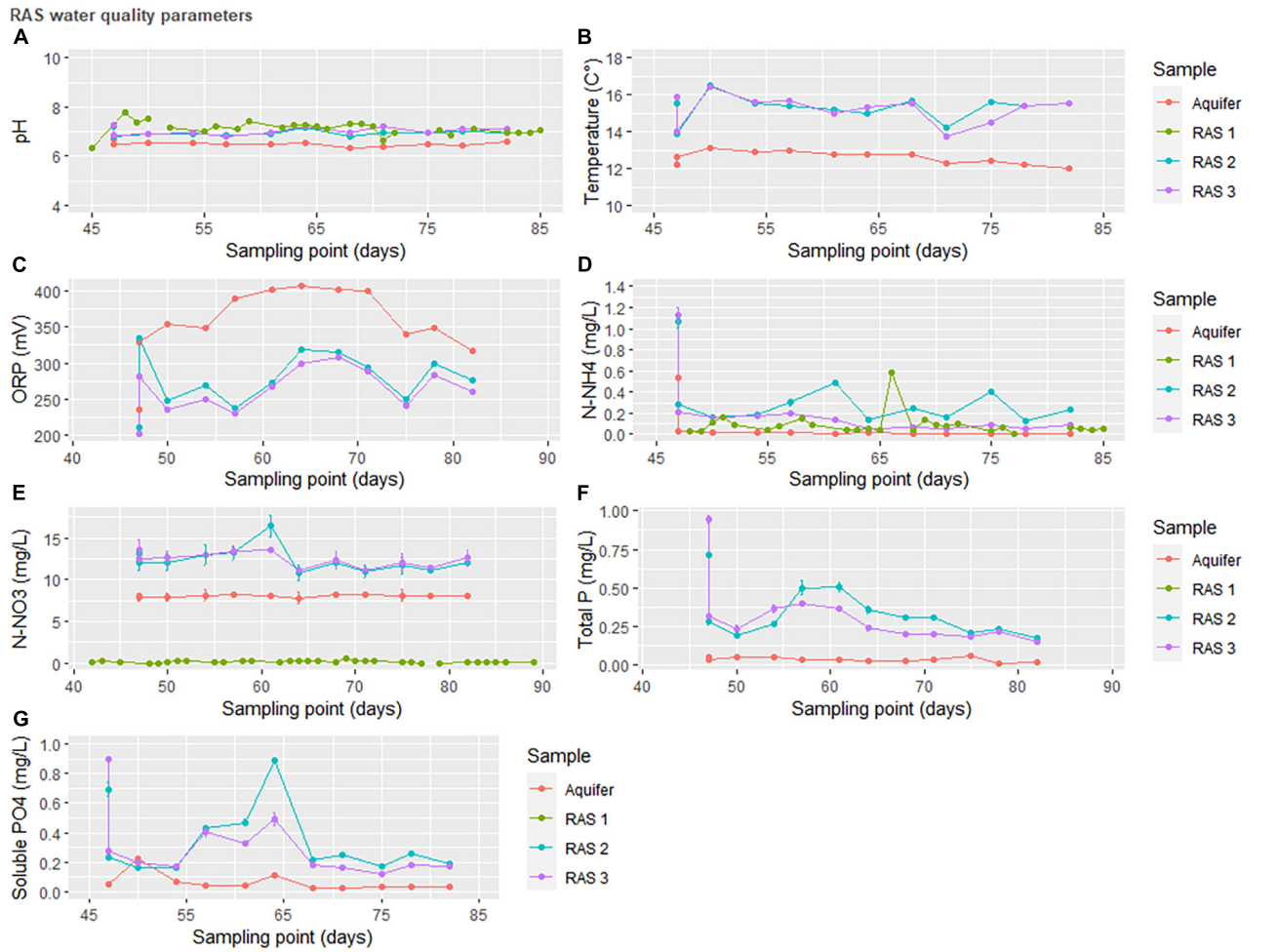

FIGURE 3 | Water quality parameters in the recirculating aquaculture system at INRAe-PEIMA between the coupling of the recirculating aquaculture system (RAS) to the greenhouse (day 45) and the end of the experiment (day 81). RAS1 was operated as a traditional RAS, RAS2 ran as a traditional coupled aquaponics circulation system, and RAS3 contained both aqueous and solid waste treatment components.

concentrations were much higher in the control solution than the coupled aquaponics solution.

EBPR in the Aquaponics Context

Ultimately, the success of the solids treatment system in adjusting the concentration of several important plant nutrients (**Figure 4B**) alongside a drastic reduction in carbon (**Figure 6**) opens a unique niche for sustainable, cost-effective aquaponics cultivation. The adaptation of the enhanced biological phosphorus removal system into the aquaponics system necessitated two fundamental changes in the design. Firstly, the heterogeneity of the fish solids as a C-source did not permit an enrichment of the PAO strains as canonical to EBPR wastewater treatment systems. As the solids treatment system resulted in extensive reductions of the C and N load, this was considered an acceptable trade-off as a high C-load would be unsuitable for use as a liquid fertilizer. Secondly, rather than accumulating phosphorus in the granules as done in EBPR wastewater treatment systems, the operational procedure was modified to promote P-release in the granules immediately prior to effluent evacuation [starvation period following the aerobic phase (**Table 1**)]. **Figure 7** suggests that most of the phosphorus leaving the system was soluble and accumulating in the downstream nutrient solution wells of the hydroponics unit. With respect to total phosphorus, a significant reduction over the duration of the solids treatment system indicated steady degree of extraction from the solids. Thus, while soluble P leaving the reactor was not significantly higher than the concentration entering the system (disproving the hypothesis), a net conversion of conjugated P to soluble P was evident. What is clear from **Figure 5**, however, is that the contribution of the solids treatment system to the phosphorus demand was low—indicating that this system would need to be scaled up before the nutrient demands from a greenhouse of this size will be met.

Nonetheless, the high degree of variation in influent COD was initially exacerbated by physical obstacles. These included clumping of the incoming fish solids, reduced flow in the tubing in part due to fish scale and mucous accumulation as well as

biofilm growth, a problem that was later solved by diluting the influent sludge and prolonging anaerobic fermentation. Due to this practice, regular wasting of the SBR (removal of accumulated settled solids on the order of ca. 100 mg/week) is not represented in the graph. These problems are likely irrelevant at greater production capacities where fish solids are more bioavailable with a decreased proportion of scales and mucous, as well as larger piping diameters to handle greater flows (Lobanov et al., unpublished).

Plant Nutrient Concentrations

The plant sap analysis was chosen as a tool to confirm the successful acquisition of nutrients by the plants from the surrounding aqueous milieu. Old and young leaves were sampled from the plants 2 weeks before harvest, as per standard NovaCrop Control protocols often used in the hydroponics industry to measure plant health. At harvest, old and young leaves were again sampled along with roots from the same plants to provide a comparative measure of nutrient distribution over time.

Of the physiochemical parameters, pH levels were constant for all three sample types: young leaves, old leaves, and root mass corresponding to **Figures 8A,B**, and **9**, respectively. In terms of EC, all treatments were similar for young leaves, RM was slightly lower than the rest for old leaves, however, the HNS root EC was twice that of other treatments. In terms of sugar content, RM was an outlier with the highest percentage while other treatments averaged similarly together.

Plant nutrient concentrations varied drastically across treatments (**Figures 8A,B**, **9**). Of the primary macronutrients, nitrogen (TN, NH_4^+, and NO_3^-) and phosphorus were more concentrated in the HNS control group than other treatments in old leaves and roots. N and P concentrations in young leaves were more balanced across all treatments, indicating that aquaponics-fertilized treatments could meet their nutritional needs but were not in excess of either nutrient. RM tended to be lowest in K, although HNS was significantly higher than other treatments only in the roots. Despite this, RM was the most balanced in terms

FIGURE 4 | Continued

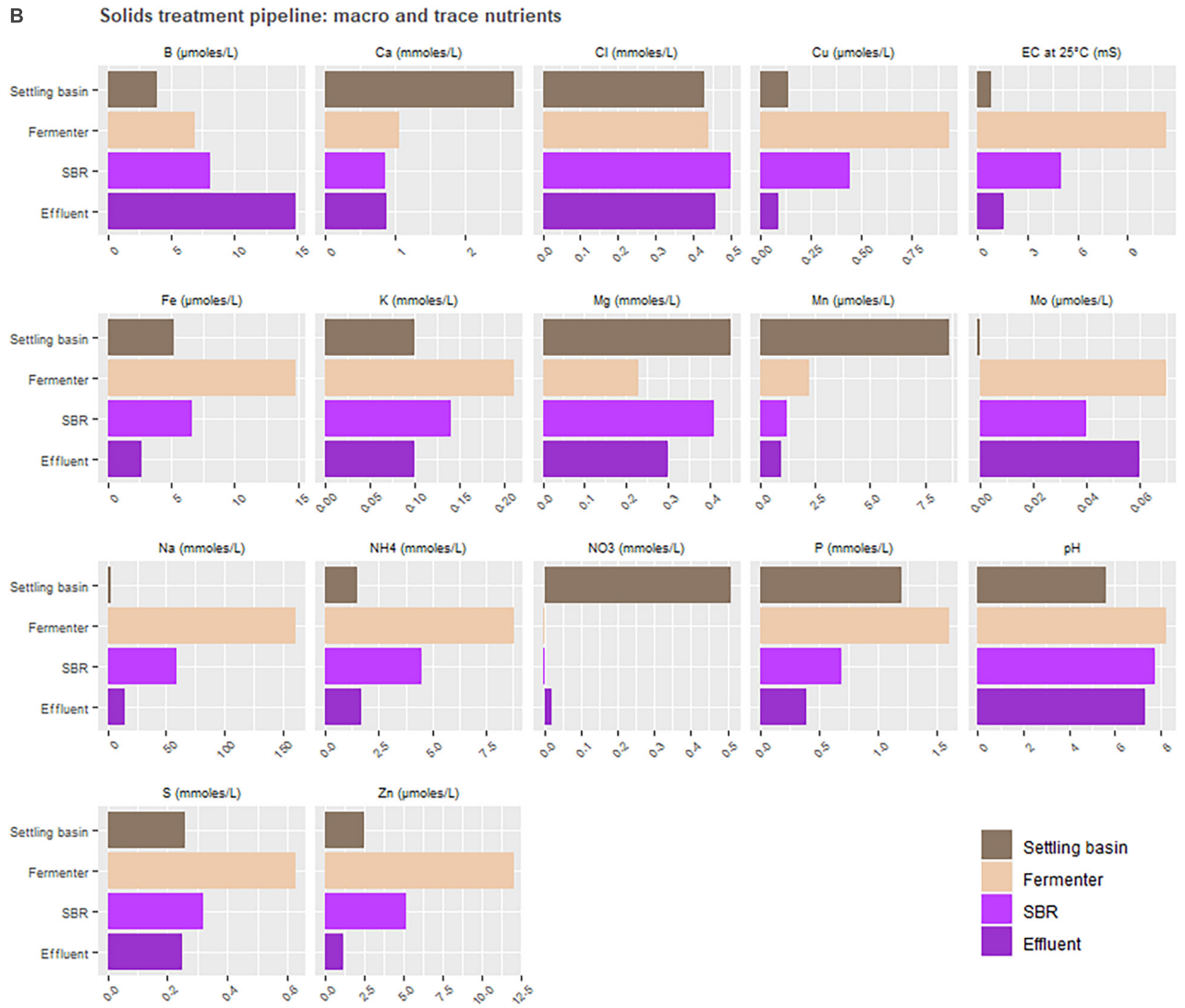

FIGURE 4 | Nutrient load in the recirculating aquaculture system (RAS) **(A)** and solids treatment pipeline **(B)** at steady state conditions.

of K:Ca, while HNS was heavily skewed toward K across all sample types.

For many nutrients, RM and HNS were opposite, with BF and RM+BF treatments falling in between. RM was generally higher in Ca, Mg, Na, Cl, S, SiO_2, Cu, and Al although this was not universal for each nutrient at all sample types. The K/Ca ratio, often used as a general monovalent/divalent cation ratio, was most balanced in RM and most skewed toward K in HNS. Besides N and P, HNS had twice as much Fe in young leaves (0.255 ± 0.05 ppm vs. 0.158 ± 0.02 ppm for all other treatments). This was not the case for older leaves where all treatments were similar (averaging 0.158 ± 0.03 ppm) and was the opposite scenario in the roots (HNS = 1.29 ppm, RM = 4.27 ppm, BF = 4.59 ppm, and RM+BF = 3.13 ppm). It is thus difficult to correlate iron uptake efficiency to the treatment, however, it is clear that the nutrient rich solution did not result in consistently better uptake. In the water quality analysis (**Figure 4A,B**), Mo was not shown to be present in the RAS and solid waste treatment system but was present in the HNS control.

Harvest

The harvest was carried out after 8 weeks of cultivation as plants were beginning to crowd each other on the rafters. Lettuce heads and roots were weighed at harvest. Crop fresh and dry weight varied significantly across treatments, with the HNS control achieving the highest weight yield (**Figure 10**). Shoot yield varied most considerably across treatments, with the HNS treatment significantly larger than the others at $p < 0.05$ (**Table 2**). BF and RM+BF formed the next yield category, with the RM treatment trailing behind. While relatively abundant in micronutrients, the RM treatment was specifically deficient in N and K, likely responsible for the stunted growth.

Root metrics reflected the nutrient saturation of the HNS treatment, with HNS root (fresh and dry) weighing about half

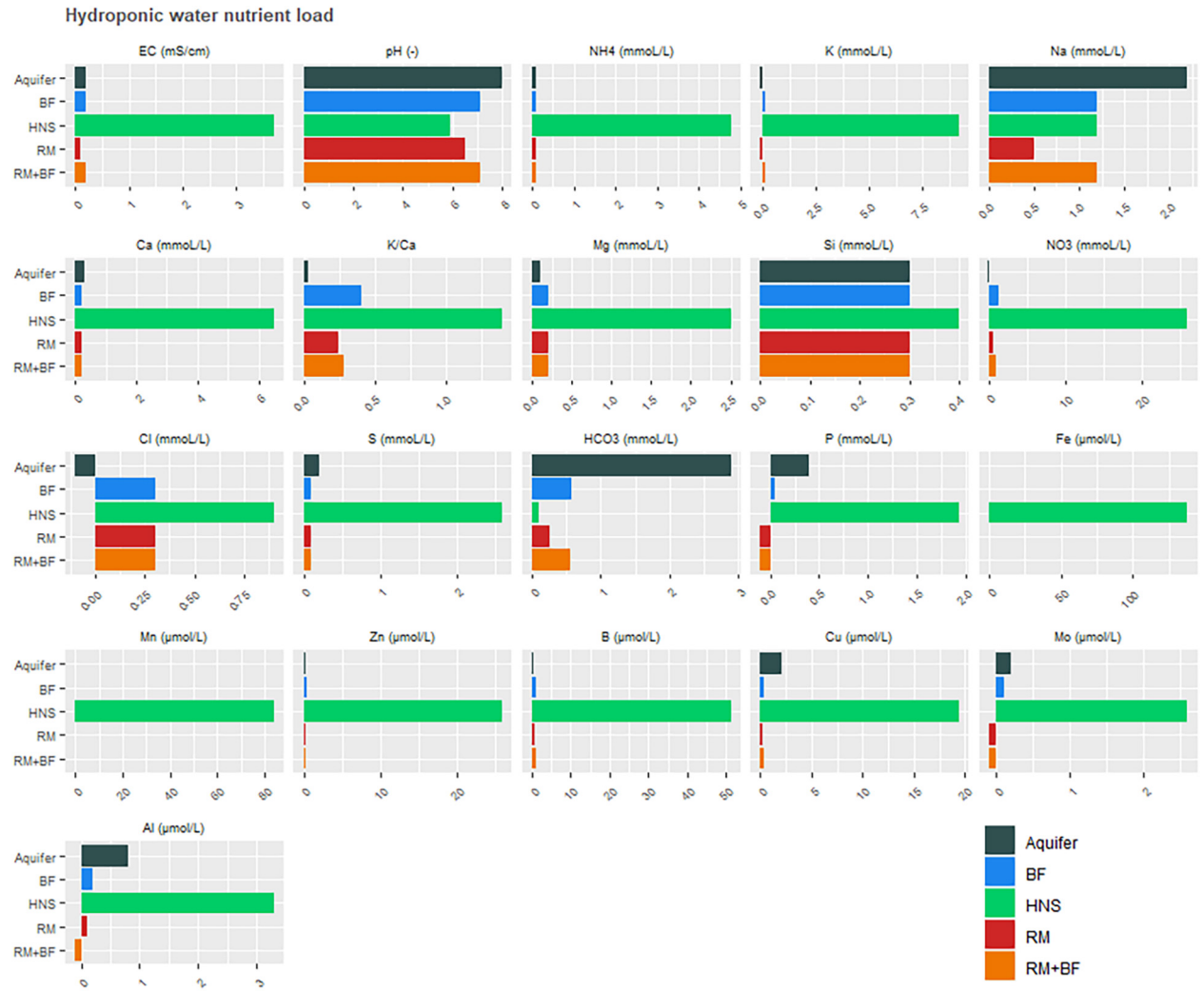

FIGURE 5 | Nutrient loads across greenhouse nutrient solutions, all measurements were taken 1 week prior to harvest.

of the other treatments. While root length was highly variable within each treatment, HNS similarly had the shortest.

A 1-way ANOVA suggests that all treatments were divergent in both shoot and root weights at $p < 0.05$. Removing the HNS treatment, BF, RM, and RM+BF diverged only in shoot dry and fresh weights but not in root mass. The Tukey multiple pairwise-comparisons test demonstrated that the HNS treatment was indeed the outlier, with the RM+BF and BF treatments being the most similar in harvest parameters (**Table 2**). The ratio between fresh and dry weights across all treatments and within each treatment is described in **Table 3**. Root lengths were not significantly divergent across any of the treatments as indicated by ANOVA and Tukey multiples tests.

Disease Prevalence

With respect to disease, 16 of the 52 HNS lettuce heads had mold growth that developed shortly before harvest. No signs of disease were seen in other treatments. The treatment RM was severely deficient in nitrogen, likely explaining the yellowish coloration of the leaves commonly associated with a nitrogen-deficient state.

DISCUSSION

Although EBPR is a firmly established strategy for nutrient recuperation from municipal wastewater it has not yet been investigated in the context of solids treatment for freshwater aquaculture. This study is the first of its kind to assess the suitability of the technology as well as the impact of remineralization on the availability of trace nutrients in the downstream hydroponics unit.

Balancing Macronutrient Excess With Micronutrient Deficiencies

Often, the concept of high-output yield (e.g., fast growth, with inherent economic implications) is prioritized

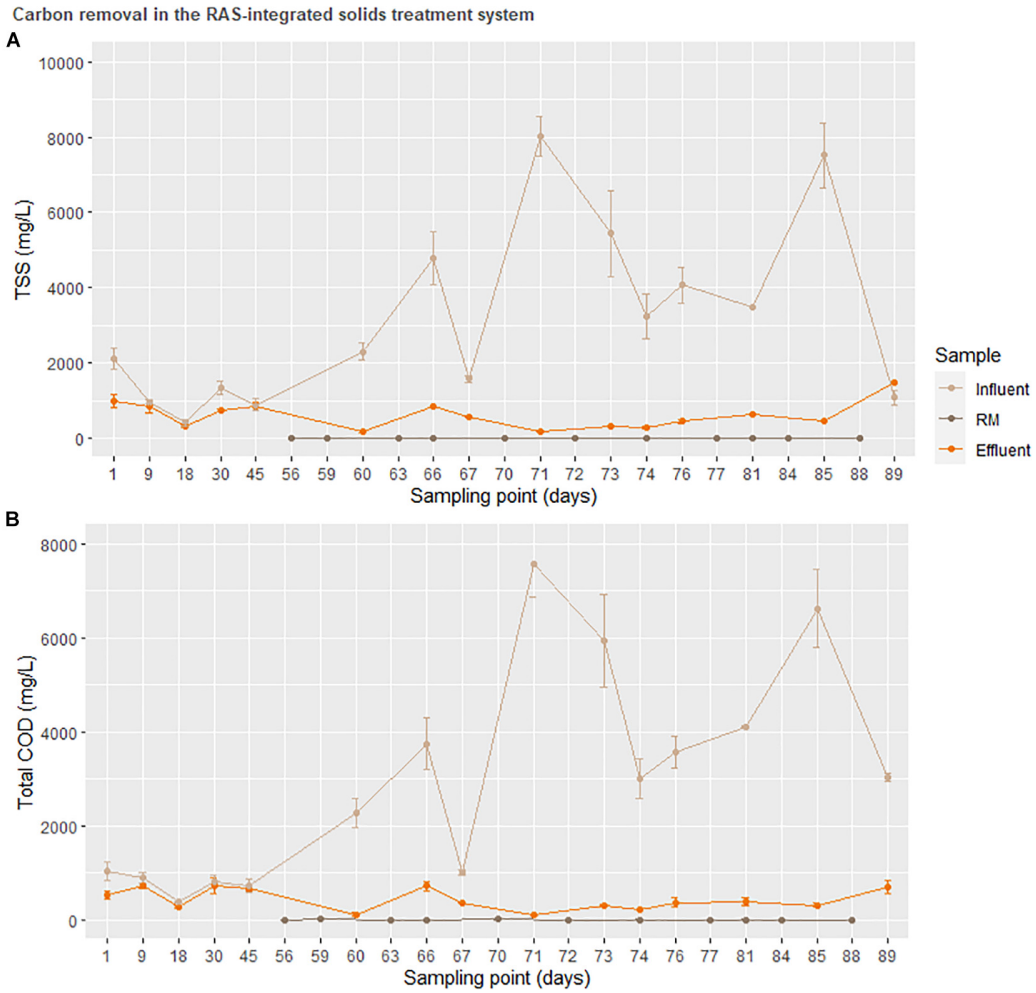

FIGURE 6 | Total suspended solids and total carbon oxygen demand of sludge prior to entering the sequential batch reactor (SBR). The treatment RM was used to represent accumulation in a downstream hydroponic (HP) unit.

over plant health. This view may need to be revised considering the relative abundance of trace nutrients in the lettuce across treatments. The chronic deficiencies present in the HNS control (**Figures 8A,B**, **9**) suggest an internal triage response to manage the excesses of other nutrients, a phenomenon known as nutrient lockout (Du Laing et al., 2009; Enez et al., 2018; Pertiwi and Prastya, 2020). As reviewed by Marles (2017), several studies have indicated that the nutritional content of vegetables available to consumers has decreased in important mineral nutrients (e.g., Fe, Zn, Cu, and Ca) although the review stresses the lack of consensus surrounding potential causes (Marles, 2017). Nutrient-deficient vegetables is an issue of public health concern (Maggini et al., 2010; Keatinge et al., 2011). Understanding the differences between the hydroponic nutrient solution control and the aquaponics-derived nutrient streams can shed light on possible mechanisms underlying observed discrepancies in bioavailability.

Comparing the Commercial Nutrient Solution to the Remineralization/Biofilter Effluent Solution

In comparing aqueous concentrations, it is evident that the control group receiving HNS had access to all relevant macro- and micronutrients at greater quantities in the water supply than was available for other treatments (**Figure 5**). Water originating from the RAS was deficient in Cu, Fe, Mn, and Mo, however, all of these elements were recovered from the solids treatment system (**Figures 4**, **5**). One of the key findings of this work is the importance of incorporating a solid treatment system into aquaponics systems, that are traditionally reliant primarily on the dissolved nutrient fraction in the water that circulates between the fish and plants. The beneficial impact of integrating solids treatment into aquaponics cultivation were demonstrated by several researchers in the past (Delaide et al., 2018; Goddek et al., 2018; Khiari et al., 2019). Sharing fundamental objectives, the solids treatment system discussed in this study improves

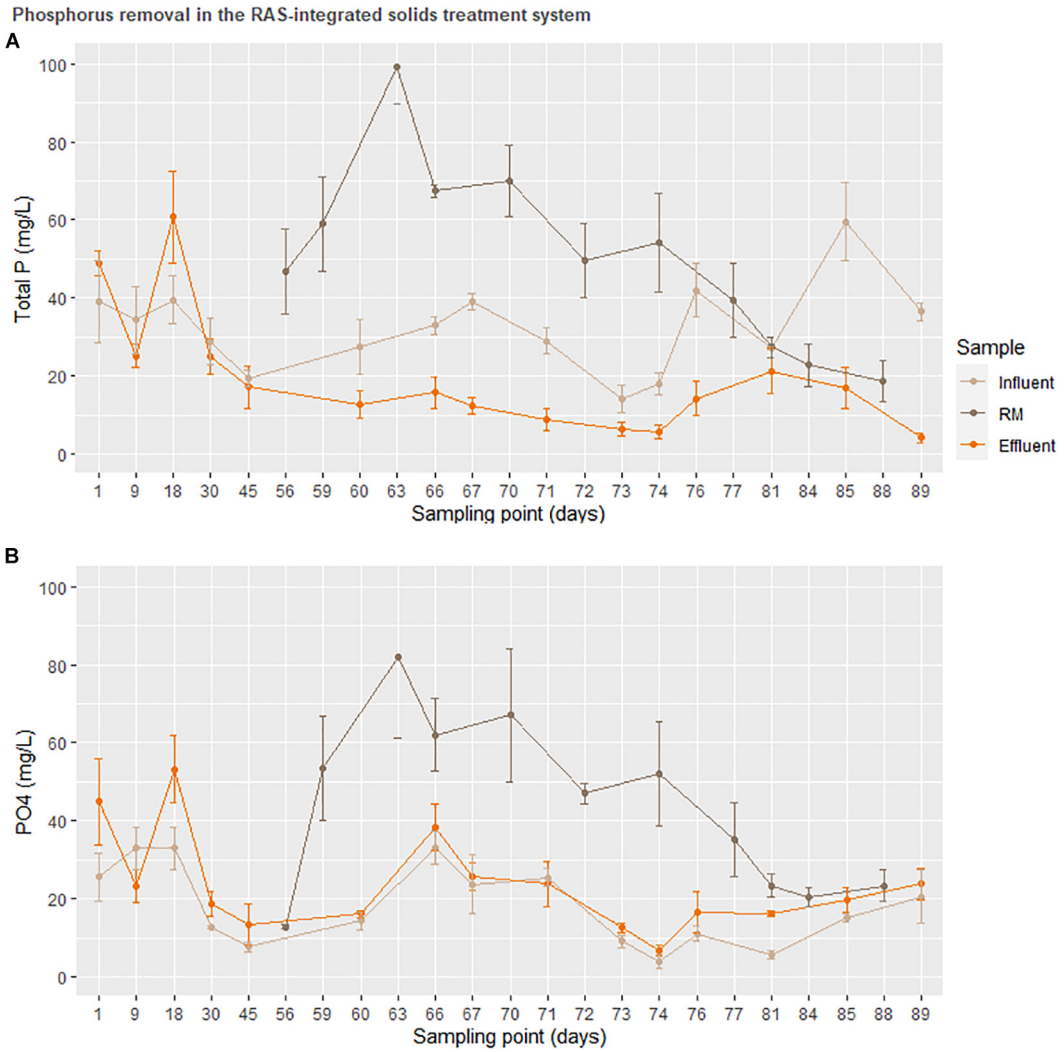

FIGURE 7 | Total (top) and soluble (bottom) phosphorus remineralization in the solids treatment system normalized to total mass transferred. The treatment RM was used to represent accumulation in a downstream hydroponic (HP) unit.

upon these systems by addressing the primary goal of nutrient remineralization alongside efficient waste treatment (C- and N-removal from bulk sludge to prevent eutrophication) alongside minimal endogenous biomass production. Previous studies, however, restricted their discussion of nutrient remineralization to macronutrients and a small number of micronutrients (Goddek et al., 2016b; Khiari et al., 2019).

With respect to the macronutrient phosphorus, it was assumed that approximately a third of the total system P would be carried downstream as soluble phosphate in the circulating water (Davidson et al., 2013; Cerozi and Fitzsimmons, 2017; Jaeger et al., 2019), and that the solids treatment system would further augment this quantity. However, no such trend could be observed in the nutrient solution wells (**Figure 5**). In terms of phosphorus, HNS, RM, and RM+BF had similar concentrations in young leaves. While soluble P was transferred to the greenhouse in the traditional aquaponics setup (BF), it appears that it was insufficient to meet plant needs. Surprisingly, water concentrations of P were nearly identical across RM, RM+BF, and BF treatments. It seems that the RM and RM+BF treatments were able to adapt to more efficiently acquire phosphorus, or perhaps that the phosphorus supplied by the solid waste treatment system was exceptionally bioavailable. The HNS treatment with 38.6× more P than the other nutrient solutions had significantly higher P accumulation in roots and old leaves in addition to the slight gain in young leaves (**Table 4**). Ultimately, based on literature recommendations for lettuce sap P concentration (deficiency below 0.43% P/DW, sufficiency at 0.55–0.76% P/DW) we note that all treatments had their demand satiated (Barker and Pilbeam, 2015). Zn and Cu blocking, associated with an excess of P, seems to have affected the HNS treatment only in terms of Zn, where it was deficient (<1 ppm) in older leaves and borderline deficient in young leaves and roots (Forsee and Allison, 1944; Chapman, 1966;

TABLE 2 | Tukey multiple pairwise-comparisons indicate that the hydroponic nutrient solution (HNS) crop was significantly different from other treatments in both shoot and root weights, although other treatments were more similar for certain metrics.

Multiples	Harvest parameter *p*-values adjusted to multiples				
	Shoot dry weight	Shoot fresh weight	Root dry weight	Root fresh weight	Root length
HNS-BF	0.00	0.00	0.00	0.00	0.49
RM-BF	0.02	0.00	0.95	0.68	0.98
RM+BF-BF	0.43	0.98	0.97	1.00	0.94
RM-HNS	0.00	0.00	0.00	0.00	0.62
RM+BF-HNS	0.00	0.00	0.00	0.00	0.24
RM+BF-RM	0.43	0.00	0.77	0.56	0.74

Cakmak and Marschner, 1987). No visible signs of Zn deficiency were observable, however (Barker and Pilbeam, 2015).

The relationship between pH and nutrient bioavailability has long been a challenge in chemical fertilization as it quickly leads to unideal nutrient solubilities regardless the value. Below a pH of 6, Mn, Zn, and Fe become more soluble at the cost of Ca, Mg, and K. The pH of the solids treatment system did not strongly deviate from upstream or downstream components. While acidity in the RAS (pH 7.18 ± 0.04) dropped to 5.61 in the primary treatment, effluent leaving the pipeline had returned above 7, before stabilizing around 6.23 ± 0.5 across all hydroponic well measurements. From this we conclude that acidification was not responsible for the increased solubility of easily complexed nutrients such as P and especially Fe across the solid waste treatment system.

The overapplication of nitrogen (esp. nitrate) remains the most common detrimental impact of fertilizer misuse on crop health (Addiscott, 2005). Excess nitrate in plants leads to

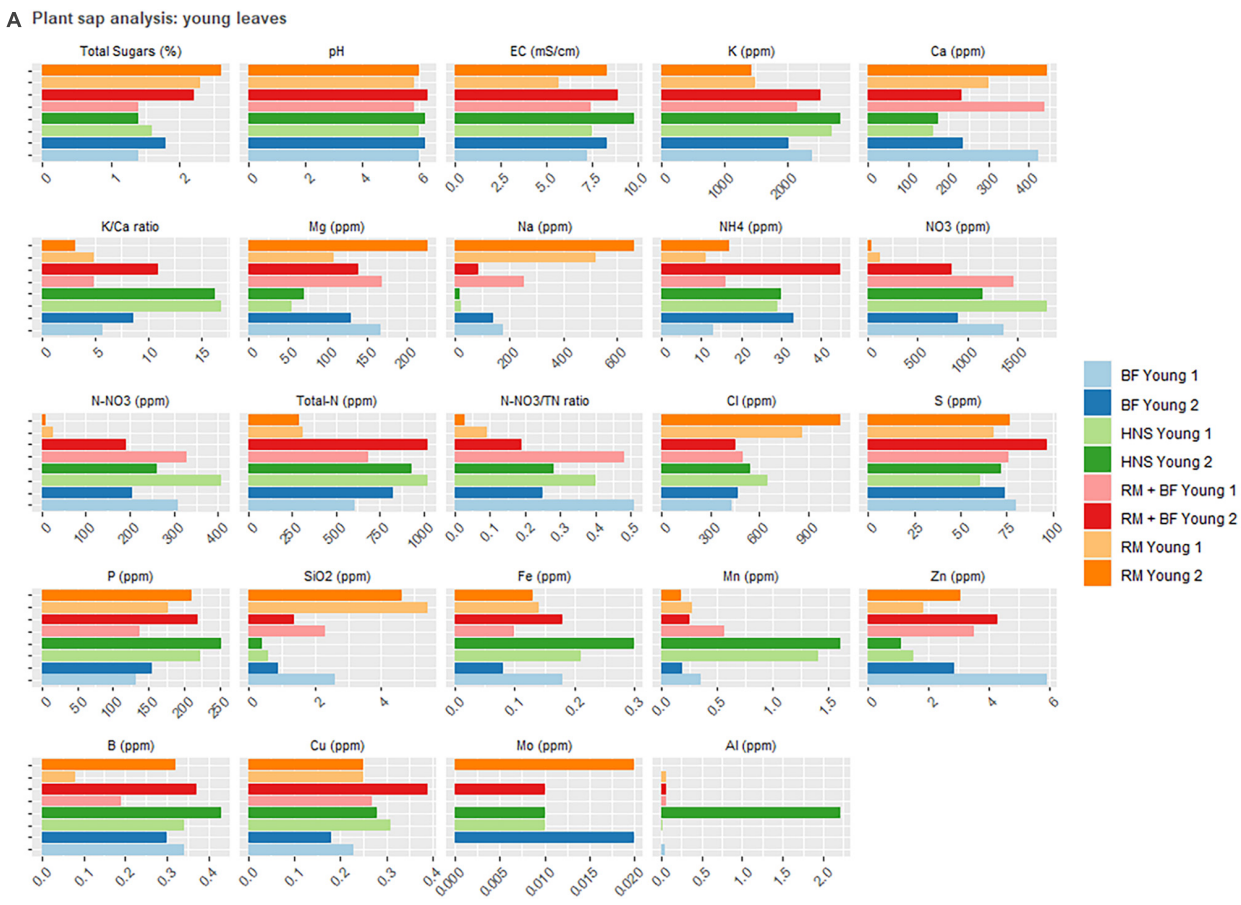

FIGURE 8 | Continued

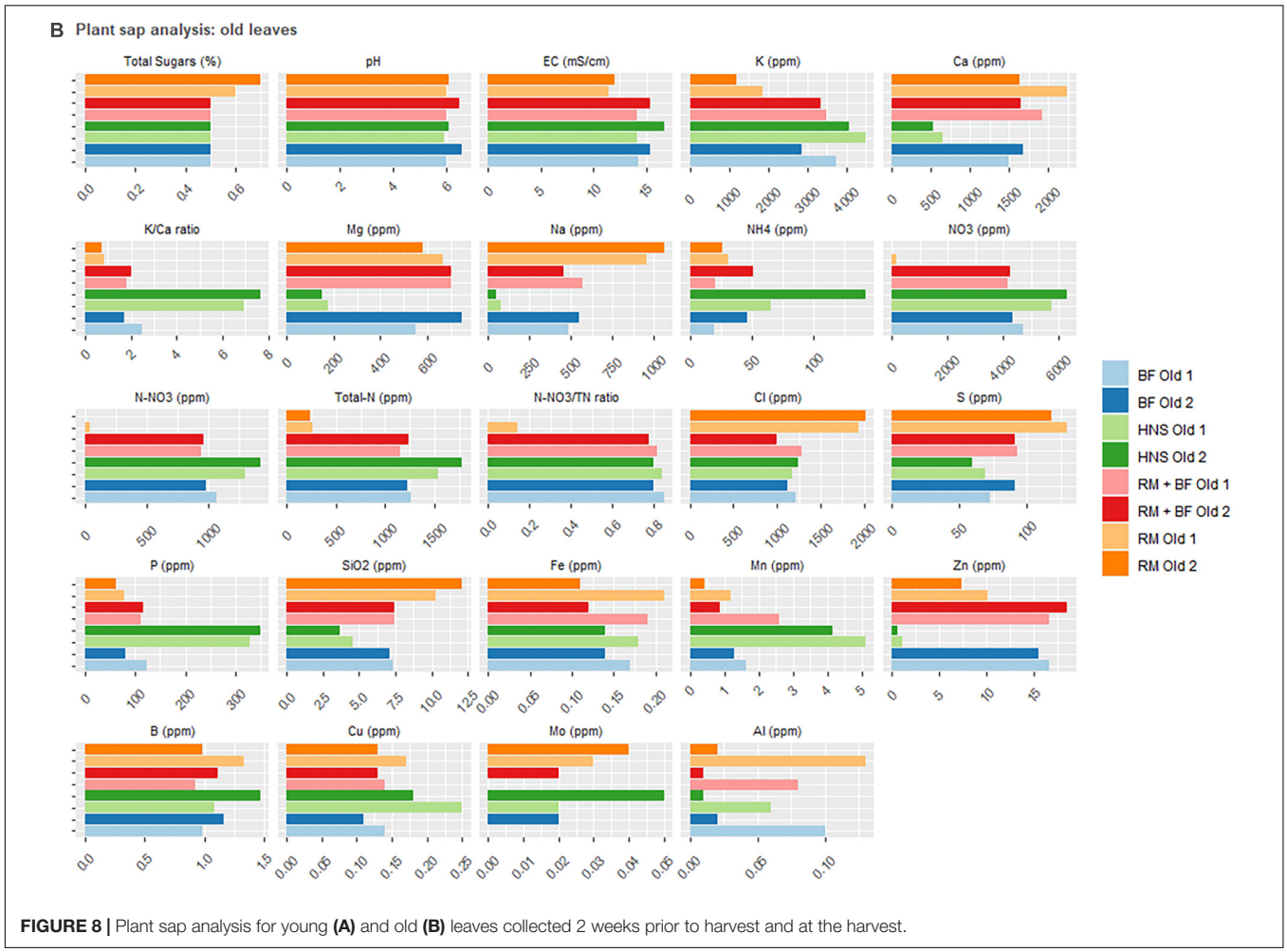

FIGURE 8 | Plant sap analysis for young **(A)** and old **(B)** leaves collected 2 weeks prior to harvest and at the harvest.

TABLE 3 | Ratio between fresh and dry weights for shoots and roots at harvest.

Treatment	Samples	Median fresh/dry ratio (%)	Mean fresh/dry ratio (%)
All treatments	Root weight	4.31	4.22
BF		4.29	4.52
HNS		2.73	2.88
RM		4.04	4.49
RM+BF		4.54	4.4
All treatments	Shoot weight	4.71	4.76
BF		4.78	4.8
HNS		4.26	4.44
RM		5.52	5.65
RM+BF		4.61	4.53

TABLE 4 | Phosphorus as percentage weight over the average dry weight for each treatment.

	Old leaves	Young leaves
Treatment	% P	% P
BF	1.5	2.1
HNS	4.9	3.4
RM	1.0	2.8
RM+BF	1.7	2.6

consistent disease symptoms such as excess intracellular moisture uptake, cell elongation, decreased total sugars content, and a weakening of the cell wall (Timmermans and Van De Ven, 2014). The commercial HNS was highly charged in both ammonium and nitrate (47× and 30× more concentrated than other treatments, respectively). While this disparity directly translated into higher ammonia and nitrate concentration in all plant sap samples, it led to only a slightly higher (1.7×) total nitrogen concentration, leaving N-NO_3^-/TN ratios to be similar to other treatments. Total nitrogen, calculated as the sum of inorganic and organic sources, is indicative of internal protein concentrations [about 85% of TN consists of protein (Barker and Pilbeam, 2015)]. Nitrogen needs were satisfied in all treatments except for RM, which displayed clear signs of N-deficiency both visually as described in the literature (Marschner, 1995; Barker and Pilbeam, 2015) and as well indicated plant sap analysis (**Figures 8A,B**).

Nitrate reductase requires Mo as a cofactor in the conversion of nitrates to amino acids, although it is difficult to assign a

FIGURE 9 | Plat sap analysis of the roots at harvest.

target threshold at which point this need is met. Barring potential blocking from other nutrients, this need appears to be satisfied when Mo nutrient solution concentrations exceed 0.06 mg/L as the case in across all treatments within this study. There was no significant difference in Mo concentrations in young leaves, however, HNS contained higher concentrations in old leaves and root samples (Rocha et al., 2020). None of the treatments appear to have suffered from Mo deficiency (<0.01 ppm).

Iron is also required along with Mo for healthy nitrogenase activity among other essential enzymatic functions (Marschner, 1995). While the hydroponic nutrient solution was 460× higher in Fe than other nutrient solutions in our treatments, the extra supplementation resulted in only a 1.6× increase (from 0.1575 ± 0.02 to 0.255 ± 0.05 ppm Fe) in young leaves, no significant difference in old leaves, and a decreased concentration in the roots compared to other treatments. It is important to note that we are unable to comment on the speciation (and thus the bioavailability) of iron. However, as the HNS control was prepared weekly and contained EDTA-chelated iron, we can at least maintain that iron was soluble and flowing through the roots. The reduction of iron from insoluble Fe^{III} to Fe^{II} occurs most commonly under anaerobic conditions. Recorded ORP values in the 200–400 mV range suggest that if this occurs, it is done in rhizospheric microenvironments or through the action of siderophore producing microorganisms (Barker and Pilbeam, 2015; Singh et al., 2019).

Magnesium is required for the production of chlorophyll at a Mg:N ratio of 1:4, and deficiencies can lead to nitrate hyperaccumulation (Schwartzkopf, 1972; Bousset et al., 2019). Despite the HNS having the highest concentration of Mg (14× greater than found in other treatments), young HNS leaves were deficient for Mg (<100 ppm), although this was not the case for older leaves. K concentrations in the HNS treatment reflected the priority given to this nutrient by commercial fertilizers (92× increase in the nutrient solution). RM plants, which had the lowest K concentration in young and old leaves, simultaneously had the highest total sugar percentage of all treatments, and thus were likely not symptomatic of K deficiency as described elsewhere (Haeder, 1974; Lawlor et al., 2004). The total sugars percentage is a widely used measure of plant health in terms

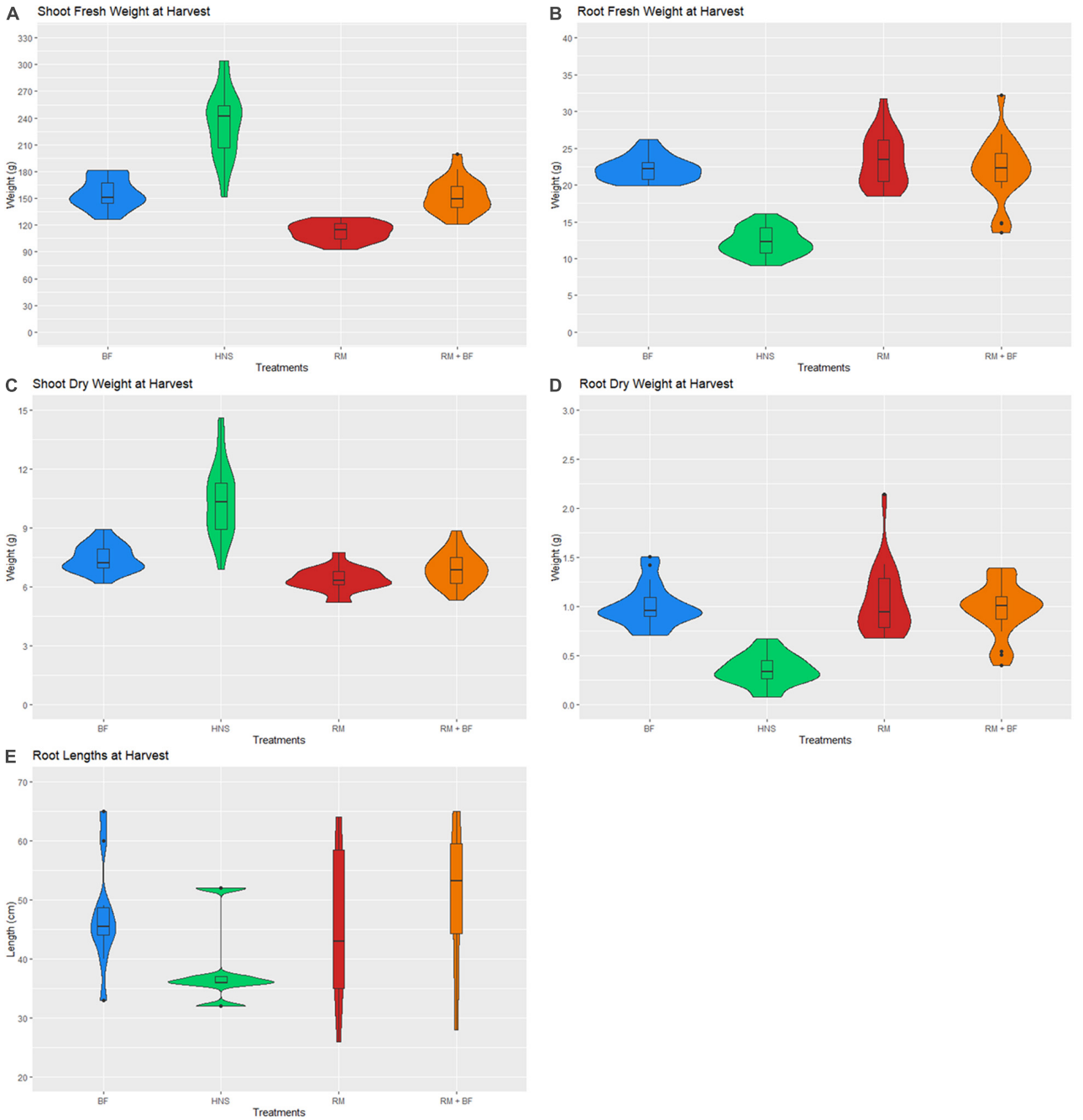

FIGURE 10 | Lettuce head fresh **(A,B)** and dry **(C,D)** weights for shoots and roots at harvest. Root lengths are depicted in **(E)**.

of biotic and abiotic stress resistance. No treatments were considered deficient in total sugars (<0.5%), however, there was much variability across treatments and sample types.

The counterbalance of K against Mg, Ca, and Na is a well-established example of nutrient blocking (Schwartzkopf, 1972; Timmermans and Van De Ven, 2014; Barker and Pilbeam, 2015). The HNS treatment was richest in K across all sample types (young and old leaves, roots) while containing the least amount of Mg, Ca, and Na compared to all other treatments (**Figures 8A,B**). This contrasts heavily to the available concentrations of K (<0.1 mmol/L in other treatments, 9.2 mmol/L for HNS), Mg (14× more concentrated in the HNS than other treatments), and Ca (29× more concentrated in the HNS than other treatments), although Na was in a similar range (0.5–2.2 mmol) across all treatments. A relative low concentration of Ca in the HNS treatment was observed across all sample types, despite an abundance (29× greater than other treatments) in the water supply,

however, this was not below recommended values for lettuce (Barker and Pilbeam, 2015).

Chloride levels were similar across the HNS, BF, RM+BF treatments for sample type (although varied significantly across sample type). RM Cl concentrations (6.9 mg Cl/g DW), likely elevated as a reaction to nitrate deficiency, were well below toxicity thresholds (>23.0 mg Cl/g DW) (Johnson et al., 1957; Van der Boon et al., 1990). Sulfur was not deficient for any of the treatments, although the HNS treatment had the least across all sample types. While a known relationship between S and N concentrations has already been established, it is not well understood how N-excess impacts S metabolism; synergistic effects with P and K uptake have been suggested (Barker and Pilbeam, 2015). Boron aluminum, and copper were not deficient for any treatment. Silicon, widely associated with disease suppression (Rodrigues and Datnoff, 2015), was deficient in HNS young leaves, with low values reported for BF and RM+BF young leaves. No deficiency was seen in other sample types.

Of all the nutrients, Mn was definitively deficient (<1.2 ppm) in all treatments except for the HNS for young leaves, and at the limit of deficiency for RM lettuce in their old leaves and roots. As Mn deficiency is associated with retarded growth, this may have played a role in the yield discrepancy (Hannam and Ohki, 1988). However, none of the common indications of Mn deficiency were visible across any of the treatments (Barker and Pilbeam, 2015).

Nutrient Concentration Comparison Between the Three Alternative Liquid Fertilizer Solutions

As the entire cultivation system (RAS-greenhouse coupling, with solids treatment) was in continuous operation, the hydroponic well nutrient concentrations (**Figure 5**) were considered representative of the concentrations that the plants were exposed to in the respective treatments. From this, we were able to determine the extent to which plants were able to satisfy their nutritional needs at these concentrations.

Condensing the above analysis across study results reveals a surprising set of trends. Firstly, all treatments were below the recommended threshold for iron, according to suggestions for instance by commercial companies routinely assessing hydroponic crop health (e.g., NovaCrop Control, Netherlands) who were used for the analysis and conduct testing for the well-established Dutch hydroponics industry. The topic of iron supplementation in aquaponics was reviewed recently by Kasozi et al. (2019), who highlighted the lack of consensus around optimal concentrations for vegetal and fruit-bearing plants (Kasozi et al., 2019). Nonetheless, it remains perplexing that the EDTA-chelated, highly concentrated, commercial iron solution was not capable of increasing vegetal iron concentrations. In the aquaponic systems, however, the story of iron is more complicated. On the one hand, iron was not detected in any of the three RAS, nor the aquifer environment (**Figure 4**). The anaerobic fermenter had elevated iron concentrations at levels that would have been sufficient for plant needs (0.83 mg/L), however, these concentrations were not maintained in the effluent nor nutrient solution wells. Ultimately, the similarity of all four treatments in the plant sap concentrations suggests that much work needs to be done in understand iron solubilization dynamics, whether the iron needs of plants are being satisfied, and how the rhizosphere can be better recruited to fulfill this demand.

RM was manly deficient in two macronutrients, K and N, as well as the micronutrient B. The BF lettuce alone were deficient notably in P, but as well B as per NovaCrop Control guidelines. All of these requirements were met in the RM+BF treatment, suggesting that the proposed solid waste treatment system has significant potential to address plant nutritional needs. The HNS control suffered from some unique deficiencies, namely of Mg and Ca in young leaves, as well as Na and Si in both young and old leaves. On the other side of the spectrum, all other treatments suffered from Mn deficiency. Thus, while iron supplementation remains an open question, Mn must definitely be supplied in aquaponics given the insufficient access to the micronutrient through the fish feed. Likely, there is an ideal nutrient supplementation level greater than the baseline concentrations established here. Whether this demand will be satiated by an expanded solids treatment system alone will need to be established in future studies.

Sizing Up the Solids Treatment System to Match Aquaponic Needs

This study investigated whether an in-line, EBPR-inspired solids treatment system could improve nutrient remineralization while removing excess carbon and nitrogen from the system. These trends were demonstrable; however, it is likewise obvious that the efforts were insufficient to satiate all plant micro-nutrient needs.

On average, the solids treatment system resulted in a 12× removal of total COD between the anaerobic fermenter (influent) and the hydroponics unit (effluent). Although this value does not account for solids removed from the system for SRT control, it is a considerable reduction. Considering the 450 kg tons of fish in the system producing ca. Forty-five kilogram dry weight solids with a theoretical average P of 23.9 mg/g dry weight (Schumann and Brinker, 2020), the solids treatment system encountered a theoretical P load of 1.08 kg. This resulted in ca. Forty-four milligram P was provided to the greenhouse daily from the ca. 1.85 g of sludge from the anaerobic digester passing through the SBR daily. While a P uptake requirement for the plants in not possible to define here, scaling up the SBR threefold would at least provide a daily discharge of around 150 ppm, a reasonable target concentration for plant P demand.

Yields Comparison

Across all treatments, an average of ca. Ninety-five percent of the total weight (shoot and root) consisted of water. A notable exception to this rule were the HNS roots, which were ca. Ninety-seven percent water (**Table 3**). While from a mass yield perspective it is not desirable to increase the relative amount of root mass compared to marketable vegetal biomass, the essential role of the rhizosphere in plant nutrient acquisition and stress tolerance cannot be neglected. Rhizophagy has been identified as a principal mechanism for nutrient

acquisition and microbial shepherding by plants, a topic that is well reviewed in current literature (Paungfoo-Lonhienne et al., 2013; White et al., 2018; Ameer and Hussein, 2020). In addition to nutrient uptake, endophytic microorganisms are now understood to be crucial to several fundamental plant functions (growth and development, oxidative stress reduction, disease and predation prevention) (Chaudhary et al., 2017; Patel et al., 2018; White et al., 2019). Plants that naturally grow in soil-less environments (e.g., bare rock) are particularly reliant on a diverse and well-developed endophytic community, which may suggest similar patterns in hydroponic cultivation systems (Burghelea et al., 2015; Remiszewski et al., 2016; Burghelea et al., 2018). Ignoring the role of the rhizosphere is ignoring a fundamental plant organ (Lynch and Whipps, 1990; Yang and Crowley, 2000; Lynch and de Leij, 2001; Richardson et al., 2009; Garcia and Kao-Kniffin, 2018; Guyonnet et al., 2018; Zhalnina et al., 2018). In this context, the differences in root length and mass between the control and other treatments suggest an underexplored contribution of the rhizosphere to nutrient uptake in hydroponic cultivation (**Figure 10**).

The impact of microbially-suppressing agrochemicals strongly diminishes and shifts the rhizosphere community, with effects on both the effective bioavailability of nutrients and the rhizospheric reserves available to plants (Rengel and Marschner, 2005; Chen et al., 2019; Singh et al., 2019; Huang et al., 2020). An inhibited exchange of organic acids and nutrients between plants and their rhizosphere has been shown to engender drastic effects on nutrient-recycling and secondary metabolites (impacting taste, antioxidant capacity, etc. of the crop). These changes have been described in soil systems although contextualization in the hydroponic context is lacking (Mozafar, 1993; Massalha et al., 2017; Garcia and Kao-Kniffin, 2018; Guyonnet et al., 2018; Singh et al., 2019). The onset of mold in nearly a third of the HNS treatment lettuce, but not in other treatments, suggests that even while the plants obtained a better mass yield, they were potentially compromised in other aspects. Whether this could be linked to nutrient deficiencies [e.g., Si deficiency in young leaves has been linked to increased disease susceptibility (Etesami and Jeong, 2018)] or whether it is the result of a diminished rhizosphere community, was not confirmed in this experiment, but is worthy of further investigation.

CONCLUSION

Fundamentally, the challenge of closed environment agriculture is one of resource-use optimization. The exploitation of readily available, soluble aquaculture effluent expanded our conception of nutrient transfer in the hydroponic environment to include the role of microorganisms and the rhizosphere. Nutrient remineralization has not been adopted as unanimously mainly due to the challenges and carbon reduction and the additional costs associated with existing waste revalorization systems. This study contributes to the field by presenting a novel strategy for solids treatment to this base inspired from EBPR processes found in municipal wastewater treatment plants. This system permits simultaneous waste treatment (C- and N-reduction) with low residual biomass generation and a diverse trace nutrient spectrum for downstream hydroponics cultivation. To gauge the impact of the nutrient streams on agricultural yield and quality, we did not supplement for deficient nutrients. This strategy provided a unique perspective into the ability of the hydroponic crops to take up aqueous nutrients.

For this investigation, the micronutrient profiles of the remineralized effluent, traditional coupled aquaponics, and a commercial hydroponic nutrient solution were measured. Nutrient concentrations diverged significantly between the aquaculture-derived treatments and the commercial solution, which eclipsed other treatments for virtually every measured element in the water column. In contrast, plant sap analysis did not reflect a universally higher nutrient content in lettuce grown under excessive nutrient conditions.

Lettuce grown in the commercial HNS likewise experienced deficiencies of Mg and Ca (young leaves) as well as Na and Si (both young and old leaves). Uptake of certain elements (Cu, Fe, Mg, S, and Zn) was greater across aquaponic treatments than initially predicted, however, Mn was universally absent from aquaponic treatments. B and P were especially low in the standard aquaponics treatment (fertilization with soluble RAS nutrients only). Together this suggests that the solids treatment system in parallel to RAS soluble effluent may be advantageous for aquaponic facilities seeking to maximize the benefits of the fish solids for plant nutrition.

Nonetheless, iron remains the most capricious element to provide for plants. The evidence that neither the commercial solution, nor aquaponic treatments was wholly successful in increasingly iron uptake, suggest a need for future studies to determine minimal "optimal" concentrations for plants and as well the real repercussions of deficiencies on crop yield and nutritional quality.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AJ and VL developed the theoretical formalism of the study. VL carried out the experiment with support from LL, DC, and PP. VL performed the data analysis and wrote the manuscript with support from AJ. The final version of the manuscript received input from all authors. All authors contributed to the article and approved the submitted version.

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Effect of LED Spectrum on the Quality and Nitrogen Metabolism of Lettuce Under Recycled Hydroponics

Jie Li[1,2,3], Tao Wu[1,2,3], Ke Huang[1,2,3], Yubing Liu[1,2,3], Mingyue Liu[1,2,3] and Junwei Wang[1,2,3]**

[1] College of Horticulture, Hunan Agricultural University, Changsha, China, [2] Engineering Research Center for Horticultural Crop Germplasm Creation and New Variety Breeding, Ministry of Education, Changsha, China, [3] Key Laboratory for Vegetable Biology of Hunan Province, Changsha, China

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***Correspondence:**
Mingyue Liu
liumingyue58@126.com
Junwei Wang
JunweiWang87@126.com

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Light quality optimization is an efficient method for improving the growth and quality of lettuce in plant factories. In this study, lettuce seedlings were illuminated under different light-emitting diode (LED) lights, namely, red-blue (RB), red-blue-green (RBG), red-blue-purple (RBP), and red-blue-far-red (RBF) LED lights, to investigate the effect of light quality on growth, quality, and nitrogen metabolism. The combination of 75% red and 25% blue light was set as the basic light source, and 20% of green, purple and far-red light were added to basic light source, respectively. All the treatments were set to 200 μmol m^{-2} s^{-1}. Results showed that the fresh weight and dry weight of aboveground lettuce under RBG, RBP, and RBF treatments were significantly lower than those under the RB treatment because of the decrease in the effective photon flux density for chlorophyll absorption. The vitamin C content of the lettuce leaves was increased by about 23% with the addition of purple light. For nitrate reduction, the addition of green light significantly increased the nitrite content of the lettuce leaves. It also promoted the reduction from nitrite to ammonium through the activation of the nitrite reductase (*NiR*) expression and enzyme activity. The nitrate and ammonium content decreased with the addition of purple light because of the inhibited *NR* and *NiR* expression and enzyme activity. For nitrogen assimilation, individual (e.g., Asp, Glu, and Leu) and total amino acids were induced to increase by adding green, purple, and far-red light. The addition of light was hypothesized to have inhibited protein biosynthesis, thereby causing the accumulation of amino acids. Correlation analysis showed that the relative expression levels between *HY5* and *NR*/*NiR* presented a significantly negative correlation. Transcription factor HY5 might mediate the regulation of light quality on nitrogen metabolism by inhibiting *NR* and *NiR* expressions. It might also exert a negative effect on nitrate reduction. Further studies via genome editing techniques on the identification of HY5 functions for nitrate assimilation will be valuable. Nevertheless, the results of this work enrich the understanding of the effect of light quality on nitrate metabolism at the level of gene expression and enzyme activity.

Keywords: lettuce, LED, quality, nitrogen reduction, nitrogen assimilation

INTRODUCTION

With the rapid development of greenhouse agriculture, plant factory production and greenhouse light supplementation have become key technologies in protected horticulture production. Hydroponics in plant factories offers numerous advantages, such as high yield, good quality, continuous production, efficient resource utilization, and avoidance of the impacts of environment change; it has been used to solve the unstable vegetable supply and the pollution of land resources (Viršilë et al., 2020; Zou et al., 2020). However, hydroponics production can cause the accumulation of nitrate nitrogen in plants, especially in leaf vegetables, such as lettuce (*Lactuca sativa L.*) (Rouphael et al., 2018). Excessive nitrate can be converted into nitrite, which can cause methemoglobinemia and carcinogenic nitrosamine in the human body (Chan, 2011). Lettuce is one of the main vegetable crops produced by hydroponics in plant factories, and it is widely consumed in its raw state because of its taste and high nutritional value. However, lettuce is a hyperaccumulator of nitrates, that is, it easily accumulates high nitrate content in its leaves (Bian et al., 2018). In accordance with the division of nitrate content in fresh vegetables, researchers have found that the nitrate content of lettuce is ≥2,500 mg/kg of its fresh weight (FW). Such content reflects the vegetable's excessive nitrate accumulation. Therefore, an important issue is to control the nitrate content of lettuce within a reasonable range.

The physiological theory of the excessive accumulation of nitrate in vegetables can be attributed to the rate of nitrate absorption by vegetables being greater than the rate of nitrate assimilation. Nitrate is one of the most abundant nitrogen (N) sources in natural and agricultural systems, and it is the main form of nitrogen absorbed by plants (O'Brien et al., 2016). Nitrate could be reduced to ammonium through nitrate reductase (NR) and nitrite reductase (NiR). NR catalyzes the reduction of nitrate to nitrite in plants. Then nitrite is reduced to ammonium under the action of NiR. Ammonium is assimilated into organic nitrogen under the action of the glutamate synthase (GOGAT) and glutamine synthetase (GS) cycle and glutamate dehydrogenase (GDH) pathway. Several strategies can be used to reduce excessive nitrate accumulation through the regulation of enzymatic activities and gene expression-related nitrogen metabolism. Recently studies have focused on the optimization of supplemental light and the effective ways to regulate nitrate accumulation in lettuce in plant factories (Hytönen et al., 2017; Viršilë et al., 2019; He et al., 2021).

For green plants, light is an important environmental factor for life and is involved in the regulation of growth, morphology, and metabolism (Viršilë et al., 2020; Samuolienë et al., 2021). Light-emitting diodes (LEDs) are new illuminants with advantageous properties, including energy conservation, long life, small size, light weight, and high light efficiency. These advantages make LEDs a perfect light source for artificially regulating crop growth and development (Amoozgar et al., 2017; Ferreira et al., 2017). Recent studies have shown that the combination of red and blue light serves as a highly efficient light source for promoting lettuce growth (Chen et al., 2016; Amoozgar et al., 2017). The addition of certain green, purple, far-red, and UV-C light could affect the content of chemical substances in lettuce, such as nitrate, nitrite, vitamin C (Vc), amino acid content, and antioxidant capacity (Naznin et al., 2019; Riga et al., 2019; Viršilë et al., 2020; He et al., 2021). For example, the addition of green light relative to red and blue LED significantly increases NR, NiR, GOGAT, and GS activities (Bian et al., 2018). Purple light could significantly reduce nitrate accumulation and the activities of N metabolism-related enzymes, such as NR, NiR, GS, and GOGAT (Zhang et al., 2018). In sum, light quality is an important factor in the effective regulation of the growth, quality, and nitrate accumulation of lettuce in plant factories. Moreover, green, purple, and far-red light are often used as supplemental light sources and are added to basic light sources, such as white light or a combination of red and blue light.

In response to environmental changes, plants have developed a suite of photoreceptors, such as phytochromes (phy) and cryptochromes, to monitor light availability and quality. Specifically, phy are sensitive to irradiation by red and far-red light, and they uniquely function by measuring the relative quantity of each of these wavelengths (Rockwell et al., 2006). Higher plant genomes encode a suite of phytochrome proteins, including phyA, phyB, phyC, phyD, and phyE. Cryptochrome is the receptor of blue or UV-A light and encodes genes (*CRY1* and *CRY2*) in plants. Previous reports have shown that the absorption of light by different photoreceptors leads to the modulation of the core COP1/SPA complex, which further regulates downstream transcription factors to adjust plant growth and development (Gangappa and Botto, 2016; Kim et al., 2017; Roeber et al., 2021). Transcription factor HY5 (LONG HYPOCOTYL5) is a central regulator via the COP1/SPA complex, and it can be activated by photoreceptors to promote photomorphogenesis downstream to phytochromes, cryptochromes, and UV-B photoreceptors (Gangappa and Botto, 2016). However, whether HY5 mediates the regulation of light quality on the activity and expression of the NR gene in lettuce has yet to be fully studied.

According to previous research, the combination of red and blue light serves as a high-efficiency light source to promote lettuce growth. Therefore, using the combination of red and blue LEDs light as the basic light source, we investigated the effect of adding green, purple, or far-red light to basic light sources on the growth, quality, and nitrogen metabolism of lettuce. The mechanism of light quality on nitrogen metabolism was discussed on the basis of the expression level of genes related to light response and nitrogen metabolism. The results of this work should serve as a reference for supplemental light optimization in lettuce production and enable a good understanding of the effect of light quality on nitrate assimilation on the basis of the level of gene expression and enzyme activity.

MATERIALS AND METHODS

Plant Material

Experiments were conducted in an artificial climate chamber at Hunan Agriculture University (Latitude: 27.55°N, Longitude: 113.92°E), Changsha, China, from 2 May 2019 to 28 July 2019. After soaking and germination, lettuce (*Lactuca sativa*

var. cv Yidali, from Clover Seed, Co., Ltd., China) seeds were sown in rock wool (25 mm × 25 mm × 40 mm) and placed in artificial climate chamber. The climate chamber measured 4,050 mm (length) × 2,218 mm (width) × 3,000 mm (height), and comprised six three-layers shelves and a temperature, relative humidity and LED illumination control system. The growth conditions of lettuce were set as follows: white light with 12 h photoperiod (200 mmol m^{-2} s^{-1}), 25°C (daytime)/18°C (night), and 75% relative humidity. When the first true leaf was unfolded, a half unit of the garden test nutrient solution (**Supplementary Table 1**) was used (Hori, 1966). After the second true leaf was fully unfolded, seedlings on the same site were transplanted into hydroponic tanks (1,800 mm × 650 mm × 100 mm) containing half-strength garden test nutrient solution. The nutrient solution was set as follows: EC of 1.2 dS/m, pH of 6.4, depth of 8 cm, and continuously aeration with an air pump at an interval of 20 min to maintain the dissolved oxygen at 8.0 ± 0.2 mg L^{-1}. The nutrient solution was recycled every 7 days.

After 5 days of preculture under the white LED illumination with 12 h photoperiod (200 mmol m^{-2} s^{-1}), the seedlings were subjected to four experimental illumination treatments. Four light treatments were as follows: (1) red-blue (RB), 75% red + 25% blue LED light; (2) red-blue-green (RBG), 60% red + 20% blue + 20% green LED light; (3) red-blue-purple (RBP), 60% red + 20% blue + 20% purple LED light; (4) red-blue-far-red (RBF), 60% red + 20% blue + 20% infrared LED light. The light intensity of all the treatments was set to 200 ± 10 mol m^{-2} s^{-1}. The LED light source (T8) was from Shanzai Agriculture and Forestry Technology Co., Ltd., Zhongshan, China. Photon fluxes were measured with a spectroradiometer (PLA-20, Everfine Optoelectronic Information Co., Ltd., Hangzhou, China). The light intensity was adjusted on the basis of the distance between the light source and the lettuce canopy. The center wavelength of each light was as follows: red light, 660 nm; blue light, 440 nm; green light, 530 nm; purple light, 410 nm; infrared light, 750 nm. The spectral values of the four treatments are shown in **Figure 1**.

The seedlings were organized in a complete randomized block design with three replicates per treatment, for a total of 180 seedlings in the four treatments (45 seedlings per treatment). After 25 days of transplanting, the lettuce plants under different treatments were sampled, and the related indexes were determined. The sample parts were immediately frozen in liquid nitrogen and stored at −80°C until analysis.

Measurement of Lettuce Biomass and Chlorophyll Content of Leaves

At 25 days after treatment, the lettuce was harvested. The plants' shoot FW and dry weight (DW) were examined using an electronic balance. Five plants were then randomly selected from each replicate, for DW deactivated at 105°C, and then dried at 75°C for 72 h.

Chlorophyll content was measured calorimetrically according to Holden's method. Briefly, 0.2 g of fresh leaves was dipped in 10 mL of a mixture of acetone, ethanol, and water (4.5:4.5:1, v/v/v) until they turned white to extract the chlorophyll a and b content. The absorbance of the extract liquor was determined using an ultraviolet spectrophotometer at 645 nm (OD_{645}) and 663 nm (OD_{663}). The chlorophyll content was calculated as follows: chlorophyll a content (mg/g) = 12.7 × OD_{663} − 2.69 × OD_{645}; chlorophyll b content (mg/g) = 22.9 × OD_{645} − 4.86 × OD_{663}

Measurement of Lettuce Quality

The contents of soluble sugar, soluble protein, and Vc of the lettuce leaves were determined using kits (Shanghai ZCIBIO Technology Co., Ltd., Shanghai, China). The measurement of soluble sugar was based the Anthrone method (Liu et al., 2011). Exactly 0.1 g of samples was mixed with 1 mL distilled water and ground into a homogenate, soaked in a 95°C water bath for 10 min, and then centrifuged at 25°C for 10 min. The supernatant was diluted to 10 mL (distilled water). Soluble sugar was extracted with the prepared solution in the kit, and the absorbance was measured at 620 nm. The measurement of soluble protein and Vc was based on the Coomassie brilliant blue colorimetry method (Bradford, 1976) and 2,6-dichlorophenol-indophenol method (Nielsen, 2017). The absorbance of soluble protein determined using the ultraviolet spectrophotometer was 595 nm.

Measurement of Nitrate, Nitrite, Ammonium, and Amino Acids Contents

Fresh samples collected from the third-young, fully expanded leaves were used to determine the nitrate, nitrite, and ammonium content. Nitrate was extracted and quantified in accordance with a previously described method (Cataldo et al., 1975) with minor modifications. The principle is that nitrate reacts with salicylic acid to produce nitrosalicylic acid. The absorbance was determined to be 410 nm by using the ultraviolet spectrophotometer. Nitrite was extracted and determined in accordance with a previously described method (Stevens and Oaks, 1973). Briefly, the leaf sample was homogenized using sulfanilamide and *N*-(1-Naphthyl)-ethylene-diamine dihydrochloride. The absorbance was determined to be 550 nm by using the ultraviolet spectrophotometer. Ammonium was extracted and determined in accordance with a previously reported method (Solórzano, 1969). The principle is that ammonium reacts with ninhydrin to produce a blue compound. The absorbance was determined to be 580 nm by using the ultraviolet spectrophotometer.

Free amino acids were assayed in accordance with a previously reported method given by Shu et al. (2016). In brief, 0.1 g of dry samples was used to extract free amino acids by utilizing HCl at 110°C for 24 h. The cooled hydrolysate was filtered, dissolved, and evaporated under vacuum. The final dry matter was dissolved in citrate buffer (67 mM, pH = 2.2) and used for analysis. The content of free amino acid was determined with an automatic amino acid analyzer (Hitachi L-8900, Japan).

Assay of Enzyme Activity of Nitrogen Metabolism

The NR activity was determined according to the method reported by Yaneva et al. (2002). Briefly, 0.5 g of fresh leaf was

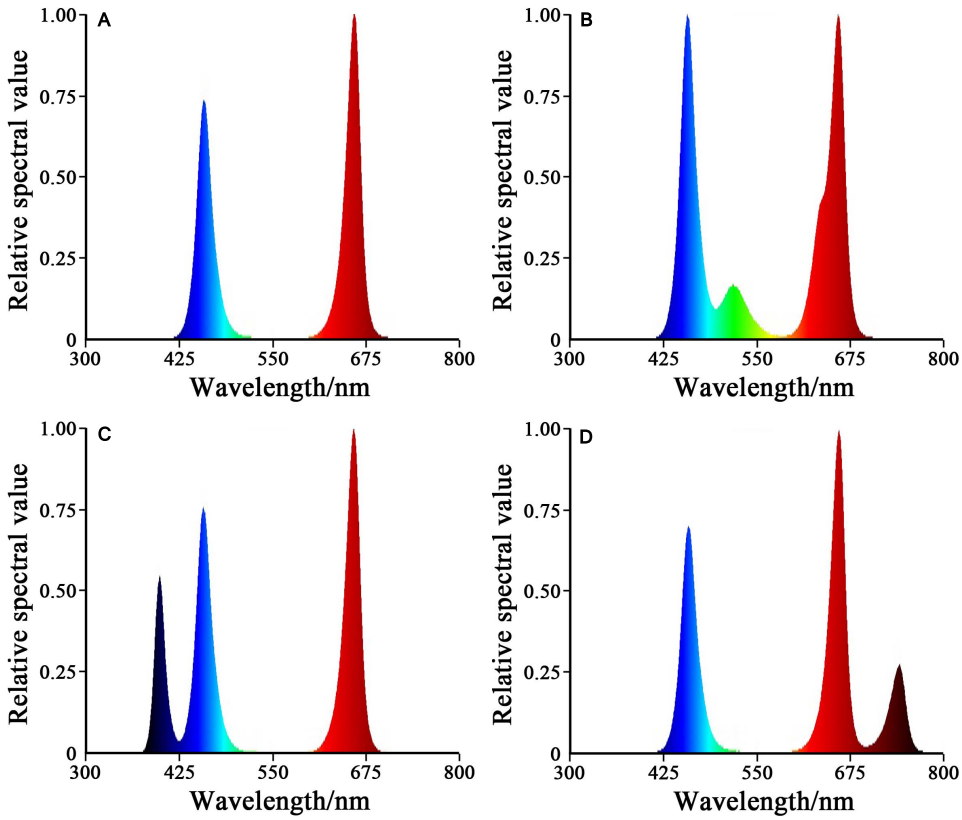

FIGURE 1 | Relative spectral value of four treatments. **(A)** RB, 75% red + 25% blue LED. **(B)** RBG, 60% red + 20% blue + 20% green LED. **(C)** RBP, 60% red + 20% blue + 20% purple LED. **(D)** RBF, 60% red + 20% blue + 20% infrared LED.

used to extract NR by utilizing 15 mL of phosphate (0.1 M, Ph = 7.5) at 4°C for 15 h. The supernatant was added to a reaction solution containing 200 μM KNO_3 and 0.2 μM NADH. The amount of catalyzed production of 1 μmol NO_2^- per gram of fresh sample per hour was regarded as one NR activity unit. The NiR activity was determined by the method given in the literature (Datta and Sharma, 1999). Enzyme was extracted with 50 mM phosphate buffer (containing 1 mM EDTA, 25 mM cysteine, and 3% BSA; pH = 8.8). In the reaction, 50 mM $NaNO_2$ was the reaction substrate, and 5 mM methyl viologen was the electron donor. The unit of NiR activity was defined as the molar mass of NO_2^- reduced per gram of leaf per minute.

The method described by Cánovas et al. (1991) was used for GS activity measurement, and the method by Singh and Srivastava (1986) was used for GOGAT activity measurement. Enzymes were extracted with 50 mM phosphate buffer (2 mM EDTA, 2 mM dithiothreitol, 1% insoluble polyvinylpyrrolidone, and 1.5% soluble casein, pH = 7.5). The supernatant was used for the GS and GOGAT activity assay. The activity of GS was expressed as μmol γ-glutamylhydroxamate formed per gram per minute. The GOGAT activity was defined as μmol NADH oxidized per gram per min. GDH was extracted using assay kits (Shanghai ZCIBIO Technology Co., Ltd., Shanghai, China), and the activity was calorimetrically measured at 340 nm by the ultraviolet spectrophotometer. The number of moles of NADH consumed per minute was defined as the unit of enzyme activity.

RNA Extraction and Genes Expression Analysis

Total RNA was extracted from fresh lettuce (0.1 g) by using the RNA prep Pure Plant Kit: Polysaccharides and Polyphenolics-rich (Tiangen, Beijing, China) in accordance with the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA by using an MMLV reverse transcriptase kit (Transgen, China) in accordance with the manufacturer's instructions. For the fluorescent quantitative PCR analysis of the gene expression, the sequences of *NR*, *NiR*, *GS*, *GOGAT*, *GDH*, *phyA*, *phyB*, *phyE*, *CRY1*, and *HY5* were amplified with primers designed on the basis of the sequences obtained from NCBI (**Supplementary Table 2**). The expression data were analyzed with the ΔΔCt method as previously described (BioRad Real-time PCR Application guide) (Kenneth and Thomas, 2002).

Statistical Analysis

Experimental data were processed with GraphPad Prism 5. Duncan's multiple comparison tests at $P < 0.05$ level of significance was used to conduct one-way ANOVAs. Principal

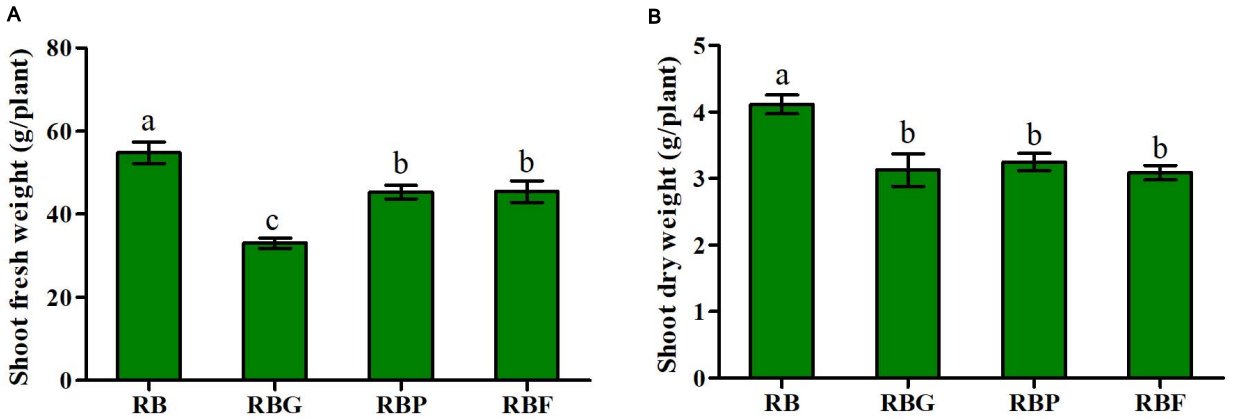

FIGURE 2 | Effects of spectra on shoot fresh weight **(A)** and dry weight **(B)** in lettuce. RB, 75% red + 25% blue LED; RBG, 60% red + 20% blue + 20% green LED; RBP, 60% red + 20% blue + 20% purple LED; RBF, 60% red + 20% blue + 20% infrared LED. The letters represent the significant difference of different treatments ($p < 0.05$).

component analysis (PCA) was used to calculate the correlation between indicators and the comprehensive effect of different light conditions on the nitrogen metabolism of lettuce.

RESULTS AND ANALYSIS

Effect of LED Illumination Spectra on Lettuce Biomass

The FW and DW of aboveground lettuce under the RBG, RBP, and RBF treatments were significantly lower than those under the RB treatment (**Figures 2A,B**). Relative to those under the RB treatment, the FW values under the RBG, RBP, and RBF treatments decreased by 39.7, 17.2, and 17.1%, respectively. Meanwhile, the DW values under the RBG, RBP, and RBF treatments decreased by 24.0, 21.2, and 25.0%, respectively. The chlorophyll content showed an insignificant change when green, purple, or far-red light was added to the red and blue light combination. The values of chlorophyll a, b, and a + b in the lettuce leaves showed a more significant increase under the RBP and RBF treatments than under the RB treatment. Meanwhile, the values of chlorophyll a and a + b under the RBG treatment showed a more significant decrease than those under the RB treatment (**Table 1**).

Effect of LED Illumination Spectra on Lettuce Quality

The soluble sugar content of lettuce leaves under the RBG and RBF treatments was significantly lower than that under the RB treatment, indicating a decrease of 20.9 and 18.2%, respectively (**Table 1**). The soluble protein content of lettuce leaves decreased more significant under RBG, RBP, and RBF light than that under the RB treatment. The percentage of decreased value was, respectively, 32.9, 23.3, and 48.1%. Relative to that under RB light, the Vc content of lettuce leaves under RBP light increased by about 23%. It decreased slightly under the RBG and RBF conditions relative to that in the RB treatment, but it did not reach the significance level.

Effect of LED Illumination Spectra on Nitrogen Reduction and Assimilation in Lettuce Leaves

The content of nitrate, nitrite, and ammonium in the lettuce shoots were shown in **Figure 3**. A significant decrease in nitrate content was determined under the RBP treatment compared with RB treatment. No significant difference was noted among the RB, RBG, and RBF treatments (**Figure 3A**). Similarly, ammonium content decreased by 18% under the RBP treatment relative to that under the RB treatment. A slight increase was determined

TABLE 1 | Effects of spectra on the quality of lettuce under different LED treatments.

Light treatments	Chl a (mg/g)	Chl b (mg/g)	Chl a + b (mg/g)	Soluble sugar (mg/g FW)	Soluble protein (mg/g FW)	Vitamin C (mg/kg FW)
RB	0.446 ± 0.026c	0.120 ± 0.015b	0.566 ± 0.034c	5.73 ± 0.28a	13.17 ± 0.62a	118.8 ± 8.64ab
RBG	0.319 ± 0.019d	0.120 ± 0.034b	0.439 ± 0.011d	4.53 ± 0.32b	8.84 ± 0.64bc	110.4 ± 7.59b
RBP	0.734 ± 0.006a	0.210 ± 0.028a	0.944 ± 0.021a	5.00 ± 0.12ab	10.10 ± 0.82b	146.3 ± 9.91a
RBF	0.585 ± 0.027b	0.173 ± 0.016a	0.758 ± 0.054b	4.69 ± 0.13b	6.830 ± 0.36c	104.7 ± 9.00b

RB, 75% red + 25% blue LED; RBG, 60% red + 20% blue + 20% green LED; RBP, 60% red + 20% blue + 20% purple LED; RBF, 60% red + 20% blue + 20% infrared LED. The letters represent the significant difference of different treatments ($p < 0.05$).

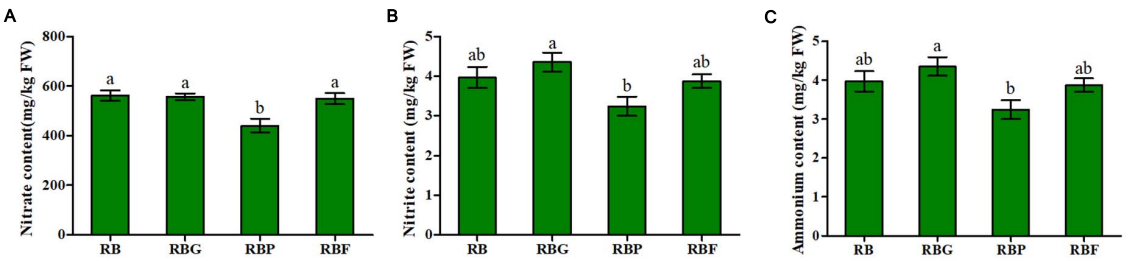

FIGURE 3 | Effects of spectra on nitrate **(A)**, nitrite **(B)**, and ammonium content **(C)** in lettuce leaves. RB, 75% red + 25% blue LED; RBG, 60% red + 20% blue + 20% green LED; RBP, 60% red + 20% blue + 20% purple LED; RBF, 60% red + 20% blue + 20% infrared LED. The letters represent the significant difference of different treatments ($p < 0.05$).

under the RBG treatment relative to RB treatment (**Figure 3C**). As for the nitrite content change of the lettuce leaves under RBG, RBP, and RBF light, it did not reach a significant level relative to that under the RB light (**Figure 3B**). The RBG treatment exerted a positive effect on the increase of nitrite content in lettuce leaves, achieving a 9.8% increase relative to that under RB light, while about 18.4% decrease under RBP light.

As shown in **Table 2**, 16 types of amino acids were analyzed by the amino acid analyzer. Overall, the contents of Asp, Glu, and Leu in lettuce leaves were relatively high, accounting for more than 35% of the total free amino acids identified. The Met content was the lowest, accounting for only approximately 1% of the total number of amino acids. Relative to those under RB light, the contents of the 16 types of amino acids under RBG and RBP light sources increased to varying degrees, thereby causing the total free amino acid content of lettuce to increase by 19.9 and 25.1%, respectively. The content of Glu under RBG, RBP, and RBF light significantly increased by 31.8, 27.1, and 23.7%, respectively, relative to that under RB light. In particular, the RBG light source resulted in the highest Glu content.

Effect of LED Illumination Spectra on Enzyme Activity of Nitrogen Metabolism

The activities of NR, NIR, GS, GOGAT, and GDH in lettuce leaves can be significantly affected by light quality. As shown in **Figure 4A**, relative to the activity of NR in lettuce under the RB light, that under the RBP light significantly decreased by 35.8%, whereas no significant changes were observed under the RBG and RBF treatments. A similar result was observed in NiR activity, which decreased by 34.8% under RBF light relative to that in the RB treatment (**Figure 4B**). As indicated by the results in **Figures 4C–E**, the quality of RBG light quality showed an inhibition effect on GS, GOGAT, and GDH activities

TABLE 2 | Effects of spectra on content (mg/g DW) of free amino acids in lettuce leaves.

Amino acids names	RB	RBG	RBP	RBF
Asp	1.575 ± 0.014c	1.980 ± 0.006a	1.970 ± 0.010a	1.697 ± 0.007b
Thr	0.720 ± 0.005c	0.830 ± 0.006b	0.897 ± 0.003a	0.717 ± 0.007c
Ser	0.455 ± 0.009d	0.537 ± 0.003b	0.590 ± 0.006a	0.487 ± 0.007c
Glu	1.800 ± 0.006d	2.373 ± 0.007a	2.287 ± 0.009b	2.227 ± 0.012c
Gly	0.880 ± 0.017c	1.027 ± 0.003b	1.100 ± 0.006a	0.887 ± 0.007c
Ala	0.970 ± 0.003c	1.147 ± 0.003b	1.210 ± 0.010a	0.977 ± 0.007c
Val	1.165 ± 0.009c	1.313 ± 0.003b	1.383 ± 0.003a	1.117 ± 0.007d
Met	0.165 ± 0.014a	0.173 ± 0.013a	0.180 ± 0.003a	0.110 ± 0.003b
Ile	0.895 ± 0.009c	1.053 ± 0.003b	1.110 ± 0.006a	0.887 ± 0.007c
Leu	1.475 ± 0.014c	1.710 ± 0.006b	1.820 ± 0.010a	1.450 ± 0.010c
Tyr	0.535 ± 0.003c	0.627 ± 0.012b	0.677 ± 0.009a	0.470 ± 0.006d
Phe	1.015 ± 0.014c	1.190 ± 0.003b	1.257 ± 0.009a	1.000 ± 0.006c
Lys	1.060 ± 0.035c	1.283 ± 0.012b	1.377 ± 0.007a	1.097 ± 0.007c
His	0.340 ± 0.006b	0.420 ± 0.003a	0.430 ± 0.006a	0.343 ± 0.003b
Arg	0.835 ± 0.003d	1.017 ± 0.007b	1.093 ± 0.003a	0.853 ± 0.003c
Pro	0.820 ± 0.006c	0.950 ± 0.006b	1.017 ± 0.003a	0.830 ± 0.006c
Free amino acids	14.705 ± 0.101d	17.630 ± 0.061b	18.397 ± 0.092a	15.147 ± 0.091c

RB, 75% red + 25% blue LED; RBG, 60% red + 20% blue + 20% green LED; RBP, 60% red + 20% blue + 20% purple LED; RBF, 60% red + 20% blue + 20% infrared LED; Asp, aspartic acid; Thr, threonine; Ser, serine; Glu, glutamic acid; Gly, glycine; Ala, alanine; Val, valine; Met, methionine; Ile, isoleucine; Leu, leucine; Tyr, tyrosine; Phe, phenylalanine; Lys, lysine; His, histidine; Arg, L-arginine; Pro, proline.
The letters represent the significant difference of different treatments ($p < 0.05$).

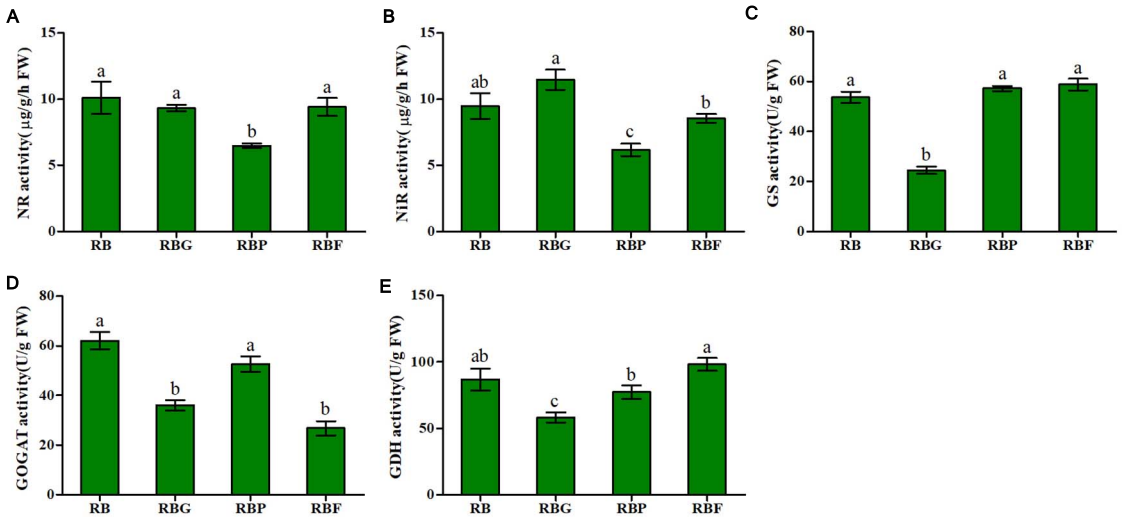

FIGURE 4 | Effects of spectra on enzyme activity of nitrogen metabolism in lettuce. **(A)** NR activity, **(B)** NiR activity, **(C)** GS activity, **(D)** GOGAT activity, **(E)** GDH activity. RB, 75% red + 25% blue LED; RBG, 60% red + 20% blue + 20% green LED; RBP, 60% red + 20% blue + 20% purple LED; RBF, 60% red + 20% blue + 20% infrared LED. The letters represent the significant difference of different treatments ($p < 0.05$).

relative to the quality of RB light, significantly decreasing them by 54.3, 41.9, and 32.9%, respectively. Relative to RB light, RBP light inhibited GDH activity, and RBF light significantly inhibited GOGAT activity, resulting in decreases of 10.9 and 15.2%, respectively.

Effect of LED Illumination Spectra on Genes Expression of Light-Response and Nitrogen Metabolism

As shown in **Figures 5A,B**, different LED light qualities will significantly affect the expression level of *NR* and *NiR*. Herein, no significant upregulation or downregulation of *NR* expression was observed under the RBG, RBP, and RBF treatments relative to the RB treatment. Relative to that under RB light, *NR* expression was slightly upregulated (an increase by 40%) under RBG light. The expression of *NiR* in lettuce leaves under the RBG treatment was significantly upregulated, exceeding that under the RB light by 3.07 times. The expression of *NiR* under the RBP and RBF treatment showed no significant difference from that under the RB treatment. The expressions of *GS*, *GOGAT*, and *GDH* under the RBG and RBF treatments were significantly downregulated relative to those in the RB treatment (**Figures 5C–E**). Relative to the quality of RB light, the quality of RBP light significantly downregulated *GOGAT* expression and had no significant effect on *GS* and *GDH* expressions.

The relative expression levels of light-response genes are shown in **Figures 5F–J**. Relative to the RB light treatment, the RBG and RBP light treatments induced significant *phyA*, *phyB*, and *phyE* down-regulation and *CRY1* upregulation. The RBF light treatment had no significant effect on the expressions of *phyA* and *CRY1*, but is significantly up-regulation on *phyB* expression and down-regulation on *phyE* expression. Relative to RB light, RBG light significantly downregulated *HY5* expression whereas RBP and RBF light did not show significant effects. Correlation analysis showed that the relative expression levels between *phyA*, *phyB*, *HY5*, and NR had a significantly negative correlation. Moreover, a significantly negative correlation existed between *HY5* and *NR* expression (**Figure 6**). By contrast, a significant positive correlation was observed between the *HY5* and *GDH* expressions.

Principal Component Analysis and Comprehensive Evaluation

Principal component analysis was used to analyze 36 indexes of lettuce nitrogen metabolism under the four treatments (**Figure 7**). According to the results of the PCA, the nitrate and nitrite contents of lettuce leaves were closely correlated with ammonium content, and NR, NiR activities. Meanwhile, the following significant differences were observed: amino acid had a stronger intercorrelation with RBG and RBP light, and Glu showed the closest connection with RBG. NR and NiR activities and nitrate, nitrite, and ammonium content were closely correlated with RBF and RBG light and negatively correlated with RBP light. The expression of *HY5* was closely related to RB treatment and negatively correlated with the expressions of *NR* and *NiR*. The comprehensive effect of light quality on nitrogen metabolism in lettuce leaves can be evaluated by a scatter plot with different colors.

DISCUSSION

Adding Green, Purple, and Far-Red Light Had Negative Effect on Lettuce Growth

Light is one of the most important environmental factors in regulating plant growth and development by serving as an

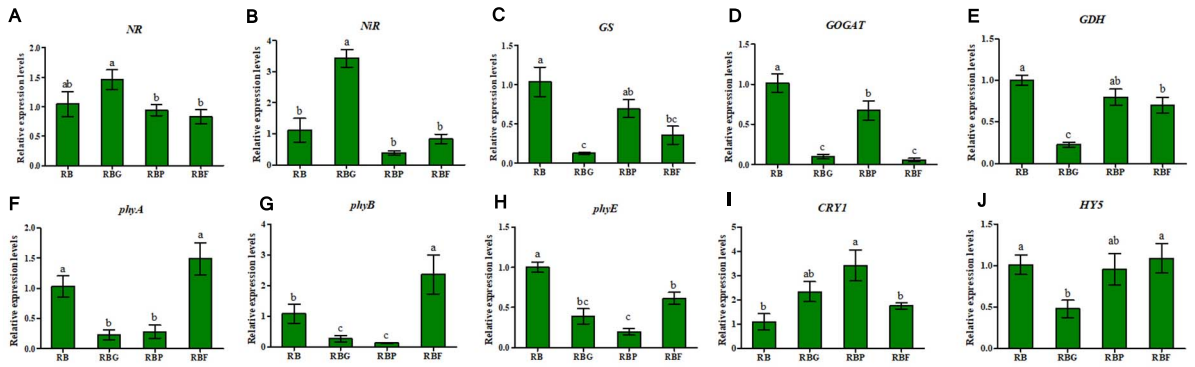

FIGURE 5 | Effects of spectra on genes relative expression level of light-response and nitrogen metabolism in lettuce leaves. **(A)** Relative expression level of *NR*. **(B)** Relative expression level of *NIR*. **(C)** Relative expression level of *GS*. **(D)** Relative expression level of *GOGAT*. **(E)** Relative expression level of *GDH*. **(F)** Relative expression level of *phyA*. **(G)** Relative expression level of *phyB*. **(H)** Relative expression level of *phyE*. **(I)** Relative expression level of *CRY1*. **(J)** Relative expression level of *HY5*. RB, 75% red + 25% blue LED; RBG, 60% red + 20% blue + 20% green LED; RBP, 60% red + 20% blue + 20% purple LED; RBF, 60% red + 20% blue + 20% infrared LED. The letters represent the significant difference of different treatments ($p < 0.05$).

	phyA	*phyB*	*phyE*	*CRY1*	*HY5*	*NR*	*NiR*	*GS*	*GAGAT*	*GDH*
phyA	1.000	0.940**	0.655*	-0.505	0.564	-0.630*	-0.429	0.276	-0.041	0.431
phyB	0.940**	1.000	0.520	-0.362	0.501	-0.606*	-0.330	0.069	-0.244	0.279
phyE	0.655*	0.520	1.000	-0.719**	0.357	-0.202	-0.080	0.510	0.358	0.491
CRY1	-0.505	-0.362	-0.719**	1.000	-0.340	-0.161	-0.043	-0.136	-0.109	-0.299
HY5	0.564	0.501	0.357	-0.340	1.000	-0.629*	-0.707*	0.415	0.212	0.830**
NR	-0.630*	-0.606*	-0.202	-0.161	-0.629*	1.000	0.731**	-0.512	-0.150	-0.609*
NIR	-0.429	-0.330	-0.080	-0.043	-0.707*	0.731**	1.000	-0.628	-0.464	-0.802**
GS	0.276	0.069	0.510	-0.136	0.415	-0.512	-0.628*	1.000	0.830**	0.796**
GAGAT	-0.041	-0.244	0.358	-0.109	0.212	-0.150	-0.464	0.830**	1.000	0.660*
GDH	0.431	0.279	0.491	-0.299	0.830**	-0.609*	-0.802**	0.796**	0.660*	1.000

1.00 0.75 0.50 0.25 0.00 -0.25 -0.50 -0.75 -1.00

FIGURE 6 | Correlation analysis of relative expression levels between light-response genes and nitrogen metabolism genes. **Correlation is significant at the 0.01 level (2-tailed), *Correlation is significant at the 0.05 level (2-tailed).

energy source for plant photosynthesis and a regulator of plant physiology (Bian et al., 2018; Zhang et al., 2018). Previous studies have shown that the combination of red (600–700 nm) and blue light (400–500 nm) is an effective lighting source for crop growth by promoting metabolism and carbohydrates, including soluble sugars (Chen et al., 2016; Amoozgar et al., 2017). In our study, the biomass of aboveground lettuce was significantly higher under RB light than that under the other light treatments, especially RBG light (**Figure 2**). Similar results were observed for the soluble sugar content of lettuce leaves (**Table 1**). In other words, green, purple, and far-red light exert a negative effect on photosynthetic products and plant synthesis and growth. For plants, chlorophyll predominantly absorbs light in the red and blue portions of the spectrum and not in the green, purple, or far-red portions. When other light quality (green, purple, and far-red) is added to the combination of red and blue light, the effective red or blue light intensity decreases. In this study, the photon flux density of red and blue light under RBG, RBP, and RBF light was only 80% of that in the RB treatment. On the one hand, red and blue light intensity decreased after the addition of other light quality. On the other hand, photosynthesis might be inhibited under green, purple, and far-red illumination. Interestingly, the chlorophyll a content of lettuce showed a more significant decrease under RBG light than under RB light. Meanwhile, the chlorophyll content of lettuce increased more significantly under RBP or RBF light than under RB light (**Table 1**). The chlorophyll content caused the decrease of the photosynthetic rate and might explained why the FW and DW of above-ground of lettuce under RBG light were significantly lower than those in the RB treatment. However, the decrease of biomass under RBP or RBF light is difficult to explain in terms of chlorophyll content.

Adding Purple Is Conducive to Vc Accumulation in Lettuce Leaves

For plants, light is not only the driving force for photosynthesis but also the transduction signal for regulating quality features, such Vc content. Previous studies have shown that blue or purple light could increase Vc content in lettuce (Chen et al., 2011; Zha et al., 2020). Consistent with previous results, our results

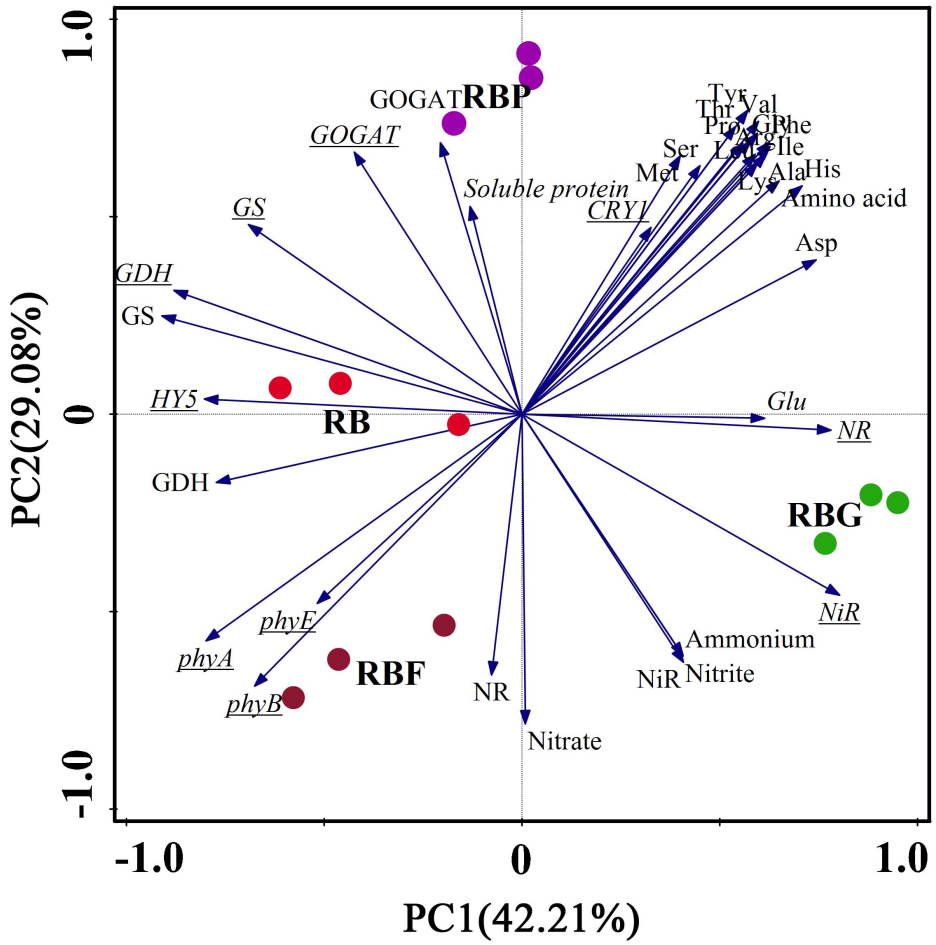

FIGURE 7 | Principal component analysis (PCA) showing differences and correlations in nitrogen assimilation in lettuce leaves under different illumination spectrum. Scatter plot with different color indicated four light treatments (n = 3 replications). Underlined letters indicate genes expression. RB, 75% red + 25% blue LED; RBG, 60% red + 20% blue + 20% green LED; RBP, 60% red + 20% blue + 20% purple LED; RBF, 60% red + 20% blue + 20% infrared LED.

indicate that the addition of purple light is conducive to Vc accumulation in lettuce leaves (**Table 1**). Compare with long-wavelength light (e.g., red or far-red light), short-wavelength light (e.g., blue, purple, or UV light) had higher energy at the same photon flux density. Under short-wavelength light, the antioxidant activity in plants is activated for adaption to increase photooxidative pressure (Gill and Tuteja, 2010; Wen et al., 2019). As a result, ascorbate, the reduced form of Vc, is synthesized by upregulating the gene expression and activity of ascorbate regeneration enzymes for cleaning up excessive reactive oxygen species (Zha et al., 2020). Thus, Vc accumulation induced by adding purple light might protect lettuce leaves form photooxidative pressure.

Effect of Adding Green and Purple Light on Nitrogen Reduction

Nitrate is the main nitrogen source for plant growth and development, and it accumulates in the vacuoles of cells to act as an osmotic adjustment substances. A large amount of nitrate accumulates in cells when nitrogen is sufficient. Nitrate is reduced to nitrite by NR and reduced to ammonium by NiR. Ammonium is assimilated into organic nitrogen through the GOGAT/GS pathway. In the process, the accumulation of nitrogen metabolism intermediates exerts a direct impact on genes expression or enzyme activity, and it may significantly affect nitrogen metabolism (Perrin et al., 2016). In this study, the addition of purple light inhibited NR and NiR activities and caused relative lower nitrate, nitrite, and ammonium content (**Figure 3**). The possible reason is that higher level amino acids was accumulated in lettuce leaves and resulted in a feedback regulation in the absorption and reduction of nitrate through the inhibition of NR and NiR activities. In additional, adding green light caused an increase in nitrite content (**Figure 3B**). Relative to nitrate, excessive nitrite could be harmful to plants. To prevent excessive nitrite accumulation in leaves, plants possibly activate a protection mechanism by increasing *NiR* expression and activity (**Figures 4B**, **5B**), and accelerated nitrite reduction, which leads to the increase in ammonium content under the RBG treatment in this work (**Figure 3C**). Bian et al. (2018)

reported that adding green light to continuous red and blue light significantly reduces the nitrate content of lettuce leaves under continuous red and blue light. However, in the current work, nitrate content did not decrease significantly after the addition of green light (**Figure 3A**). Previous studies have shown that nitrate content is negatively correlated with the soluble carbohydrates in plants (Bouly et al., 2007). Nitrate plays a complementary role with soluble carbohydrates in maintaining cell osmotic pressure (Dapoigny et al., 2000). In this study, the addition of green light decreased the photosynthetic capacity of lettuce leaves and resulted in a relatively low soluble sugar content (**Table 1**). Hence, lettuce may increase the absorption of nitrate to maintain normal osmotic pressure.

Adding Green, Purple, and Far-Red Light Had Contribution on Amino Acid Accumulation

In plants, the reduction of nitrate produces ammonium, which is then assimilated rapidly into organic nitrogen through the GS/GOGAT cycle and GDH pathway. GS has two isoenzymes, which are located in the cytoplasm and chloroplast. Synthesizing ammonium in chloroplast into glutamine is the main function of GS in chloroplast. Glutamine is synthesized under the action of GOGAT to complete the assimilation of ammonium. In this process, light quality also plays an extremely important regulatory role in enzyme activity and gene expression (Meya and Kowallik, 1995; Ning et al., 2019). The data in the current work demonstrated the positive effect of adding green, purple, and far-red light had contribution on amino acid accumulation (**Table 2**). This finding agrees with the results of previous studies (Sun et al., 2016; Wang et al., 2017; Zhang et al., 2018). Interestingly, relative to the RB treatment, the addition of green, purple, and far-red light caused a decrease in GS, GOGAT, and GSH activities and an increase in amino acid content. Moreover, the soluble protein content was lower under the RBG, RBP, and RBF treatments than under the RB treatment (**Table 1**). The speculation was that adding green, purple, and far-red light might inhibit protein biosynthesis as the precursor of amino acid and thereby cause the accumulation of amino acids.

Relationship Between HY5 Gene and Nitrogen Metabolism

Plants sense light through specific photoreceptors and by monitoring environmental characteristics; hence, their sophisticated mechanisms have been evolved to determine light availability and quality (Jones, 2018). In general, phytochromes and cryptochromes respond to light condition (light quality, density, and photoperiod) and induced photomorphogenesis or metabolism through the HY5 transcription factor (Osterlund et al., 2000; Huang et al., 2013). In the presence of red or blue light, the COP1/SPA complex is inactivated by phytochromes or cryptochromes (Yi and Deng, 2005; Lau and Deng, 2012), leading to the accumulation of HY5 and the induction of photomorphogenesis (Laubinger et al., 2004). Signore et al. (2020) pointed out that the red spectrum induces phytochrome phototransformation and results in an increased NR activity. In the current work, the correlation analysis showed that the relative expression levels between *HY5* and *NR/NiR* had a significantly negative correlation (**Figure 6**). The speculation was that green light increased the nitrite and ammonium content because of the high NR and NiR activity and expression, which was negatively regulated by *HY5* (**Figure 5**). *HY5* might be an inhibitory factor of *NR* or *NiR* expression. The inhibitory effect was weakened when *HY5* was downregulated. In additional, *CRY1* was upregulated with the addition of purple light, which did not cause a significant change in downstream *HY5* (**Figure 5**). This finding agrees with the results of previous work, that is, *phy* or *CRY* upregulation inactivates the COP1/SPA complex, and *HY5* plays a regulatory role in downstream metabolize. In sum, *HY5* is possibly associated with *NR* and *NiR* expression and exerts a negative effect on nitrogen reduction. Therefore, further studies on the identification of HY5 functions for nitrate assimilation via Y1H assay or genome editing should be valuable.

CONCLUSION

Our results indicated that LED illumination spectra exerted a significant effect on the growth and nitrogen metabolism of lettuce. Adding green, purple, and far-red light had a negative effect on lettuce growth because of the decrease in the effective photon flux density for chlorophyll absorption. However, purple was found to be conducive to Vc accumulation in lettuce leaves. For nitrate reduction, adding green light caused an increase in nitrite and promoted the reduction from nitrite to ammonium through the activation of *NiR* expression and enzyme activity. On the contrary, adding purple light inhibited NR and NiR activities and caused a low nitrate, nitrite, and ammonium content. For nitrogen assimilation, the addition of green and purple light contributed to amino acid accumulation. Transcription factor HY5 might mediate the regulation of light quality on nitrogen metabolism by inhibiting *NR* and *NiR* expression, and it might have a negative effect on nitrate reduction. The lettuce under RBP treatment had higher comprehensive properties than other light, which could be used for reference in supplemental lighting strategy greenhouse production. Further studies on the identification of *HY5* functions for nitrate assimilation via genome editing techniques will be valuable.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

JW was the recipient of funds. TW, KH, and ML conceived the experiment. JL, ML, and YL prepared the plant materials, collected samples, and undertook experiments. JL analyzed the data and prepared the manuscript. All authors contributed to the manuscript revision.

FUNDING

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SUPPLEMENTARY MATERIAL

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Organic Waste-Based Fertilizer in Hydroponics Increases Tomato Fruit Size but Reduces Fruit Quality

Dmitry Kechasov[1]*, *Michel J. Verheul*[1], *Martina Paponov*[1], *Anush Panosyan*[1] and *Ivan A. Paponov*[1,2]

[1] Division of Food Production and Society, Department of Horticulture, Norwegian Institute of Bioeconomy Research (NIBIO), Ås Municipality, Norway, [2] Department of Food Science, Aarhus University, Aarhus, Denmark

In regions with intensive agricultural production, large amounts of organic waste are produced by livestock animals. Liquid digestate from manure-based biogas production could potentially serve as fertilizer if integrated with closed horticultural irrigation systems. The aim of this experiment was to investigate how fertilizer based on liquid biogas by-products of pig manure digestion can affect the growth and production of tomato plants. Integration of a nitrification bioreactor presumes a significantly lower concentration of nutrient solutions and a higher level of oxygenation than classical mineral cultivation. Therefore, additional controls were included. We compared plant growth and fruit quality traits of tomato plants grown in a hydroponic solution with organic fertilizer with two levels of mineral fertilizer. The tomatoes grown with organic waste-based liquid fertilizer showed reduced growth rates but increased mean fruit size, resulting in no significant change in total yield compared with high-mineral cultivation. The growth rate was similarly reduced in plants cultivated with low-mineral fertilizer. Plants cultivated with organic waste-based fertilizer had high Cl^- concentration in xylem sap, leaves, and, ultimately, fruits. The leaves of plants cultivated with organic waste-based fertilizer contained higher concentrations of starch and soluble carbohydrate and low concentrations of phosphorous (P) and sulfur (S). The plants grown with organic waste-based or low-mineral medium showed significantly poorer fruit quality than the plants cultivated with the high-mineral solution. The low-mineral treatment increased xylem sap contribution to fruit weight because of higher root power. The organic waste-based fertilization did not change the root power but increased fruit size. In conclusion, organic waste-based cultivation is a possible solution for sustainable plant production in greenhouses. However, additional adjustment of nutrient supply is required to improve fruit quality.

Keywords: ***Solanum lycopersicum*, closed soilless cultivation, sustainable production, biogas digestate, nutrient recycling, xylem sap, metabolomics, moving bed bioreactor**

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Abdolhossein Edalati,
University of California, Davis, United States,
in collaboration with reviewer HEE

***Correspondence:**
Dmitry Kechasov
dmitry.kechasov@nibio.no

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INTRODUCTION

In northern latitudes, year-round cultivation of high-value vegetables, such as tomatoes, can only be conducted in greenhouses. In modern greenhouses, tomatoes are grown in soilless cultures using nutrient film techniques (NFT) or rockwool slabs with drip irrigation. Nutrient composition and supply are highly controlled to reduce fertilizer usage and water consumption. However, the average

leaching rate from open drip irrigation systems is between 20 and 40% (Van Os, 1999). Closed-cycle soilless systems, where all leachate is recycled, can further reduce fertilizer run-off by 30–40% compared with open-cycle systems (Montesano et al., 2010).

Recycling soilless systems are used in many countries. However, these systems increase the risks of disease spread and investment costs for the construction of recycling facilities and leachate disinfection. In addition, current disinfection procedures (e.g., heat treatment, ozone, hydrogen peroxide, and UV radiation) are not sustainable (Maessen and Verheul, 2016). These treatments also create a sterile root environment, making roots prone to invasive diseases and increasing potentially explosive disease outbreaks. In contrast, experiences with organic growth systems have shown that the development of diseases is seldom explosive in these systems. This might reflect the natural non-sterile biological environment that promotes a root zone that is more resistant to invasive diseases.

One potential source of organic nutrients for soilless cultivation is organic wastes produced by livestock animals. The annual world production of nitrogen (N) fertilizer as livestock animal manure is 125 billion tons, but 70% of it is left on pastures and cannot be collected. The remaining amount of this potential N-fertilizer could theoretically supplement the yearly consumption of mineral N-fertilizers in the world (11 million tons) (FAOSTAT, 2019, http://www.fao.org/faostat/). Unused or misused fertilizers can also contribute to air pollution, acidification of soil, and eutrophication of nearby aquatic ecosystems (Carpenter et al., 1998; Mallin and Cahoon, 2003; Woodward et al., 2012; Mateo-Sagasta et al., 2017). The use of organic waste-based fertilizer in a closed recirculation system in a greenhouse would, therefore, be beneficial for the reduction of N and P pollution (Martinez-Alcantara et al., 2016) and carbon emission (Favoino and Hogg, 2008).

The main challenges that currently hinder the application of organic waste-based fertilizer in tomato greenhouse systems are difficulties that arise in providing the proper level and balance of nutrients, and dealing with the presence of phytotoxic organic compounds, heavy metals, and salts (Ehret et al., 2005; Jones, 2007). The effects of phytotoxic stress factors can be alleviated in part by oxygenation (van Os et al., 2012). However, little is known regarding the effects of organic waste-based fertilizer on the development of tomato plants and quality of tomato fruits. In other crops, organic fertilization may increase root development, for example, citrus trees grown in soil (Martinez-Alcantara et al., 2016), maize seedlings (Canellas et al., 2002; Jindo et al., 2012), and lettuce in hydroponic culture (Shinohara et al., 2011). Generally, tomato plants cultivated organically (e.g., in organic waste or manure) have comparable (Verheul, 2005; Mitchell et al., 2007; Shinohara et al., 2011; Antonious et al., 2019) or slightly lower (Zhai et al., 2009) yields than plants cultivated with conventional fertilizer. However, reductions in fruit size have often been reported (Oliveira et al., 2013; Zhang et al., 2016). This suggests that the choice of organic material may be important to ensure efficient tomato production.

Hydroponic cultivation of tomatoes in greenhouses could utilize many sources of liquid organic waste by-products, such as compost, biogas effluent, soluble fish waste, or corn steep liquor (Liedl et al., 2006; Zhai et al., 2009; Shinohara et al., 2011). The liquid effluent from anaerobic digestion (digestate) also has the potential for use as a fertilizer in hydroponic cultures (Cheng et al., 2004). During anaerobic digestion, nutritionally interdependent communities of bacteria and Archaea convert biomass into energy-rich biogas and nutrient-rich liquid digestates in the absence of oxygen (Sarker et al., 2019). The resulting digestate has a high concentration of ammonium (NH_4^+) and potassium (K^+) ions and can be readily generated from locally collected household wastes or livestock manure. Since digestate is a by-product of biogas production, it can be integrated into a sustainable energy production chain to minimize waste fraction.

In areas with both greenhouse and livestock productions, liquid organic waste from livestock can be used as a fertilizer for closed soilless cultivation of greenhouse crops. Soilless cultivation requires significantly less disinfection and does not destroy the natural microbial biodiversity in the rhizosphere (Sonneveld and Voogt, 2009).

The main challenge that limits the application of these fertilizer sources is N availability (Möller and Müller, 2012), as the bulk of N in organic fertilizers occurs in organic (org-N) or reduced (NH_4^+-N) forms. Organic N occurs mostly in the form of urea and peptides. Peptides are largely unavailable for plants, while urea can be hydrolyzed to ammonium in digesters and in soils. Ammonium ions are soluble and easily taken up and utilized by plants, but they are harmful to plants in large concentrations (Magalhaes and Wilcox, 1984). Moreover, high levels of ammonium may reduce calcium uptake, thereby inducing disorders like blossom-end rot and subsequent losses in production (Hagassou et al., 2019). Deficiencies in nutrients like N in organic waste-based fertilizers can be overcome by the addition of mineral fertilizer, as has been shown for tomatoes (Poustkova et al., 2009).

The bioavailability of N for plants can also be improved by oxidation of reduced forms of N to nitrate *via* nitrification. This process naturally occurs in soil, mainly because of the activity of aerobic bacteria. In soilless cultures, bioreactors hosting nitrifying bacteria can be integrated into recirculation systems to provide plants with N (Shinohara et al., 2011; Saijai et al., 2016) with added benefit of reducing risk of pathogen infection. Moving bed biofilm reactors (MBBRs) use a large surface area in combination with aeration to produce bacterial sludge that can convert ammonia to nitrate (Rusten et al., 2006). Aerobic conditions may also help to mineralize org-N from organic waste-based fertilizers (Möller and Müller, 2012). Thus, a closed

Abbreviations: COD, chemical oxygen demand; DMC, dry matter content; DO, dissolved oxygen; DW, dry weight; EC, electrical conductivity; ED, end of dark; EL, end of light; FW, fresh weight; GC–MS, gas chromatography–mass spectrometry; HK, hexokinase; IQR, interquartile range; MBBR, moving bed bioreactor; MeOH, methanol; MSD, mass selective detector; MSTFA, N-trimethylsilyl-N-methyl trifluoroacetamide; MTBE, methyl *tert*-butyl ether; ORP, oxidation-reduction potential; PCA, principal component analysis; SLA, specific leaf area; sPLS-DA, sparse partial least squares discriminant analysis; SS, soluble solids; SSC, soluble solid content; TS, total solid; TTA, total titratable acidity; VS, volatile solid.

recirculation system with built-in nitrification bioreactor can be viewed as a reasonable setup for the cultivation of vegetables in a greenhouse.

The use of organic waste as a fertilizer can also create salinity problems, especially in closed irrigation systems. Salt toxicity may influence the uptake of nutrients, especially Ca^{2+} and K^{+} ions, thereby reducing the commercial quality and yield of tomatoes and negatively affecting the growth and photosynthetic efficiency of tomato plants (Sonneveld and Voogt, 2009). Thus, a closed recirculation system with built-in nitrification bioreactor can be viewed as a reasonable set up for the cultivation of vegetables in a greenhouse.

Although many different types of organic waste can potentially be used to grow tomatoes in soilless culture, the long-term effects of organic waste-based fertilizers on plant growth, development, and fruit quality have not been sufficiently investigated. Moreover, no studies have examined how the cultivation of tomatoes in soilless cultures using fertilizers based on organic wastes might affect the nutritional quality of their fruits.

We have analyzed nutrient content, quality, and growth of tomatoes cultivated in an industrial greenhouse using a closed irrigation system with organic waste-based fertilizer. We have estimated the effect of organic waste-based fertilizer on root activity, evaluated root pressure by measuring xylem sap exudation, and analyzed the xylem sap composition of ions and metabolites.

The objective of this study was to investigate the effect of a fertilizer derived from the liquid effluent of a pig manure-based biogas production system on the physiology of greenhouse tomato plants and the quality of their fruits.

- The taste quality and yield of tomatoes cultivated with organic waste-based fertilizer (EC 0.5 ± 0.2 $mS{\cdot}cm^{-1}$) were compared with tomatoes grown with mineral fertilizer in low (EC 0.4 ± 0.2 $mS{\cdot}cm^{-1}$) and high concentrations (EC 3.8 ± 0.6 $mS{\cdot}cm^{-1}$).
- The effect of different types of fertilizer on the size of tomato fruits was investigated.
- The differences in plant development, biomass distribution, and photosynthetic ability between plants grown with different fertilizers were investigated.
- The effect of different fertilizers on root metabolism was investigated by the analysis of xylem sap composition.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Tomato plants (*Solanum lycopersicum cv.* Dometica) were cultivated in a Venlo-type greenhouse in southwestern Norway (58°42'49.2"N 5°31'51.0"E) from September 21, 2018 to January 22, 2019. Seeds were sown on August 13, 2018, initially grown in a growth chamber in rockwool cubes (Grodan, Roermond, the Netherlands) and watered daily with high-mineral fertilizer with electrical conductivity of 3.2 mS cm^{-1}. For climate adaption, plants were transported into the greenhouse and grown for three weeks prior to transplanting them into AeroFlo growing chambers (General Hydroponics, Santa Rosa, CA, United States). In the growing chambers, the plants in rockwool cubes were fixed with expanded clay pebbles inside a plastic mesh pot. A nutrient solution was sprayed on the mesh pots, and the plant roots were not submerged in the nutrient solution until they reached the bottom of the growth chamber (**Figure 1**). The tomatoes were cultivated as one-shoot plants in a high-wire system.

High-pressure sodium (HPS) lamps (Philips GP Plus 750 W, Gavita Nordic AS, Andebu, Norway) with the intensity of 161·W m^{-2} were installed about 1.5 m above the top of the canopy. The threshold for turning on HPS lights was set to 250 $W{\cdot}m^{-2}$ of incoming solar radiation, which means that the HPS lamps were switched on continuously for 12 h per day for weeks 38–41, then for 15 h for week 42, and 18 h from week 43 to the end of the experiment. During the experiment, the following environmental conditions were measured in the greenhouse: average daily temperature of 21.3 ± 2.4°C, relative humidity of 68 ± 13%, the CO_2 concentration of 682 ± 209 ppm, and solar radiation of 30 ± 72 $W{\cdot}m^{-2}$. Tomato flowers were pollinated by bumblebees. The plants were lowered weekly by about 30 cm. Side shoots were removed, and three or more leaves were removed below the truss that first showed orange tomatoes. The trusses were pruned to seven fruits per truss.

Experimental Setup

The set up consisted of three closed irrigation systems, organic waste-based, low mineral, and high mineral. The first two systems included an MBBR. Each treatment was represented by one main reservoir with nutrient solution (550 L biofilter or 220- L barrel), five small reservoirs (60 L barrels), each barrel was connected to an AeroFlo growth chamber with two tomato plants (**Figure 1**). The total number of plants per treatment was 10 ($n = 10$). We selected low-pressure hydroponics over drip irrigation because of (1) elimination of clogging due to sludge/particle buildup in hoses and rockwool slabs and (2) the possibility to investigate intact root architecture and take samples.

The main reservoirs were placed outside of the greenhouse compartment and were connected to the small reservoirs through a system of hoses and pipe manifolds (**Figure 1**). The nutrient solution flowed gravimetrically from the main reservoir to the small reservoirs and was pumped back by a water pump. A small pump was used to transport the nutrient solution to the AeroFlo growing chambers located above the small reservoirs. The nutrient solution was sprayed continuously onto the roots. The growing chambers were placed at an angle to allow the nutrient solution to return to the small reservoirs through a drain tube located at the lowest point of the growth basket (**Figure 1**). In other words, the nutrient solution circulated simultaneously between the main and small reservoirs, and between the small reservoirs and the greenhouse.

Fertilizer was added after regular monitoring of nitrate level two to three times per week with the help of semi-qualitative test strips (Quantofix, 91313, Sigma-Aldrich, Darmstadt, Germany). The minimum nitrate level in the nutrient solution was set to 1 $mmol{\cdot}L^{-1}$. The plants in the control treatment (i.e., high-mineral) received a complete nutrient solution based on standardized recommendations containing the following:

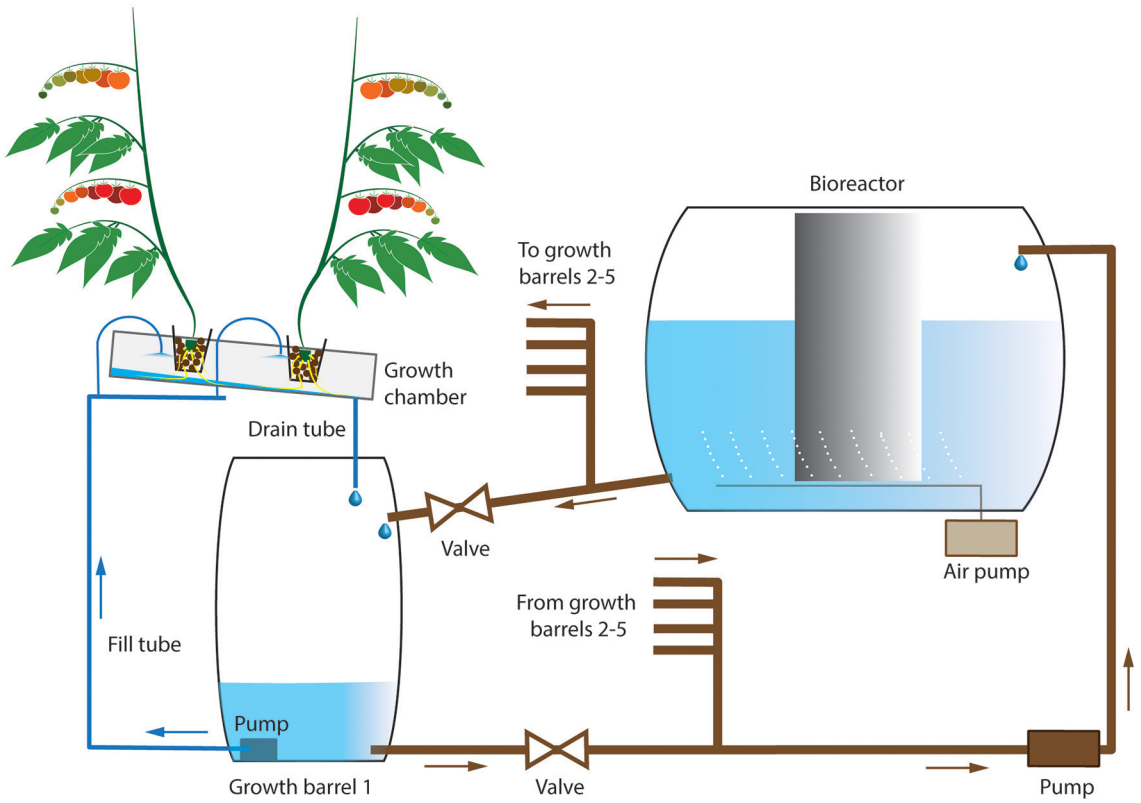

FIGURE 1 | Experimental setup for the organic waste-based and low-mineral treatments. The nutrient solution was mainly contained in a large reservoir (e.g., bioreactor) and gravimetrically transported to each of five growth barrels. The nutrient solution from each growth barrel was pumped into the growth chamber and sprayed on roots. The nutrient solution returned to the barrel through the drain pump, and was collected from all five pumps and returned to the large reservoir. For the high-mineral treatment, the large barrel was used instead of the bioreactor. The plants in rockwool cube were placed in expanded clay pebbles.

17.81 mM NO_{3-}, 0.71 mM NH_{4+}, 1.74 mM P, 9.2 mM K, 4.73 mM Ca, 2.72 mM Mg, 2.74 mM S; and micronutrients: 15 μmol Fe, 10 μmol Mn, 5 μmol Zn, 30 μmol B, 0.75 μmol Cu, and 0.5 μmol Mo. The electrical conductivity of the nutrient solution was about 3.5 $mS{\cdot}cm^{-1}$, and the pH was 5.9. The same stock nutrient solution was added to the low-mineral treatment as fertilizer. Its initial concentration was ∼20-fold lower than that of the high-mineral treatment. Tap water was used to compensate for plant water uptake. In the organic waste-based treatment, the liquid digestate was used as the main fertilizer. However, some mineral salts (Ca, Mg, P, and S) were added to correct for nutrient unavailability. The initial physiochemical parameters and ionic composition of all tested treatments can be found in **Supplementary Table 1**. Nitric acid (HNO_3) was used to adjust the pH to about 7 to ensure bacterial growth. A mixture of essential microelements Pioneer red (Yara, Oslo, Norway) was also added to the organic waste-based treatment before transferring the plants (4.5 ml) and 56 days after transferring (6 ml).

Liquid digestate was produced from pig manure by anaerobic mesophilic process (average reactor temperature 37°C, filtration with 200-micron filter, 10 days hold time). The digestate was autoclaved at 120°C for 15 min, and its pH was 7.5 before autoclaving and 9.7 after autoclaving, EC 2.7 $mS{\cdot}cm^{-1}$. The average chemical oxygen demand (COD) of the digestate was 5.4 ± 0.8 $g{\cdot}L^{-1}$, total solids (TS) 8 ± 0.7 $g{\cdot}L^{-1}$, and volatile solids (VS) 52 ± 4%. The digestate was diluted ∼20 times and was stored indoors at 7–15°C in closed plastic barrels.

An MBBR (Nexus220, Evolution Aqua, Wigan, United Kingdom) was used for biological nitrification of biogas effluent. The biofilter was 1 m in diameter and had a water depth of 1 m, and it contained 150 L of K1 micro Kaldnes media (Evolution Aqua, Wigan UK) with a specific surface of 950 $m^2{\cdot}m^{-3}$. The digestate was manually added into the inner chamber, where it was mixed with recycled nutrient solution collected from the small barrels and mechanically filtered. A well-mixed solution was passed to the outer chamber containing Kaldnes media with resident nitrifying bacteria. The nitrified solution was then gravimetrically transported into the greenhouse for fertigation. Organic waste-based treatment had the following characteristics ($n = 4$): soluble solids (SS) 86 ± 28 $mg{\cdot}L^{-1}$, TS 605 ± 127 $mg{\cdot}L^{-1}$, and VS 79 ± 10%.

Physiochemical parameters of the nutrient solution (temperature, pH, EC, etc.) were monitored two times each

week with a multimeter (HI 98194, Hanna Instruments, Woonsocket, RI, United States). Samples of the nutrient solution were taken every week from the main reservoir before adding fertilizer and frozen at −20°C until analysis.

Harvest

Fruits were harvested two times per week. The harvested fruits were weighed individually, and their position in the truss and the number of trusses were recorded. Each position for every treatment was represented by 47–70 fruits. Final harvests were performed on February 22 and 24, 2019. To estimate dry matter content, leaves, stems, and fruits were completely dried at 70°C (∼2 weeks for fruits, otherwise 96 h).

Xylem Sap Collection

Xylem sap was collected on February 22 and 24, 2019 from four randomly selected shoot plants using a root pressure method adapted from Alexou and Peuke (2013). Stems of selected plants were cut with garden scissors at a position about 10 cm above the root-shoot interface. The cut surface was cleaned with deionized water, and a silicon tube was fixed over the stump and sealed with silicone grease. The exudate collected from the silicone tubes during the first 60 min period was discarded with a pipette. The xylem exudate collected during the next 60 min period was stored in plastic vials on ice and subsequently frozen in liquid N, and stored at −80°C.

Quality Analysis of Fruits

Samples for fruit quality assessment were collected on January 1, 2019 (harvest 1), January 9, 2019 (harvest 2), and January 21, 2019 (harvest 3). Each replicate consisted of six tomato fruits selected from a pool of fruits collected from 10 plants per treatment. To ensure that the tomatoes had equal ripeness, tomato fruits were sampled at grade 8, determined visually based on a scale provided by Bama AS (Oslo, Norway) (from 1, green to 12, deep red).

For each harvest, three replicates ($n = 3$) per treatment were investigated for firmness, SS content (SSC), and total titratable acidity (TTA). For one tomato, firmness was measured three times using a Durofel firmness tester (Agro Technologies, Forges-les-Eaux, France) and averaged on a scale from 1 to 100, where 100 meant full firmness and 1 meant complete lack of firmness (i.e., Durofel units). The tomatoes were then cut into four parts. One quarter from each of six fruits was combined to make a replicate (a total of six quarters) and homogenized with a handheld blender. The resulting homogenate was used for determination of SSC and TTA on the same day as harvesting, following the procedures published by Mitcham et al. (1996) and Verheul et al. (2015). The SSC (expressed as Brix) was measured with a digital PR-101α refractometer (ATAGO, Tokyo, Japan) set to zero with deionized water. The TTA was determined using 794 Basic Titrino (Metrohm, Herisau, Switzerland), an automatic titrator, with potentiometric detection (pH_{final} 8.2) and expressed percent of citric acid equivalents (CAE) per 100 g FW (Verheul et al., 2015). An aliquot of tomato fruit homogenate was transferred into a 1.5-ml centrifuge tube, weighed, immediately frozen in liquid N, and stored at −80°C until further.

Analysis of Soluble Ions

The ion composition analysis of the nutrient solution, fruits, and xylem sap was performed by using ion chromatography with conductive detection, as described in Paponov et al. (2020).

Leaf Area Measurements

The fresh weights of proximal leaflets of leaves at the bottom of the canopy were measured, and the area was determined using LI-3100 Area Meter (LI-COR, Inc., Lincoln, NE, United States). The leaves were dried at 65°C for 48 h.

Extraction and Separation of Polar and Non-polar Metabolites From Leaves

The extraction of metabolites from leaves and their separation were performed using a two-phase separation method described by Salem et al. (2016). In short, distal leaflets of mature undamaged leaves at the bottom of the canopy were collected at the end of the day (ED, i.e., 12 midnight) and the end of the night (EN, i.e., 6 a.m.). The leaves were immediately frozen in liquid N and stored at −80°C. Prior to the analysis, leaf samples were lyophilized for ∼48 h without heating in freeze dryer BK-FD10S (BIOBASE, Jinan, China). Thereafter, the leaves were ground in a grinding mill (Star-Beater, VWR, Radnor, USA) in a pre-cooled holder using pre-cooled stainless-steel balls (0.5 g) for 2 min at max frequency (30 Hz). The ground samples were pre-cooled with liquid N spatulas and transferred in new 2 ml centrifuge tubes. Approximately 20 ± 0.5 mg of the dried leaves were aliquoted in the new tubes and used for sample extraction. In each tube, 1 ml of a pre-cooled mixture of methyl *tert*-butyl ether (MTBE) and methanol (MeOH) in proportion 3:1 (v/v) was added. The tubes were incubated in an orbital shaker at 100 rpm for 45 min at 4°C. Thereafter, the tubes were sonicated for 15 min in an ice-cooled sonication bath (USC300TH, VWR, USA). To initiate phase separation, 650 μl of H2O:MeOH (3:1, vol/vol) was added to each sample tube. The mixture was vortexed for 1 min (Vortex Genie 2, Scientific Industries, Inc., Bohemia, USA) and spinned in a centrifuge (MicroStar 17R, VWR, USA) at 17,000 × g for 15 min at 4°C, and 450 μl of the solvent from the upper, non-polar, phase was transferred into a 1.5-ml microcentrifuge tube for pigment analysis. The remaining upper phase and interphase were discarded, and 400 μl of the solvent from the lower phase containing polar and semi-polar metabolites was transferred into a new 1.5-ml microcentrifuge tube. The extracts were used directly or stored at −80°C. For further starch analysis, the remaining liquid was carefully removed by pipetting. The obtained pellet was washed with 500 μl MeOH by vortexing for 1 min. The samples were centrifuged at 10,000 × g for 5 min at 4°C.

Quantification of Glucose, Fructose, and Sucrose

The quantification of glucose, fructose, and sucrose was performed by sequential enzymatic assays with photometric detection in a spectrophotometric plate reader (Multiscan GO,

Thermo Fisher Scientific, Waltham, USA) according to Zhao et al. (2010).

To estimate glucose concentration, 10 μl of the polar solvent phase was diluted eight times with 80% ethanol in a 1.5 centrifuge tube, and 20 μl of the resulting mixture was transferred into a 96-well-plate in triplicate. The ethanol was evaporated from the wells in an oven at 60°C for ∼40–50 min. The dried material was resuspended by the addition of 20 μl of deionized water, then 100 μl of the glucose hexokinase (HK) assay reagent (G3293, Sigma) was added into each well. The 96-well-plate (UV-STAR, Greiner Bio-One, Kremsmünster, Austria) was covered with a lid and incubated inside the plate reader for 15 min at 30°C. The absorbance of samples, blanks, and standards was measured at 340 nm at 30°C and in precision mode.

The amount of fructose was determined by phosphoglucose isomerase (PGI) assay, and 10 μl of the PGI assay reagent (0.2 M HEPES with pH 7.8) was added into each well previously used for glucose quantification. The absorption was measured at 340 nm after incubation inside the spectrophotometer for 15 min at 30°C.

The amount of sucrose was determined by adding 10 μl of the invertase assay reagent (10 mg ml^{-1}, I4504, 300 units·mg^{-1}, Sigma) in 1 M Na-citrate buffer with pH 6) into each well. Absorption was measured at 340 nm after incubation inside of the spectrophotometer for 60 min at 30°C.

Extraction of Starch

The analysis of starch content was performed using the remaining pellet after liquid extraction according to Salem et al. (2016) with modifications, and 1 ml of 80% ethanol was added to each tube. The tubes were incubated for 3 min at 80°C in a water bath and then cooled at RT. The insoluble material was spinned down (5 min, 10,000 × g, RT). By the end of the third washing step, the pellet had a light brown color. The samples were placed in a chemical hood to allow ethanol to evaporate. The dried pellet was resuspended in 0.5 ml of deionized water and vortexed for 1 min for homogenization. The starch was gelatinized at 100°C for 15 min in the water bath and vortexed for 1 min, and 200 μl of homogenate pellet was transferred to each of two 1.5-ml microcentrifuge tubes, containing: (a) 200 μl of digestion mix and 200 μl of buffer solution (200 mM sodium acetate–acetic acid, pH 4.8). The digestion mix contained 6 U·ml^{-1} of α-amyloglucosidase from *Aspergillus niger* (6 U·mg^{-1} 11202367001, Roche, Basel, Switzerland) and 10 μl per 20 mg sample DW of α-amylase (A4582, Sigma). The tubes were incubated at 60°C for 30 min in the water bath, and then centrifuged at 14,000 × g for 10 min at RT to remove undigested material. The glucose content from starch was quantified using the previously described HK enzymatic assay. Starch concentration was determined by multiplying glucose concentration by dilution factor and by the glucose equivalent of 0.9 (Chow and Landhausser, 2004; Hostettler et al., 2011).

Analysis of Pigments

Chlorophyll (a and b) and carotenoid content in the tomato leaves was determined spectrophotometrically. A 35- μl volume of the upper polar phase was diluted 20 times with MeOH, and 200 μl of the diluted mixture was pipetted in triplicate into a 96-well-plate. Absorbance was measured at 470, 652, and 665 nm. The concentration of carotenoids was measured using the formula for pure MeOH from Lichtenthaler and Buschmann (2001). Path length correction factors for the 96-well-plate were measured and applied as specified by Warren (2008). The concentration of chlorophylls a and b, and total carotenoids was calculated as mg·g^{-1} DW.

Total N Determination

The total N content of the tomato leaves was determined by using persulfate digestion (Purcell and King, 1996; De Borba et al., 2014) coupled with ion chromatography. Approximately 20 mg of lyophilized leaves were mixed with 4 ml of a digestion mixture (0.475 M NaOH and 0.167 M $K_2S_2O_8$) in glass centrifuge tubes and closed with Teflon-lined caps. The tubes were incubated in a heating block at 120°C for 60 min. The N content was determined by suppressed ion chromatography. Elution parameters were similar to those previously described (Paponov et al., 2020), except that acetone was not used for eluent preparation to avoid peak coelution. Recovery of N extraction was 78% for inorganic ammonium salt and glycine standards. Organic N was calculated by subtraction of inorganic N (NH_4-N, NO_2-N, and NO_3-N) from the total N concentration. Values of N materials were presented as g·100 g^{-1} leaf DW.

Metabolic Analysis of Xylem Sap

The extraction of polar metabolites was performed according to Fiehn (2003). In short, frozen xylem sap was thawed, and 100 μl of it was added to water:chloroform mixture (500 μl water and 300 μl chloroform with 10 μl ribitol standard with concentration 0.1 mg·ml^{-1}). The mixture was vortexed for 30 s and centrifuged at 17,000 × g for 2 min at 4°C, and 450 μl of the upper phase was transferred into a new 1.5-ml tube and lyophilized overnight.

Metabolite derivatization was performed according to Hill and Roessner (2013) with minor modifications. After lyophilization, the samples were quickly spinned, and 40 μl of methoxamine in pyridine (20 mg·ml^{-1}) was added. The tubes were incubated for 90 min at 37°C with a shaking speed of 750 rpm. Thereafter, 80 μl of N-Trimethylsilyl-N-methyl trifluoroacetamide (MSTFA, 394866, Sigma-Aldrich) containing a mixture of alkanes (C8-C40, 40147-U, SUPELCO, Sigma-Aldrich) for retention index calculation was added, and the tubes were incubated for 30 min at 37°C with the same shaking speed. After incubation, the tubes were centrifuged for 1 min at 17,000 × g at RT, and 50 μl of the sample was transferred into a GC vial with a micro insert and closed with a screw cap containing Teflon septa.

Derivatized samples were immediately transferred to a GC instrument. The samples were analyzed with Agilent 6890 Gas Chromatograph coupled with Agilent 5975 Mass Selective Detector (MSD, Agilent Technologies, Santa Clara, CA, United States). Chromatographic separation was performed on Rxi-5Sil MS with an Integra-Guard column (30 m long, 0.25 mm inner diameter, 0.25 μm film thickness, Restek, Bellefonte, PA, United States), and 1 μl of the sample was automatically injected by the MPS autosampler (Gerstel, Mülheim, Germany) equipped with a 10-μl syringe (Gerstel, Germany) in a pulsed splitless

injection mode at 230°C. Pulse was held at 200 kPa for 2 min. Helium was used as a carrier gas at a constant flow rate of 1 ml·min^{-1}. The temperature of the GC oven was ramped from initial 70°C to final 325°C at rate 5°C·min^{-1} and held at final temperature for 3 min (54 -min run in total). MSD was tuned with perfluorotributylamine before the analysis. The MSD was operated in electron ionization mode at 70 -eV electron ionization energy. The temperatures of the transfer line, the ion source, and the quadrupole were set to 280, 230, and 150°C. Mass spectra were recorded at frequency 5.5 scans/s with a scanning range from 50 to 550 m/z.

Data acquisition was carried out by MassHunter GC-MS software (version B.07.00, Agilent Technologies, Santa Clara, CA, United States). Data analysis was performed with the MS-DIAL software version 4.38, compound identification was performed using an integrated compound library (GCMS DB_AllPublic-KovatsRI-VS2) using both spectrum and retention index similarity (Tsugawa et al., 2015: Lai et al., 2018). Peak heights present in blank samples were subtracted from corresponding peak heights in samples. Normalization (sum of peak heights of identified metabolites, i.e., mTIC), data filtering [default setting for interquartile range (IQR) filter] transformation (log and Pareto scaling), and statistical analysis [ANOVA, heatmap, principal component analysis (PCA), and sPLS-DA] were performed in MetaboAnalyst version 5.0 (Chong et al., 2019).

Statistics

Each treatment consisted of 10 plants grown in pairs. Biomass accumulation traits of above-ground parts (plant length, number of trusses, leaves, etc.) were collected individually for each plant ($n = 10$). The roots of the plants grown in pairs were interconnected, and their weight was determined pairwise ($n = 5$). Total biomass accumulated during growth was calculated for each plant pair. Leaf samples for chemical analysis were collected from two plants grown in pairs ($n = 5$). Fruit numbers and their weight were recorded for each plant. The quality analysis of fruits was performed with fruits pooled from different plants within the same treatment. Statistical differences were evaluated using the general linear model (ANOVA) followed by Tukey's multiple comparisons test using the Minitab 19 software. Datasets were tested in the presence of normal distribution and identified outliers were removed. The level of significance was set as 0.05 ($p = 0.05$). Data were presented as mean values with SD.

RESULTS

Physiochemical Parameters of the Nutrient Solution

The physiochemical parameters of the nutrient solution and its ionic composition were monitored weekly for the entire cultivation period. The average values obtained during cultivation are presented in **Table 1**, **Supplementary Figure 1**, **Supplementary Table 1**.

The high-mineral treatment was a solution with high nutrient concentration typically used for hydroponic tomato cultivation. The organic waste-based treatment was characterized by significantly lower concentrations of nutrients in the solution because nutrient influx in this system occurs continuously because of digestion of organic compounds and nitrification by bioreactor. These conditions prevent the accumulation of nutrients at the high levels found in the high-mineral nutrient solution. We addressed the effects related to differences in ion concentrations between the high-mineral and organic waste-based treatments by including an additional control, consisting of diluted mineral concentrations (the low-mineral treatment) that mimicked the nutrient concentrations found in the organic waste-based treatment. This experimental design meant that the high-mineral solution had the highest concentration of major nutrients (**Table 1**) and therefore the highest conductivity (3.8 $\pm$ 0.6 mS·cm^{-1}), whereas the organic waste-based and low-mineral solutions had conductivity values of 13 and 11% of the high-mineral solution, respectively (**Table 1**). The high-mineral solution was not aerated; therefore, the dissolved oxygen (DO) was significantly lower in the high-mineral treatment (63.5 $\pm$ 15.5 %) than in the organic waste-based and low-mineral treatments (83.4 $\pm$ 5.5 and 83.2 $\pm$ 6.2, respectively). None of the other monitored parameters differed significantly, except for pH, which was 7 in the low-mineral, 6.4 in the high-mineral, and 6.3 in the organic waste-based treatments (**Table 1**).

The organic waste-based treatment had 3.8- and 7.5-fold higher concentrations of Na^+ than the high- or low-mineral treatment, respectively, and the concentration of Cl^- in the organic waste-based treatment was 5.5- and 6.6-fold higher than in the low-mineral and the high-mineral treatments, respectively (**Table 1**). The average concentrations of other major soluble inorganic ions (NH_4^+, K^+, Ca^{2+}, Mg^{2+}, NO_2^-, NO_3^-, PO_4^{3-}, and SO_4^{2-}) were similar for the organic waste-based and low-mineral treatments throughout the cultivation.

The estimated average water use for the organic waste-based, low-mineral, and high-mineral treatments was 1.02, 1.33, and 0.83 L·plant^{-1}·day^{-1}, respectively. This was equivalent to 79, 103, and 64% of the water used when growing tomato plants in rockwool slabs with drip irrigation and without recirculation.

The estimation of relative N distribution showed that the low-mineral treatment had the highest percentage of used N from the nutrient solution (74%, compared with 60% for organic waste-based and 46% for high-mineral treatments, **Supplementary Figure 3**).

Fruit Yield and Tomato Size

The differences in plant nutrition affected the total plant yield, total number of fruits and trusses, and individual tomato fruit size (**Table 2**). The plants cultivated with low-mineral solutions had significantly lower yields than the plants grown with the high-mineral treatment. The plants grown with the organic waste-based treatment also had lower yields than the plants grown with the high-mineral solution. However, this difference was not statistically significant. The plants grown with the organic waste-based and low-mineral treatments had lower numbers of fruits compared to those grown with the high-mineral treatment (38.7 $\pm$ 10.1, 39.6 $\pm$ 9.7, and 54.4 $\pm$ 10.6, respectively), whereas the total number of trusses and number of trusses with ripened

TABLE 1 | Physiochemical parameters and ionic composition of the tested treatments.

Fertilizer	Organic waste-based	Low-mineral	High-mineral
Physiochemical parameters *n* = 28–31			
Temperature (°C)	18.4 ± 2.3^{a}	18.4 ± 2.3^{a}	17.1 ± 2.3^{a}
ORP (mV)	273 ± 57^{a}	271 ± 40^{a}	291 ± 44^{a}
DO (% saturation)	83.4 ± 5.5^{a}	83.2 ± 6.2^{a}	63.5 ± 15.5^{b}
pH	6.3 ± 1.0^{a}	7.0 ± 0.5^{b}	6.4 ± 0.3^{a}
EC ($mS \cdot cm^{-1}$)	0.5 ± 0.2^{a}	0.4 ± 0.2^{a}	3.8 ± 0.6^{b}
Water use ($L \cdot plant^{-1} \cdot day^{-1}$)	1.02	1.33	0.83
Cations ($mmol \cdot L^{-1}$) *n* = 17			
Na^{+}	1.5 ± 0.6^{a}	0.2 ± 0.1^{b}	0.4 ± 0.2^{b}
NH_4^{+}	0.181 ± 0.334^{ab}	0.003 ± 0.007^{b}	0.370 ± 0.479^{a}
K^{+}	0.79 ± 0.63^{a}	0.28 ± 0.44^{a}	10.36 ± 0.94^{b}
Ca^{2+}	0.42 ± 0.11^{a}	0.91 ± 0.46^{a}	7.74 ± 3.39^{b}
Mg^{2+}	0.2 ± 0.08^{a}	0.34 ± 0.18^{a}	3.95 ± 1.28^{b}
Anions ($mmol \cdot L^{-1}$) *n* = 18–19			
Cl^{-}	1.05 ± 0.19^{a}	0.16 ± 0.2^{b}	0.19 ± 0.19^{b}
NO_2^{-}	0.03 ± 0.03^{a}	0.02 ± 0.01^{a}	0.17 ± 0.22^{b}
NO_3^{-}	1.5 ± 1.1^{a}	1.5 ± 1.1^{a}	23.5 ± 5.4^{b}
PO_4^{3-}	0.28 ± 0.18^{a}	0.20 ± 0.16^{a}	1.52 ± 0.53^{b}
SO_4^{2-}	0.12 ± 0.12^{a}	0.26 ± 0.11^{a}	2.63 ± 0.72^{b}

ORP, oxidation-reduction potential; DO, dissolved oxygen; EC, electrical conductivity. Mean values ± sd are shown. Different letters indicate statistically significant differences between treatments at p = 0.05.

TABLE 2 | Commercial yield and yield components of tomato grown under different nutrient conditions.

Fertilizer	Yield per plant ($kg \cdot plant^{-1}$) *n* = 10	Yield per area ($kg \cdot m^{-2}$) *n* = 10	Mean fruit weight (g) *n* = 384-537	Number of fruits ($fruits \cdot plant^{-1}$) *n* = 10	Total number of trusses ($truss \cdot plant^{-1}$) *n* = 10	Number of trusses with ripen tomato ($truss \cdot plant^{-1}$) *n* = 10
Organic waste-based	3.7 ± 1.3^{ab}	15.9 ± 5.5^{ab}	94.2 ± 22.1^{a}	38.7 ± 10.1^{a}	14.5 ± 1.3^{ab}	6.3 ± 1.3^{ab}
Low-mineral	3.5 ± 1.0^{b}	15.0 ± 4.5^{b}	87.7 ± 23.2^{b}	39.6 ± 9.7^{a}	13.7 ± 1.7^{a}	5.8 ± 1.0^{a}
High-mineral	4.7 ± 0.9^{a}	20.286 ± 4.0^{a}	86.2 ± 18.6^{b}	54.4 ± 10.6^{b}	15.8 ± 1.0^{b}	7.5 ± 1.4^{b}

Mean values ± sd are shown. Different letters indicate statistically significant differences between treatments at p = 0.05.

tomatoes were significantly different only between the low-mineral and high-mineral treatments (**Table 2**).

Plants that received the organic waste-based fertilizer had the highest fruit fresh weight compared with the high-mineral (+10%) or low-mineral (+8%) treatment. Significantly fewer fruits werev produced with both the organic waste-based and low-mineral treatments. The number of fruits in a plant is dependent on the number of developed trusses, indicating that low nutrient supply might be the main factor delaying plant development. However, the organic waste-based treatment showed a weak tend toward a higher number of trusses than the low-mineral treatment, indicating that some other factor common only to the organic waste-based treatment might partly restore plant development under low nutrient availability.

The analysis of fruit weights with fruit position in the trusses also showed that tomatoes at positions 1, 3, 5, and 6 were significantly larger in the organic waste-based treatment than in the high-mineral treatment (**Figure 2**). The absence of a significant interaction between treatment and the cluster position of the fruits indicate that the organic waste-based fertilizer did not alter the competition between basal and apical tomatoes in a truss. The fruit weight for the low-mineral treatment was close to that of the high-mineral treatment. However, at positions 4 and 6, the fruit size tended to be higher for the low-mineral than for the high-mineral treatment. We also analyzed tomato weight distribution among trusses and found a significant effect of treatment on fruit size (**Figure 3**).

Tomato Plant Biomass and Dry Matter Partitioning

Tomato plants that received organic waste-based nutrition showed the lowest total dry biomass. However, the difference in plant biomass between the organic waste-based and low-mineral treatments was not statistically significant. The different

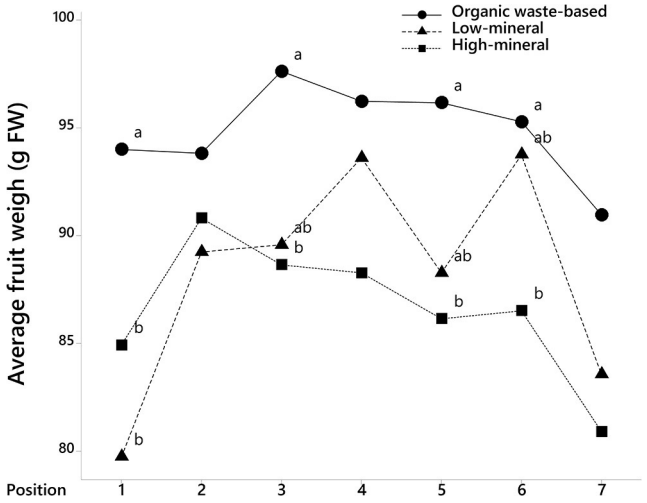

FIGURE 2 | The average weight of tomato fruits at different positions on truss, where 1 is the first to bear fruits on the truss. Mean values are shown. Different letters indicate statistically significant differences at $p = 0.05$, $n = 47–70$.

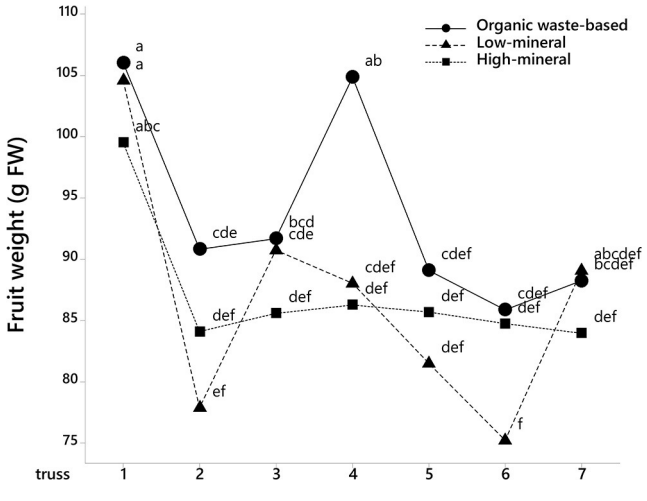

FIGURE 3 | Average tomato fruit weight depends on truss position and treatment, where 1 is the first to develop truss. Mean values are shown. Different letters indicate statistically significant differences at $p = 0.05$, $n = 23–69$.

treatments also altered dry matter distribution, with organic waste-based cultivation preferentially stimulating dry matter partitioning to the roots (**Table 3**). This trend was not observed for the low-mineral treatment, indicating that the greater allocation to the roots was not a consequence of low-mineral nutrient concentrations. Dry matter allocation to the leaves and fruits was similar between the organic waste-based and high-mineral treatments. However, the low-mineral treatment stimulated greater distribution of dry matter to the leaves and a smaller distribution to the fruits. These differences indicate that low concentrations of nutrients diminish dry matter allocation to generative organs. However, the organic waste-based treatment, despite low level of essential nutrients, restored the dry matter distribution from vegetative to generative organs (**Table 3**).

The number of leaves correlated with the total biomass values for all treatments, with the highest value observed in the high-mineral treatment and the lowest in the organic waste-based treatment. Specific leaf area (SLA) did not show significant differences between the organic waste-based and high-mineral treatments. However, the SLA was significantly higher for the organic waste-based than for the low-mineral treatment, indicating that low nutrient supply decreased the efficiency of leaf area expansion per unit leaf dry matter, whereas the organic waste-based treatment recovered this trait. The differences in SLA were related to the dry matter percentage in the leaves, indicating that modulation of SLA was not due to differences in leaf thickness but to differences in dry matter content. The plant height and xylem sap flow rates were also more similar between the organic waste-based and high-mineral treatments than for the low-mineral treatment. Taken together, these data indicated that although the plant biomass was significantly lower in the organic waste-based than in the high-mineral treatment, many other plant traits were more phenotypically similar between these two treatments than in plants grown with the low-mineral treatment.

Fruit Quality

The basic parameters of tomato fruit quality were related to nutrient concentrations in the nutrient solution (**Table 4**): both the low-mineral and organic waste-based treatments showed significantly lower SSC (15 and 18% lower, respectively), TTA (18 and 20% lower, respectively), and DMC of red fruits (20%). This indicates that low nutrient concentrations in the growth solution directly affect the quality traits of tomato fruits. The tomatoes had 4% lower average firmness when grown with the organic waste-based fertilizer (**Table 4**) than those grown by using the high-mineral or low-mineral fertilization; this probably reflected the larger fruit size (**Table 2**).

The analysis of fruit ionic composition showed that plants accumulated higher concentrations of Cl^- and Na^+ ions when grown with the organic waste-based treatment compared with the other two treatments (**Figure 4**, **Supplementary Table 2**). The tomato fruits grown by organic waste-based fertilization had an ∼5-fold higher concentration of Cl^- compared with the other treatments, but they showed a near absence of NO_3^- and the lowest concentrations of PO_4^{3-}, and SO_4^{2-} (57 and 12%, respectively, of the fruit content in the high-mineral treatment). The proportion of inorganic anions to total inorganic ions was significantly lower in the tomato fruits from the high-mineral treatment (36%) than from the organic waste-based or low-mineral treatments (45 and 44%, respectively). This could be linked to the higher accumulation of organic acids in the high-mineral treatment.

Chemical Composition of Leaves

To characterize leaf function, leaves at the bottom of the canopy were analyzed for their quantities of soluble inorganic

TABLE 3 | Biomass parameters of tomato grown under different nutrient conditions.

Fertilizer		Organic waste-based	Low-mineral	High-mineral
Mean total biomass weight (g DW) $n = 5$		1,126 ± 147^{a}	1,251 ± 140^{a}	1,695 ± 154^{b}
Mean root weight (g DW) $n = 5$		40.8 ± 7.4^{a}	36.6 ± 4.6^{a}	46.7 ± 10.6^{a}
DMC of roots (%) $n = 5$		5.6 ± 0.9^{a}	4.2 ± 0.3^{b}	4.6 ± 0.8^{ab}
Partition coefficients $n = 5$	Leaves	30.9 ± 4.4^{a}	36.1 ± 2.3^{b}	32.1 ± 1.8^{ab}
	Stems	16.3 ± 1.6^{a}	17 ± 1.0^{a}	17.2 ± 0.7^{a}
	Roots	3.6 ± 0.3^{a}	2.9 ± 0.3^{b}	2.7 ± 0.5^{b}
	Fruits	49.2 ± 5.7^{a}	43.9 ± 3.4^{a}	47.9 ± 2.6^{a}
Number of leaves at harvest (leaves·plant^{-1}) $n = 10$		28.9 ± 3.0^{a}	31.6 ± 2.8^{a}	36.3 ± 2.1^{b}
SLA (cm^{2}·g^{-1} DW) $n = 5$		335.4 ± 45.4^{a}	238.4 ± 16.8^{b}	309.3 ± 20.6^{a}
DMC of leaves (g·100 g^{-1} DW) $n = 10$		9.1 ± 0.5^{a}	12.6 ± 0.5^{b}	10.6 ± 0.3^{c}
Plant length (cm) $n = 10$		381 ± 20^{ab}	360 ± 16^{a}	396 ± 42^{b}
Xylem sap exudation rate (mL·h^{-1}) $n = 4$		6.5 ± 3.2^{a}	13.0 ± 1.5^{b}	5.2 ± 1.2^{a}

Mean values ± sd are shown. Different letters indicate statistically significant differences between treatments at $p = 0.05$.

TABLE 4 | Average dry matter content, soluble solids content, total titratable acidity measured in fruits from plants grown under different nutrient conditions, $n = 9$.

Fertilizer	SSC (°Brix)	TTA (CAE)	DMC (%)	Fruit DW (g)	Firmness (Durofel units)	pH
Organic waste-based	4.1 ± 0.1^{a}	0.40 ± 0.01^{a}	5.2 ± 0.2^{a}	4.9 ± 0.7^{a}	0.86 ± 0.02^{a}	4.7 ± 0.4^{a}
Low-mineral	4.2 ± 0.1^{a}	0.38 ± 0.03^{a}	5.3 ± 0.1^{a}	4.5 ± 0.4^{a}	0.89 ± 0.02^{b}	4.8 ± 0.4^{a}
High-mineral	5.0 ± 0.1^{b}	0.51 ± 0.03^{b}	6.5 ± 0.2^{b}	5.6 ± 0.6^{b}	0.90 ± 0.01^{b}	4.7 ± 0.4^{a}

SSC, soluble solid content; TTA, total titratable acidity; DMC, dry matter content. Mean values ± sd are shown. Different letters indicate statistically significant differences between treatments at $p = 0.05$.

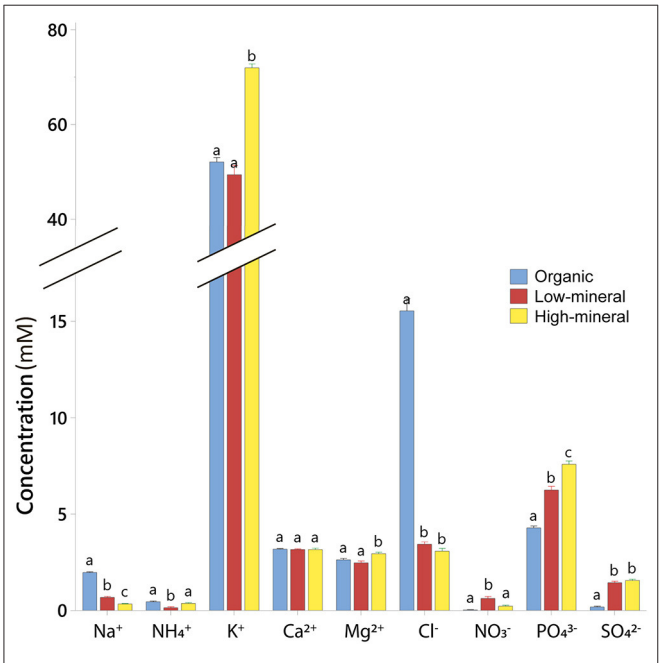

FIGURE 4 | Ionic composition of tomato fruits harvested from plants cultivated with different fertilizers. Mean values ± SD are shown. Different letters indicate statistically significant differences at $p = 0.05$, $n = 9$.

ions, pigments, major carbohydrates, and N (**Table 5**). The leaves showed large differences in ionic composition in the organic waste-based treatment compared with the other two treatments, as the concentration of Na^{+} and Cl^{-} were 7 and 76-fold higher, respectively, in plants from the organic waste-based treatment than plants from the high-mineral treatment. By contrast, PO_4^{3-} and SO_4^{-2} concentrations were the lowest for the organic waste-based treatments at 21 and 5%, respectively, of the levels in leaves from the high-mineral treatment. These low concentrations indicate that PO_4^{3-} and SO_4^{-2} can be limiting nutrients in leaves of organic waste-based cultivated plants. Interestingly, the highest concentration of NO_3^{-} was found in the leaves of organic waste-based cultivated plants, indicating that deficiency of other nutrients can inhibit NO_3^{-} assimilation and cause NO_3^{-} accumulation in leaves. The concentration of K^{+} was lower in the organic waste-based and low-mineral treatments than in the high-mineral treatment, confirming that the K^{+} concentration in the nutrient solution defines the ultimate concentration of K^{+} in the leaves. The leaves of organic waste-based cultivated plants were also characterized by the lowest concentration of NH_4^{+}, which might also be related to the reduction in nitrate assimilation to NH_4^{+}, as indicated by the highest concentration of nitrate in the leaves.

TABLE 5 | Ionic and carbohydrate composition of tomato leaves grown under different nutrient conditions. Chla (b), Chlorophyll a (b); Car, carotenoids.

Component	Organic waste-based	Low-mineral	High-mineral
Inorganic ions (mmol·kg^{-1} DW) $n = 10$			
Na^+	125 ± 37^a	18 ± 9^b	40 ± 43^b
NH_4^+	0.4 ± 0.8^a	2 ± 1.7^b	2.1 ± 1.5^b
K^+	217 ± 59^a	202 ± 27^a	341 ± 61^b
Ca^{2+}	143 ± 46^a	106 ± 22^{ab}	84 ± 34^b
Mg^{2+}	192 ± 43^{ab}	201 ± 22^a	152 ± 32^b
Cl^-	515 ± 139^a	7 ± 2^b	6 ± 2^b
NO_3^-	272 ± 100^a	66 ± 30^b	132 ± 50^b
PO_4^{3-}	21 ± 9^a	78 ± 11^b	99 ± 28^c
SO_4^{2-}	8 ± 8^a	162 ± 37^b	150 ± 37^b
Pigments (mg·g^{-1} DW) $n = 10$			
Chla	12.9 ± 1.0^a	11 ± 0.7^b	13.7 ± 1.4^a
Chlb	3.7 ± 0.4^a	3.2 ± 0.1^b	4.0 ± 0.4^a
Chla+Chlb	16.6 ± 1.3^a	14.2 ± 0.7^b	17.6 ± 1.7^a
Chla/Chlb	3.2 ± 0.4^a	2.6 ± 0.3^b	3.3 ± 0.3^a
Car (x+c)	3.5 ± 0.2^a	3.4 ± 0.2^a	3.5 ± 0.1^a
(Chla+Chlb)/Car	5.2 ± 0.5^a	5.6 ± 0.6^b	5.4 ± 0.2^{ab}
Nitrogen content (g·100 g^{-1} DW) $n = 10$			
N-NH_4^+	0.0007 ± 0.0004^a	0.0003 ± 0.0004^b	0.0003 ± 0.0006^b
N-NO_3^-	0.38 ± 0.14^a	0.09 ± 0.04^b	0.18 ± 0.07^b
Organic Nitrogen	2.4 ± 0.2^a	2.1 ± 0.1^{ab}	2.5 ± 0.2^b
Total Nitrogen	2.8 ± 0.3^a	2.2 ± 0.1^b	2.7 ± 0.3^a
Carbohydrates (mg·g^{-1} DW) $n = 8$–10			
Glucose	9.6 ± 2.5^a	5.0 ± 1.7^b	6.0 ± 2.2^b
Fructose	4.8 ± 1.0^a	2.8 ± 0.6^b	4.2 ± 1.1^a
Sucrose	9.7 ± 1.1^a	6.8 ± 1.6^b	7.7 ± 1.0^b
Starch	24.8 ± 12.4^a	8.8 ± 6.1^b	5.0 ± 1.2^b
Total carbohydrates	46.2 ± 13.5^a	23.5 ± 7.9^b	22.9 ± 3.6^b

Mean values ± sd are shown. Different letters indicate statistically significant differences between treatments at $p = 0.05$.

Plants in the low-mineral treatment had significantly lower leaf contents of chlorophylls a and b compared with the other two treatments (**Table 5**), although all the three treatments showed the same content of carotenoids (∼3.5 mg·g^{-1} DW).

Leaves from the organic waste-based and high-mineral treatments had similar total N content, which was significantly higher than the N content in leaves from the low-mineral treatment. The organic N was, therefore, a major contributor to the observed differences in total N content. The organic waste-based and low-mineral treatments showed similar total N distribution to leaves (73.5 and 73 g N, correspondingly) and fruits (56 and 55 g N, correspondingly). The total N distribution in the high-mineral treatment was 113 g N to leaves and 81 g N to fruits (**Supplementary Figure 3**).

The leaves of plants grown with the organic waste-based fertilizer had significantly larger concentrations of all analyzed carbohydrates compared with the other two treatments (**Table 5**). The only exception was a similar concentration of fructose between the leaves from the organic waste-based and high-mineral treatments (4.8 ± 1 and 4.2 ± 1.4 mg·g^{-1} DW, respectively). Glucose, fructose, sucrose, and starch concentrations were 93, 68, 42, and 182% higher in leaves from the organic waste-based treatment than from the low-mineral treatment. The higher accumulation of carbohydrates in leaves from the organic waste-based treatment also coincided with the lowest total biomass in the whole plants, indicating that the reduction of biomass accumulation might arise due to negative feedback inhibition of photosynthesis and accumulation of carbohydrates in the leaves.

The analysis of the carbohydrate content of leaf samples taken at the end of the light period (EL) and the end of the dark period (ED) showed that only glucose concentrations were significantly different between these time periods in leaves from the organic waste-based and high-mineral treatments (**Supplementary Figure 2**).

Ionic and Metabolic Composition of Xylem Sap

The xylem sap of tomato plants grown with organic waste-based fertilizer had the highest concentration of NH_4^+, Cl^-, and Na^+ ions, and exceeded the concentrations found in the high-mineral treatment by 6.6-, 4.6-, and 20-fold, respectively

(**Figure 5**, **Supplementary Table 3**). The xylem sap of plants from the organic waste-based treatment also had the lowest concentrations of SO_4^{2-} and PO_4^{3-} among all treatments, 5.4 and 3-fold lower, respectively, than in the high-mineral treatment. NO_3^- and K^+ were dominant in all the treatments, and their concentration was not significantly different between the organic waste-based and low-mineral treatments.

Untargeted metabolic analysis was performed with GC-MS on the xylem sap from the three treatments (**Figure 6**, **Supplementary Table 4**). An unsupervised multivariate analysis (PCA, **Figure 6A**) showed significant differences in the xylem sap from the low-mineral and high-mineral treatments, while the xylem sap from the organic waste-based treatment shared a high degree of similarity with both of the other treatments. the supervised analysis clearly separated the treatments (sPLS-DA, **Figure 6B**) similar to the separation achieved with a heatmap plot, which showed that treatments were correctly separated based on the 50 most significant features according to ANOVA testing (**Figure 6C**). The organic waste-based treatment had a compositional heterogeneity that was correlated with the cultivation conditions (i.e., samples from plants grown in the same box have similar compositions). Among the significantly different features, the plants in the organic waste-based treatment had the highest concentration of amino compounds (e.g., asparagine and putrescine) and of some organic acids (e.g., malate) (**Figure 6D**). The high-mineral treatment had the highest concentrations of several compounds, especially those involved in galactose metabolism (e.g., ribose and galactinol) (**Figure 6D**). The low-mineral treatment generally had the lowest concentrations of the analyzed compounds, such as sucrose (**Figure 6D**). However, the levels of some amino acids (e.g., serine and alanine) were significantly higher than in the other treatments (**Figure 6D**).

DISCUSSION

Tomato cultivation in a closed re-circulating hydroponics system using organic waste-based fertilizer and an integrated nitrification bioreactor was confirmed in this study to produce yields comparable with those achieved with current hydroponic technology. Moreover, we also identified the principal factors that drive the formation of the major components of yield and that modulate fruit quality under organic waste-based plant cultivation. Specifically, we found that (I) organically cultivated plants had a lower growth rate compared to plants cultivated with conventional nutrition, and this was related to the low concentrations of essential nutrient elements in the organic waste-based nutrient solution; (II) fruits of organically cultivated plants were larger in size, that was related to greater availability of Cl^- in the nutrient solution of organic hydroponics and subsequently higher Cl^- accumulation in the fruits; (III) tomato plants grown with an organic waste-based nutrient solution accumulated more carbohydrate in the leaves; and (IV) fruits cultivated with organic waste-based fertilizer or a low-mineral treatment had lower quality than fruits cultivated with the high-mineral nutrient solution. However, different mechanisms were responsible for the reductions in fruit quality in response to organic waste-based and low-mineral treatments.

Hydroponics With Organic Waste-Based Fertilizer Decreases Plant Growth, Development, and Yield Because of the Low Concentration of Essential Mineral Elements in the Nutrient Solution

The classical nutrient solution used in industrial hydroponics contains high concentrations of nutrients, exceeding 10 mM for macronutrients like nitrate and potassium (Jones, 2007; Sonneveld and Voogt, 2009), which is the concentration that corresponds to the saturation of the second system of nutrient uptake (George et al., 2012). However, these high concentrations are not necessary for hydroponically grown plants, at least during the early growth stage when plant growth is exponential (Ingestad and Lund, 1986). Numerous experiments examining unlimited, optimal, and suboptimal supplies of nutrients have shown that growth reduction under low concentrations of nutrients in hydroponic solution does not occur only because the concentration is too low for sufficient nutrient uptake but also because of the restricted volume of the nutrient solution: low concentrations result in more rapid and complete depletion of nutrients that occurs with the solution with higher concentrations. Thus, the control of nutrient supply by external concentration is considered to be inadequate because it offers two options for concentration-controlled culture: excess supply or uncontrolled deficiency (Macduff et al., 1993). Taking into account the importance of excluding the complete depletion of nutrients from the solution, we used a large-sized 550 L reservoir and conducted regular monitoring of the concentrations of essential elements in the solution. These approaches allowed us to maintain the continuous presence of essential nutrient elements in solution throughout the entire plant cultivation period in both the organic waste-based and the low-mineral treatments. The fact that plant development (based on the number of trusses, **Table 2**) decreased in the organic waste-based and low-mineral treatments (despite the verified continuous presence of essential elements in nutrient solution, **Table 1**) indicates that tomato plants, throughout long-term cultivation, require high concentrations of nutrients typically found in the high-mineral solution used in industrial hydroponics.

Low nutrient concentrations are typical in close-circulation systems, where the main source of essential mineral elements is the digestion of organic fertilizers (Bittsanszky et al., 2016). This type of closed-circulation system is represented by aquaponics, which combines hydroponics and fish production, using the fish effluent, after digestion in a bioreactor, as a source of plant nutrients. In agreement with our data, the low concentration of nutrients in aquaponics solutions is also considered a major challenge that hinders the sustainability of aquaponics as a mode of plant production (Yep and Zheng, 2019). These low concentrations of essential nutrients are less of a problem for leafy vegetables, such as lettuce (Pantanella et al., 2012; Delaide et al., 2016), but can restrict the yield potential for larger plants, such as tomato, which have a longer production season.

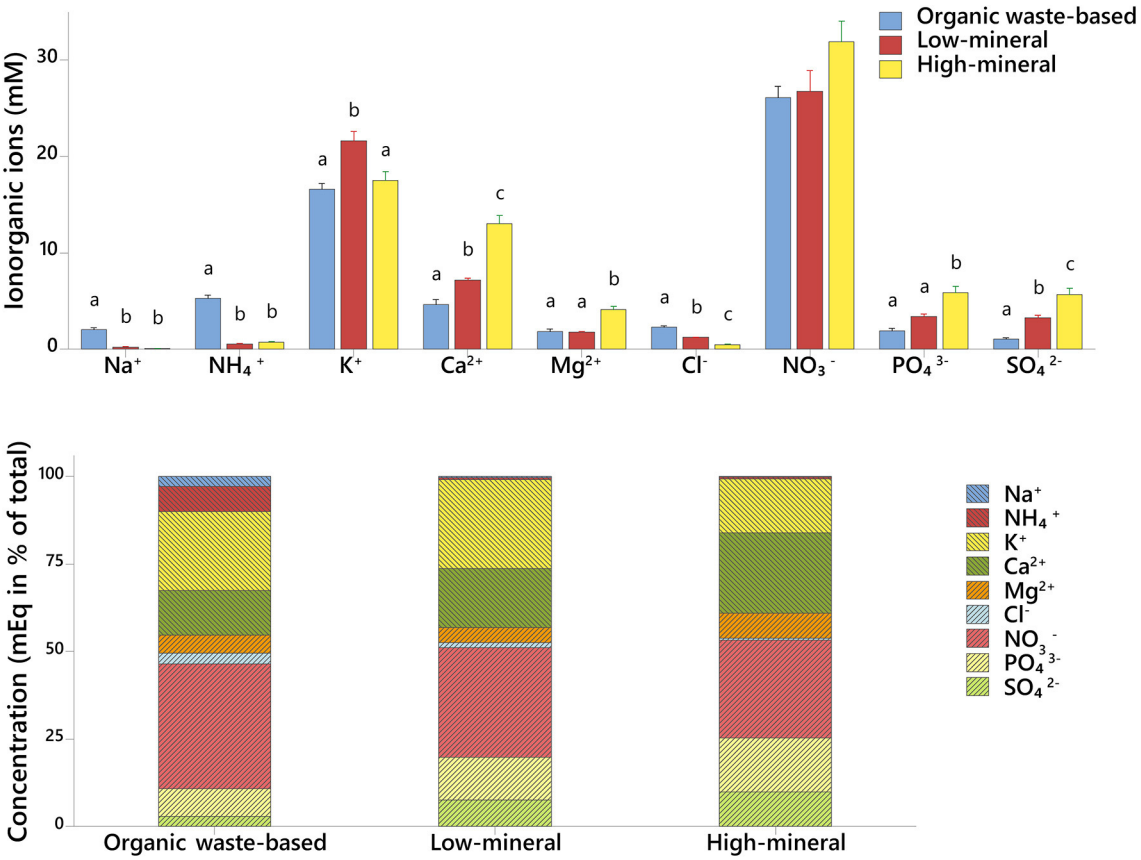

FIGURE 5 | Ionic composition of xylem sap of plants cultivated with different fertilizers. Upper graph, concentration of individual inorganic ions in mmol. Lower graph, ratios of individual inorganic ions in mEq. Mean values ± SD are shown. Different letters indicate statistically significant differences at $p = 0.05$, $n = 4$.

Tomato productivity was similar to mineral one when using organic fertilizer in soil (Mitchell et al., 2007; Antonious et al., 2019) and short-term hydroponic cultivation (Shinohara et al., 2011). However, only this study compares tomato productivity in soilless hydroponics in long-term cultivation.

Interestingly, the response of tomato plants may not always be explained by differences in mineral composition of nutrient solution. For example, Knaus and Palm (2017) found that the use of tilapia effluent gave higher tomato yield when compared to common carp effluent. The reason for this difference is not clear, but we can assume that the presence of specific biostimulants and/or enrichment of the population of plant growth-promoting bacteria could play a role in this difference. Overall, however, the suboptimal nutrient concentrations in organic hydroponics systems appear to be a major limitation for growing crops that have high nutrient requirements.

Both the organic waste-based and the low-mineral treatments decreased plant growth (**Table 3**). However, the reason for the growth decrease was not identical, because many agronomic and physiological plant traits showed different responses depending on the nutrition supply. Moreover, the values of many plant traits were more similar to traits of high-mineral plants when the plants were grown in the organic waste-based treatment than when grown in the low-mineral treatment. Specifically, plants cultivated with organic waste-based fertilizer were similar to the high-mineral plants in such traits as distribution of dry matter to fruits and leaves, SLA, plant height, and xylem sap exudation rate from decapitated plants. By contrast, the plants subjected to the low-mineral treatment differed significantly in these traits when compared with either the organically grown or the high-mineral cultivated plants (**Table 3**). The metabolomic analysis of the xylem sap also showed that metabolite composition under organic waste-based cultivation tended to fall between the high-mineral and low-mineral treatments (**Figure 6**), further supporting the observations on morphological traits that some as yet unspecified factor available in the organic waste-based system was able to trigger a partial recovery from the "negative" effects of low concentrations of essential elements. However, these factors were not able to recover plant growth in general, as the plants grown with organic waste-based fertilizer had the smallest plant biomass (**Table 3**).

The low biomass in the organic waste-based treatment might be related to the imbalance of plant nutrition in the organically cultivated plants because the functional leaves of these plants showed very low phosphate and sulfate concentrations (**Table 5**). The fact that these leaves also accumulated excess

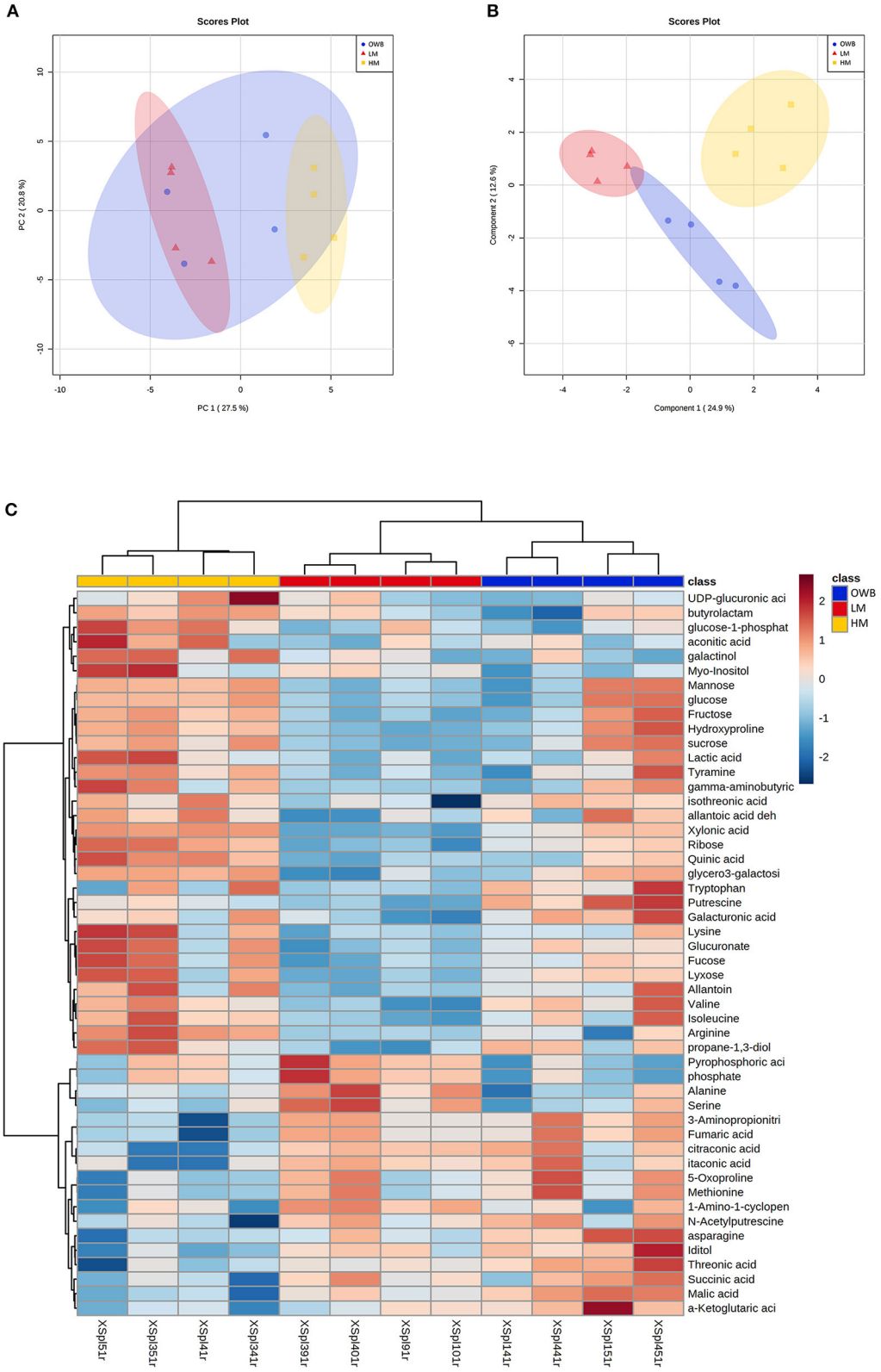

FIGURE 6 | (Continued)

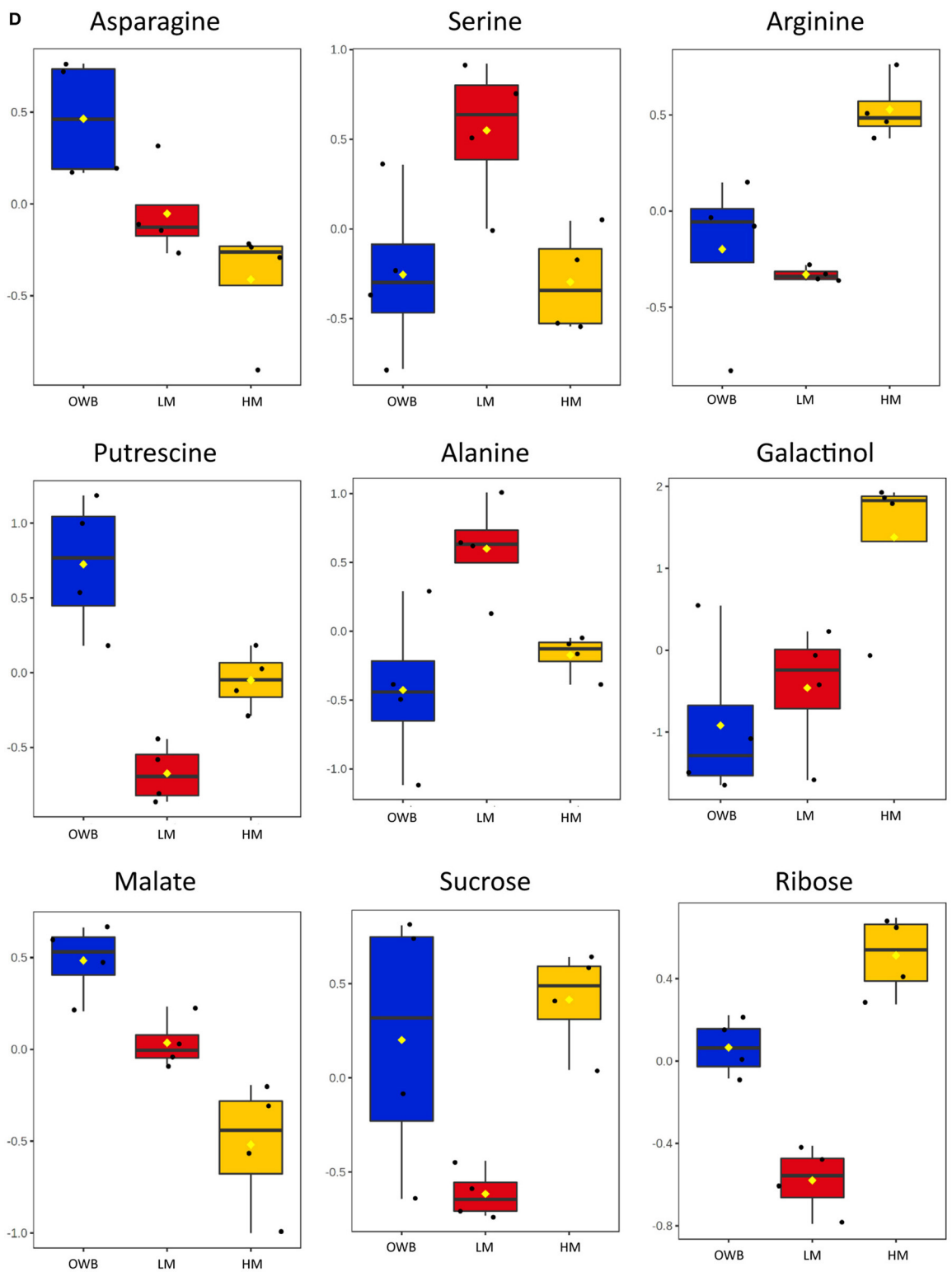

FIGURE 6 | Selected metabolites from the xylem sap of plants cultivated with different fertilizers. Identification is performed in MSDIAL, statistics performed with MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/). Concentrations are shown as log-transformed and normalized mean values. **(A,B)**: Unsupervised and supervised analysis of xylem sap composition, where **(A)** is principal component analysis (PCA) loading plot and **(B)** is sPLS-DA loading plot. **(C)**: Heatmap of 50 most significant metabolites based on ANOVA test. **(D)**: Fold change in selected metabolites for three treatments, $n = 4$. OWB, Organic waste-based; LM, Low-mineral; HM, High-mineral treatments.

carbohydrates supports the assumption that phosphate and/or sulfate deficiency might inhibit assimilate export from the leaves and potentially induce a negative feedback inhibition of photosynthesis under these deficiency conditions. Indeed, phosphate deficiency induces an accumulation of non-structural carbohydrates in leaves (Hammond and White, 2008) that ultimately can decrease photosynthesis and plant growth. Furthermore, the observation that the plants cultivated on organic waste-based fertilizer allocated more dry matter to the roots than plants cultivated in the high-mineral and low-mineral treatments supports the hypothesis that organically cultivated plants experienced nutrient deficiency (**Table 3**). The trait of dry matter allocation to the roots is very sensitive to nutrient deficiency, as confirmed by numerous investigations (Paponov et al., 1999; Kang and van Iersel, 2004). However, the increased dry matter allocation to the roots in the plants cultivated with organic waste-based fertilizer might also be related to other factors, such as the presence of specific organic compounds. Indeed, stimulatory effects on root growth by organic wastes have been observed in soil-grown citrus trees (Martinez-Alcantara et al., 2016), maize seedlings (Canellas et al., 2002; Jindo et al., 2012), and lettuce in hydroponic culture (Shinohara et al., 2011). Some authors, for example, Compant et al. (2005) and Chinta et al. (2015), have associated this with enhanced root hair formation due to auxin and ethylene produced by microorganisms, whereas Baldi et al. (2010) have linked it to the auxin-like properties of certain humic acids present in organic waste. In addition, humic acids also possess chelating properties that enhance the availability of certain elements, like Fe or Zn. Humic acids, microorganisms, and root hairs are all interconnected, as the initial enhancement of root hair formation improves microbial biofilm development, which further increases root hair growth because of the hormone production by the microorganisms.

Another reason for lower biomass accumulation in organic waste-based treatment may be the presence of organic waste phytotoxins. Plants grown by organic fertilization often develop stress symptoms related to the presence of phytotoxins in organic fertilizer or the accumulation of phytotoxins released by plants. For instance, bok choi plants showed reduced biomass accumulation when cultivated in a closed hydroponic system with plant-derived digestate as a fertilizer (Pelayo Lind et al., 2020).

Oxygenation was provided to both treatments with low EC to improve the nitrification ability of a bioreactor (Ebeling and Timmons, 2012). Saturation of nutrient solution with oxygen was reported to reduce growth self-inhibition of roses in re-circulating cultivation systems (van Os et al., 2012).

Regulation of Fruit Size Under Organic Waste-Based Production

One interesting observation in this study was that the fruits from organic treatments were larger in size than the fruits from the high-mineral and low-mineral treatments (**Table 2**). Tomato fruit size is strongly dependent on water flux from the phloem and xylem, because mature tomato fruits have a water content of 92–95% (Guichard et al., 2001), and the extent of water contribution from the phloem and xylem to the fruits depends on plant nutrition. Indeed, reduction in the nutrient concentrations in the low-mineral treatment strongly increased root power (based on the xylem sap rate of decapitated plants, **Table 3**), indicating a higher contribution from water moving through the xylem. This observation is in agreement with numerous other observations of the effects of nutrient concentrations on xylem sap flow rate (Ehret and Ho, 1986). Interestingly, the fruit size for the low-mineral treatment was not significantly increased beyond that seen in high-mineral nutrition, suggesting that the higher xylem flux to the fruits was compensated by a lower flux from the phloem, resulting in unchanged final fruit size.

Surprisingly, plants cultivated with the organic waste-based fertilizer, despite the similarities in the ion concentrations in the nutrient solution to those in the low-mineral treatment, had a xylem sap flow rate half that observed in the low-mineral plants and was more similar to the xylem sap flow rate in the high-mineral solution. This indicates that some other factor specific to the plants cultivated with organic waste-based fertilizer was offsetting the positive effect of low ion concentrations on root power. The root power, and therefore the water flux through the xylem, was not enhanced in the plants cultivated with organic waste-based nutrition compared with the plants in the high-mineral nutrition, and yet the size of the fruits was significantly larger in the plants cultivated with organic waste-based fertilizer. This suggests that some alternative form of regulation of fruit size is occurring under the organic nutrition conditions, independent of root power regulation.

Detailed ionic and metabolomics analysis, as well as determination of the xylem sap rate of decapitated plants, strongly pointed to a potential role of Cl^- in the fruit size increase in the plants growing in organic hydroponics. Indeed, fruit analysis showed about a 5-fold higher Cl^- accumulation in the plants cultivated in the organic waste-based treatment than in the plants cultivated in the low-mineral and high-mineral treatments (**Figure 4**). High salinity is often responsible for reductions in fruit size (Oliveira et al., 2013; Zhang et al., 2016), but at intermediate concentrations, the replacement of NO_3^- with Cl^- causes no effects of Cl^- on fruit size (Chapagain et al., 2003). However, the effect of low chloride concentrations on the nutrient solutions with low electrical conductivity (EC) has not been investigated before. A recent investigation of the role of Cl^- in shoot growth showed that under non-saline conditions (up to 5 mM Cl^-) and with no water limitations, Cl^- specifically stimulated an increase in leaf cell size and led to a moderate increase in biomass because of greater cell expansion (Franco-Navarro et al., 2015). Application of Cl^- in a 1–5 mM range indicated specific roles for Cl^- in regulating leaf osmotic potential and turgor. Clearly, these effects of Cl^-, if also occurring in tomato fruits, could reduce the fruit quality of tomatoes produced with organic waste-based fertilizer.

Quality of Tomato Fruits Under Organic Waste-Based Production

Organically cultivated fruits are generally perceived to have higher quality than conventionally grown fruits. In this experiment, tomatoes cultivated in hydroponics with organic waste-based fertilizer had lower fruit quality, based on common characteristics such as SSC, TTA, and DMC (**Table 2**).

Accumulation of soluble carbohydrates, which are the main component of tomato fruit quality, is strongly dependent on the contribution of phloem and xylem fluxes to the fruits. Direct comparison of the two mineral treatments (high-mineral vs. low-mineral) showed that an increased contribution of xylem flux over phloem flux due to higher root power in the low-mineral treatment (because of increased xylem sap rate) might be the main cause of decreased fruit quality because the concentration of sucrose is much higher in the phloem than in the xylem (Nakamura et al., 2008; Jacobs et al., 2012).

The lower quality of fruits of the plants cultivated with the organic waste-based fertilizer, in contrast, appears to arise *via* other mechanisms than increased xylem flux, because the xylem sap flux in decapitated plants was the same as that of organically cultivated and high-mineral plants. The lower quality might be related to a dilution effect that gave rise to the larger fruits. As mentioned before, the higher concentration of Cl^- in the nutrient solution, and ultimately in the fruits, might be responsible for the larger fruit size. Moreover, high concentration of Cl^- in the fruits may have osmotic effects that can result in reduced demands for the accumulation of low-molecular-weight organic compounds (organic acids and monosaccharides) required to maintain fruit turgor.

The effect of NaCl on fruit quality is strongly dependent on the NaCl concentration in the nutrient solution. High NaCl concentrations (above 100 mM) that cause salt stress typically increase the concentrations of soluble carbohydrates (Zhang et al., 2016) but may also cause blossom end rot in tomatoes because of reduction in the uptake of K, Ca, Mg, and P (Sonneveld and Voogt, 2009; Hagassou et al., 2019). Investigations of moderate NaCl applications (17 mM) have shown no deleterious effects of Cl^- on fruit quality (Montesano et al., 2010).

Interestingly, low concentration of soluble carbohydrates was observed in the fruits under organic waste-based cultivation, despite the highest accumulation of non-structural carbohydrates in the leaves with this treatment. This indicates that regulation of the export of carbohydrates from the leaves participates in the regulation of soluble sugar accumulation in fruits.

Common Recommendations for Organic Waste-Based Fertilizers Used in Hydroponics

In this study, organic waste was used as a base for fertilizer formulation. However, its elemental composition was not sufficient for normal growth of tomato plants over 6 months. Therefore, the liquid digestate was supplemented with essential microelements, magnesium, calcium, sulfate, and phosphate ions. When necessary, the pH was adjusted with nitric acid or potassium hydroxide.

Maintaining an optimal pH (5.5–6.5) of the nutrient solution is very important for nutrient availability in the hydroponic cultivation of tomatoes (Hosseinzadeh et al., 2017). In particular, the pH (7.5–8.5) will reduce the availability of Fe, Mn, and Zn to the plants (Resh, 2012). The average pH of the organic waste-based and high-mineral solutions was below the recommended upper limit (6.3 and 6.4, correspondingly), while the low-mineral solution showed a higher average value of pH 7 (**Table 1**), which might only partially limit nutrient availability. At the same time, pH values of up to 7 did not affect yield and DMC of cucumber in fertilization systems that incorporated an integrated nitrification bioreactor (Tyson et al., 2008).

Despite constant recirculation of the nutrient solution, the variation in pH level was highest for the organic waste-based fertilizer (SD = 0.99, **Table 1**). One explanation is the batch addition of the high-pH organic waste (9.7), which temporarily increased the pH of the fertilization solution. With time, as ammonium was nitrified to nitrate, the pH again declined. A large variation in pH was also reported in the cultivation of bok choi in a closed system with an integrated MBBR (Pelayo Lind et al., 2020).

This strong pH variation in the nutrient solution might be responsible for the chlorosis observed in the upper leaves in the canopy of plants grown with organic waste-based fertilizer, assuming this chlorosis was a consequence of deficiency in non-recirculating nutrients. The pH fluctuations after the addition of new organic fertilizer could potentially influence the uptake of microelements (e.g., Fe and Zn, Marschner, 1995), as these microelements have a low reutilization rate from previously accumulated sources in plants, such as old leaves. Consequently, their deficiency appears on the upper, most recently expanded leaves. However, we cannot exclude the potential influence of the organic component of the organic waste-based fertilizer, as this had a high concentration of sterols, lipids, fatty acids, and especially phenolic compounds (data not shown).

Overall, this study showed that fertilizer based on a liquid by-product of biogas production from livestock manure can be used for the hydroponic cultivation of tomatoes in a greenhouse. However, the nutrient content requires optimization by the selective addition of minerals or by the addition of a combination of different organic wastes for optimal plant development. The use of organic waste-based fertilizer is favorable from the circular economy point of view but has several limitations, such as production of lower quality fruit, need for nitrification, and control of the nutrient composition of fertilizer. The use of this fertilizer should therefore be policy-driven or should operate from a niche in the market where it will not compete with tomatoes produced by cultivation with mineral fertilizers.

We have designed and constructed a closed re-circulating cultivation system with an integrated nitrification bioreactor. We have compared plant development, fruit yield, and quality of tomatoes grown with a fertilizer based on organic waste (the liquid by-product of biogas production from pig manure) with greenhouse tomatoes grown with mineral fertilizers. The tomatoes grown with the organic waste-based fertilizer had a similar yield but poorer taste characteristics when compared with tomatoes grown with the high-mineral fertilizer. The plants grown with organic waste-based fertilizer had larger average fruit weight, smaller proportion of leaves, and lower DMC of the leaves compared to plants grown with the low-mineral fertilizer. The plants grown with the organic waste-based

treatment accumulated higher amount of salts, especially Cl^-, in their tissues. Overall, fertilizers based on organic wastes change plant development toward a generative state and can partially recover the physiological and biochemical responses seen in plants grown under suboptimal fertilization conditions, suggesting that these fertilizers could be favored over mineral fertilizers with similar inorganic compositions. However, the use of organic waste-based fertilizers is less feasible than high-mineral fertilizers because of the lower quality of tomato fruits produced.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

DK, IP, and MV planned and designed the experiment. DK performed the experiment and analysis of samples. MP and AP substantially contributed to the establishment of analytical procedures for chemical analysis of leaves and to sample collection and analysis. DK drafted the manuscript with a substantial contribution from IP, MV, and MP. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.680030/full#supplementary-material

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frontiers in Plant Science

ORIGINAL RESEARCH
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Evaluating the Aqueous Phase From Hydrothermal Carbonization of Cow Manure Digestate as Possible Fertilizer Solution for Plant Growth

Silvia Celletti[1], Maximilian Lanz[1], Alex Bergamo[1], Vittoria Benedetti[1], Daniele Basso[2], Marco Baratieri[1], Stefano Cesco[1] and Tanja Mimmo[1,3]**

[1]Faculty of Science and Technology, Free University of Bolzano-Bozen, Bolzano, Italy, [2]HBI S.r.l., Bolzano, Italy, [3]Competence Centre for Plant Health, Free University of Bolzano-Bozen, Bolzano, Italy

Improving the agronomic use of recycled nutrients derived from organic waste is one of the priorities within the measures adopted by the European community to reduce environmental issues but remains an unexplored area of research. This study focused on investigating the possibility of using innovative fertilizer solutions in hydroponic systems for the growth of agricultural plants. To this purpose, a liquid fraction [aqueous hydrothermal carbonization (HTC) liquid (AHL)] derived from HTC of cow manure digestate was chemically characterized (pH, electrical conductivity, mineral elements, and organic compounds such as phytotoxins), diluted with distilled water (1:30, 1:60, and 1:90, v/v) to reduce its potential phytotoxicity, and used to grow hydroponic maize (*Zea mays* L.) plants instead of the classical full-strength nutrient solution. The results indicated that the dilution ratio 1:30 of the AHL solution maintained a high level of toxicity for the plants (phytotoxic substances, especially Na and alkalinity), inducing the arrest of their growth. Differently, the two other dilution ratios (i.e., 1:60 and 1:90) seemed to considerably limit the levels of toxicity, since they allowed the plants to develop. However, these dilution ratios were poor in nutrient elements, inducing alteration in photosynthesis and an onset of deficiency symptoms such as pronounced leaf chlorosis. In view of an eco-friendly approach, future studies are, therefore, needed to identify the correct species-specific dilution ratio to supply both low levels of phytotoxins and adequate content of essential nutrients for appropriate plant growth and development. Furthermore, in order to lower specific Na phytotoxicity, treatments are of utmost importance before using AHL as a fertilizer solution.

Keywords: digestate management, hydrothermal carbonization, liquid phase, maize, nutrients, sustainability

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Texas A&M University, United States

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Daniele Del Buono,
University of Perugia, Italy
Joe Masabni,
Texas A&M University, United States

****Correspondence:***
Silvia Celletti
silvia.celletti@unitus.it
Tanja Mimmo
tanja.mimmo@unibz.it

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INTRODUCTION

There is a clear need for waste management actions aimed at encouraging restraint of waste volumes and efficient recovery and use of resources still present in waste (Directive 2006/12/EC, 2006). Anaerobic digestion (AD) is a biological process applied to biodegradable wastes (e.g., animal manure, sewage sludge, organic fraction of municipal solid waste, and aquaculture

residues) for their conversion into biogas to be exploited for energy purposes (Weiland, 2010; Appels et al., 2011). In the context of natural resource recycling and reuse, digestate, the solid/liquid by-product of AD, should also be valorized. Although digestate is used in agriculture for its high mineral nutrient content [mainly nitrogen (N), phosphorus (P), and potassium (K)] and organic matter (Baştabak and Koçar, 2020), it can be considered as an effective organic soil amendment or fertilizer only if managed properly (Nkoa, 2014). In this respect, it is important to highlight that N input must be limited to 170 kg ha^{-1} $year^{-1}$ on agricultural soils in vulnerable areas according to European Directives 2016/2284/EU and 91/676/EEC (Celletti et al., 2021). Consequently, in specific agricultural contexts/areas, digestate must be disposed of as waste with relevant operational transport and energy consumption for drying (mainly related to its high water content that can reach up to 90–95%, w/w) and, subsequently, additional environmental costs (Timonen et al., 2019).

An innovative technological solution, proposed to treat and valorize digestate and wet biomass in general, is represented by hydrothermal carbonization (HTC; Mumme et al., 2011; Hitzl et al., 2015; Pecchi and Baratieri, 2019). Unlike conventional dry thermochemical processes (e.g., combustion, pyrolysis, and gasification), HTC does not require an expensive or energy-intensive preliminary drying step, as it directly exploits the water retained in digestate as a solvent during the process. In particular, the HTC process takes place between 180 and 250°C and 10 and 80 bars, with residence time ranging from few minutes to several hours. The HTC process converts wet feedstock into gas (mainly CO_2), a carbonaceous solid fraction (termed hydrochar), and a liquid phase [termed aqueous HTC liquid (AHL); Funke and Ziegler, 2010; Maniscalco et al., 2020].

In the last 20 years, hydrochar has gained attention because of its chemical and physical properties, which can be exploited for numerous purposes (Hu et al., 2008; Fang et al., 2018). For instance, hydrochar can be used as (i) solid fuel for energy production (Lucian and Fiori, 2017); (ii) adsorbent material to remediate polluted soils and water because of toxic substances (Elaigwu et al., 2014; Fornes et al., 2015; Fang et al., 2018; Parlavecchia et al., 2020); and (iii) organic soil amendment, being rich in carbon (C) and nutrients (Maniscalco et al., 2020). Recently, it has also been suggested as either a stand-alone substrate or a constituent of growing media for plants in soilless culture systems (Belda et al., 2016; Gruda, 2019). However, the evaluation of the effectiveness of hydrochar in the aforementioned applications is still ongoing, since it depends on the initial biomass type/composition and on operating parameters (i.e., temperature, residence time, and pressure).

The valorization of AHL is much more challenging than that of hydrochar, and the literature regarding the knowledge at the level of chemical characterization and subsequent possible applications of AHL is remarkably scarce (Vozhdayev et al., 2015; Mau et al., 2019; Langone and Basso, 2020). Similar to hydrochar, the differences in the chemical compositions of AHL depend on the type/composition of the feedstock and operating conditions adopted during HTC, which affects the distribution of compounds between the solid and liquid phases (Langone and Basso, 2020).

Overall, AHL is naturally composed of water and a mixture of organic and inorganic compounds (Lucian and Fiori, 2017): organic acids, carbohydrates, nutrients (especially nitrogen and phosphorus), dissolved salts, and heavy metals (Nakhshiniev et al., 2014; Huang and Yuan, 2016). In addition, most potentially phytotoxic organic compounds (mainly furan derivatives and aromatic compounds; Bargmann et al., 2013; Puccini et al., 2018; Celletti et al., 2021), which are formed during the HTC process from biomass polymers (Funke and Ziegler, 2010), are water-soluble and, therefore, concentrated in the AHL (Karagöz et al., 2005; Bargmann et al., 2013; Elaigwu and Greenway, 2016).

Some routes have been suggested for AHL exploitation as a recirculation substrate in a closed-loop system for AD (Pecchi and Baratieri, 2019) or HTC (Stemann et al., 2013; Weiner et al., 2014; Catalkopru et al., 2017) to reduce large AHL volumes. Alternatively, considering its high nutrient content, AHL can be used as a nutrient source for microalgae growth (Levine et al., 2013; Belete et al., 2019), and for irrigation in agricultural fields (Nicolae et al., 2020), fertilizer production (Yahav Spitzer et al., 2018; Wang et al., 2019), or recovery of chemicals such as N and P (Becker et al., 2019; Ovsyannikova et al., 2019).

Among the few studies that evaluated the possibility of valorizing AHL using it as a fertilizer for plant species, several other ones considered the effects of AHL on plant growth by mixing it with hydrochar or other organic substrates (Busch et al., 2013; Jandl et al., 2013; Sun et al., 2014). To the best of the knowledge of the authors, only two studies (Vozhdayev et al., 2015; Mau et al., 2019) investigated the effect of AHL alone on plant growth as a liquid fertilizer added to inorganic substrates (e.g., silica or quartz sand), showing, in some cases, inhibitory and, in others, stimulatory plant responses according to the origin and levels of AHL applied. However, none of these studies examined plant growth responses using the AHL solution alone as a substitute for the nutrient solution commonly adopted in soilless systems. This study is the first to fill this knowledge gap. Specifically, it aims to evaluate the possibility of using AHL from cow manure digestate by testing it at three different dilution ratios (1:30, 1:60, and 1:90, v/v) with distilled water as a possible nutrient solution to support the hydroponic growth of maize (*Zea mays* L.), which is selected because of its fast growth.

MATERIALS AND METHODS

Digestate Collection, Moisture, and Microbiological Analysis

Semi-liquid cow manure anaerobic digestate was used as feedstock for the HTC process. It was provided by the company "Biogas Wipptal Srl," located in Vipiteno, Italy.[1]

Total moisture content of the digestate was determined in accordance with ISO 18134-3:2015 (EN ISO 18134-3:2015, 2015).

[1]https://www.biogas-wipptal.com/en/

Briefly, the fresh digestate was weighed, placed in aluminum crucibles inside an oven, and heated up to 105°C for 24 h. Subsequently, it was cooled in a desiccator and weighed again to calculate the moisture content.

Detection and enumeration of fecal coliforms (*Escherichia coli*) and Salmonella spp. in the digestate were performed according to the procedure ISO 7251:2005 (ISO 7251:2005, 2005) and USEPA 1682:2006 [USEPA (U.S. Environmental Protection Agency), 2006].

Chemical Characterization of Digestate

Prior to chemical analyses, the digestate was oven-dried at 105°C until a constant weight was reached, and then it was finely pulverized and homogenized with a ball mill (Mixer Mill, MM400, RETSCH, Bergamo, Italy).

The pH and electrical conductivity (EC) of the water extract were measured by immersing the electrode of a portable pH meter (pH 70 + DHS, Giorgio·Bormac Srl, Modena, Italy) and a conductivity meter (EC-meter, edge™ HI2020-02, HANNA Instruments Srl, Salerno, Italy), respectively. The water extract was obtained from 2-g dry weight (DW) of pulverized digestate diluted in 40 ml Milli-Q water (1:20, w/v), after 30 min of shaking and 5 min of centrifugation at 4,000 rpm at room temperature.

Total C and total N contents were determined by weighting approximately 2.5 mg DW of pulverized digestate into tin capsules (5 × 9 mm, Säntis Analytical AG, Teufen, Switzerland), carefully closed with tweezers and inserted into the sample tray of a Flash Elemental Analyzer (Flash EA 1112, ThermoFisher Scientific, Dreieich, Germany). The Flash EA had an oxidation furnace temperature of 1,020°C and a reduction furnace temperature of 900°C. In an integrated gas chromatograph, the gas mixture was separated and measured by means of a thermal conductivity detector. The results were expressed as a percentage of C and N.

The concentrations of the main mineral elements were determined by mineralizing approximately 0.3 g DW of pulverized digestate with 4 ml of concentrated ultrapure nitric acid (HNO_3, 650 ml L^{-1}, Carlo Erba, Milan, Italy), using a single reaction chamber microwave digestion system (UltraWAVE, Milestone, Shelton, CT, United States). After cooling, the digested sample was diluted with Milli-Q water to 20 ml. Finally, the concentrations of the elements were analyzed with an inductively coupled plasma-optical emission spectroscopy (ICP-OES, Spectro Arcos, Spectro Ametek, Kleve, Germany) instrument using spinach leaves (SRM 1570a) and tomato leaves (SRM 1573a) as external certified reference materials.

The ash content of the digestate was determined in accordance with EN ISO 18122:2015 (EN ISO 18122:2015, 2015). Briefly, the dry and pulverized digestate was weighed and then placed in ceramic crucibles inside a muffle furnace. It was heated according to the following temperature program: (a) ramp from room temperature to 250°C at 5°C min^{-1}; (b) hold at 250°C for 60 min; (c) ramp to 550°C at 10°C min^{-1}; (d) hold at 550°C for 120 min; (e) allow the temperature to drop to 105°C, and (f) hold at 105°C until it was removed. After the digestate was taken out of the muffle, it was cooled in a desiccator and weighed again to calculate the ash content.

Hydrothermal Carbonization Experiment and Sampling of AHL

Hydrothermal carbonization of the digestate was conducted in a 4-L stainless steel batch reactor in order to produce the liquid fraction (AHL). The detailed description of the experimental procedure is reported elsewhere (Celletti et al., 2021). Briefly, 2 L of the digestate, previously stored at 4°C, was placed in the reactor and heated up to 180°C at a rate of 4°C min^{-1}. The set process temperature was kept constant for a residence time of 3 h, and then the reactor was left to cool overnight. The latter was opened at room temperature to collect the resulting semi-solid product. Subsequently, AHL was obtained by separating it from the solid fraction (hydrochar) by centrifugation at 5,000 rpm for 5 min at 4°C and collected in dark glass bottles. The AHL was stored at 4°C before being chemically characterized and used in the plant experiment.

Chemical Characterization of AHL

The AHL was thoroughly vortexed prior to being used for chemical characterization.

The pH and electrical conductivity (EC) of the AHL were measured directly by immersing the electrode of a portable pH-meter (pH 70 + DHS, Giorgio·Bormac Srl, Modena, Italy) and a conductivity meter (EC-meter, edge™ HI2020-02, HANNA Instruments Srl, Salerno, Italy), respectively.

The total organic carbon (TOC) and total nitrogen (TN) of the AHL were simultaneously measured with TOC-L Analyzer equipped with TNM-L TN Unit and ASI-L Autosampler (Shimadzu Corporation, Kyoto, Japan). This apparatus adopts the 680°C combustion catalytic oxidation method to also efficiently oxidize hard-to-decompose insoluble and macromolecular organic compounds. Before inserting the aqueous phase in the autosampler collector, it was filtered through 0.45-µm regenerated cellulose syringe filters (Phenex™-RC 26 mm 0.45 u, Phenomenex, Castel Maggiore, Bologna, Italy). Afterward, the filtrates were diluted 1:200 (v/v) with Milli-Q water to a final volume of 8 ml.

Two milliliter of AHL was mineralized with 2 ml of concentrated ultrapure HNO_3 (650 ml L^{-1}; Carlo Erba, Milan, Italy), using a single reaction chamber microwave digestion system (UltraWAVE, Milestone, Shelton, CT, United States). After cooling, the digested sample was diluted with Milli-Q water to 20 ml. Finally, the concentrations of the main mineral elements were determined by ICP-OES (Spectro Arcos, Spectro Ametek, Kleve, Germany) analysis, using spinach leaves (SRM 1570a) and tomato leaves (SRM 1573a) as external certified reference materials.

Sugars (glucose), organic acids (lactic acid, formic acid, acetic acid, and fumaric acid), and furan compounds [5-hydroxymethylfurfural (HMF) and furfural] in the AHL were separated and quantified simultaneously by high performance liquid chromatography (HPLC) using a cation exchange column Aminex 87-H column (300 × 7.8 mm, 9 µm, Bio-Rad Laboratories, Segrate, Milano) and an isocratic elution

with 10 mM H_2SO_4 as a carrier solution at a flow rate of 0.6 ml min^{-1}. Sugars were detected with a refractive index detector (2414 RI, Waters Spa, Milan, Italy), while organic acids and furan compounds were detected at 210 and 280 nm, respectively, using a photodiode array detector (2998 PDA, Waters Spa, Milan, Italy). Standards for each analyte were prepared as individual stock solutions, using reagent-grade compounds (Merck, Darmstadt, Germany) and then combined to give diluted reference standards. All compounds were identified by comparing retention times of unknowns to pure compounds.

Each analysis was repeated three times (technical replicates, $n = 3$).

Plant Growth

Maize (*Z. mays* L) seeds were soaked in distilled water for 24 h. Subsequently, they were transferred on a narrow mesh net placed in a container with an aerated solution of 0.5 mmol L^{-1} $CaSO_4$ and left to germinate for 6 days in the dark at room temperature. Six-day-old seedlings were selected on the basis of their size uniformity to be transferred into 1.5-L plastic pots (10 seedlings/pot) filled with a continuously aerated nutrient solution (NS) with the following composition (mM): $Ca(NO_3)_2x$ $4H_2O$ 2, KCl 0.1, KH_2PO_4 0.1, K_2SO_4 0.7, $MgSO_4x$ $7H_2O$ 0.5, $CuSO_4x$ $5H_2O$ 0.2×10^{-3}, Fe(III) – EDTA 0.1, H_3BO_3 1×10^{-3}, $MnSO_4x$ H_2O 0.5×10^{-3} $(NH_4)_6Mo_7O_{24}x$ $4H_2O$ 0.01×10^{-3}, and $ZnSO_4x$ $7H_2O$ 0.5×10^{-3} (slightly modified by Zhang et al., 1991). The pH of the NS was titrated at 6 with 0.1 mM MES-KOH. After 3 days of growing in the NS, the maize plants were transferred into aerated solutions of the AHL diluted with distilled water at a ratio of 1:30, 1:60, and 1:90 (v/v) and grown for additional 12 days. The control plants were grown simultaneously using the NS instead of the AHL. The plants were cultivated for a total of 15 days in a growth chamber under controlled climatic conditions with a day/night cycle of 14/10 h, temperature regime of 24/19°C, light intensity of 250 μmol m^{-2} s^{-1}, and relative humidity of 70%. Growing solutions were changed twice a week, and the pots were rotated randomly to a different position within the block each day for the duration of the experiment.

Measurement of Plant Growth and Root Morphological Parameters

At the end of the experimental growing period (15 days after sowing), the leaf chlorophyll content of the maize plants was measured using a portable non-destructive tool, the Soil Plant Analysis Development (SPAD – 502 Plus, Konica Minolta, Osaka, Japan). Specifically, three SPAD values were taken from the base to the apex (along the proximal, central, and distal portions) of the youngest fully expanded leaf of each plant, resulting in a total of 36 measurements (12 plants × 3 repeats) per treatment, and were averaged and expressed as SPAD index. Subsequently, the maize plants were harvested by separating shoots from roots. The roots were gently rinsed with distilled water. Shoot and root fresh weights (FWs) were recorded, and root-to-shoot ratios were assessed. Digital scans of the root morphological and architectural features (i.e., total length and number of tips) were analyzed with a WinRHIZO™ system (EPSON1680, WinRHIZO Pro2003b Software; Regent Instruments Inc., Quebec, Canada). The roots and shoots were consequently dried at 65°C until a constant weight was reached.

Analysis of Main Essential Nutrients and Sodium in Plant Tissues

Dried plant tissues were finely ground and homogenized with a ball mill (Mixer Mill, MM400, RETSCH, Bologna, Italy). Approximately 0.3 g DW of each ground sample was mineralized in 4 ml concentrated ultrapure HNO_3 (650 ml L^{-1}; Carlo Erba, Milan, Italy) using a single reaction chamber microwave digestion system (UltraWAVE, Milestone, Shelton, CT, United States). After cooling, the digested samples were diluted with Milli-Q water to 20 ml and analyzed by ICP-OES (Spectro Arcos, Spectro Ametek, Kleve, Germany) as previously described.

Statistical Analysis

The data are expressed as means ± SE of technical replicates ($n = 3$) for both the digestate and the AHL analysis and of biological replicates ($n = 12$) for plant analysis. The significance of differences among the means was calculated by one-way ANOVA with LSD test at $p < 0.05$ using the R software (version 3.6.3). R packages, ggplot2 (version 3.3.2), and agricolae (version 1.3-3) were used for data visualization and statistical analysis.

RESULTS

Digestate Properties

Table 1 includes the physical (i.e., moisture content) and microbiological (i.e., *E. coli* and Salmonella spp.) parameters of the digestate, which was used as feedstock for the HTC process. The digestate had a relatively high moisture content (approximately 90% on FW basis) and had, therefore, a semi-liquid consistency, and it did not contain fecal bacteria potentially harmful to human health and the environment. Indeed, the number of *E. coli* (on DW basis) resulted in less than 3, i.e., below the limit of quantification of the method, and Salmonella species (on FW basis) were absent (**Table 1**).

Figure 1A shows that the digestate was characterized by alkaline $pH_{(1:20)}$ (8.37 ± 0.03), $EC_{(1:20)}$ value of 9.27 ± 0.06 mS cm^{-1}, and contents of total C, total N, and ash (in percentage on DW basis) were 35.07 ± 0.10, 2.27 ± 0.04, and 25.64 ± 0.19, respectively. **Figure 1B** shows the composition

TABLE 1 | Physical and microbiological properties of the digestate.

Parameter	
Moisture (%, w/w)	90.34 ± 0.04
Escherichia coli (MPN/g_{DW})	<3
Salmonella spp. (MPN/$25g_{as\text{-}is}$)	Absent

MPN, most probable number; DW, dry weight.

and total concentration of each of the 16 mineral elements detected in the dried digestate. Specifically, the concentration values of these elements are detailed in **Supplementary Table 1**. The concentration of Ca (13 ± 0.3 mg g_{DW}^{-1}) prevailed over the remaining macronutrients, such as Mg > P > and S, whose levels ranged from 3 to 6 mg g_{DW}^{-1} (**Figure 1B**; **Supplementary Table 1**). Among the micronutrients (i.e., B, Cu, Fe, Mn, Mo, and Zn), the highest concentration was observed for Fe (1.4 ± 0.1 mg g_{DW}^{-1}), followed by Mn > Zn > B ≈ Cu >> Mo (**Figure 1B**; **Supplementary Table 1**). Regarding the heavy metals (i.e., Cd, Co, Cr, Ni, and Pb), Cr was the most abundant (4.7 ± 0.2 μg g_{DW}^{-1}), whereas the Cd concentration was below the limit of detection (LOD = 0.177 μg L^{-1}; **Figure 1B**; **Supplementary Table 1**). Finally, Na content was equal to 6.7 ± 0.1 mg g_{DW}^{-1} (**Figure 1B**; **Supplementary Table 1**).

AHL Properties

Figures 2A,B show the same chemical parameters examined for the digestate. As observed earlier for the digestate, the pH of the AHL was alkaline (9.24 ± 0.07; **Figure 2A**). In particular, the HTC process increased the pH by one unit compared with the digestate (**Figure 1A**). In addition, the process also increased the EC value (i.e., the concentration of dissolved ions and salts), which was approximately twice that of the digestate and equal to 16.46 ± 0.47 mS cm^{-1} (**Figure 2A**). Moreover, the AHL had TOC and TN contents of 10.53 ± 0.35 and 1.99 ± 0.05 g L^{-1}, respectively (**Figure 2A**). Thus, with the HTC process, the C content was reduced by about slightly more than three times, while the N content remained almost constant (**Figure 2A**). Overall, as for the digestate, the C content in the AHL was always higher than the N content (**Figures 1A, 2A**). Specifically, the TOC was five and 15 times higher than the TN content in the AHL (**Figure 2A**) and the digestate (**Figure 1A**), respectively. **Figure 2B** displays the composition and total concentration of the 16 mineral elements also analyzed in the digestate. Specifically, the concentration values of these elements are detailed in **Supplementary Table 2**. Among the macronutrients (i.e., Ca, Mg, P, and S), the S concentration in the AHL was the highest (200.3 ± 10.1 mg L^{-1}), followed closely by Ca and then in the following order by P > Mg (**Figure 2B**; **Supplementary Table 2**). With regard to the micronutrient (B, Cu, Fe, Mn, Mo, and Zn) contents, Fe was the most abundant (20.8 ± 8.3 mg L^{-1}) and Mo (570 ± 5.0 μg L^{-1}) was the least abundant as observed in the digestate. In particular, the B content was close to that of Fe (16.2 ± 0.2 mg L^{-1}), while Zn, Mn, and Cu contents varied between approximately 2 and 1 mg L^{-1} (**Figure 2B**; **Supplementary Table 2**). Among the heavy metals (i.e., Cd, Co, Cr, Ni, and Pb), unlike in the digestate, the most abundant was Ni (546 ± 49.1 μg L^{-1}) and then in the following order Cr > Co, with the Cd and Pb contents below LOD (0.177 and 6.730 μg L^{-1}, respectively). Specifically, the Cd content was lower than LOD not only in the AHL (**Figure 2B**; **Supplementary Table 2**) but also in the digestate (**Figure 1B**). Finally, the Na content was equal to 628.3 ± 34.2 mg L^{-1}, and this value was even greatly higher than that of the macronutrients measured in the AHL (**Figure 2B**; **Supplementary Table 2**).

In order to evaluate the effect of the HTC process on the distribution of nutrients between the liquid and solid phases, the ratio between the concentration in the digestate and the concentration in the AHL of the 16 mineral elements has been measured (**Figure 2C**). Apart from Cd, which was not detected by the ICP-OES analysis of the digestate and, therefore, obviously not in the AHL, in general, all the element concentrations decreased from the digestate to the AHL, although with very different variations (**Figure 2C**). Specifically, two groups, in terms of the extent of reduction, can be clearly distinguished: the first group, with Mg > Mn > Ca > Fe > Cr > Zn > P > Cu, showed reductions varying from 82 to 44 times, and the second group, with S > Co > Na > Ni > Mo > B, showed reductions varying from 15 to three times (**Figure 2C**). On the other hand, Pb was present in the digestate

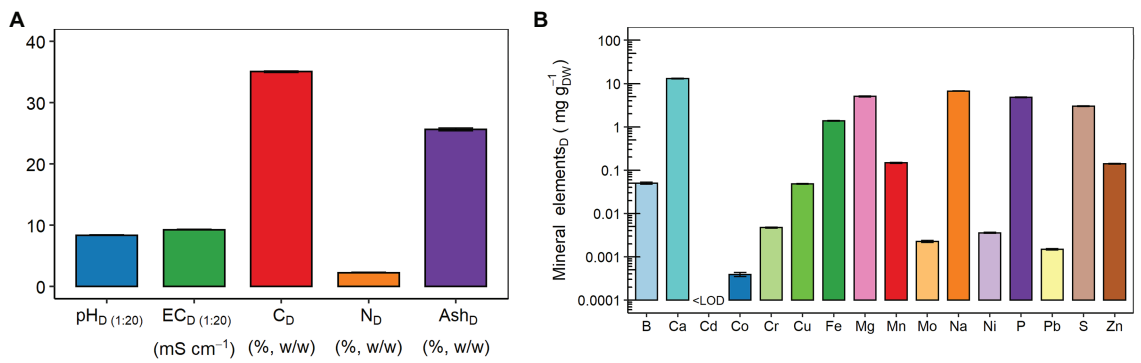

FIGURE 1 | Chemical properties of the digestate (D): **(A)** pH, electrical conductivity (EC), carbon (C), nitrogen (N), and ash contents; and **(B)** mineral element concentrations: boron (B), calcium (Ca), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), sodium (Na), nickel (Ni), phosphorus (P), lead (Pb), sulfur (S), and zinc (Zn). LOD, limit of detection. The LOD of Cd was 0.177 μg L^{-1}. Data are presented as means ± SE (n = 3). Data of mineral element concentrations are presented on a logarithmic scale (Log_{10}) for better graphical display, and the values are detailed in **Supplementary Table 1**.

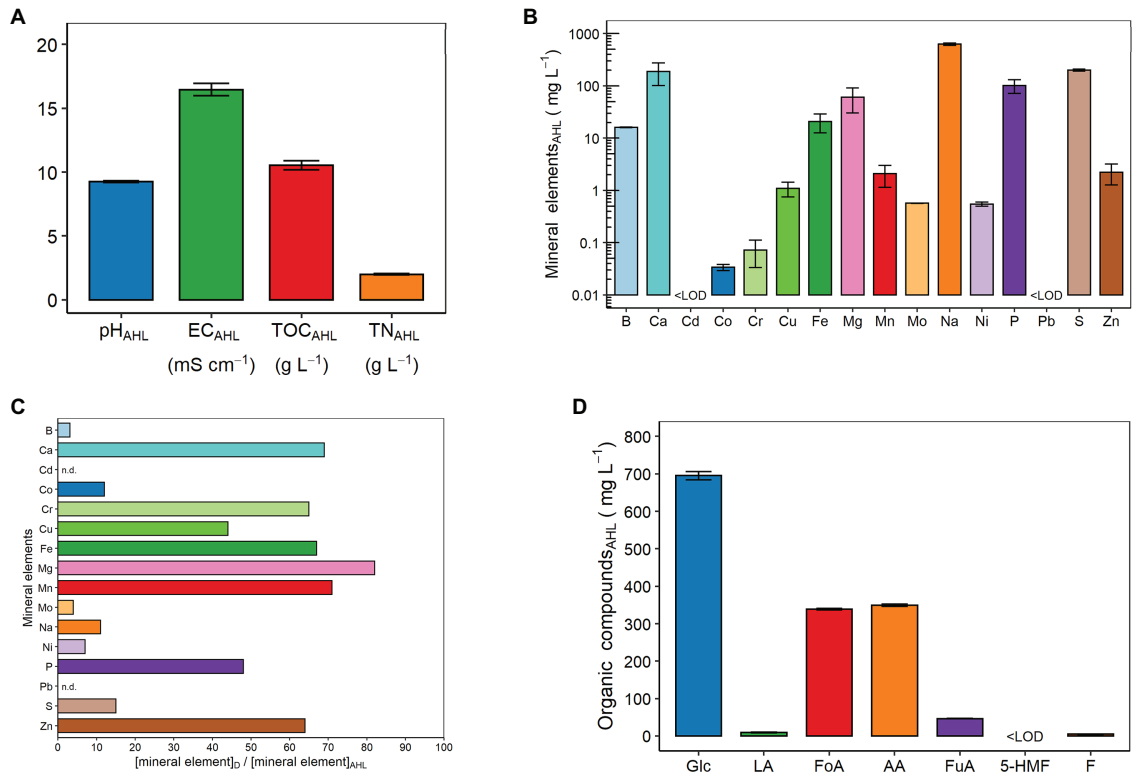

FIGURE 2 | Chemical properties of the aqueous liquid fraction of hydrothermal carbonization [aqueous HTC liquid (AHL)]: **(A)** pH, electrical conductivity (EC), total organic carbon (TOC), and total nitrogen (TN) content; **(B)** mineral element concentrations: boron (B), calcium (Ca), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), sodium (Na), nickel (Ni), phosphorus (P), lead (Pb), sulfur (S), and zinc (Zn); **(C)** ratio between the concentration in the digestate (D) and the concentration in the AHL of each of the following mineral elements: boron (B), calcium (Ca), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), sodium (Na), nickel (Ni), phosphorus (P), lead (Pb), sulfur (S), and zinc (Zn). For Cd and Pb, the ratio was not determinable (n.d.), as the mineral element concentration was below the LOD (i.e., 0.177 and 6.730 μg L^{-1}, respectively) in D and/or AHL; and **(D)** organic compound concentrations: glucose (Glc), lactic acid (LA), formic acid (FoA), acetic acid (AA), fumaric acid (FuA), 5-hydroxymethylfurfural (5-HMF), and furfural (F). LOD, limit of detection. The LODs of Cd and Pb by inductively coupled plasma-optical emission spectroscopy (ICP-OES) were 0.177 and 6.730 μg L^{-1}, respectively. The LOD of 5-HMF was 0.706 mg L^{-1} and was determined using the method described by Miller and Miller (2010). Data are presented as means ± SE (n = 3). Data of mineral element concentrations are presented on a logarithmic scale (Log$_{10}$) for better graphical display, and the values are detailed in **Supplementary Table 2**.

(1.5 ± 0.1 μg g_{DW}^{-1}), but in the AHL, its concentration was it lower than that of instrumental LOD (**Figure 2C**).

Figure 2D shows the seven organic compounds identified within the AHL, namely, sugars [i.e., glucose (Glc)], acids [i.e., lactic (LA), formic (FoA), acetic (AA), and fumaric (FuA)], and furans [i.e., 5-hydroxymethylfurfural (5-HMF) and furfural (F)]. The most abundant was Glc (695.07 ± 10.64 mg L^{-1}; **Figure 2D**). Among the four organic acids identified in the AHL, the concentration of AA prevailed, being 349.14 ± 3.33 mg L^{-1}. However, this concentration was not much higher than that of FoA, which reached a concentration of 338.86 ± 2.60 mg L^{-1}. In contrast, these concentrations were about seven times higher than that of FuA and about 38 times higher than that of LA. In particular, the concentrations of FuA and LA were 46.31 ± 0.64 and 9.19 ± 0.87 mg L^{-1}, respectively (**Figure 2D**). In the case of the two furan compounds, a higher concentration was observed for F (4.87 ± 0.22 mg L^{-1}), since the 5-HMF concentration was below LOD (0.706 mg L^{-1}) in the AHL (**Figure 2D**).

Effect of Different AHL Dilution Ratios on Maize Plant Growth and Related Traits

Significant differences in leaf color and growth were visually evident in the 15-day-old maize plants grown either with the full-strength NS (control condition – C) or with the three different diluted AHL solutions (1:30, 1:60, and 1:90; **Figure 3A**). These visual differences were confirmed by the leaf chlorophyll contents (**Figure 3B**) and biomass accumulation (i.e., fresh weight) of both the shoots and the roots (**Figure 3C**). None of the three AHL dilution ratios allowed the plants to achieve chlorophyll contents similar or equivalent to that of the control plants (33.2 ± 0.5 SPAD index; **Figure 3B**). Moreover, this reductive effect on chlorophyll content was significantly more pronounced as the dilution ratio decreased (1:30 > 1:60 > 1:90). Indeed, the chlorophyll content for 1:30 plants was reduced by about half, for 1:60 plants by almost 3-fold, and for 1:90 plants by slightly more than 4-fold when compared with the control (**Figure 3B**). The control plants also exhibited the highest fresh weight at both

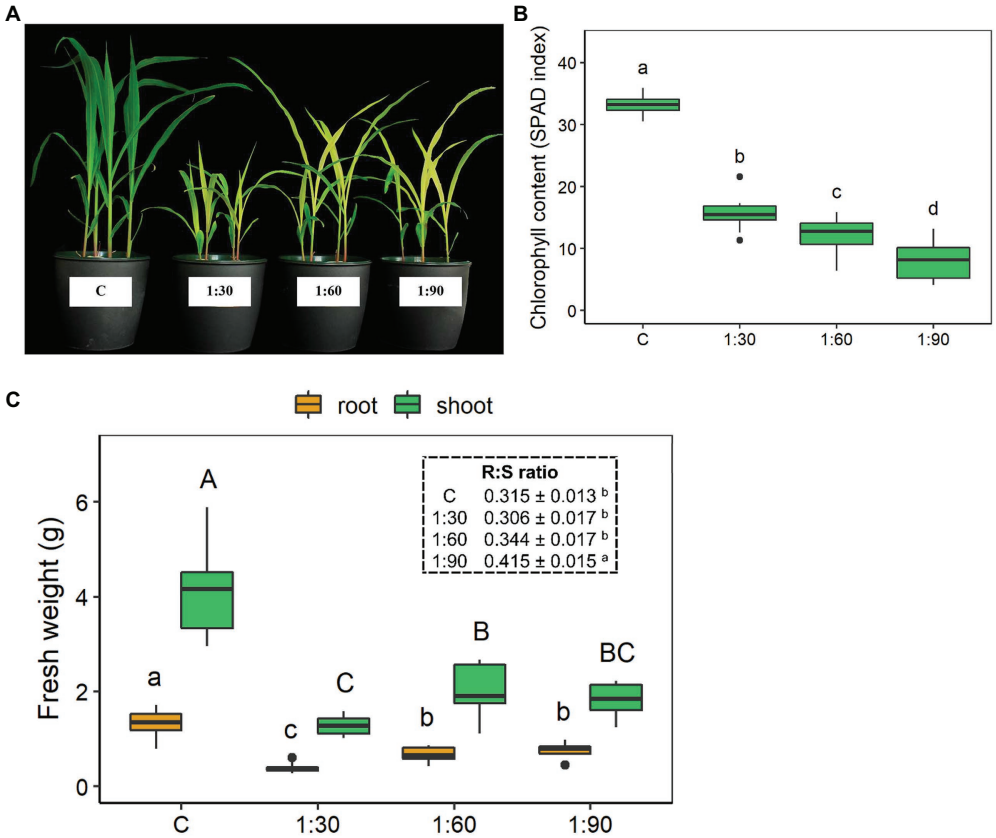

FIGURE 3 | Growth parameters of 15-day-old maize seedlings grown in the full-strength nutrient solution (C, control) and in the AHL diluted with distilled water in a ratio of 1:30, 1:60, and 1:90 (v/v): **(A)** representative shoots, **(B)** leaf chlorophyll content, and **(C)** shoot and root fresh weight (FW). Insert: root (R) to shoot (S) ratio. Data are presented as means ± SE (n = 12). Statistical significance was tested by one-way ANOVA analysis with LSD post-test ($p < 0.05$). Statistically significant differences among the four different growth conditions are indicated by different letters: different lower case letters indicate significant differences among the growth conditions in roots; different upper case letters indicate significant differences among the growth conditions in shoots.

shoot and root levels compared with the plants grown with the diluted AHL solutions (**Figure 3C**). In this case, the 1:30 treatment had the most dramatic reducing effect on the shoot and root fresh biomass accumulation (−70% vs. C in both shoots and roots) than the 1:60 and 1:90 treatments (**Figure 3C**). However, the root:shoot ratio increased by 32% only with the 1:90 dilution level, as no statistically significant changes were observed for the two other treatments compared with the control (insert of **Figure 3C**). Among the three different AHL dilution ratios, there was a 35% decrease in shoot fresh weight only for 1:30-treated compared with the 1:60-treated plants, whereas the shoot fresh weight of the 1:90-treated plants did not show significant differences from those of the 1:30- and 1:60-treated plants. On the other hand, the root fresh weights of the 1:60- and 1:90-treated plants did not differ statistically from each other, but both were significantly higher (+88%) than that of the 1:30-treated plants (**Figure 3C**).

At the root morphological level, significant visual differences were also clearly distinguished among the four different growth conditions of the maize plants (**Figure 4A**). Visually, the root systems of the treated plants increased with increasing dilution ratio and resulted more similar to the control. However, the treated plants had a less developed root system, especially in terms of lateral root length (**Figure 4A**). These differences were confirmed by two morphological parameters such as total root length and number of root tips (**Figure 4B**). Overall, both parameters were greatly reduced in plants grown with the AHL compared with the control plants (C). In particular, there were no significant differences in total root length between the 1:60 and 1:90 treatments, whereas the effect of the 1:30 treatment was remarkably evident on the reduction of this parameter (−74% vs. 1:60 and −80% vs. 1:90; **Figure 4B**). For the number of root tips, no statistically significant change was observed between the 1:30, 1:60, and 1:90 treatments, although a reduction to a greater extent was observed for the 1:30 treatment (−57% vs. 1:60 and −59% vs. 1:90; **Figure 4B**). Accordingly, the reductions in both morphological parameters were clearly sharpened especially when comparing plants supplemented with the most concentrated AHL (i.e., 1:30-treated plants) with the control (−93% for total root length and −83% for number of root tips; **Figure 4B**).

Figure 5 shows maize root and shoot concentrations of the main essential nutrients (macronutrients: Ca, Mg, P, and S;

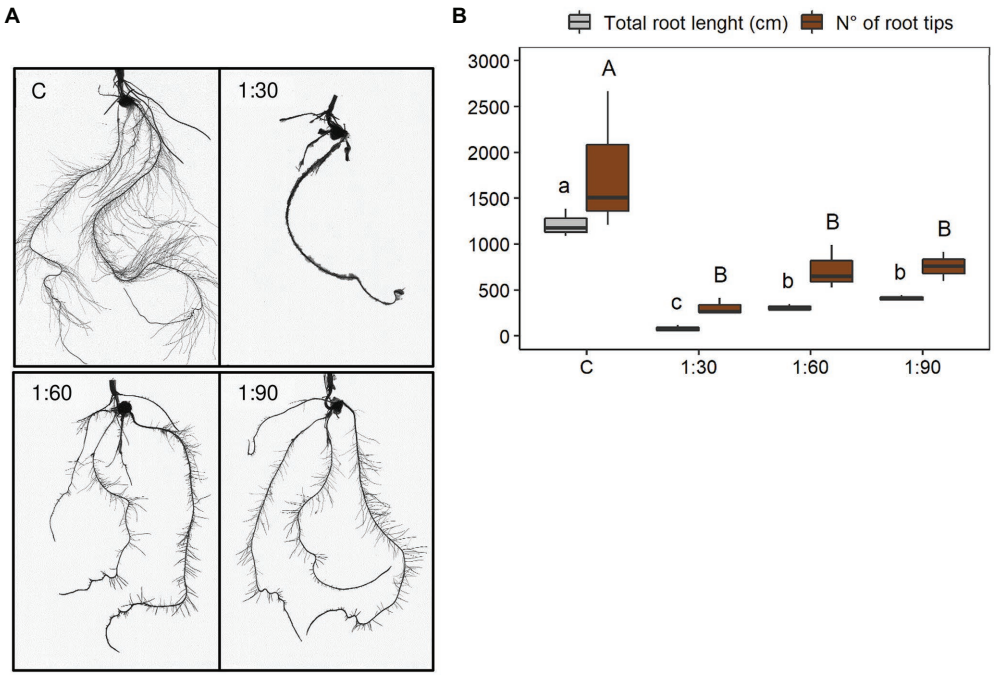

FIGURE 4 | Morphological parameters of root systems of 15-day-old maize seedlings grown in the full-strength nutrient solution (C, control) and in the AHL diluted with distilled water in a ratio of 1:30, 1:60, and 1:90 (v/v): **(A)** WinRHIZO images and **(B)** total root length and number of root tips. Data are presented as means ± SE (n = 12). Statistical significance was tested by one-way ANOVA analysis with LSD post-test ($p < 0.05$). Statistically significant differences among the four different growth conditions are indicated by different letters: different lower case letters indicate significant differences among the growth conditions in total root length; different upper case letters indicate significant differences among the growth conditions in a number of root tips.

and micronutrients: B, Cu, Fe, Mn, Mo, and Zn) and of one of the beneficial non-essential elements (Na) for plant nutrition. The different dilution ratios of the AHL did not result in any significant change in the root contents of Ca, Mg, P, Cu, and Fe. However, the contents of Ca, Mg, P, Cu, and Fe were lower in the roots grown with the different AHLs than in control roots (**Figure 5**). In contrast, the root contents of Mn and Zn, although presenting values lower than those of the control, varied depending on the AHL dilution ratio. Specifically, Mn, which was reduced by 72% in the 1:30 condition compared with the control, was reduced even more (−88%) in the 1:60 and 1:90 treatments, both showing values equal to approximately half the value of the 1:30 treatment. Instead, the Zn content decreased by 28% in both the 1:60 and 1:90 conditions compared with the control, but the greatest reduction (−74%) occurred in the roots of plants grown with the 1:30 diluted AHL (thus the more concentrated one) when compared with the control (**Figure 5**). On the contrary, the roots of plants treated with 1:30 differed from all the other conditions for the highest content of S (26.1 ± 10.4 mg g_{DW}^{-1}), B (138.7 ± 3.4 μg g_{DW}^{-1}), and Na (3.2 ± 0.1 mg g_{DW}^{-1}) contents. In particular, the 1:60, 1:90, and control conditions did not show significant differences for the S content and the decrease was, on average, −75% compared with the 1:30 treatment. B and Na gradually decreased as the dilution ratio increased, but in the case of B, its content with the 1:90 treatment did not differ statistically from that with the control, whereas for Na, its content further decreased with the control (**Figure 5**). For the Mo content, the 1:30 and 1:60 conditions presented values below the detection limit (<10.107 μg L^{-1}), whereas the 1:90 condition was not statistically different from that of the control.

On the other hand, in the shoots, the different dilution ratios of AHL did not affect significantly only P and Fe contents and both were lower than those measured in shoots of control plants (**Figure 5**). In contrast, the Ca, Cu, and Mn contents of the shoots, although presenting values lower than those of the C condition, varied according to the type of dilution ratio. Specifically, the Ca content was similar to that in the 1:60 and 1:90 conditions and decreased by 38 and 29%, respectively, compared with the control. However, the most severe reduction (−67%) was observed in the shoots of the plants grown with the 1:30 diluted AHL compared with the control shoots. Cu, which was reduced by about half in the 1:90 condition compared with the control, was reduced even more (by approximately slightly more than half) by the 1:30 and 1:60 treatments compared with the 1:90 treatment. Finally, for the Mn content, there were significant differences only between the shoots of the 1:30- and 1:90-treated plants (with 1:30 < 1:90), since the Mn content in the 1:60 treatment did not differ statistically from either the 1:30 or 1:90 treatment (**Figure 5**). The shoots from the 1:90-treated plants differed from those of the control in higher Mg (2.7 ± 0.1 mg g_{DW}^{-1}), Mo (5.4 ± 2 μg g_{DW}^{-1}), and Zn (90.8 ± 8.5 μg g_{DW}^{-1}) contents. Exactly, there was no statistically significant variation between the Mg content in

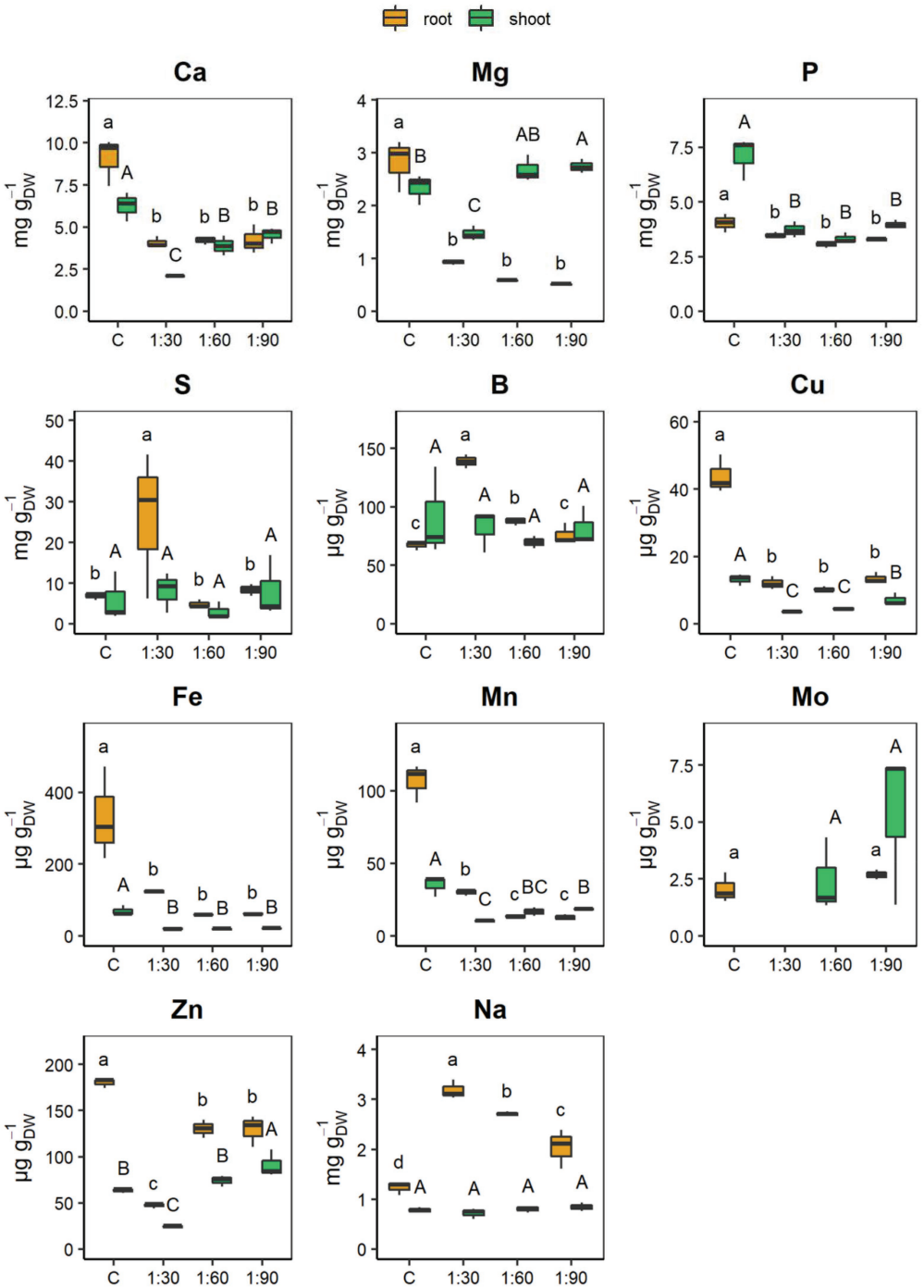

FIGURE 5 | Concentrations of main macro- [calcium (Ca), magnesium (Mg), phosphorus (P), and sulfur (S)] and micronutrients [boron (B), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), and zinc (Zn)], and sodium (Na) in roots and shoots of 15-day-old maize seedlings grown with the full-strength nutrient solution (C, control) and with the AHL diluted with distilled water in a ratio of 1:30, 1:60, and 1:90 (v/v). Boxplots were omitted when the concentration values are below the limit of detection (LOD). The LOD of Mo was 10.107 µg L^{-1}. Data are presented as means ± SE (n = 12). Statistical significance was tested by one-way ANOVA analysis with LSD post-test ($p < 0.05$). Statistically significant differences among the four different growth conditions are indicated by different letters: different lower case letters indicate significant differences among the growth conditions in roots; different upper case letters indicate significant differences among the growth conditions in shoots.

the 1:60 and 1:90 shoots. In turn, however, the Mg present in the 1:60 shoots did not differ significantly from that of the control shoots. Instead, the Mg content was greatly reduced (−44%) in the 1:30 shoots compared with the 1:90 shoots. The Mo content in 1:60 statistically equaled the high levels measured in the 1:90 shoots, while it was below the detection

limit (10.107 μg L^{-1}) in both 1:30 and C conditions. Finally, Zn gradually decreased as the AHL concentration increased (1:90 > 1:60 > 1:30), with the 1:60 treatment exhibiting values similar to those of the control. Thus, the shoots of the 1:30-treated plants differed from all the other conditions by lowest contents not only of Ca (2.1 ± 0 mg g_{DW}^{-1}) but also of Mg (1.5 ± 0.1 mg g_{DW}^{-1}) and Zn (24.4 ± 1.0 μg g_{DW}^{-1}; **Figure 5**). Lastly, for the shoot contents of elements, such as S, B and Na, there was no statistically significant variation between the maize plants grown with the dilute AHL solutions and the control (**Figure 5**).

DISCUSSION

Considering increasing water scarcity, urbanization, and decline of arable lands due to climate change (FAO, 2017), the development and use of value-added products from wastes, such as AHLs, is a relevant aspect not only for the scientific community but also for agricultural producers (Gruda, 2019). Studies aimed at investigating the effects of AHLs by the HTC process used as agricultural liquid fertilizers have only recently begun to be conducted (Vozhdayev et al., 2015; Mau et al., 2019). Specifically, the possibility of using AHL as a fertilizer solution in soilless horticultural systems is an entirely new field of study that, therefore, needs to be explored in the upcoming future. However, it is well-known that HTC products could also have a certain level of phytotoxicity. Toxicity might be derived from the type of feedstock as well as from the formation of a variety of harmful substances because of thermochemical conditions and reactions of biomass conversion, which affect their further utilization in the real world (Fang et al., 2018; Usman et al., 2019; Celletti et al., 2021). In this study, an eco-friendly approach was followed based on the evaluation of feedstock properties (i.e., cow manure digestate) and the chemical composition of the AHL and then its impact on maize growth.

The microbiological data presented for the digestate (**Table 1**) clearly demonstrated that the feedstock was free of bacteria (namely, *E. coli* and Salmonella spp.) pathogenic to human beings and the environment. Therefore, if the AD process has a potential for sanitation (Seruga et al., 2020), it can be assumed that the AHL of the corresponding digestate, obtained at a HTC temperature (180°C) and a time (3 h) higher than those commonly used for heat sterilization (Block, 2000), is consequently free of hazardous microbial load. In the digestate, the values of the microbiological parameters as well as the concentrations of the heavy metals (i.e., Cd, Cr, Cu, Ni, Pb, and Zn; **Table 1**; **Figure 2B**, respectively) respected the limits set by the current European legislation for sewage sludge use in agriculture (Council Directive 86/278/EEC, 1986) and the Italian regulation for fertilizers [Mipaaf (Ministero delle politiche agricole alimentari e forestali), 2010]. As a result, the concentrations of these heavy metals together with the remaining mineral elements were further reduced with the AHL compared with the solid phase (hydrochar; **Figure 2B**).

In view of using AHL as a fertilizer solution, the ratio between the concentration in the digestate and the AHL of each inorganic element has provided useful indications to understand their behavior in the distribution between the AHL and hydrochar (**Figure 2C**). In agreement with recent observations (Sun et al., 2013; Ekpo et al., 2016), this study showed that the HTC process tended to concentrate all the elements (**Figures 1B, 2B,C**), such as C (**Figures 1A, 2A**), in the hydrochar with respect to the AHL. For this reason, hydrochar, being rich in C and nutrients, has been reported to have the potential to be used as a soil amendment (Bento et al., 2019; Kalderis et al., 2019) or as growing substrate for plants, after some operationally feasible adjustments (Roehrdanz et al., 2019; Celletti et al., 2021). The P binding to Ca, Fe, Mg, and Mn, forming insoluble phosphate salts that precipitate and accumulate in hydrochar has been extensively studied and described (Heilmann et al., 2014; Shi et al., 2014; Huang and Tang, 2015; Wang et al., 2017). This could explain why in our conditions, the AHL became mainly depleted of these essential nutrients for plants compared with the digestate (**Figure 2C**). Different behaviors were observed only for Cd (keeping constantly <LOD = 0.177 μg L^{-1}) and N, the concentrations of which did not vary after the HTC process. Unlike some studies reporting that Pb is almost entirely released from the solid phase (Sun et al., 2013; Ekpo et al., 2016), in our case, it was the only mineral element to exhibit concentrations below LOD (i.e., <6.73 μg L^{-1}) in the AHL, suggesting that it precipitates totally in the hydrochar.

Since the conditions of the HTC process further decreased the contents of heavy metals in the AHL, already below legal limits, as discussed above, this phenomenon can be considered a positive aspect. Nevertheless, all the other mineral elements were also reduced from the digestate to the AHL. The contents of the main essential plant nutrients (in decreasing order: Mg > Mn > Ca > Fe > Zn > P > Cu) were greatly reduced (in a range of 82 to 44 times; **Figure 2B**). On the other hand, the Na and B contents were decreased only slightly (by 11 and three times, respectively). In particular, Na was the most abundant mineral element in the AHL (**Figure 2B**; **Supplementary Table 2**).

However, comparing AHL with solutions commonly used to grow plants hydroponically, such as the Hoagland solution (Hoagland and Arnon, 1950), AHL was still more concentrated in all the essential plant nutrients, apart from Ca and Mg, which were about the same. Indeed, the AHL contained about three times more P and S, four times more Mn, seven times more Fe, 32 times more B, 44 times more Zn, 55 times more Cu, 57 times more Mo, and even about 524 times more Na than the Hoagland solution. From this, it can be clearly deduced that the main problem was related to the extremely high concentrations of Na in the AHL, not adequate for plant growth in hydroponics. The calculation of the sodium adsorption ratio (SAR), i.e., a common indicator of the suitability of water of irrigation, showed that the AHL had a value of 14.3 meq L^{-1}, almost double the value considered safe (i.e., 8 meq L^{-1}), in order not to cause sodicity problems (Zaman et al., 2018). In addition, the results that emerged from the

preliminary experiments with the maize plants grown with AHL have suggested the need to dilute the AHL at different ratios (1:30, 1:60, and 1:90, v/v) to prevent plant death phenomena (data not shown). Therefore, these previous detrimental effects on maize plants could be most likely attributable to high EC (16.46 ± 0.47 mS cm^{-1}; **Figure 2A**) and high Na content (628.3 ± 34.2 mg L^{-1}; **Figure 2B**; **Supplementary Table 2**), rather than the presence of potentially phytotoxic furan compounds (i.e., 5-HMF and furfural) in the AHL (**Figure 2D**). In fact, the 5-HMF concentration was found to be below the instrumental limit of detection (LOD < 0.706 mg L^{-1}), and the furfural concentration was equal to 4.87 ± 0.22 mg L^{-1}, a concentration that has been shown not to inhibit the germination of seeds of cress, which is a plant species highly sensitive to the presence of phytotoxins (Celletti et al., 2021). Furthermore, the potential phytotoxicity originated by the presence of organic acids (i.e., lactic, formic, acetic, and fumaric acid) or sugars (i.e., glucose) detected in the AHL can be excluded since they are part of the exudates synthesized by plants and released from their roots into the external medium (Warren, 2016). Overall, organic acid release into the rhizosphere is a mechanism adopted by plants to cope with situations of low inorganic nutrient availability (such as Fe and P), in order to solubilize nutrients (either by acidification or chelation) and consequently to enhance their availability for root uptake (Adeleke et al., 2017).

It is well-described in the literature that Na is considered a beneficial element but not essential for plants and mainly responsible for salt stress (Gómez-Merino and Trejo-Téllez, 2018). When the salt concentration exceeds certain concentrations (e.g., 14.61 g L^{-1} NaCl for maize plants; Farooq et al., 2015), it leads to deleterious effects on plants, such as impaired growth, reduced chlorophyll content, impeded ability to acquire water supply, induced nutritional imbalances, and toxicity phenomena (Ashraf and Harris, 2013; Machado and Serralheiro, 2017). Furthermore, the salinity threshold (EC_t) of the majority of crops is low (ranging from 1 to 2.5 mS cm^{-1}; Machado and Serralheiro, 2017) and dependent on the growth stage and plant species (Kaddah and Ghowail, 1964; Celletti et al., 2021). Although maize is classified as a plant that is moderately tolerant to salinity (EC_t = 1.7 mS cm^{-1}; Bernstein and Ayers, 1949; Kaddah and Ghowail, 1964), the EC values of the three diluted solutions of the AHL were presumably too high for its equilibrate growth and especially for its early phase of development. On one hand, as the concentration of the AHL in the fertilizer solution increased, the biomass growth and accumulation, chlorophyll content, and the number of root tips were reduced (**Figures 3A–C, 4B**); on the other, visual root morphological deformations were increased in the 15-day-old maize seedlings compared with those grown with the full-strength nutrient solution (control condition; **Figure 4A**). It is interesting to note that within the plant organs, Na was accumulated to a greater extent in the roots than in the shoots of the maize plants and increased linearly with the amount of the AHL present in the growth solutions (1:30 > 1:60 > 1:90; **Figure 5**). An accumulation trend similar to that of Na was also observed for B, since high concentrations of B are often associated with salinity (Del Carmen Martínez-Ballesta et al., 2008). These greater Na and B accumulations in the roots represent a survival and resistance strategy to minimize or avoid the transport of these two elements toward photosynthetic organs (Farooq et al., 2015). Moreover, this finding suggests that Na may be the primary cause of toxicity by causing morphological deformations, reducing the growth of the root systems, and consequently leading to limited development of the whole plant, interfering with the uptake and assimilation of other nutrients. Therefore, treatments regarding, for instance, the use of ion-exchange resins (Lukey et al., 1999; Subban and Gadgil, 2019) or hydro-extraction or re-crystallization methods (Sumada et al., 2018), would be required to remove undesirable dissolved salts (mainly Na) and thus to lower the phytotoxicity and to improve the fertilizing power of the AHL-containing solutions.

Besides the presence of phytotoxic compounds, pH is one of the main factors affecting the mobility of nutrients, and consequently their acquisition by plants (Fageria and Baligar, 2004). Most nutrients are optimally available to plants in the pH range of 6.5–7.5. The pH of the AHL was very strongly alkaline (9.24 ± 0.07; Ramírez-Rodríguez et al., 2007), and it can be considered as a side effect of the high Na concentration (Machado and Serralheiro, 2017). Alkaline pH limits especially the availability of micronutrients (i.e., Cu, Fe, Mn, and Zn; Rengel, 2015) to the plants but also of some macronutrients (i.e., P; Ramírez-Rodríguez et al., 2007; Gentili et al., 2018). Among the micronutrients, the only exception is Mo, which appears to be less available at acidic pH and more available under alkaline pH (Rutkowska et al., 2017).

In this study, the alkalinity of AHLs compared with the control solution (which was kept at pH 6) may have reduced the bioavailability of micronutrients, such as Cu, Fe, and Mn, and macronutrients, such as Ca and P. In fact, the maize plants grown with the solutions with AHL accumulated less of these nutrients in both shoots and roots than plants grown with the control solution (**Figure 5**). In turn, this phenomenon probably explains why the leaves of the plants grown with the three different diluted AHLs appeared clearly chlorotic (**Figure 3A**) and why their chlorophyll content (**Figure 3B**) was significantly reduced compared with the control plants. Indeed, leaf yellowing is a characteristic symptom of nutrient deficiencies, in this case mainly due to the simultaneous deficiencies of Fe, Cu, and Mn, which play a crucial role in the photosynthetic process (Marschner, 2012). However, it is interesting to observe that the shoots of the 1:60 and 1:90 plants were significantly richer in some essential nutrients, such as Ca, Mg, Mn, and Mo, than the shoots of the 1:30 plants; despite having grown with the two more diluted solutions and thus certainly containing a lower amount of nutrients, the reduced alkalinity might have increased the availability of these nutrients. In particular, the shoots of the 1:90 plants contained much more Cu (+97% and +58%, respectively) and Zn (+272 and 23%, respectively) compared with the shoots of the 1:30 and 1:60 plants (**Figure 5**). Consequently, since nutrients provide essential building blocks for plant growth, the higher accumulation of these nutrients

in 1:60 and 1:90 maize plants could explain their higher biomass accumulation compared with the 1:30 plants. In addition, the growth of the 1:30 plants was further hindered by the additive toxic effects of Na-B interaction. Both elements were not translocated to the leaves but were stored defensively in the roots (**Figure 3C**; Ismail, 2003).

CONCLUSION

The results presented here contribute to expanding the current rather scarce knowledge on the composition and level of phytotoxic substances in AHL as a consequence of the type of feedstock and thermochemical reactions of the HTC process. In addition, the physiological responses of maize plants, when grown at different AHL dilution ratios, have been also evidenced. On the one hand, the 1:30 AHL solution was too rich in potentially phytotoxic substances (mainly Na) and with a very alkaline pH that reduced the bioavailability of the nutrients and thus led to growth arrest. On the other, the 1:60 and 1:90 solutions were very similar in terms of composition and impact on plant growth. In particular, while the less concentrated phytotoxicity load, which allowed plant growth, has to be considered an advantage, the levels of nutrient concentration are surely a disadvantage, because they hindered the proper photosynthetic functionality, as demonstrated by the more pronounced chlorosis appearance and more reduced chlorophyll content.

Therefore, it is necessary to carry out further research to identify the right dilution ratio, which represents a good compromise between not causing phytotoxicity damage and providing enough amounts of essential nutrients for proper plant growth and development. In addition, *ad-hoc* treatments of Na limitation or removal are absolutely required before using AHL as a fertilizer solution. Finally, given the high genetic variation between plant species, future studies are undoubtedly needed to identify the most suitable species for growth in fertilizer solutions composed partly of AHLs by the HTC process. As an example, the aptitude of tomato to grow and produce even under high-salt conditions makes this species a good candidate.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

SCl and TM designed the study. SCl, ML, AB, and VB performed the experiments, and collected and analyzed the data. SCl wrote the original draft of the manuscript. VB contributed to the manuscript preparation. VB, MB, SCs, and TM revised and edited the original draft of the manuscript. DB, MB, SCs, and TM supervised and acquired the funds. All the authors gave their final approval of the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.687434/full#supplementary-material

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