SOILLESS CULTIVATION THROUGH AN INTENSIVE CROP PRODUCTION SCHEME. MANAGEMENT STRATEGIES, CHALLENGES AND FUTURE DIRECTIONS

EDITED BY: Nikos Tzortzakis, Silvana Nicola, Dimitrios Savvas and Wim Voogt

PUBLISHED IN: Frontiers in Plant Science

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88963-742-3
DOI 10.3389/978-2-88963-742-3

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SOILLESS CULTIVATION THROUGH AN INTENSIVE CROP PRODUCTION SCHEME. MANAGEMENT STRATEGIES, CHALLENGES AND FUTURE DIRECTIONS

Topic Editors:
Nikos Tzortzakis, Cyprus University of Technology, Cyprus
Silvana Nicola, University of Turin, Italy
Dimitrios Savvas, Agricultural University of Athens, Greece
Wim Voogt, Wageningen University and Research, Netherlands

Citation: Tzortzakis, N., Nicola, S., Savvas, D., Voogt, W., eds. (2020). Soilless Cultivation through an Intensive Crop Production Scheme. Management Strategies, Challenges and Future Directions. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-742-3

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frontiers in Plant Science

EDITORIAL
published: 03 April 2020
doi: 10.3389/fpls.2020.00363

Editorial: Soilless Cultivation Through an Intensive Crop Production Scheme. Management Strategies, Challenges and Future Directions

Nikolaos Tzortzakis[1]*, *Silvana Nicola*[2]*, *Dimitrios Savvas*[3]* and *Wim Voogt*[4]*

[1] Department of Agricultural Sciences, Biotechnology and Food Science, Cyprus University of Technology, Limassol, Cyprus, [2] DISAFA, Department of Agricultural, Forest and Food Sciences, University of Turin, Turin, Italy, [3] Laboratory of Vegetable Crops, Department of Crop Science, Agricultural University of Athens, Athens, Greece, [4] Wageningen University and Research, Greenhouse Horticulture, Wageningen, Netherlands

OPEN ACCESS

Edited by:
Antonio Elia,
University of Foggia, Italy

Reviewed by:
Francesco Di Gioia,
Pennsylvania State University, United States
Maria Gonnella,
Institute of Food Production Sciences, Italy
Cherubino M. Leonardi,
University of Catania, Italy

***Correspondence:**
Nikolaos Tzortzakis
nikolaos.tzortzakis@cut.ac.cy
Silvana Nicola
silvana.nicola@unito.it
Dimitrios Savvas
dsavvas@aua.gr
Wim Voogt
wim.voogt@wur.nl

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *27 January 2020*
Accepted: *12 March 2020*
Published: *03 April 2020*

Citation:
Tzortzakis N, Nicola S, Savvas D and Voogt W (2020) Editorial: Soilless Cultivation Through an Intensive Crop Production Scheme. Management Strategies, Challenges and Future Directions. Front. Plant Sci. 11:363. doi: 10.3389/fpls.2020.00363

Keywords: hydroponics, biofortification, abiotic stress, induced resistance, nutrient management

Editorial on the Research Topic

Soilless Cultivation Through an Intensive Crop Production Scheme. Management Strategies, Challenges and Future Directions

INTRODUCTION

Soilless culture can increase not only yield but also quality and safety of fresh produce and thus meet the demands of modern society. Soilless cultivation generally refers to any method of growing plants without soil as a rooting medium. The major advantage of soilless cultivation is the uncoupling plant growth from problems associated with soil, such as soilborne pests and diseases, non-arable soil, soil salinity, poor soil quality e.g., The increased interest in the commercial application of soilless cultivation in the last decades has encouraged intensive research activity focusing on the development of new growing systems and a better understanding of the crop physiology and its impact on quality aspects.

The cultivation on substrates is worldwide the primarily used soilless technique for fruiting vegetables and cut flowers. Water culture systems such as deep float techniques (DFT), nutrient film technique (NFT) and aeroponics— referred also as "hydroponics"— are often used for leafy vegetable production. The complete control of nutrition via the nutrient solution (NS) provides efficient tools for physiological and nutritional studies, improving product quality. The recycling and the control of the excess NS that drains off brings a considerable reduction in leaching of nutrients and plant protection products to the environment and saves water.

SOILLESS CULTIVATION THROUGH AN INTENSIVE CROP PRODUCTION SCHEME

One of the topics addressed in the current special issue on soilless culture is the nutrient needs and the quality characteristics of specialty vegetable crops when grown hydroponically, for which the available information in the scientific literature is scarce. In this context, Chatzigianni et al. evaluated two contrasting *Chichorium spinosum* ecotypes originated from a montane and a coastal-marine habitat and provided insights on their **salinity** tolerance and nutrient needs but also on the possibility to be used as promising germplasm resources for future breeding programs.

Salinity affects not only crop yield but also produce quality. To that direction, Chrysargyris, Tzionis et al. studied the *Tagetes patula* response to short-term exposure to moderate (50 mM) or high (100 mM) salinity. Results indicated that salinity decreased at one hand plant biomass but at the other the induction of non-enzymatic and enzymatic antioxidant mechanisms and short-term exposure to salinity and/or ethanol application during flower stage resulted in higher carotenoids and anthocyanins levels of flowers, which might be a new source of nutraceuticals.

In addition, Rouphael and Kyriacou reported **on salinity eustress** (positive stress) and **biofortification** as tools to improve product quality. Effective application of eustress, can elicit tailored plant responses involving activation of physiological and molecular mechanisms that can result in strategic accumulation of bioactive compounds necessary for adaptation to suboptimal environments. Chrysargyris, Michailidi et al. studied the effects of salinity on the physiological and biochemical responses of medicinal and aromatic plants focusing on lavender (*Lavandula angustifolia*). In that study, high (100 mM NaCl) salinity decreased plant growth, polyphenols, antioxidant capacity and essential oil yield, while low-moderate salinity levels maintained the volatile oil profile in lavender.

Ropokis et al. examined the **nutrient and water uptake** by *Capsicum annuum* as impacted by the cultivar and grafting and observed that different pepper cultivars may take up nutrients and water at different ratios under the same conditions. However, such variation was not found when pepper was grafted onto a *C. annuum* rootstock. They concluded that cultivar "Sondela" require higher Ca, Mg, and B concentrations than standard and higher K for cultivar "Bellisa." In another paper dealing with the application of new technologies in hydroponics, Moon et al. studied the EC fluctuation of root-zone nutrient solutions in closed-loop soilless cultures using recurrent neural network (RNN), recording EC every 10 s for a period of 2.5 months in hydroponically grown sweet peppers (*C. annuum*). It was concluded that a single-layered algorithm showed the highest test accuracy, while deep learning algorithms could be applied with the addition of other environmental factors or plant growth.

In another paper exploring the possibilities of utilizing soilless culture to produce **functional food**, Asaduzzaman et al. investigated in four cultivars the production of low-potassium melon, by restricting potassium concentration in the supplied nutrient solution which resulted in production of melon fruits with 55–58% lower potassium content compared standard. Biofortification in soilless culture was the topic of a Incrocci et al., who studied iodine accumulation in hydroponically grown sweet basil (*Ocimum basilicum*). Different cultivars of sweet basil were screened with respect to their tolerance to iodine, since biofortification with iodine entails the use of tolerant cultivars to iodine, due to their ability to withstand higher concentrations of iodine in leaf tissues, rather than due to efficient exclusion of this element from the leaves.

Plant protection and chemical application in soilless culture is limited and alternative means of **disease control** are attracting research interest. Yin et al. assessed the effectiveness of essential oils of *Zingiber officinale* Roscoe as a biological pesticide toward root-rot diseases of a highly valuable medicinal herb (*Panax notoginseng*) when grown in a soilless cultivation system. The findings reveal that essential oils from plants might serve as promising sources of eco-friendly natural pesticides.

SOILLESS CULTURE: CONCLUDING REMARKS AND FUTURE ISSUES

The benefits of soilless systems, i.e., higher yields, high produce quality and the potential of control over emission of nutrients and plant protection products are greatly achieved in high-tech greenhouses which enable year-round production. In contrast, in low-tech greenhouses in countries characterized by a mild climate, the costs of soilless systems are not always recouped by higher yields because some other factor may limit production. Hence, in those countries soilless culture is mainly adopted when the problems originating from the soil become critical, water resources are limited, or the environmental pollution by nutrient leaching is serious (Savvas and Gruda, 2018). This is likely the main reason for the less extensive expansion of commercial soilless culture in most Mediterranean countries compared to north European countries.

Soilless culture is not merely a modern technology for greenhouse production of vegetables and ornamentals. The inherent feature of soilless culture to decouple cultivation of plants from the soil can be used in more sophisticated plant cultivation systems, such as vertical farming, in which plants are grown in multiple layers mounted in closed constructions using artificial lighting and full control of all climate parameter, which enable crop production on locations usually not suited for horticulture, like inner cities or deserts. The ultimate cropping systems are the bioregenerative life-support systems for production of fresh food to nourish astronauts in space colonies (Paradiso et al., 2014). Another application of hydroponics is aquaponics, which couples hydroponic production of vegetables or ornamentals with fish production, by utilizing fish excrement for crop nutrition (Tyson et al., 2011). Finally, soilless culture can be applied for urban agricultural production (Eigenbrod and Gruda, 2015).

AUTHOR CONTRIBUTIONS

NT prepared the outline of the manuscript and analyzed the special issue topics. DS wrote the concluding remarks and future issues. All authors wrote parts of the manuscript, improved the draft and revised the final version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer FD declared a past collaboration with one of the authors NT to the handling editor.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 20 April 2018
doi: 10.3389/fpls.2018.00489

Physiological and Biochemical Responses of *Lavandula angustifolia* to Salinity Under Mineral Foliar Application

*Antonios Chrysargyris, Evgenia Michailidi and Nikos Tzortzakis**

Department of Agricultural Sciences, Biotechnology and Food Science, Cyprus University of Technology, Limassol, Cyprus

Saline water has been proposed as a solution to partially cover plant water demands due to scarcity of irrigation water in hot arid areas. Lavender (*Lavandula angustifolia* Mill.) plants were grown hydroponically under salinity (0–25–50–100 mM NaCl). The overcome of salinity stress was examined by K, Zn, and Si foliar application for the plant physiological and biochemical characteristics. The present study indicated that high (100 mM NaCl) salinity decreased plant growth, content of phenolics and antioxidant status and essential oil (EO) yield, while low-moderate salinity levels maintained the volatile oil profile in lavender. The integrated foliar application of K and Zn lighten the presumable detrimental effects of salinity in terms of fresh biomass, antioxidant capacity, and EO yield. Moderate salinity stress along with balanced concentration of K though foliar application changed the primary metabolites pathways in favor of major volatile oil constituents biosynthesis and therefore lavender plant has the potential for cultivation under prevalent semi-saline conditions. Zn and Si application, had lesser effects on the content of EO constituents, even though altered salinity induced changings. Our results have demonstrated that lavender growth/development and EO production may be affected by saline levels, whereas mechanisms for alteration of induced stress are of great significance considering the importance of the oil composition, as well.

Keywords: antioxidants, essential oil, *Lavandula angustifolia*, salinity, cation application, soilless culture

OPEN ACCESS

Edited by:
Stefania De Pascale, University of Naples Federico II, Italy

Reviewed by:
Petronia Carillo, Università degli Studi della Campania "Luigi Vanvitelli" Caserta, Italy
Giancarlo Fascella, Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria (CREA), Italy

****Correspondence:***
Nikos Tzortzakis
nikolaos.tzortzakis@cut.ac.cy

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *08 December 2017*
Accepted: *29 March 2018*
Published: *20 April 2018*

Citation:
Chrysargyris A, Michailidi E and Tzortzakis N (2018) Physiological and Biochemical Responses of Lavandula angustifolia to Salinity Under Mineral Foliar Application. Front. Plant Sci. 9:489. doi: 10.3389/fpls.2018.00489

INTRODUCTION

Salinity is the condition in soil characterized by a high concentration of soluble salts and is one of the major factors that affects plant growth, causing considerable losses in agricultural production (Wu et al., 2007). The harmful effects of salinity on plant growth are related with (a) low osmotic potential of soil solution (water stress), (b) nutritional imbalance (synergism-antagonism), (c) specific ion effect (salt stress), or (d) a mixture of these factors (Yildirim and Taylor, 2005). Saline soils are generally dominated by sodium, chloride and sulfate ions, with high sodium absorption rate and with high pH and electrical conductivity (EC $>$ 4.0 dS/m) (Flowers and Flowers, 2005).

The high saline condition determines high osmotic pressure in the rhizosphere and eventually reduces plant water and nutrients availability, which in turn affect crops' primary and secondary metabolism (Hendawy and Khalid, 2005). Plants utilize molecular O_2 as a terminal electron acceptor. For this reason, reactive oxygen species (ROS), such as singlet oxygen ($O_2{}^1$), superoxide (O_2^-), and hydrogen peroxide (H_2O_2) are normally produced by metabolism in all cellular

compartments. In particular, the electron transport chain is responsible for most of the superoxide produced through partial reduction of oxygen (Bolisetty and Jaimes, 2013). They can destroy normal metabolism through oxidative damage of lipids, proteins, and nucleic acids when they are produced in excess as a result of oxidative stress (Gill and Tuteja, 2010). In order to overcome oxidative-related stress, together with non-enzymatic antioxidant molecules (ascorbate, glutathione, α-tocopherol etc.), plants detoxify ROS by up-regulating antioxidative enzymes like superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), glutathione peroxidase (GPX), glutathione *S*-transferases (GST), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione reductase (GR), and monodehydroascorbate reductase (MDHAR) (Turkan and Demiral, 2009). SOD provides the first line of defense against the toxic effects of ROS-elevated levels. The SODs convert $O_2^{-\cdot}$ to H_2O_2 while H_2O_2 is a strong nucleophilic oxidizing agent and the oxidation of SH-group is one of the major mode of its toxicity. The produced H_2O_2 is then scavenged by catalase and a variety of peroxidases (Tarchoune et al., 2010). Catalase dismutates H_2O_2 into water and molecular O_2, whereas POX decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants. In plant cells the ascorbate/glutathione (ASH-GSH) cycle represents an alternative and more efficient detoxification mechanism against H_2O_2, operating both in the chloroplasts and the cytosol (Sgherri and Navari-Izzo, 1995). It may remove H_2O_2 in a series of enzymatic reactions involving APX and GR (Ahmad et al., 2010; Gill and Tuteja, 2010). Therefore, the two likely harmful superoxide and hydrogen peroxide are converted to water.

In saline stress condition, both SOD and CAT activities are reduced and malondialdehyde (MDA) accumulates rapidly in plants (Fadzilla et al., 1997; Woodrow et al., 2017), resulting in higher plasma membranes permeability. Additionally, the production of antioxidant compounds serves as a prominent defense trait under environmental stress (de Abreu and Mazzafera, 2005). Actually the biosynthesis of polyphenols may be accelerated in response to abiotic constraints (Naczk and Shahidi, 2004) and saline-stressed plants might represent a promising source of polyphenols (Taarit et al., 2012).

Sodium (Na) excess in roots disrupt plant nutrition, especially potassium (K) absorption. Potassium deficiency inevitably reduce plant growth as it plays a critical role in maintaining cell turgor, membrane potential and enzyme activity (Jouyban, 2012). Once Na gets into the cytoplasm, it inhibits several enzymes activity, whereas a high Na/K ratio cause more damage (Jouyban, 2012). Potassium affects plant physiological processes such as photosynthesis, proteins metabolism, phloem transport, enzymes activity, and osmotic potential maintenance (Chérel, 2004). The monovalent cations, such as K, play a role in enzyme activation lowering energy barriers in the ground and/or transition states rather than being the agents themselves of causing catalysis (Page and Di Cera, 2006). In addition, K increases the vegetation and essential oils (EO) yield in numerous aromatic crops (Singh et al., 2007; Said-Al Ahl et al., 2009) and EO constituents (Hussien, 1995).

Zinc (Zn) is an essential component for over 300 enzymes in plants and makes up an integral constituent of the enzyme structure (Hendawy and Khalid, 2005). It is involved in the auxin synthesis, photosynthesis, cell division, membrane structure, and function maintenance, sexual fertilization and is thus linked to photosynthesis and carbohydrate metabolism (Said-Al Ahl and Omer, 2009; Marschner, 2012). Parker et al. (1992) suggested that root cell membrane permeability is increased under zinc deficiency which might be associated to the zinc functions in cell membranes. Alpaslan et al. (1999) reported that the application of zinc could alleviate possible Na and Cl injury in salt-stressed plants by preventing Na and/or Cl uptake or translocation. Foliar spraying (micro- and macronutrients) under saline conditions could be much more efficient than any other application of nutrients to the soil (El-Fouly et al., 2001). Several reports are reported to the zinc application in plants highlighting its stimulatory effects, such as on basil (Said-Al Ahl and Mahmoud, 2010), geranium (Misra et al., 2005), and coriander (Said-Al Ahl and Omer, 2009). Misra et al. (2005) stated that EO biosynthesis in geranium was greatly influenced by zinc acquisition or deficiency.

Silicon (Si) was disclosed to reduce the hazardous effects of various biotic and abiotic stresses counting salt and drought stress, metal toxicity, radiation damage, several pests, and diseases caused by both fungi and bacteria, nutrients imbalance, high, and low temperature-freezing (Ma, 2004). Silicon reduces the transpiration rate by suppressing the salt translocation from the rhizosphere to the shoot and thereby Si alleviates salt stress (Matlou, 2006). Si application increases phosphorus, calcium, and magnesium uptake but decreases the uptake of nitrogen and potassium (Liang et al., 2007). Salinity tolerance in plants by Si application include mechanisms related to the plant water status increase (Romero-Aranda et al., 2006), ROS stimulation (Zhu et al., 2004), toxic Na^+ ion immobilization (Liang et al., 2003), and higher K^+:Na^+ selectivity by reduced Na^+ uptake in plants (Hasegawa et al., 2000). In order to alleviate salinity induced stress, several protective (priming, develop salt-tolerant crops through breeding) and curative (cation and anion enrichment, plant growth-promoting rhizobacteria, controlled irrigation schedule, cultivation system) means have been examined (Gill and Tuteja, 2010; Tzortzakis, 2010; Chondraki et al., 2012; Ibrahim, 2016).

Nowadays, a great interest of Medicinal and Aromatic Plants exploitation is taking place due to their high antioxidant and antimicrobial activity, which surpassed many commonly used natural and synthetic antioxidants. Lavender is cultivated either as ornamental crop or aromatic crop for EO production and pharmaceutical uses. Therefore, lavender is used widely in medicines, balms, salves, perfumes, cosmetics and constitute a model plant for isoprenoid studies (Biswas et al., 2009). Several studies examined the effects of abiotic stress on lavender plants, including salinity (Cordovilla et al., 2014; Garcia-Caparros et al., 2017), water (Chrysargyris et al., 2016a), and mineral (Santos et al., 2016) stress.

Medicinal and Aromatic Plants properties are related to several vitamins, carotenoids, chlorophylls, catechins, phytoestrogens, minerals, etc. and contribute with plants and/or

their antioxidant components for food preservation (Parejo et al., 2002). Recently, we have optimized the nutrition levels in lavender grown hydroponically, despite the less expansion of lavender intensive cultivation in soilless culture, in order to achieve a constant and repeatable EO composition during crop production (Chrysargyris et al., 2016b, 2017a). Moreover, little is known about salinity interaction with potassium, zinc and silicon deprivation. The objective of the present study was to examine the effects of saline levels and means for salinity induced stress overcome by mineral foliar application, when lavender is cultivated as (a) ornamental crop, (b) crop for essential oil production, or (c) crop for the leaves antioxidant quality. Therefore, it was studied several growth parameters, nutrient content, antioxidant activity as well as quality and quantity of essential oil of *Lavandula angustifolia* plant, just before flowering, as quite often plants are early harvested in an intermediate vegetative and flowering stage.

MATERIALS AND METHODS

Plant and Experimental Conditions

The experiment was carried out at the Hydroponic Infrastructures (non-heated fully controlled plastic greenhouse) of the Experimental Farm, Cyprus University of Technology, Limassol, Cyprus, during spring-summer (15 of April−29 of June) season of 2014. Average minimum and maximum air temperatures were 18 and 28°C respectively (Figure S1), as temperature reached up to 32–35°C during sunny hours in early summer.

Lavender (*L. angustifolia* Mill.) cuttings were purchased from the Cypriot National Centre of Aromatic Plants in trays at the stage of 3–4 leaves and 4–5 cm height. Cuttings were transplanted into pots (1 plant/pot) with perlite (5 L/pot). Pots arranged in twin rows (twin rows were 0.5 m apart and plants were separated by 0.2 m).

Experimental Design and Treatments

Once lavender plants were adapted to soilless culture conditions, they were exposed for up to 60 days to four saline treatments and four foliar mineral applications. Each salinity treatment was divided into three complete randomized blocks, fertigated from the same nutrient solution tank of 180 L capacity. Each treatment consisted of six biological replications (three plants in each replication; 18 plants in total for each treatment) and were considered as experimental and measured further. The different nutrient solutions were applied to the plants considering four salinity levels (0–25–50 mM and 100 mM NaCl) with individual foliar spraying with dH_2O, K (1,250 mg/L) using K_2O, Zn (144 mg/L) using $ZnSO_4$, and Si (725 mg/L) using SiO_2 resulted in 16 treatments (Supplementary Presentation 1). The concentration used for foliar sprays were based on preliminary studies and/or previous reports (Tzortzakis, 2010). Foliar spays took place once every 2 weeks.

The soilless culture system was open or run-to-waste system, i.e., the drainage solution was thrown out of the system after fertigation and drainage. Nutrient solution used was based on previous findings (Chrysargyris et al., 2016b, 2017a). A solution (1:100 v/v) in water containing the following concentration of nutrients was used: NO_3^--N = 14.29, K = 8.31, PO_4-P = 2.26, Ca = 7.48, Mg = 5.76, SO_4^{-2}-S = 1.56, and Na = 1.91 mmol/L, respectively; and B = 18.21, Fe = 71.56, Mn = 18.21, Cu = 4.72, Zn = 1.53, and Mo = 0.52 μmol/L, respectively. Fertigation was applied during daytime through a timer (eight times with 1 min every time at a flow rate of 30 mL/min, due to low water holding capacity of perlite medium) with a drip irrigation system (via emitters; one emitter/plant) by means of pressure pumps (Einhell BG-GP 636, Germany). Fertigation was adjusted appropriately according to the plant needs and climatic conditions as fertigation management has been described previously (Chrysargyris et al., 2016b, 2017a). Pots were placed on swallow plates in order to collect partly the drainage solution and therefore, drainage solution was available to roots through capillary suction. Periodically, plates were washed out to eliminate any salt accumulation. The pH of the nutrient solution was set to 5.8 for all treatments and adjusted with diluted nitric acid (5% v/v). The standard EC of the nutrient solution was 2.1 mS/cm for the control treatment (0 mM NaCl), 5.0 mS/cm for the 25 mM NaCl, 9.0 mS/cm for the 50 mM NaCl and 14.0 mS/cm for the 100 mM NaCl.

Measurement of Growth Parameters

Plant height, leaf length, stem thickness, shoot number, root length, plant fresh and dry weight for upper and root part were observed after 60 days of plant growth in six plants from each treatment. For measurement of fresh and dry weights of upper biomass (leaves and stems) and roots, respective plant parts were excised from control, NaCl-treated and foliar-treated plants and the fresh weight was noted immediately. Later, these plant parts were wrapped in pre-weighed aluminum foils and kept in an incubator at 75°C for 72 h before the dry weight was recorded.

Measurement of Physiological Parameters

Stomatal conductance in six leaves (2nd–3rd from the top, fully mature sun-exposed leaf) in different individual plants per treatment was measured using a ΔT-Porometer AP4 (Delta-T Devices-Cambridge, UK) according to the manufacturer's instructions at the end of the experiment. For leaf chlorophyll determination, lavender leaf tissues (six replications/treatment; each replication was pooled out of two individual plants; leaf: 0.1 g) were incubated in heat bath at 65°C for 30 min, in the dark, with 10 mL dimethyl sulfoxide (DMSO, Sigma Aldrich, Germany) for chlorophyll extraction. The extract absorbance was measured at 645 and 663 nm (TECAN, infinite M200PRO, Männedorf, Austria). Photosynthetic leaf pigments, chlorophyll a (Chl a), chlorophyll b (Chl b), and total chlorophyll (t-Chl) concentrations were calculated (Chrysargyris et al., 2016b).

Estimation of Plant Mineral Ion Contents

Leaf (six replications/treatment) and root (three replications/treatment) plant tissue samples were dried at 75°C for 4 d, weighted, and grounded in a Wiley mill to pass through a 40 mesh screens. Sub samples (0.2–0.3 g) were acid digested (2 N HCl). Determination of K, P, Ca, Mg, Fe, Cu, Mn, Zn, Na, and B was done by inductively coupled plasma optical

spectrometry [ICP-OES; PSFO 2.0] (Leeman Labs INC., Hudson, USA) and N by the Kjeldahl (BUCHI, Digest automat K-439 and Distillation Kjelflex K-360) method.

Essential Oil Extraction and Analysis

Lavender plant harvested and three biological samples (pooled of three individual plants/sample) for each treatment were air-dried at 42°C in oven, chopped and were hydrodistilled for 3 h, using Clevenger apparatus for EO extraction. The EO yield was measured (%) and oils were analyzed by Gas Chromatography-Mass Spectrometry (GC/MS- Shimadzu GC2010 gas chromatograph interfaced Shimadzu GC/MS QP2010plus mass spectrometer) and constituents were determined (Chrysargyris et al., 2016b).

Polyphenol Content and Antioxidant Activity of Lavender

Polyphenols were extracted from six samples (two individual plants were pooled/sample) for each treatment. Plant tissue (0.5 g) was milled (for 60 s) with 10 mL methanol (50% v/v) and extraction was assisted with ultrasound for 30 min. The samples were centrifuged for 15 min at 4,000 g at 4°C (Sigma 3–18 K, Sigma Laboratory Centrifuge, Germany). Extracts were stored at −20°C until use for analysis of total phenolic and total antioxidant activity by the 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and 2,2′-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method.

Total phenols content was determined using Folin-Ciocalteu method at 755 nm according to Tzortzakis et al. (2011) and results were expressed as equivalents of gallic acid (Scharlau, Barcelona, Spain) per g of fresh weight (mg of GAE/g Fwt). DPPH and FRAP radical-scavenging activity was determined as described previously (Chrysargyris et al., 2016b). In details, DPPH radical scavenging activity of the plant extracts was measured at 517 nm from the bleaching of the purple-colored 0.3 mM solution of DPPH. Standard curve was prepared using different concentrations of trolox [(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid], and results were expressed as mg trolox/g Fwt. FRAP radical scavenging activity of the plant extracts was measured at 593 nm and the results were expressed as mg trolox/g Fwt (Chrysargyris et al., 2016b). The antioxidant capacity using the ABTS method was carried out according to Wojdylo et al. (2007) and results were expressed as mg trolox/g Fwt.

Damage Index: Determination of Content of H_2O_2 and Lipid Peroxidation

The content of H_2O_2 was determined according to Chrysargyris et al. (2017b), from six samples (two individual plants were pooled/sample) for each treatment. Leaf tissue (0.2 g) was ground in ice cold 0.1% trichloroacetic acid (TCA) and centrifuged at 15,000 g for 15 min. Aliquot (0.5 mL) of the supernatant was mixed with 0.5 mL of 10 mM potassium-phosphate buffer (pH = 7.0) and 1 mL of 1 M potassium iodide. The H_2O_2 concentration was evaluated using standards of 5–1,000 μM of H_2O_2 and calibration curve plotted accordingly. The absorbance of samples and standards was measured at 390 nm and results were expressed as μmol H_2O_2/g Fwt.

Lipid peroxidation was assessed according to Azevedo Neto et al. (2006) and measured in terms of malondialdeyde content (MDA). Leaf tissue (0.2 g) was homogenized in 0.1% TCA and the extract was centrifuged at 15,000 g for 10 min. The reaction mixture of 0.5 mL extract and 1.5 mL of 0.5% thioarbituric acid (TBA) in 20% TCA was incubated at 95°C for 25 min and then cooled on ice bath. The absorbance was determined at 532 nm and corrected for non-specific absorbance at 600 nm. MDA amount was determined using the extinction coefficient of 155 mM/cm. Results were expressed as nmol of MDA/g Fwt.

Activities of Antioxidant Enzymes and Proline Content

Leaf tissue was homogenized in a chilled mortar using ice cold extraction buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1% (w/v) polyvinylpyrrolidone (PVPP), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.05% Triton X-100 in 50 mM potassium-phosphate buffer (pH = 7.0) (Chrysargyris et al., 2017b). Protein content in the enzyme extracts was determined according to Bradford assay (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

Catalase (EC 1.11.1.6) and SOD (EC 1.15.1.1) activity were assayed as described previously (Chrysargyris et al., 2017b). Catalase activity was assayed in a reaction mixture (1.5 mL) containing 50 mM K-phosphate buffer (pH = 7.0), 10 mM H_2O_2 and an enzyme aliquot. The decomposition of H_2O_2 was followed at 240 nm. The results were expressed as CAT units/mg of protein (1 unit = 1 mM of H_2O_2 reduction/min). SOD was assayed using the photochemical method. Reaction mixture (1.5 mL) containing 50 mM K-phosphate buffer (pH = 7.5), 13 mM methionine, 75 μM nitro blue tetrazolium (NBT), 0.1 mM EDTA, 2 μM riboflavin and an enzyme aliquot. Reaction started with the addition of riboflavin and placing tubes with the reaction mixture below a light source of two 15-watt fluorescent lamps for 15 min. Reaction stopped by placing the tubes in the dark. Mixtures without enzyme extract developed maximal color (control) and non-irradiated mixture used as blank. The absorbance was determined at 560 nm and activity was expressed as units/mg of protein. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the NBT photoreduction rate. Peroxidase activity (EC 1.11.1.6) was determined as described by Tarchoune et al. (2012) following the increase in absorbance at 430 nm. Calculations were performed using the coefficient of extinction of 2.47 mM/cm. One POD unit was defined as the amount of enzyme to decompose 1 μmol of H_2O_2 per minute. Results were expressed as units of peroxidase/mg of protein. The activity of APX (EC 1.11.1.11) was determined according to Zhu et al. (2004), by the decrease in absorbance of ascorbate at 290 nm. Results were expressed as units APX/mg of protein.

Proline was measured with the method of acid-ninhydrin and toluene at 520 nm, as described by Khedr et al. (2003). The amount of proline was calculated using a standard curve of proline and results were expressed as μg proline/g Fwt.

Statistical Methods

Data were statistically analyzed using analysis of variance (ANOVA) by IBM SPSS v.22, and presented as treatment mean ± SE of six biological measurements. Pairwise metabolites effects correlations were calculated by Pearson's correlation test using the R program. The components chemical structure and the relationship among treatments were determined by Linear Discriminate analysis (LDA) as described previously (Chrysargyris et al., 2016b), and performed at the percentages of all identified compounds for all treatments by SPSS program. Duncan's multiple range tests were calculated for the significant data at $P < 0.05$.

RESULTS

Plant Growth

Data presented in **Tables 1**, **2** indicated that plant growth variables were mainly influenced by salinity and less by mineral foliar applications. Plant height and stem thickness were significantly decreased at NaCl concentrations >50 mM, while no differences were observed at lower salinity levels (i.e., 50 mM NaCl) (**Table 1**). Also, the salinity conditions (25–50–100 mM NaCl) reduced leaf length, fresh upper biomass and biomass dry matter content up to 16, 53, and 27%, respectively. Middle (50 mM NaCl) and high (100 mM NaCl) salinity reduced root fresh weight while root length was reduced only at a NaCl concentration of 100 mM (**Table 2**). As a consequence, the ratio of biomass:root was greater at NaCl concentrations >50 mM respect to the low (i.e., 25 mM NaCl) or non-saline treatment, mainly due to the lower root development (lower root fresh weight). No differences were found for shoot number (averaged in 3.54) and root dry matter content (averaged in 11.03%).

Considering the effects of foliar mineral application on the plant growth, the application of K, Zn, and Si reduced the fresh upper biomass in non-saline treatments, whereas this was not evident in saline treatments (**Table 1**). The application of K at NaCl concentration of 0 and 25 mM (including Si) reduced plant height. Interestingly, foliar application of K, Zn, and Si at highest (100 mM NaCl) salinity reduced further the stem thickness and shoot number produced. The application of K at a NaCl concentration of 50 mM reduced the root dry matter content compared to the relevant control treatment (50 mM NaCl without foliar application) (**Table 2**).

Summarizing in **Tables 1**, **2**, two-way ANOVA revealed that salinity significantly ($P < 0.01$; $P < 0.001$) affected plant growth parameters, both in upper and root part of the plant, while foliar application affected plant height ($P < 0.05$); biomass fresh weight ($P < 0.001$); and root fresh weight ($P < 0.05$). The interaction of salinity × foliar application affected ($P < 0.05$) root dry matter content through salinity impacts.

Physiological Parameters

Examining the effect of salinity and/or foliar mineral application on physiological parameters, it was found that salinity at NaCl concentrations >50 mM reduced (up to 73%) the content of Chl a and Chl b, and as a consequence, the total Chl content (**Table 3**). In general, foliar application did not have any profound effect on chlorophyll content, with the exception of the Si treatment at a NaCl concentration of 25 mM, that caused reduction in Chl a, Chl b, and total Chl of 28, 29, and 28%, respectively. However, neither salinity nor minerals affected leaf stomatal conductivity (averaged in 1.51 cm/s). Two-way ANOVA revealed that salinity affected ($P < 0.001$) the content of chlorophylls, while neither the foliar application nor the salinity × foliar interaction affected physiological parameters in any way.

The application of salinity affected the content of total phenols and antioxidative activity of lavender plants (**Figure 1**). Thus, total phenols, FRAP and ABTS radical scavenging activity were significantly reduced at NaCl concentrations >50 mM comparing with the control (0 mM NaCl) and 25 mM NaCl applications. The same results were obtained for the DPPH radical scavenging activity respect to the control and NaCl concentrations >50 mM.

K application gave similar results following salinity effects. i.e., the application of NaCl concentrations >50 mM caused total phenols and antioxidant activity reductions. However, foliar with K increased the FRAP activity compared to relevant control at low (25 mM) salinity treatments but not at middle (50 mM) and high (100 mM NaCl) salinity (**Figure 1**).

The application of Zn on lavender reduced the total phenols, DPPH and FRAP radical scavenging activity at NaCl concentrations >50 mM respect to the 0 and 25 mM NaCl applications, while this reduction was evident even in low (25 mM NaCl) salinity levels for the ABTS activity (**Figure 1**). Similar to K application, Zn foliar increased the FRAP activity at NaCl concentration of 25 mM. However, it's worth noting that Zn application at NaCl concentration of 100 mM reduced DPPH radical scavenging activity further.

The FRAP and ABTS activity were reduced at NaCl concentrations >50 mM with Si application, while the DPPH activity and total phenols were reduced even at 25 mM NaCl treatment (**Figure 1**). Si application increased FRAP activity and total phenols in lavender plants grown in non-saline conditions. However, similar to Zn foliar, Si application at NaCl concentration of 100 mM reduced further the DPPH radical scavenging activity.

Two way ANOVA revealed that salinity significantly ($P > 0.001$) affected total phenols and antioxidant activity in lavender, while foliar application affected DPPH ($P < 0.05$) and FRAP ($P < 0.001$) activity (**Table 4**). Salinity × foliar interaction affected ($P < 0.05$) the content of total phenols and DPPH activity, resulting by the salinity treatment impacts.

The effects of salinity into nutrient solution on damage index, enzymes activity and proline content in lavender plants are presented in **Figure 2**. The APX activity increased at NaCl concentrations >50 mM, while the opposite occurred in SOD activity. Moreover, the increased salinity levels affected the proline content and POD activity, with greater values to be found at NaCl concentration of 100 mM, while no differences were found among 25 and 50 mM of NaCl treatment. Plants grown under salinity had lower CAT values, which were independent of the salinity levels applied. Neither H_2O_2 nor MDA production got significantly affected by salinity treatments (**Figures 2E,F**).

TABLE 1 | Effect of salinity levels (0–25–50–100 mM NaCl) and foliar applications (no foliar, K, Zn, and Si) on lavender plant height (cm), leaf length (cm), stem thickness (mm), shoot number, biomass fresh weight (FW; g/plant), and biomass dry matter (DM; %) in plants grown hydroponically in perlite.

Salinity (NaCl)	Foliar applic.	Plant height	Leaf length	Stem thickness	Shoot number	Biomass FW	Biomass DM
0 mM	0	40.83 ± 1.53a[Y]	6.12 ± 0.15ab	5.49 ± 0.15ab	4.33 ± 0.71abc	27.63 ± 1.19a	31.21 ± 0.51a
	+K	36.50 ± 2.04bcd	6.03 ± 0.09abcd	5.40 ± 0.22ab	4.67 ± 0.55ab	20.20 ± 1.52bc	32.94 ± 0.39a
	+Zn	39.00 ± 0.36ab	6.08 ± 0.13abc	5.81 ± 0.31a	5.17 ± 0.65a	20.84 ± 1.66bc	33.21 ± 0.81a
	+Si	38.00 ± 1.29ab	6.15 ± 0.08a	5.47 ± 0.25ab	3.67 ± 0.33abcd	23.58 ± 1.53b	31.98 ± 1.25a
25 mM	0	37.33 ± 1.52abc	5.72 ± 0.16cde	5.37 ± 0.36ab	4.17 ± 0.54abcd	23.54 ± 2.07b	25.38 ± 1.28bc
	+K	33.00 ± 1.41de	5.75 ± 0.18bcde	5.38 ± 0.37ab	3.83 ± 0.54abcd	20.44 ± 1.50bc	27.32 ± 0.41b
	+Zn	33.67 ± 1.28cde	5.68 ± 0.11def	4.82 ± 0.19bcd	3.67 ± 0.71abcd	19.71 ± 1.33bc	27.39 ± 1.36b
	+Si	31.83 ± 1.53ef	5.67 ± 0.18def	5.14 ± 0.20abc	4.67 ± 0.33ab	20.32 ± 1.17bc	27.50 ± 1.24b
50 mM	0	27.67 ± 0.95g	5.32 ± 0.09fgh	4.31 ± 0.21de	3.67 ± 0.42abcd	18.37 ± 0.81cd	24.80 ± 0.71bc
	+K	28.83 ± 1.16fg	5.40 ± 0.12efg	4.55 ± 0.15cde	3.50 ± 0.88abcd	17.34 ± 1.23cd	25.17 ± 0.98bc
	+Zn	27.00 ± 1.39g	5.05 ± 0.11gh	4.32 ± 0.27de	2.67 ± 0.21cdef	15.59 ± 1.31de	25.31 ± 0.22bc
	+Si	26.50 ± 0.72g	5.05 ± 0.05gh	4.57 ± 0.19cde	3.50 ± 0.42abcd	19.69 ± 1.08bc	23.67 ± 0.91cd
100 mM	0	21.17 ± 1.08h	5.13 ± 0.04gh	3.93 ± 0.20e	3.33 ± 0.21bcde	13.03 ± 1.09ef	22.71 ± 0.93cd
	+K	19.58 ± 1.34h	5.02 ± 0.19gh	3.19 ± 0.21f	1.50 ± 0.34f	9.72 ± 0.87f	21.25 ± 0.85d
	+Zn	21.17 ± 2.16h	4.63 ± 0.06i	2.98 ± 0.18f	1.83 ± 0.40ef	11.63 ± 0.87ef	21.02 ± 0.87d
	+Si	19.92 ± 0.71h	4.97 ± 0.06hi	2.85 ± 0.10f	2.50 ± 0.34ef	11.68 ± 0.80ef	22.61 ± 0.80cd
SIGNIFICANCE							
Salinity (S)		***	***	***	***	***	***
Foliar (F)		*	ns	ns	ns	***	ns
S × F		ns	ns	ns	ns	ns	ns

[Y] *values (n = 6) in columns followed by the same letter are not significantly different, P < 0.05.*

*ns, *, and *** indicate non-significant or significant differences at P < 5, and 0.1%, respectively, following two-way ANOVA.*

APX activity and proline content were increased but CAT activity was reduced in plants grown under salinity and K foliar (**Figures 2A,C,G**). Indeed, the K application increased the CAT activity in 25 mM NaCl-treated plants comparing to the no foliar treatment (**Figure 2C**). POD activity did not differ in plants grown in salinity <50 mM NaCl+K while POD activity almost doubled in 100 mM of NaCl+K. The H_2O_2 production significantly increased in plants grown at NaCl concentrations >50 mM. K application increased the H_2O_2 production in 50 and 100 mM NaCl-treated plants comparing to no foliar treatments. No significant differences were found on SOD activity and MDA production.

Considering the Zn foliar application, the APX activity was increased (up to 80%) in plants grown at NaCl concentration 100 mM comparing with the activity of plants grown at NaCl concentration 25 mM or non-saline treatment (**Figure 2**). Additionally, plant grown at NaCl concentrations <50 mM did not differ on POD values (despite the increased tendency related to the increased salinity levels) and CAT values (despite the reduced tendency related to the increased salinity levels). However, the Zn application at a NaCl concentration of 100 mM, resulted in significant higher POD value and lower CAT value comparing with the control treatments (no foliar). Similar to K, the Zn application increased the proline content in relation to the salinity level increments. Neither H_2O_2 nor MDA production got significantly affected by salinity treatments (**Figures 2E,F**). SOD activity varied among Zn treatments without specific trend for the Zn effect.

Foliar application of Si on plants grown under different salinity levels had similar effects with the application of K as mentioned previously (**Figure 2**). Therefore, APX remained in similar levels in Si-treated plants grown at NaCl concentrations <50 mM, but increased at NaCl concentration of 100 mM. Interestingly, Si foliar reduced the APX values at NaCl concentration of 50 mM comparing with the no foliar treatment. CAT activity was reduced in plants grown under moderate salinity (>50 mM NaCl). Indeed, the Si application increased the CAT activity in 25 mM NaCl-treated plants comparing to the no foliar treatment. To the contrary, Si application reduced CAT activity in non-saline treated plants comparing to the no foliar treatment. POD activity increased in plants grown in different salinity levels with greater (two-times) values to be found at NaCl concentration 100 mM. The H_2O_2 production significantly increased in Si-treated plants grown at NaCl concentration 100 mM respect to the non-saline treatment. No significant differences were found on SOD activity among treatments. MDA production varied among Si treatments without specific trend for the Si effect. However, the Si foliar application increased the MDA production in plants grown in non-saline conditions comparing to the no foliar treatment. The proline content following Si foliar was increased at NaCl concentration of 100 mM comparing with 0–25 mM NaCl+Si.

TABLE 2 | Effect of salinity levels (0–25–50–100 mM NaCl) and foliar applications (no foliar, K, Zn, and Si) on lavender root fresh weight (FW; g/plant), root dry matter (DM; %), biomass:root ratio, and root length (cm) in plants grown hydroponically in perlite.

Salinity (NaCl)	Foliar applic.	Root FW	Root DM	Biomass:root	Root length
0 mM	0	15.85 ± 0.85a [Y]	12.16 ± 0.31abcd	1.81 ± 0.13cd	26.50 ± 1.68ab
	+K	15.33 ± 1.60a	10.69 ± 0.42abcd	1.54 ± 0.14d	26.00 ± 1.71ab
	+Zn	12.35 ± 0.52ab	12.61 ± 0.93abc	2.01 ± 0.15cd	27.33 ± 1.25a
	+Si	14.93 ± 1.95a	13.29 ± 1.01ab	1.73 ± 0.14cd	27.00 ± 1.63ab
25 mM	0	15.33 ± 1.28a	9.09 ± 1.85d	1.88 ± 0.10cd	27.00 ± 0.96ab
	+K	14.63 ± 2.00a	11.20 ± 0.57abcd	1.60 ± 0.13cd	24.50 ± 1.87abc
	+Zn	12.25 ± 1.61ab	9.94 ± 1.49cd	1.92 ± 0.24cd	27.33 ± 1.85a
	+Si	13.23 ± 0.68a	9.27 ± 0.56d	1.65 ± 0.17cd	23.67 ± 0.61abc
50 mM	0	7.38 ± 1.06cd	13.44 ± 0.82a	2.79 ± 0.32ab	22.67 ± 1.68bcd
	+K	9.17 ± 0.78bc	9.45 ± 0.57d	2.29 ± 0.22abcd	22.83 ± 1.01bcd
	+Zn	6.90 ± 0.45cd	12.12 ± 1.29abcd	2.39 ± 0.34abc	22.83 ± 1.27bcd
	+Si	9.22 ± 0.61bc	9.89 ± 0.48cd	2.05 ± 0.15bcd	21.33 ± 1.05cde
100 mM	0	5.38 ± 0.69d	10.52 ± 0.36abcd	2.96 ± 0.34a	16.75 ± 0.96f
	+K	5.07 ± 0.58d	10.40 ± 0.56abcd	2.37 ± 0.36abc	18.92 ± 0.89def
	+Zn	4.38 ± 0.68d	12.01 ± 0.74abcd	2.78 ± 0.29ab	17.83 ± 0.98ef
	+Si	4.33 ± 0.81d	10.33 ± 0.86bcd	2.88 ± 0.26a	16.83 ± 0.79f
SIGNIFICANCE					
Salinity (S)		***	**	***	***
Foliar (F)		*	ns	ns	ns
S × F		ns	*	ns	ns

[Y] *values (n = 6) in columns followed by the same letter are not significantly different, P ≤ 0.05.*
*ns, *, **, and *** indicate non-significant or significant differences at P < 5, 1, and 0.1%, respectively, following two-way ANOVA.*

Two-way ANOVA revealed that both salinity ($P < 0.001$) and foliar ($P < 0.01$) but not their interaction, affected APX activity. CAT activity and H_2O_2 production were significantly affected either by salinity, foliar or their interaction (salinity × foliar) as presented in **Table 5**. MDA activity was affected by salinity ($P < 0.05$) or foliar ($P < 0.05$) application, but not by their interaction. Salinity affected ($P < 0.001$) POD activity, while foliar application did not cause any significant effect. Salinity affected ($P < 0.001$) the content of proline, and this effect was also evident in saline × foliar interaction. Interestingly, neither salinity nor foliar application affected SOD content, while their combined effect (salinity × foliar interaction) resulted in significant ($P < 0.05$) impact.

Mineral Nutrient Content

Salinity level and foliar application affected micro- and macro-nutrient content in both leaves and roots (**Figures 3**, **4**, Figure S2). In leaves, salinity at a NaCl concentration of 100 mM reduced (up to 24%) nitrogen content (**Figure 3A1**). Foliar K application alleviated the high salinity negative effect as the leaf nitrogen content was similar among treatments (**Figure 3A2**). The Zn and Si foliar application reduced nitrogen content at NaCl concentration of 100 mM compared with the non-saline treatments (**Figures 3A3,A4**). The leaf potassium content decreased (up to 37%) in plants grown under salinity (**Figure 3B1**) while neither K (**Figure 3B2**) nor Zn and Si application alleviated the adverse salinity effects (**Figures 3B3,B4**). Leaf phosphorus content was increased (5.89 and 6.47 g/kg tissue) in plants grown within NaCl concentrations of 25–50 mM, respectively (**Figure 3C1**). The K application reduced the leaf phosphorus content at NaCl concentration of 100 mM (**Figure 3C2**). The foliar application with Zn or Si did not change the leaf phosphorus content (**Figures 3C3,C4**). Salinity did not affect (**Figure 3D1**) but Zn application reduced leaf calcium content in saline-grown plants (**Figure 3D3**). However, K or Si application reduced leaf calcium content at NaCl concentration of 50 mM (**Figures 3D2,D4**). Magnesium content in leaves was reduced in plants grown at NaCl concentration of 50 mM (**Figure 3E1**). Cation application reduced the leaf magnesium content in saline-treated plants (**Figures 3E2–E4**). As expected, increasing salinity levels resulted in increasing sodium content in leaves (**Figure 3F1**). The same trend was found in plants grown under saline with the foliar (K, Zn, Si) application (**Figures 3F2–3F4**).

Salinity increased aluminum content in leaves and reached the highest content at a NaCl concentration of 50 mM (**Figure 4G1**). Indeed, K and Zn foliar application alleviated the aluminum inductions in saline-treated plants, as the aluminum content did not differ among plants grown in saline and non-saline conditions (**Figures 4G2,G3**). However, the Si application posed same effects, to relieve aluminum content in leaves only at NaCl concentrations of 25 and 50 mM, but not at 100 mM

TABLE 3 | Effect of salinity levels (0–25–50–100 mM NaCl) and foliar applications (no foliar, K, Zn, and Si) on lavender leaf stomatal conductivity (cm/s), chlorophylls (Chl a, Chl b, Total Chl) content (mg/g fresh weight) in plants grown hydroponically in perlite.

Salinity (NaCl)	Foliar applic.	Stomatal conductivity	Chl a	Chl b	Total Chl
0 mM	0	1.25 ± 0.44a [Y]	1.60 ± 0.10a	0.55 ±0.04a	2.15 ±0.14a
	+K	1.66 ± 0.42a	1.62 ± 0.07a	0.56 ± 0.02a	2.19 ± 0.09a
	+Zn	1.38 ± 0.56a	1.52 ± 0.07ab	0.53 ± 0.02a	2.06 ±0.10ab
	+Si	1.00 ± 0.45a	1.37 ± 0.13abc	0.48 ± 0.05ab	1.85 ±0.19abc
25 mM	0	1.21 ± 0.62a	1.31 ± 0.12abc	0.45 ± 0.05ab	1.77 ±0.17abc
	+K	1.71 ± 0.29a	1.15 ± 0.12cde	0.36 ± 0.04bcd	1.52 ±0.16cde
	+Zn	2.25 ± 0.40a	1.21 ± 0.09bcd	0.41 ± 0.03bc	1.61 ±0.12bcd
	+Si	2.11 ± 0.43a	0.95 ± 0.14def	0.32 ± 0.05cd	1.27 ±0.19def
50 mM	0	1.31 ± 0.58a	0.81 ± 0.12efg	0.25 ± 0.04def	1.07 ±0.17efgh
	+K	1.63 ± 0.51a	0.94 ± 0.10def	0.30 ± 0.03cde	1.24 ±0.13def
	+Zn	2.12 ± 0.61a	0.75 ± 0.09fgh	0.25 ± 0.03def	1.01 ±0.13fgh
	+Si	0.93 ± 0.45a	0.87 ± 0.17def	0.29 ± 0.05cde	1.16 ±0.23defg
100 mM	0	1.15 ± 0.23a	0.42 ± 0.05h	0.14 ± 0.01f	0.57 ± 0.06i
	+K	1.74 ± 0.70a	0.71 ± 0.10fgh	0.24 ± 0.03def	0.96 ±0.13fghi
	+Zn	0.87 ± 0.30a	0.52 ± 0.20gh	0.18 ± 0.02ef	0.71 ±0.10ghi
	+Si	1.77 ± 0.56a	0.45 ± 0.08h	0.15 ± 0.02f	0.60 ±0.10hi
SIGNIFICANCE					
Salinity (S)		ns	***	***	***
Foliar (F)		ns	ns	ns	ns
S × F		ns	ns	ns	ns

[Y] *values (n = 6) in columns followed by the same letter are not significantly different, P < 0.05.*
*ns and *** indicate non-significant or significant differences at P < 0.1%, respectively, following two-way ANOVA.*

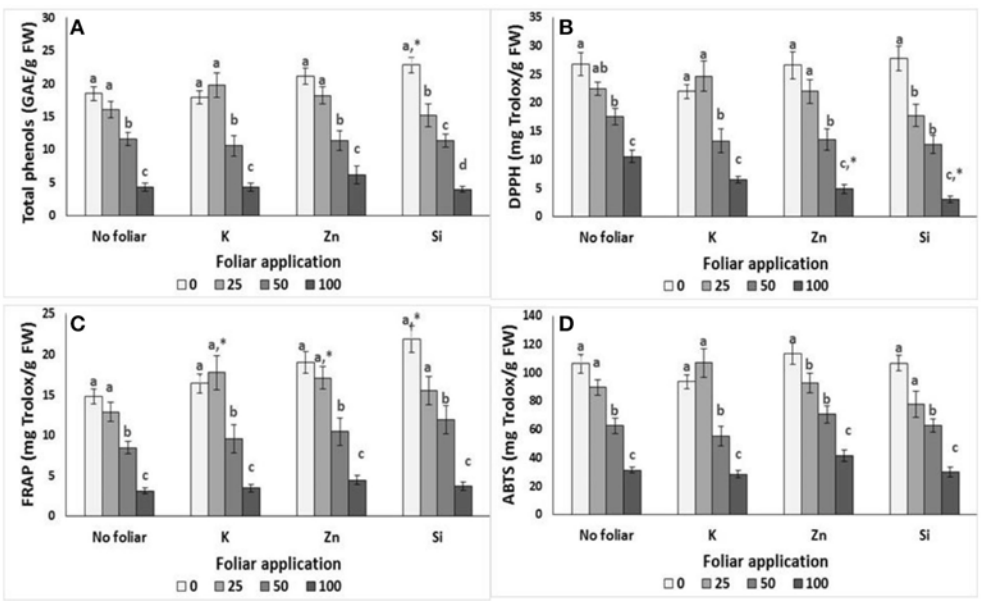

FIGURE 1 | Effect of salinity levels (0–25–50–100 mM NaCl) and foliar applications (no foliar, K, Zn, Si) on the content of total phenols and antioxidant activity in lavender. **(A)** Total phenols, **(B)** DPPH, **(C)** FRAP, and **(D)** ABTS. Significant differences ($P < 0.05$) among salinity treatments are indicated by different letters. Star (*) symbol indicated significance differ among no foliar and equivalent cation foliar. Error bars show SE ($n = 6$).

TABLE 4 | Effect of salinity levels (0–25–50–100 mM NaCl) and foliar applications (no foliar, K, Zn, and Si) on the content of total phenols and antioxidant activity in lavender grown hydroponically in perlite.

Significance	Total Phenols	DPPH	FRAP	ABTS
Salinity (S)	***	***	***	***
Foliar (F)	ns	*	**	ns
S × F	*	*	ns	ns

*ns, *, **, and *** indicate non-significant or significant differences at $P \leq 5$, 1, and 0.1%, respectively, following two-way ANOVA.*

(**Figure 4G4**). Neither salinity nor K or Zn application affected iron content in leaves (**Figures 4H1–4H3**) while Si application increased iron content in plants grown at NaCl concentration 100 mM (**Figure 4H4**). The leaf boron content reduced in plants grown at NaCl concentrations 25 and 50 mM compared to the control plants (**Figure 4I1**). Zn application reduced boron content in plants grown under salinity, and this was evidenced only for the NaCl concentration 50 mM with Si application (**Figures 4I3,I4**). The application of Zn and Si reduced the copper content in leaves at NaCl concentrations 25 and 50 mM

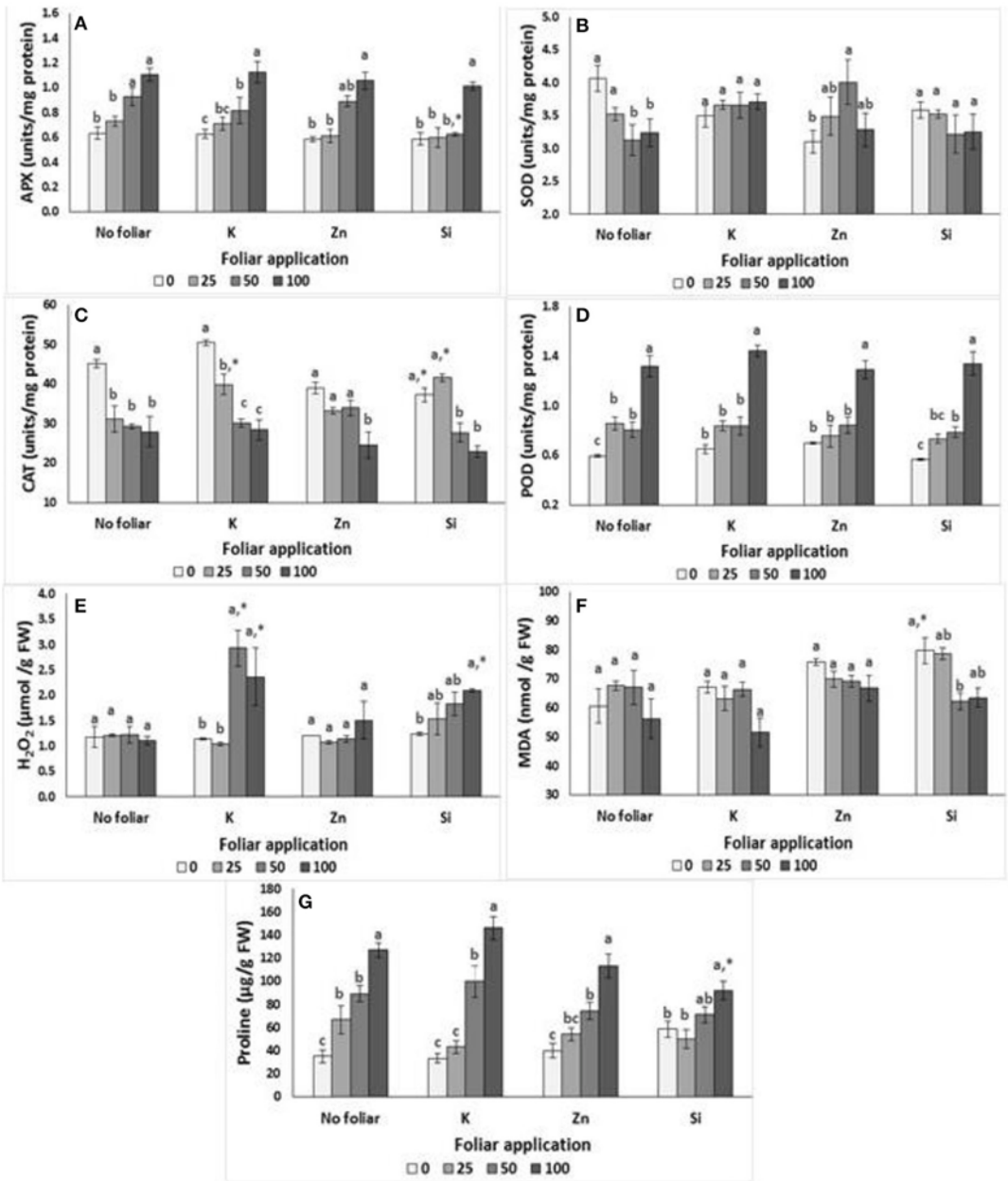

FIGURE 2 | Effect of salinity levels (0–25–50–100 mM NaCl) and foliar applications (no foliar, K, Zn, Si) on the damage index and antioxidant enzymes activities in lavender. **(A)** APX, **(B)** SOD, **(C)** CAT, **(D)** POD, **(E)** H_2O_2, **(F)** Lipid peroxidation (MDA), and **(G)** proline. Significant differences ($P < 0.05$) among salinity treatments are indicated by different letters. Star (*) symbol indicated significance differ among no foliar and equivalent cation foliar. Error bars show SE ($n = 6$).

TABLE 5 | Effect of salinity levels (0–25–50–100 mM NaCl) and foliar applications (no foliar, K, Zn, and Si) on the damage index and antioxidant enzymes activities in lavender grown hydroponically in perlite.

Significance	APX	SOD	CAT	POD	MDA	H_2O_2	Proline
Salinity (S)	***	ns	***	***	*	**	***
Foliar (F)	**	ns	*	ns	*	***	ns
S × F	ns	*	**	ns	ns	**	**

*ns, *, **, and *** indicate non-significant or significant differences at $P \leq 5$, 1, and 0.1%, respectively, following two-way ANOVA.*

(**Figures 4J3,J4**) while this was also found for the highest salinity level (100 mM NaCl) following K application (**Figure 4J2**). No clear observation can be stated for the effect of salinity on leaf copper content (**Figure 4J1**). Plants grown at NaCl concentration of 50 mM (including Si application) increased manganese content (**Figures 4K1,K4**). K or Zn application in saline-treated plants did not affect manganese content in leaves (**Figures 4K2,K3**). The zinc content in leaves increased in plants grown at NaCl concentrations >50 mM (**Figure 4L1**). Both the application of K or Si increased the zinc content

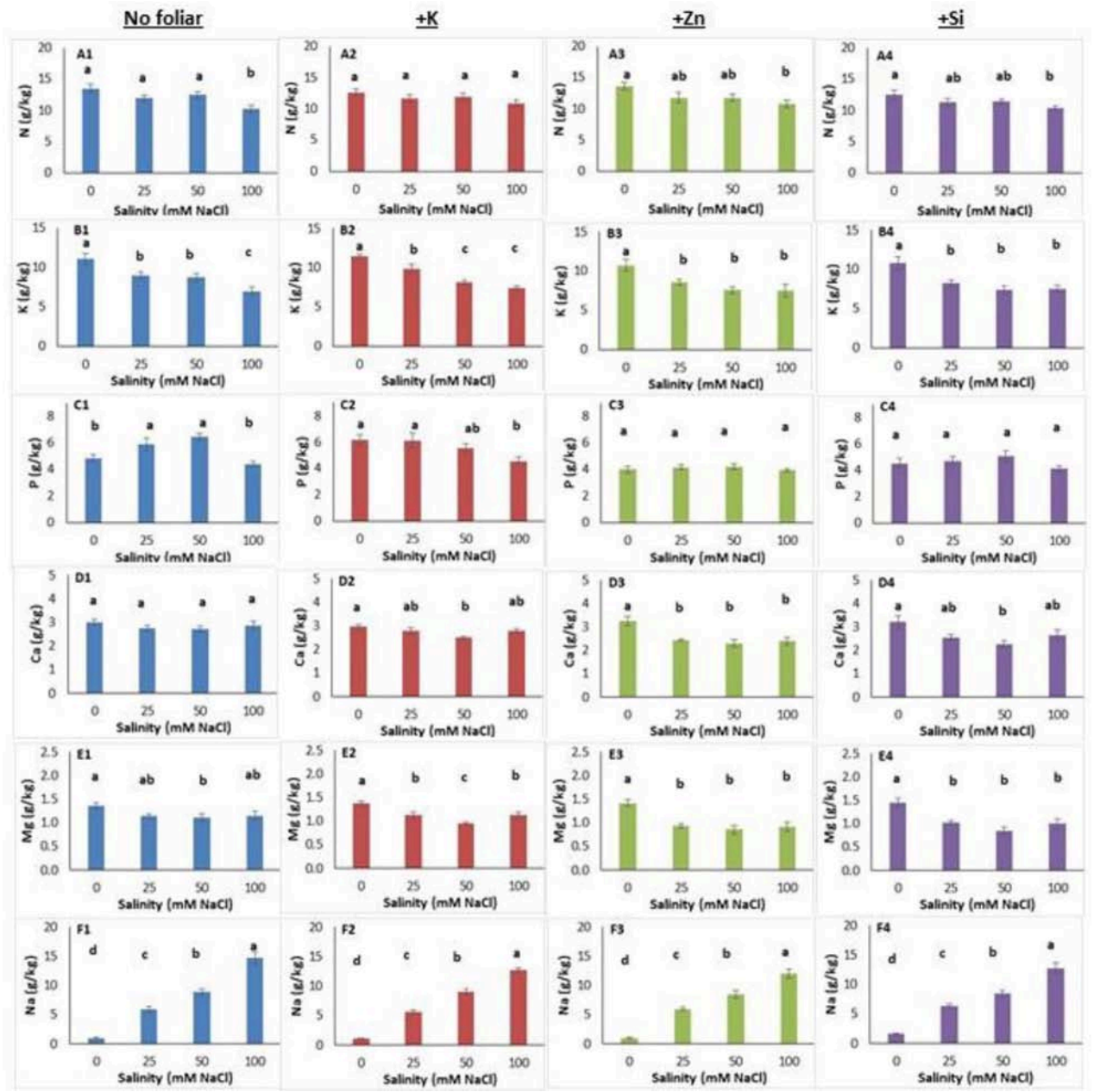

FIGURE 3 | Lavender leaf analysis (macronutrient) of plants grown hydroponically in perlite under different salinity levels (0–25–50–100 mM NaCl) and foliar applications (no foliar, K, Zn and Si). Sub-figures **(A–F)** referring to different macronutrients and numbering **(1–4)** referring to the no foliar, K, Zn and Si, respectively. Significant differences ($P < 0.05$) among treatments are indicated by different letters. Error bars show SE ($n = 6$).

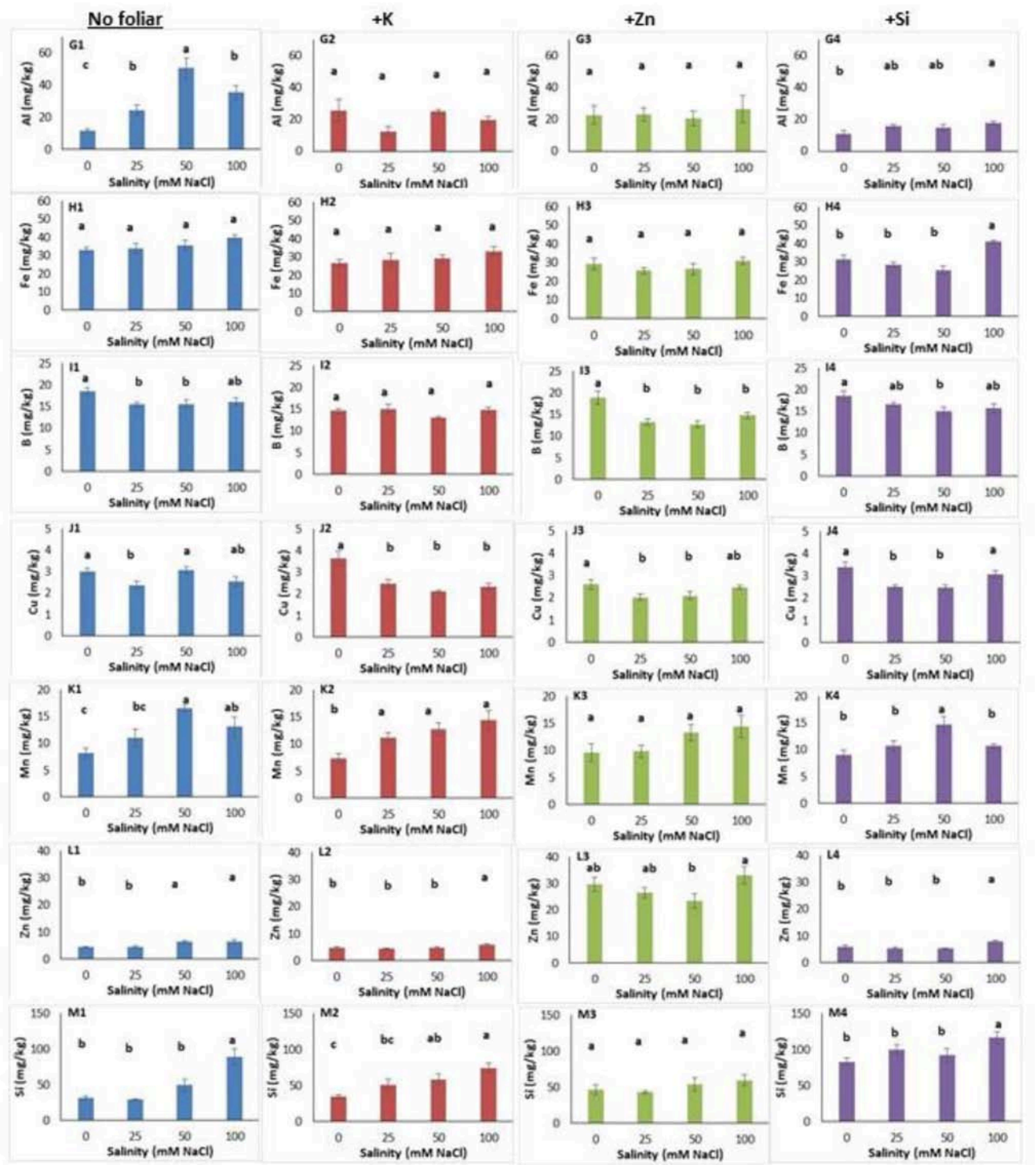

FIGURE 4 | Lavender leaf analysis (micronutrient) of plants grown hydroponically in perlite under different salinity levels (0–25–50–100 mM NaCl) and foliar applications (no foliar, K, Zn and Si). Sub-figures **(G–M)** referring to different micronutrients and numbering **(1–4)** referring to the no foliar, K, Zn and Si, respectively. Significant differences ($P < 0.05$) among treatments are indicated by different letters. Error bars show SE ($n = 6$).

at NaCl concentration of 100 mM (**Figures 4L2,L4**). Zn foliar application, as expected, increased (up to 5.8 times) the zinc leaf content comparing to no foliar applications (**Figure 4L3**). Plants grown at a NaCl concentration of 100 mM had higher silicon content (**Figure 4M1**). Additionally, K application increased silicon content in plants grown at NaCl concentration of 100 mM

comparing with plants grown at 25 mM and non-saline (control) conditions (**Figure 4M2**). Zn application did not alter the silicon content in leaves (**Figure 4M3**). The Si application increased the silicon content in plants grown at NaCl concentration of 100 mM (**Figure 4M4**), similar trend as it was found for non-foliar applications (see **Figure 4M1**). Obviously, Si application increased (up to 1.5 times) the silicon content in leaves, ranged from 83.29 to 116.70 mg/kg.

Following two-way ANOVA, salinity significantly affected ($P < 0.01$; $P < 0.001$) the leaf macro- and micronutrients (except for aluminum), while foliar application significantly affected the content of phosphorus, aluminum, iron, copper, zinc and silicon at level of $P < 0.001$, the content of boron at level of $P < 0.01$ and magnesium content at level of $P < 0.05$ (**Table 6**). Salinity × foliar interaction affected mainly micronutrients content, such as aluminum, copper and silicon at level of $P < 0.05$, 0.01, and 0.05 respectively.

The effects of salinity and foliar application on root mineral content is presented in Figure S2. Salinity (NaCl) decreased potassium and calcium (at 100 mM), iron and silicon (at ≥ 25 mM), boron (at ≥ 50 mM) but increased phosphorous, sodium, manganese and zinc content in roots. Plants grown under salinity with K application decreased root content for potassium and silicon (at ≥ 25 mM), calcium, boron, copper and iron (at ≥ 50 mM), aluminum (at 100 mM) but increase phosphorus (at 100 mM), sodium and manganese (at ≥ 50 mM) root content compared to control treatment. In case of Zn application, plants grown under salinity decreased root content for potassium, calcium and boron (at ≥ 50 mM), iron (at 100 mM), silicon (at ≥ 25 mM) but increase phosphorus and zinc (at ≥ 50 mM), as well as sodium (at ≥ 25 mM) root content respect to the equivalent control treatment. Plants grown under salinity with Si application decreased root content for potassium, magnesium, silicon and boron (at ≥ 25 mM), but increase nitrogen (at 50 mM), phosphorus, manganese and zinc (at ≥ 50 mM), and sodium (at 100 mM) content in roots.

TABLE 6 | Effect of salinity levels (0–25–50–100 mM NaCl) and foliar applications (no foliar, K, Zn, and Si) on the leaf analysis in lavender grown hydroponically in perlite.

Significance	N	K	P	Ca	Mg	Na	
Macronutrients							
Salinity (S)	***	***	***	***	***	***	
Foliar (F)	ns	ns	***	ns	*	ns	
S × F	ns	ns	ns	ns	ns	ns	
Significance	**Al**	**Fe**	**B**	**Cu**	**Mn**	**Zn**	**Si**
Micronutrients							
Salinity (S)	ns	***	***	***	***	**	***
Foliar (F)	***	***	**	***	ns	***	***
S × F	*	ns	ns	**	ns	ns	*

*ns, *, **, and *** indicate non-significant or significant differences at P ≤ 5, 1, and 0.1%, respectively, following two-way ANOVA.*

Following two-way ANOVA, salinity affected ($P < 0.01$; $P < 0.001$) the root macro- and micronutrients (except for aluminum) significantly, while foliar application significantly affected the content of potassium at level $P < 0.01$ and the content of zinc at level of $P < 0.05$ (Table S1). Indeed, the interaction of salinity × foliar application did not cause any effect on root mineral content.

Essential Oil Yield and Constituents

Lavender EO yield was reduced in plants grown under NaCl concentration of 100 mM comparing with plants grown in non-saline or low saline levels (**Figure 5**). The foliar application of minerals (K, Zn, and Si) did not affect the EO yield among treatments, but alleviated the reduced yield which was observed under the high salinity (without any foliar application). Two-way ANOVA revealed that salinity ($P < 0.01$) and foliar application ($P < 0.05$) (but not their interaction) affected the EO yield (**Table 7**).

The effect of different salinity levels as well as mineral foliar application on chemical composition of the EO of *L. angustifolia* are given in **Figure 6** and Tables S2−S5. Considering the EOs analysis, 29 components for salinity, 30 components (excluding o-cymene and including caryophyllene oxide and muurola-5-en-4-one) for K foliar, and 31 (including caryophyllene oxide and muurola-5-en-4-one) components for Zn and Si applications were identified in the EOs of lavender that

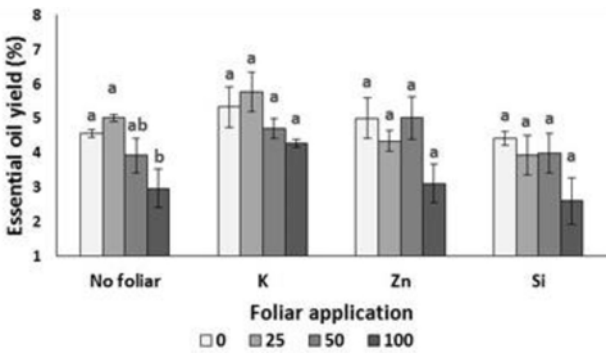

FIGURE 5 | Effect of salinity levels (0–25–50–100 mM NaCl) and foliar applications (no foliar, K, Zn, and Si) on essential oil yield (%) in lavender plant grown hydroponically in perlite. Significant differences ($P < 0.05$) among treatments are indicated by different letters. Error bars show SE ($n = 6$).

TABLE 7 | Effect of salinity levels (0–25–50–100 mM NaCl) and foliar applications (no foliar, K, Zn, and Si) on essential oil yield (%) in lavender grown hydroponically in perlite.

Significance	Essential oil yield
Salinity (S)	**
Foliar (F)	*
S × F	ns

*ns, *, and ** indicate non-significant or significant differences at P < 5 and 1%, respectively, following two-way ANOVA.*

underwent different treatments that represented 98.86–99.62% of the oils. It can be noticed that, hydrocarbon (monoterpenes and sesquiterpenes) compounds were ranged from 16.74 to 24.80% and 0.48 to 1.13%, respectively, while oxygenated (monoterpenes and sesquiterpenes) compounds were ranged from 69.58 to 79.82% and 2.13 to 4.19%, respectively (Tables S2−S5). The major components were 1.8-cineole (alcohol), D-limonene (monoterpene hydrocarbon), β-pinene (monoterpene hydrocarbon), camphor (ketone), α-pinene (monoterpene hydrocarbon), borneol (alcohol), α-terpineol (alcohol), β-myrcene (monoterpene hydrocarbon), sabinene (monoterpene hydrocarbon), *cis*-lanceol (alcohol), and α-bisabolol (alcohol). Other components were present in amounts <1% in most treatments (Tables S2−S5).

The correlation matrix of EO metabolites is presented in **Figure 6**. Among metabolites that activated specific terpenoids backbone biosynthesis related to a positive correlation are α-pinene and β-pinene, sabinene through sabinene hydrate, α-terpineol, β-myrcene through linalool, and D-limonene. The above metabolites were negative correlated with 1.8-cineole, or the formation of borneol and camphor thorough (+)-bornyl-diphosphate.

Salinity affected oil constituents as β-myrcene but also α-pinene and α-terpineol (including control treatment) increased in 25 mM NaCl-treated plants compared to 100 mM NaCl application (Table S2; **Figure 7**). Plants grown at NaCl concentration of 100 mM had significantly higher borneol content. Camphor content was significantly low at NaCl concentration 25 mM comparing with the control or NaCl concentrations 50 mM and 100 mM. Thus, the application of 25 mM NaCl concentration increased monoterpenes hydrocarbons (averaged in 24.80%) compared with the application of NaCl concentration 100 mM (averaged in 21.59%). The foliar K application increased α-pinene (at <25 mM NaCl) and D-limonene, β-pinene, β-myrcene, sabinene, α-bisabolol and *cis*-lanceol content in plants grown at NaCl concentrations <50 mM (Table S3; **Figure 7**). Contrarily, 1.8-cineole reached its maximal percentage (62.53%) as a result of NaCl concentration 100 mM. The content of α-terpineol was increased in plants subjected to NaCl concentration 25 mM compared with the plants subjected to higher salinity levels. The foliar application of Zn and Si did cause minor changes in lavender EO composition (Tables S4, S5; **Figure 7**).

To identify possible relationships between volatile compounds and salinity with or without the mineral foliar application, LDA was applied for salinity and each individual mineral application (**Figure 8**). LDA, performed on average contents of all compounds for each salinity level and/or foliar application, showed that the first two principal axes represented 95.9–99.7% of the total variation.

For salinity, the first axis (88.1% of the total variation) was mainly correlated with β-pinene, β-myrcene, camphene, and α-pinene. The second axis represented 7.8% of the total variation, and sabinene, α-terpinene and p-mentha-2,4(8)-diene were the main compounds contributing to its definition. The plot of the projection of the average values of all the compounds onto the first two principal axes, revealed a high chemical dispersion among saline concentrations (0–25–50–100 mM NaCl). Therefore, according to the LDA, three [(a) 0 mM; (b)25 mM; and 50 mM; (c) 100 mM NaCl] concentration groups in relation to the saline levels could be observed.

LDA for salinity and K application showed the first axis (97.8% of the total variation) was mainly correlated with β-myrcene, β-pinene, α-pinene, camphene and *cis*-ocimene. The second axis represented 1.9% of the total variation with sabinene to consist the main compounds contributing to its definition. LDA showed three [(a) 0 mM + K and 50 mM NaCl + K; (b) 25 mM NaCl + K; (c) 100 mM NaCl + K] concentration groups in relation to the saline levels with K foliar could be distinguished.

In case of Zn application LDA showed that the first axis (77.7% of the total variation) was mainly correlated with α-pinene, β-pinene, sabinene, β-myrcene, and o-cymene. The second axis represented 21.6% of the total variation, and camphene, cumic aldehyde and α-phellandrene were the main compounds contributing to its definition. According to the LDA, three [(a) 0 mM + Zn and 25 mM NaCl + Zn; (b) 50 mM NaCl + Zn; (c) 100 mM NaCl +Zn] concentration groups in relation to the saline levels with Zn foliar could be found.

LDA for salinity and Si application showed the first axis (76.4% of the total variation) was mainly correlated with sabinene and β-pinene. The second axis represented 20.1% of the total variation, and β-myrcene, α-pinene and camphene were the main compounds. According to the LDA, three [(a) 0 mM + Si and 25 mM NaCl + Si; (b) 50 mM NaCl + Si; (c) 100 mM NaCl + Si] concentration groups in relation to the saline levels with Zn foliar could be distinguished.

DISCUSSION

Plants subjected to saline conditions undergo many metabolic changes in order to reduce their ability to absorb water and demonstrate rapid reductions in growth rate. The reduction in growth was attributed to lower osmotic potential in the soil, which leads to decreased water uptake, reduced transpiration and stomata closure (Ben-Asher et al., 2006). The mechanisms of salinity on plant growth are highly related to the following points: (a) salinity affects root and stomatal resistance to water flow, (b) the balance between root and shoot hormones shifts greatly under saline conditions, (c) salinity changes the structure of the chloroplasts and mitochondria and such changes may interfere with normal metabolism and growth, (d) salinity increases respiration and decreases photosynthetic products (Said-Al Ahl and Mahmoud, 2010).

Plant Growth

Plant growth inhibition is a common response to salinity and might be related to salt osmotic effects, which cause cell turgor and expansion (Hendawy and Khalid, 2005). In general, salinity and, to a lesser extent, mineral foliar applications have a pronounced effect on plant growth related parameters. In the present study, levels of salinity at NaCl concentrations >50 mM reduced plant height, stem thickness and root biomass,

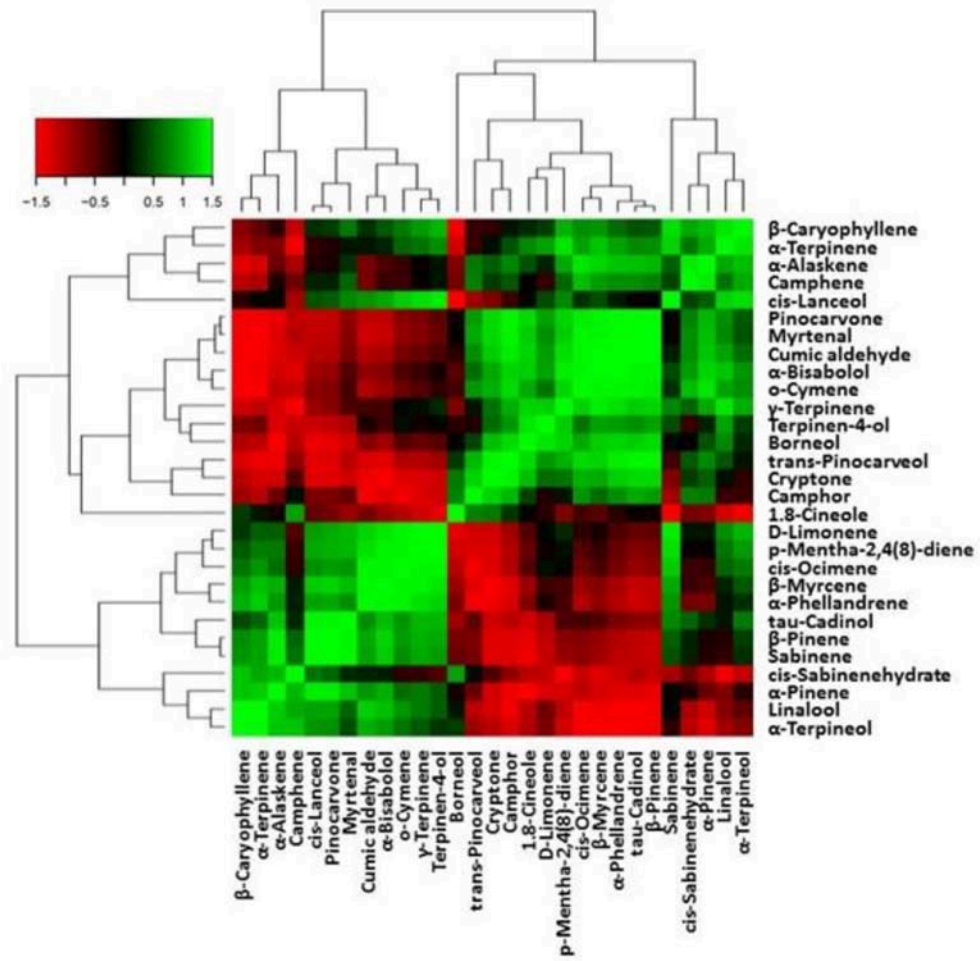

FIGURE 6 | Heat-map matrices of the correlation between EO metabolites in leaves of lavender. Each square indicates *r* (Pearson's correlation coefficient of a pair of metabolites).

while on top of that, 25 mM NaCl reduced leaf length, fresh upper biomass and biomass dry matter, being in agreement with previous reports on basil (Tarchoune et al., 2010), lavender and thyme (Cordovilla et al., 2014), chamomile (Nasrin et al., 2012), and parsley (Chondraki et al., 2012). Hejazi Mehrizi et al. (2012) reported that the application of 50 mM of NaCl concentration in the nutrient solution caused only 7% reduction in the shoot and root dry matter production in rosemary, while an equivalent NaCl concentration of 50 mM caused 21% reduction in lavender biomass dry matter indicating that *L. aguistifolia* is less salt tolerant than rosemary. However, Garcia-Caparros et al. (2017) reported that *Lavandula multifida* is better adapted to salinity compared to other members of the Lamiaceae.

Several studies have highlighted the beneficial effects of mineral application either in common plant growing conditions or in saline stressed plants. Considering the effects of foliar mineral application on lavender growth it was found that the application of K, Zn, and Si reduced the fresh upper biomass in non-saline treatments, possibly considered as overdose application of minerals. However, mineral applications alleviated the reduced fresh biomass caused by salinity, as no differences were evident among saline (<50 mM NaCl) and non-saline grown plants with the foliar application. Occasionally, foliar application caused further stress than the individual saline dose, as in the case of K-Zn-Si + 100 mM NaCl application that caused a further decrease in stem thickness and shoot number produced, being unable to alleviate induced stress caused by salinity. Despite the potassium benefits in plant metabolism -promoting carbohydrates synthesis- in the present study, K application did not improve lavender growth, or even reduced plant biomass in non-saline conditions. This might be related to the well balanced nutrient solution as optimized by Chrysargyris et al. (2016b, 2017a), applied in lavender plants under hydroponic conditions. In this sense, K application could be of redundant in the present study. Basil plants sprayed with zinc under normal and saline soils conditions were superior when compared to unsprayed plants (Said-Al Ahl and Mahmoud, 2010). The stimulatory effect

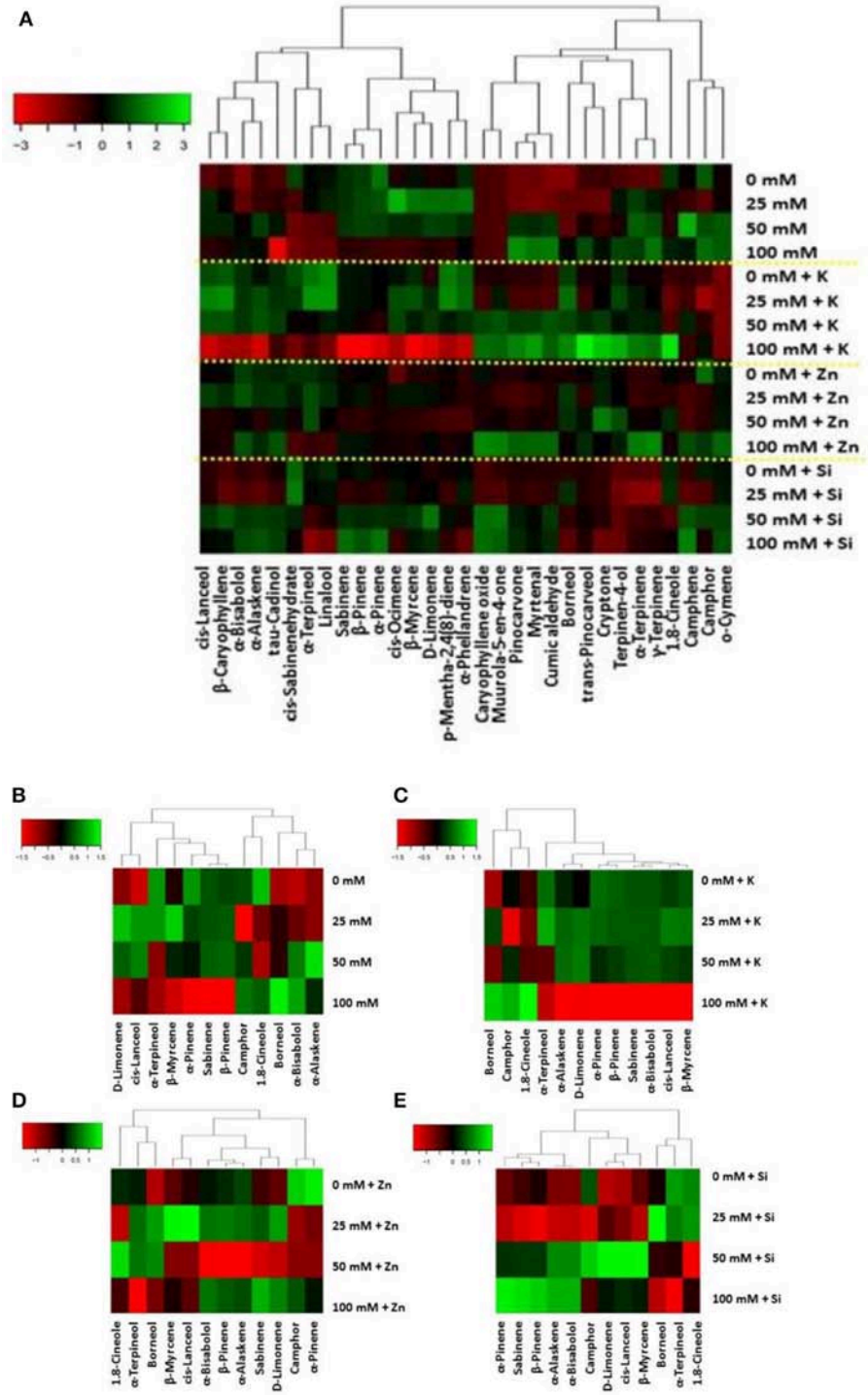

FIGURE 7 | Metabolite changes in leaves of lavender. Heat map representing relative expression of volatiles in total **(A)** elicited in leaf tissue following **(B)** salinity (25–50–100 mM NaCl) and foliar **(C-E)** applications (no foliar, K, Zn, and Si) as compared to control (no saline) plants.

of zinc on plant growth was recorded by several researchers (Said-Al Ahl and Omer, 2009) and is attributed to the well-known functions of zinc in CO_2 assimilation. More specifically, being a component of carbonic anhydrase, several dehydrogenases and auxin production it consequently enhanced the elongation processes (Marschner, 2012). As suggested by Parker et al. (1992) Zn deficiency increased the membrane permeability in root cells indicating the Zn effects in cell membranes. Zn is

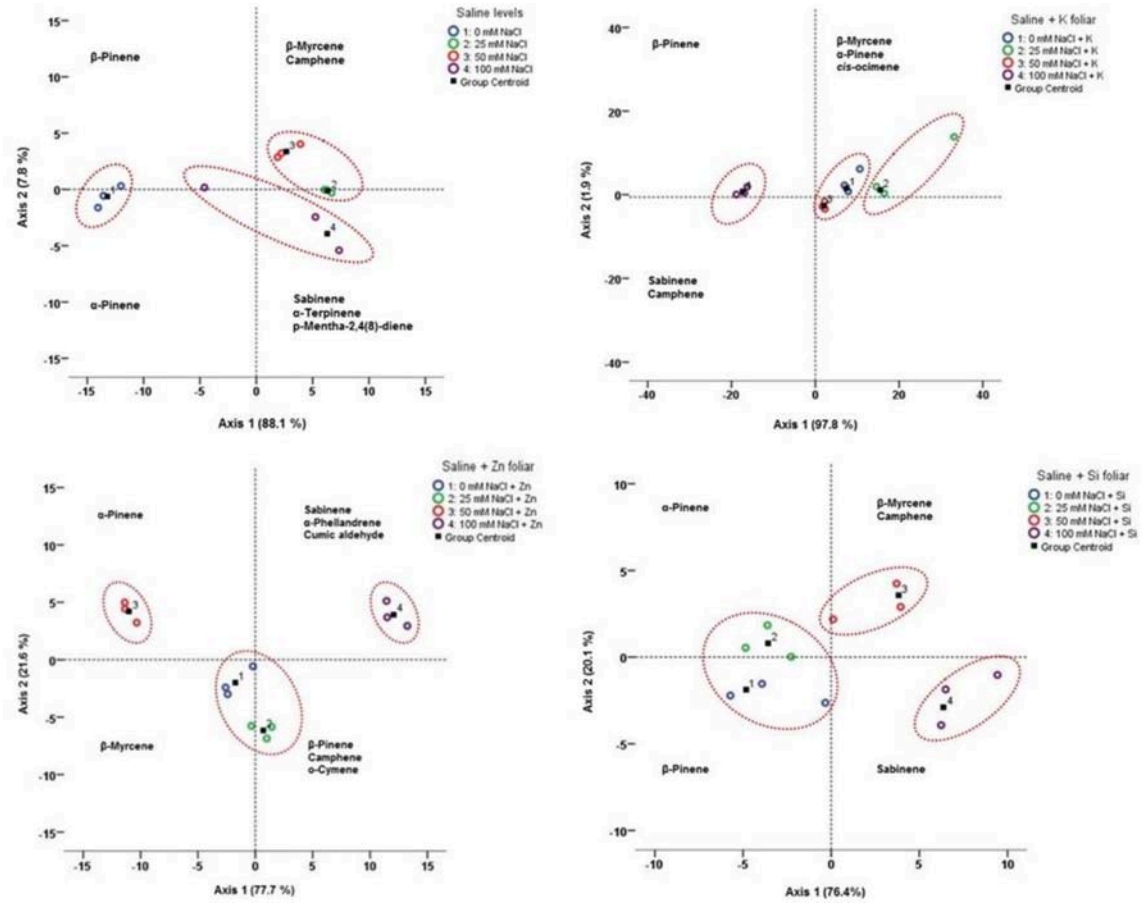

FIGURE 8 | Linear discriminant analysis (LDA) for the lavender essential oil compounds under different saline levels with K, Zn, Si foliar applications. Projection of the average contents of the essential oil compounds onto the first two principal axes (+ and – indicate positive and negative correlations with axes, respectively). Coding numbers referred to mM NaCl + foliar applications.

necessary for root cell membrane integrity and it is this aspect which prevents excessive phosphorus uptake by roots and the translocation of phosphorus from roots to leaves. From this point of view, external Zn concentrations could mitigate the negative effect of NaCl by inhibiting sodium and/or chloride uptake or translocation (Alpaslan et al., 1999). Ramezani et al. (2012) have indicated a trend to alleviate salinity effects by means of iron or iron and zinc application, but no effects on plant growth were noticed after zinc application alone, being in accordance with the present findings.

Silicon has been reported to enhance plant growth particularly under biotic and abiotic stress due to the activation of certain mechanisms. Thus, silicon can increase plant resistance toward salinity stress, which is a major yield limiting factor in arid and semiarid areas. Silicon deposition on the roots exodermis and endodermis reduces Na uptake in plants (Gong et al., 2003). Al-Aghabary et al. (2004) reported dry matter increase in saline environments and indicated beneficial effects of Si application in alleviating salinity stress. The foliar application of 250 up to 1,000 ppm SiO_2 increased the growth parameters of faba bean in saline soils (Hellal et al., 2012). Foliar application of Si was reported in gerbera (*Gerbera* hybrid L.) with a considerable increase in growth and flower quality related parameters (Kamenidou et al., 2010).

Salinity adaptation can be obtained through Na and Cl elimination which requires either high tissue tolerance to Na and Cl or preventing ions accumulation in plant tissue. Generally, according to Gupta and Gupta (1997), positive interaction between salinity and nutrients is reduced by increasing salinity and what is more, the plant's response to additional fertilization may be neutral or even lead to a reduction of yield, growth etc.

Physiological Parameters

The reduction in growth and chlorophyll level in foliage of lavender plants exposed to salt as compared to control plants follows the pattern observed in several other species (Koocheki et al., 2008; El-Danasoury et al., 2010). The decrease in chlorophylls under salt stress may be due to reduction in pigment biosynthesis or enzymatic chlorophyll degradation (Yang et al., 2009). Lee et al. (2010) reported that Si alleviated the negative effects of NaCl on chlorophyll reduction. However, this alleviating property of Si was not found in the current

study, possibly due to the different species (soybean crop), Si concentration (2.5 mM Si) used and Si application through the nutrient solution rather than in the foliar application. The similar leaf stomatal conductance among treatments might be related to the salinity stress harmonization (prevention) by the well-balanced nutrient solution in the current hydroponic study as well as the low mineral needs that lavender crop has in general.

Salinity reduced the content of total phenols as well as the antioxidative activity of the lavender plants. According to Wong et al. (2006) photosynthesis is declined (chlorophyll content as well) due to disturbance of enzymatic activities after salinity application, and as a result, polyphenols decrease. As a result, antioxidant activity is reduced too. Basil grown in saline (50 mM NaCl) conditions did not demonstrate any changes in the antioxidant activity of plant tissue (Tarchoune et al., 2010), being in agreement with the present study, in terms of lower salt concentration i.e., 25 mM NaCl. In coriander fruits, polyphenol content decreased in response to salinity (Neffati et al., 2011).

Phenolics are well-known antioxidants acting as powerful radical scavengers and ions chelators (Balasundram et al., 2006). The phenolics content increased under salinity in response to the oxidative stress generated (prevention of stress-induced oxidative damage or maintenance of osmotic balance) by the formation of ROS in these hostile environments (Navarro et al., 2006). In the DPPH system, the antioxidant activity in control plants was superior to all the examined samples. Under saline conditions, the scavenging activity was strongly diminished in comparison to the control. Our results are similar to those obtained in coriander under saline conditions, as in that study it was suggested that in coriander fruits an imbalance between ROS generation and scavenging systems might have occurred under saline treatment (Neffati et al., 2011). In our study, a significant lower reducing activity of lavender extracts was detected at NaCl concentrations $>$50 mM and this paralleled the lower total phenolic amount, being in accordance with findings by Neffati et al. (2011) in coriander.

Mineral foliar application in general did not change the salinity effects (negative trend as salinity increased) on total phenols and antioxidative activity, with some exceptions. More specifically, the positive effects of minerals in antioxidant activity were noticed in low salinity (25 mM NaCl) levels as K and Zn application increased the FRAP activity. However, this scavenging mechanism was not detected in higher ($>$50 mM NaCl) saline levels, probably due to the plant's lost ability to scavenge ROS (detoxification). Interestingly, the Si application seems to enhance the scavenging mechanism in control treatments, without saline, as both phenolics and FRAP activity significantly increased under the Si foliar treatment, indicating Si induced stress.

Differences in antioxidant enzyme activation could be related to stress intensity (Sgherri and Navari-Izzo, 1995) which depends on the kind of salts and treatment duration (Tarchoune et al., 2010) as well as the species' salt tolerance. In clary sage, the accumulation of phenolics at NaCl concentrations of 25 and 50 mM suggests a relative tolerance of this species to salinity in these two saline concentrations. At a NaCl concentration of 75 mM, there is lower efficiency to eliminate ROS due to imbalance between ROS and antioxidants formation leading to the installation of the oxidative stress (Taarit et al., 2012).

Plants' scavenging ROS capacity is directly related with the activity/content of the antioxidant enzymes (SOD, APX, GR, and CAT) which increase under stress conditions (Foyer and Noctor, 2011). H_2O_2 can be removed through the ascorbate-glutathione cycle AsA-GSH, whereas APX and SOD are the key enzymes in this cycle (Pasternak et al., 2005). Salt stress is related to the increased generation of H_2O_2 responsible for lipid peroxidation in the absence of any protective mechanism (Xiong and Zhu, 2002). In the present study, the APX enzyme activity increased significantly at NaCl concentrations $>$50 mM while the opposite occurred for SOD activity. Proline and POD activity also increased at a NaCl concentration of 100 mM. Plants grown under salinity demonstrated lower CAT enzymatic activity. Indeed, NaCl did not significantly change the levels of H_2O_2, possibly because of triggered APX activity. Similar findings were reported in basil grown under salinity conditions (Tarchoune et al., 2010).

In plants under salt stress, metabolic shifts to the pentose phosphate pathway lead to an increase in proline synthesis and to a decrease in proline degradation, resulting in higher levels of proline that ultimately enhances the level of erythrose-4-phosphate available to the shikimic acid pathway (Al-Amier and Craker, 2007). High accumulation of proline in leaves is an important adaptive mechanism of salt tolerance as proline is considered to be a source of energy, carbon and nitrogen for the recovering tissues. Proline acts as an osmolyte and reduces the osmotic potential, thus reducing toxic ion uptake (Hare et al., 1998). Similarly to our results, proline content increased in sage and wheat plant grown under saline conditions (Hendawy and Khalid, 2005; Annunziata et al., 2017). These findings are in accordance with those obtained by Woodrow et al. (2017) since they indicated that proline is involved in osmotic adjustment and ROS scavenging. In saline conditions, rosemary plants accumulated Na^+ to maintain leaf turgor, although they need synthesis of organic solute, especially proline. The accumulation of proline and Na^+ is a mechanism used for maintaining turgor and reducing the adverse effect of salt stress (Bandeh-Hagh et al., 2008). Proline may act as a radical scavenger and protects cells against salt-induced oxidative stress (Hong et al., 2000). Since total phenolic content and antioxidant activity of salt stressed plants reduced, proline content significantly increased probably acting as a radical scavenger against salt stress.

MDA is used as a marker for evaluation of lipid peroxidation which increases in saline stress conditions. In the presence of oxidative stress more lipid peroxidation products are formed due to cell damage. The increase in lipid peroxidation may be due to the incapability of antioxidants to scavenge ROS results from salt stress, which is not the case for our study. For that reason, MDA was almost unchanged in saline and non-saline treatments. Low antioxidant non enzymatic activity could probably not produce additional hydrogen peroxide from scavenging ROS, so there is no increased H_2O_2 to cause high lipid peroxidation. But even if the case was of increased H_2O_2, APX activity under high

salinity would raise, so it could eliminate any additional H_2O_2 coming from another scavenging machine. Several studies found a positive correlation between salt stress and the APX activity (Jahnke and White, 2003; Mehr et al., 2012). In the present study, by increasing NaCl concentration from 0 to 100 mM, POD activity was increased between 34 and 120% in leaves, being in accordance with previous studies (Venkatesan and Chellappan, 1999).

The increased enzymes antioxidant activity in the current study suggests that in both cases the activation of an efficient free radical scavenging system could have minimized the negative effects of a general peroxidation, thus contributing to the maintenance of membrane structure and integrity. Plant tolerance to salinity is related to unchanged general peroxidation level and cell membrane stability (Pérez-López et al., 2009). Therefore, salt tolerance is correlated with the stimulation of antioxidant enzymes and their enhanced ability to scavenge active oxygen species (Tarchoune et al., 2010). This seems to be the most enlightening explanation for the findings of the present study.

The K foliar application increased APX and POD antioxidant activities and proline content, as a result of the increased H_2O_2 production in plants grown at NaCl concentrations >50 mM. However, CAT activity was reduced in plants grown under salinity. The increased proline accumulation after K spray at higher salinity concentrations, compared to the unsprayed samples, may be the cause of the increase in H_2O_2 content. That could justify the augmentation in enzyme activity of APX and POD after K application as well as SOD activity at levels of samples with 0 mM NaCl. The same trend in enzyme activity after K application was also noticed by Umar et al. (2011). Almost similar results to K application, were found for Zn application (except that H_2O_2 production did not vary among salinity levels) and for Si foliar application.

The application of Si in 100 mM NaCl-treated plants alleviated the saline induced stress by means of proline content decrease. Similar to our findings, the NaCl application significantly increased free proline contents in soybean, while, under Si treatment, proline considerably decreased. Earlier reports suggested that silicate partly offsets the adverse impact of NaCl stress, as silicate application increased tomato and soybean tolerance to salinity by raising SOD and CAT activities (as they were increased in this study), chlorophyll content and photochemical efficiency of PSII (Al-Aghabary et al., 2004; Lee et al., 2010). The impact of Si was also noticed via APX activity, where even though it appeared higher in high salinity, activity levels were significantly lower compared to the unsprayed saline plants. It seems that Si decreased lipid peroxidation in salt-stressed plants via enhancing antioxidant enzyme activity and non-enzymatic antioxidants. This has recently been confirmed in experiments with cucumber (Zhu et al., 2004) and tomato (Al-Aghabary et al., 2004). Adding Si decreased the plasma membrane permeability (affecting structure, integrity, and functions of plasma membranes by influencing the stress-dependent peroxidation of membrane lipids) of leaf cells and significantly improved the ultra-structure of chloroplasts (Liang et al., 2005).

Our findings showed an increased H_2O_2 production in 100 mM NaCl-treated plants following Si application compared to the non-mineral foliar, indicating that Si acted as an additional stress factor on top of the salinity effects or salinity toxification effects were irreversible. Improvement of salt tolerance by Si addition has been reported in barley (Liang et al., 2003) and cucumber (Zhu et al., 2004), and its role in medicinal plants of the Lamiaceae family, such as lavender, needs to be reconsidered as an assumed means of salinity induced stress alleviation.

In general, oxidative stress might occur through decrease of key antioxidative enzymes (i.e., SOD, CAT) as salinity increases; however, oxidative damage was not evident in the present study as neither H_2O_2 nor MDA production increased. The lesser degree of membrane damage (as indicated by low MDA content) and the higher activity of APX and POD observed in NaCl-treated lavender indicated that this particular plant species had a high capacity of scavenging ROS generated by salt stress.

Mineral Content and Uptake

Salinity caused a decrease in lavender K^+ content and K^+/Na^+ ratio in treated plants. However, Na^+ content was increased at all salt levels. Under salinity stress, high Na^+ uptake competes with the uptake of K^+ and leads to K^+/Na^+ ratio and Na^+ toxicity decrease. Othman et al. (2006) found that K concentration was reduced by increasing salinity, in accordance with our findings. Na^+ accumulation in salt stressed plants led to low water potential, changed the ions uptake, and reduced leaf expansion, photosynthetic rate, and plant growth (Zaho et al., 2007). Potassium interacts with almost all essential elements and a synergistic role of K with either N or P has previously been noted (Ranade-Malvi, 2011).

Nutrient disturbances under salinity conditions cause a reduction in plant growth by affecting the availability, transport, and partitioning of the nutrients. Salinity may cause nutrient deficiencies or imbalances because of the competition of Na^+ and Cl^- with nutrients such as K^+, Ca^{2+}, and NO_3^-. Several studies indicated that under salinity conditions, there has been an increase in Na and Cl but a decrease in nitrogen, phosphorus, calcium, potassium, and magnesium levels such as in fennel (Abd El-Wahab, 2006), peppermint and lemon verbena (Tabatabaie and Nazari, 2007), and *Matricaria recutita* (Baghalian et al., 2008).

Salt-stressed root growth is restricted by osmotic effects and toxic effects of ions, which reduce nutrient uptake and inhibit the nutrient translocation, especially K^+. As a result of the similarities in physicochemical properties, Na^+ could compete with K^+ for major binding sites in key metabolic processes, including both low-affinity (e.g., non-selective cation channels) and high-affinity (e.g., K^+ uptake permeases and high-affinity K^+ transporter) transporters and could also disturb plant metabolism (Marschner, 2012). Plants use both low and high-affinity systems for potassium uptake. Sodium ions have a more damaging effect on the low-affinity system which has low potassium/sodium selectivity. Under sodium stress, it is necessary for plants to operate the more selective high-affinity potassium uptake system in order to maintain adequate potassium nutrition.

Potassium foliar application did not cause great changes in macronutrients and micronutrients. Potassium levels were increased neither in leaves nor in roots after foliar application of K, in a way to alleviate sodium accumulation in plants' tissue, although potassium levels were increased in roots at 0 mM NaCl+K treatment. This maybe imply that higher concentrations of K could be used in order to manage sodium levels, but that, in this case, that was not possible due to leaf damage, after the exogenous application of higher K concentrations, during the trial versions (preliminary trials) of the experiment. No changes in nutrient content after K foliar application were also reported by Akram and Ashraf (2009), in minerals as Na^{+}, Ca^{+2}, and Mg^{+2}, in sunflower.

Zn application had no specific effects on mineral, although potassium content increased in leaves in high salinity after Zn application. Additionally, zinc content in leaves was found to increase up to six times. Weisany et al. (2014) reported the same changes, among others, after Zn application in soybean grown under salinity. Improving zinc nutritional status of plants growing under saline conditions is of great importance for plants to be protected against toxicity, since zinc is ascribed to have a protective role controlling Na uptake (Cakmak and Marschner, 1988).

Sodium content in the leaves was reduced up to 13% in NaCl concentration 100 mM + Si, suggesting that Si suppresses the translocation of Na from the root to the shoot. Silicon application enhanced K/Na selectivity ratio in *Faba bean* thus enhancing pod and shoot yield (Hellal et al., 2012). The exclusion of Na^{+} ions and a higher K/Na ratio in bean plants grown under saline conditions have been confirmed as a substantial selection criterion for salt tolerance (Abd El-Hamid et al., 2010). In the present study, the ratio of K/Na at NaCl concentration 100 mM averaged at 0.48 while in Si foliar under saline condition (100 mM NaCl) averaged at 0.59 (23% increment). However, in non-saline condition, Si antagonizes the potassium role (appr. of a 45% value), as the K/Na in control (non-saline) averaged 11.58 and in non-saline+Si foliar averaged 6.37. The respective Si:Na in control (non-saline) averaged 33.31 and in non-saline+Si foliar averaged 49.28. The beneficial effect of silicon has been related to the prevention of excessive water loss through transpiration (Savant et al., 1999) or with silicate crystals deposition beneath the epidermal cells of leaves and stems (Trenholm et al., 2004), which may reduce water loss through the cuticles (Lee et al., 2010).

Essential Oil Yield and Constituents

The essential oil yield in aromatic plants may be affected positively or negatively by the salinity levels (Hendawy and Khalid, 2005; Said-Al Ahl and Omer, 2009; Said-Al Ahl and Mahmoud, 2010; Neffati et al., 2011; Taarit et al., 2012) as well as by the type and amount of fertilizers and cultivation practices applied (Chrysargyris et al., 2016b). Lavender EO yield was reduced in plants grown under NaCl concentration of 100 mM compared to plants grown in non-saline or low saline levels. The foliar application of minerals (K, Zn, and Si) counterbalanced the reduced yield which was observed under the high salinity. The findings of the present study are in agreement with Al-Amier and Craker (2007) on marjoram and with Said-Al Ahl and Mahmoud (2010) and Haddanpouraghdam et al. (2011) on basil, who indicated that saline application in high levels reduced EO yield.

The stimulation of EO production under salinity could be due to a higher oil gland density and an increase in the absolute number of glands produced prior to leaf emergence (Charles et al., 1990). Salt stress may also affect the EO accumulation indirectly through its effects on either net assimilation or assimilate partitioning among growth and differentiation processes (Charles et al., 1990). Morales et al. (1993) suggested that an increase in oil content in some of the salt stressed plants might be attributed to the decline of the primary metabolites due to the salinity effects, causing intermediary products to become available for secondary metabolites synthesis. Additionally, reductions in growth and chlorophylls could be expected to reduce the yield of EO due to fewer metabolites being available for conversion into oil (El-Danasoury et al., 2010) as this was demonstrated in our study.

Said-Al Ahl et al. (2009) reported that potassium-humate increases EO contents and yields in *Thuja orientalis* and oregano, respectively. Similarly, Heidari et al. (2014) reported that foliar K application (in KNO_3 form) can noticeably improve productivity traits, EO yield, and composition of tarragon plant. On the other hand, other researcher's findings showed that application of K fertilizer did not influence the EO contents of patchouli (Singh and Rao, 2009) and rosemary (Singh et al., 2007). Taking under consideration that zinc is involved in photosynthesis and saccharide metabolism, and since CO_2 and glucose are the most likely sources of carbon utilized in terpene biosynthesis, the role of zinc in influencing EO accumulation seems principally important (Marschner, 2012).

Analyzing the lavender essential oils, 29–31 individual components were identified in saline and/or foliar treatments. The majority of the EO constituents were oxygenated (monoterpenes and sesquiterpenes) compounds ranged from 69.58 to 79.82% and 2.13 to 4.19%, respectively. It has been reported in *L. aguistifolia* that 1.8-cineole, borneol and camphor were the predominant components of leaf volatile oil, while linalool, 1.8-cineole, borneol and camphor were the major components of inflorescence oil (Haddanpouraghdam et al., 2011; Chrysargyris et al., 2016b), being in accordance with the current leaf oil composition, indicating the lavender chemotype (CT) of CT-1.8 cineole. Oil quality decreases with increasing camphor ratios (Biswas et al., 2009) and the NaCl concentration of 25 mM is considered as the most appropriate one regarding the lowest camphor content. Borneol is easily oxidized to the camphor (ketone) and this was evidenced for the NaCl concentrations 0 and 50 mM. Salinity may change the content of several oil constituents by altering biosynthetic processes. Hendawy and Khalid (2005) reported that 2,500 ppm soil salinity increased α-thujone, camphor and 1.8-cineole, but decreased β-thujone compared with the control treatment in *Salvia officinalis*. The application of saline levels ranging from 0 to 100 mM NaCl did not change the 1.8-cineole and D-limonene content, the most abundant constituents in lavender leaf EO. The 1.8-cineole, also known as eucalyptol, due to its pleasant

spicy aroma and taste is extensively used in cosmetics, fragrances and flavorings as well as an insecticide and insect repellent (Sfara et al., 2009).

Interestingly, the K foliar application in plants subjected to different salinity levels changed the chemical composition of the lavender EO, and occasionally altered the salinity effects on oil composition. For example, borneol content remained similar among treatments which actually resulted in similar camphor content. Another issue is that K application enhanced the 1.8-cineole content at a NaCl concentration of 100 mM due to the reduced α-terpineol, as a precursor stage of 1.8-cineole. In this direction, K application in high salinity (100 mM NaCl) affected the geranyl diphosphate biosynthesis by altering the formation of D-limonene, α-pinene, β-pinene, and sabinene, by reducing their content, preventing limonene and pinene degradation (Sell, 2003). Similarly, K application in high salinity (100 mM NaCl) affected the geranyl diphosphate biosynthesis by reducing the formation of β-myrcene, and as a consequence delaying the formation of (+)-linalool. K application at NaCl concentrations <50 mM delayed the 1.8-cineole formation and enhanced D-limonene, α-pinene, β-pinene, and β-myrcene biosynthesis.

In the present study, salinity (100 mM NaCl) had negative effects on the biosynthesis and accumulation of α-pinene and β-myrcene, while both Zn and Si foliar applications had influential potential to compensate for the deteriorative effects of salinity depression on these compounds. Coolong et al. (2004) suggested that Zn fertility can influence changes in glucosinolates that may affect related plants flavor or medicinal features. Apparently, moderate salinity levels when combined with raising Zn levels through foliar application had synergistic effects on production of some compounds (Haddanpouraghdam et al., 2011). Misra and Sharma (1991) mentioned that Zn application stimulated menthol concentration in Japanese mint.

Based on our findings, monoterpenes were predominant compared to the sesquiterpenes, indicating the 1-deoxy-D-xylulose-5-phosphate (DXP) pathway activation, which is localized in plastids, compared to the classical mevalonic acid (MVA) pathway—it operates in cytocol and produces precursor for the biosynthesis of sesquiterpenes (which were in low percentages in the present study) and triterpenes, being in accordance with previous findings in lavender (Lane et al., 2010). High yielding lavender varieties (e.g., *Lavandin*; *Lavandula intermedia*) produce lower quality oil. Therefore, plant biomass and EO yield are not the only parameters that should be considered by investigators, although great importance is given to the oil composition, as well.

CONCLUSION

Our results have demonstrated that lavender growth/development and EO production may be affected by saline levels, whereas mechanisms for alteration of induced stress are of great importance. High levels of salinity seem to reduce lavender growth and EO yield, and this is directly related to the crop reduced marketability when used as either ornamental or for essential oil production. In general, low-moderate (20–50 mM) salinity maintained the oil composition profile for lavender. The combined foliar application of K and Zn ameliorated the apparent salinity negative effects on fresh biomass produced, EO yield and maintained, to some extent, the antioxidant properties of the saline-stressed plants. Evidently, lavender primary metabolites affected by moderate salinity stress along with K foliar operated in favor of major volatile oil components biosynthesis. Thus, lavender crop has the potential to be expanded and cultivated in semi-saline conditions. Through the current study, it has been shown that Zn and Si application, seems to have a smaller impact on the composition of EO, despite the fact that minerals alleviated salinity induced changes. The efficiency of Zn and Si should be approached in the context of optimum growing conditions and balanced micronutrients availability, while the role of iron and manganese could also be considered in future studies.

AUTHOR CONTRIBUTIONS

AC, EM, and NT: designing and performing the experiments; AC: GC/MS, ICP-OES analysis; EM and NT: physiological measurements; AC and NT: data analysis and critical discussion of the data; NT and AC: paper preparation; NT: research coordination.

ACKNOWLEDGMENTS

Thanks to Dr N. Nikoloudakis for helping greatly with the Heat map presentation and to Mrs Maria Koutroumani (Ministry of Education, Research and Religious Affairs, Athens, Greece) for language editing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00489/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 21 June 2018
doi: 10.3389/fpls.2018.00859

Forecasting Root-Zone Electrical Conductivity of Nutrient Solutions in Closed-Loop Soilless Cultures via a Recurrent Neural Network Using Environmental and Cultivation Information

*Taewon Moon†, Tae In Ahn† and Jung Eek Son**

Department of Plant Science, Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul, South Korea

OPEN ACCESS

Edited by:
Nikos Tzortzakis, Cyprus University of Technology, Cyprus

Reviewed by:
Daniele Massa, Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria (CREA), Italy
Antonios Chrysargyris, Cyprus University of Technology, Cyprus

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

† These authors have contributed equally to this work.

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *06 November 2017*
Accepted: *04 June 2018*
Published: *21 June 2018*

Citation:
Moon T, Ahn TI and Son JE (2018) Forecasting Root-Zone Electrical Conductivity of Nutrient Solutions in Closed-Loop Soilless Cultures via a Recurrent Neural Network Using Environmental and Cultivation Information. Front. Plant Sci. 9:859. doi: 10.3389/fpls.2018.00859

In existing closed-loop soilless cultures, nutrient solutions are controlled by the electrical conductivity (EC) of the solution. However, the EC of nutrient solutions is affected by both growth environments and crop growth, so it is hard to predict the EC of nutrient solution. The objective of this study was to predict the EC of root-zone nutrient solutions in closed-loop soilless cultures using recurrent neural network (RNN). In a test greenhouse with sweet peppers (*Capsicum annuum* L.), data were measured every 10 s from October 15 to December 31, 2014. Mean values for every hour were analyzed. Validation accuracy (R^2) of a single-layer long short-term memory (LSTM) was 0.92 and root-mean-square error (RMSE) was 0.07, which were the best results among the different RNNs. The trained LSTM predicted the substrate EC accurately at all ranges. Test accuracy (R^2) was 0.72 and RMSE was 0.08, which were lower than values for the validation. Deep learning algorithms were more accurate when more data were added for training. The addition of other environmental factors or plant growth data would improve model robustness. A trained LSTM can control the nutrient solutions in closed-loop soilless cultures based on predicted future EC. Therefore, the algorithm can make a planned management of nutrient solutions possible, reducing resource waste.

Keywords: black box modeling, environmental factor, long short-term memory, machine learning, sweet pepper

INTRODUCTION

Due to benefits including improved crop yield and quality, soilless cultures in greenhouses have been growing rapidly in popularity. However, most open-loop soilless cultures release drainage nutrient solutions without treatment, causing environmental pollution such as eutrophication and accumulation of heavy metals (Fargašová, 1994; Siddiqi et al., 1998; Le Bot et al., 2001; Nicoletto et al., 2017). To resolve this problem, closed-loop soilless cultures are being studied as sustainable crop cultivation systems. In commercialized closed-loop soilless cultures, nutrient solutions are controlled based on electrical conductivity (EC) because it is easily measured by sensors. Since

EC of solutions shows a linear relationship with total equivalents of ions in solutions (Griffin and Jurinak, 1973), EC-based systems have been used to control nutrient solution supply.

In soilless culture systems, root-zone EC should be controlled within target range because it significantly influences the growth and quality of crops (Sonneveld and Voogt, 2009). In general, root-zone EC dynamically varies due to environmental changes and can be controlled by adjusting the concentration of nutrient solutions. In open-loop soilless culture, these EC control processes only consider the resource usage of the system (Ku and Hershey, 1991). However, in closed-loop soilless culture, the discharge of drainage is restricted. Therefore, changes in the EC and the drainage amount are directly affected by the available concentration range of supplying nutrient solutions and the amounts of water and stock solutions for replenishment (Savvas and Manos, 1999). In the EC-based closed-loop soilless culture, which conducts minimal nutrient calibration with EC, these features may affect the stability of nutrient control (Savvas and Manos, 1999; Savvas, 2002; Massa et al., 2011). In order to maintain system reliability under these limited conditions, current control processes should be determined based on the prediction of future changes, which requires an appropriate predictive model (Draeger et al., 1995). Therefore, predicting EC is important for nutrient management of closed soilless cultures.

Although various prediction methods have been developed, nutrient control systems are usually based on contemporary EC monitoring and are vulnerable to ion balance in root-zone nutrient solutions (Neto et al., 2014; Kinoshita et al., 2016). These limits result from crops influencing changes in EC and from growth environments (Dewir et al., 2005; Stutte, 2006; Shin and Son, 2016). Because root-zone nutrient solutions are affected by environmental changes within greenhouses, predicting future changes in the EC of root-zone nutrient solutions is not easy. Prediction of EC needs various environmental data and system parameters; however, EC is greatly affected by environments in a wide range of climate changes (Savvas and Manos, 1999; Savvas, 2002; Lykas et al., 2006; Massa et al., 2011; Shin et al., 2016). It is difficult to apply a control system developed in a specific region to another region of different climate conditions. In fact, no studies have attempted to predict and forecast future EC in various climate conditions.

Deep learning has been used to draw meaningful interpretations from complicated nonlinear data (Mnih et al., 2015; Silver et al., 2016). Deep learning can be used for high-level abstraction from raw data (LeCun et al., 2015). As a part of deep learning, recurrent neural networks (RNNs) are used to analyze chronological data such as for voice and video recognition and natural language processing; this method shows better accuracy than previous algorithms (Adavanne et al., 2017; Ororbia et al., 2017).

Recurrent neural network has an advantage of inputting big data of relatively long period and the length of output values is also unlimited theoretically (Hochreiter and Schmidhuber, 1997). EC in soilless culture is a chronological factor which is difficult to predict because the future EC changes are affected by the accumulation data of past environments and plant growth. To improve EC-based nutrient controls in various climate conditions, prediction of EC should be conducted based on previous environmental factors in closed-loop soilless culture systems. The objective of this study was to predict the EC of root-zone nutrient solutions in closed-loop soilless cultures using RNN algorithms.

MATERIALS AND METHODS

Cultivation Conditions

A Venlo-type greenhouse at the experimental farm of Seoul National University, Suwon, Korea (37.3° N 127.0° E) was used for experiments. Three sweet pepper (*Capsicum annuum* L.) plants were grown in a rockwool slab and seven slabs were used per row. In this study, four cultivation lines were installed in the greenhouse, each of which is an independent closed-loop soilless culture system having mixing tank, drainage tank, and stock solutions (**Figure 1**). The stock solution was divided into A and B based on the PBG nutrient solution of Netherlands. One of the cultivation lines was used for the experiment. In the greenhouse, daytime temperature was maintained at 25–35°C and nighttime temperature at 17–22°C (**Figure 2**). Outside temperature during the experiment was at −10.8–23°C. EC of nutrient solutions was maintained at 2.6–3.0 $dS{\cdot}m^{-1}$ and pH at 5.5–6.5. Integrated solar radiation method was applied for irrigation control. Nutrient solutions' composition was 14.17 $meq{\cdot}L^{-1}$ of NO_3^-, 1.14 $meq{\cdot}L^{-1}$ of $H_2PO_4^-$, 5.92 $meq{\cdot}L^{-1}$ of K^+, 8.85 $meq{\cdot}L^{-1}$ of Ca^{2+}, 3.17 $meq{\cdot}L^{-1}$ of Mg^{2+}, and 3.20 $meq{\cdot}L^{-1}$ of SO_4^{2-} as macro elements; and 0.038 $meq{\cdot}L^{-1}$ of Fe^{2+}, 0.020 $meq{\cdot}L^{-1}$ of Zn^{2+}, 0.003 $meq{\cdot}L^{-1}$ of Cu^{2+}, 0.021 $meq{\cdot}L^{-1}$ of Mn^{2+}, and 0.001 $meq{\cdot}L^{-1}$ of MoO_4^{2-} as micro elements. After irrigation event, the drainage was returned to the reservoir tank (52 cm × 26 cm × 26 cm). EC and pH in the reservoir tanks were monitored every 3 days by using a multimeter (Multi 3420 SET C, WTW, Germany). EC and water content in the root media were measured by using a TDR sensor (Grodan, WCM-control, Denmark). EC and pH of fresh water were 0.17 $dS{\cdot}m^{-1}$ and 7.11, respectively, containing 0.21 $meq{\cdot}L^{-1}$ of Na^{2+}, 0.29 $meq{\cdot}L^{-1}$ of Cl^-, 0.04 $meq{\cdot}L^{-1}$ of K^+, 0.71 $meq{\cdot}L^{-1}$ of Ca^{2+}, 0.21 $meq{\cdot}L^{-1}$ of Mg^{2+}, 0.19 $meq{\cdot}L^{-1}$ of SO_4^{2-}, 0.39 $meq{\cdot}L^{-1}$ of NO_3^-, and 0.04 $meq{\cdot}L^{-1}$ of PO_4^{3-}. Drainage ratios were maintained at 20–30% during experimental period. Plants were grown to maintain two main stems, which were vertically trellized to a "V" canopy system (Jovicich et al., 2004).

Data Collection

Data on nutrient solutions and growth environments were measured to train the algorithm (**Table 1**). The ECs of nutrient solutions in the mixing tank and drainage tank were measured by EC sensors (SCF-01A, DIK, Korea). The EC and moisture content of substrates were measured by a FDR sensor (CoCo 100B, Mirae Sensor, Korea). CO_2 concentration and light intensity in the greenhouse were measured by using a nondispersive infrared CO_2 sensor (KCD-AN300, Sensecube, Korea) and by a pyranometer (SP-110, Apogee, United States), respectively. Data were measured every 10 s from October 15 to December 31, 2014.

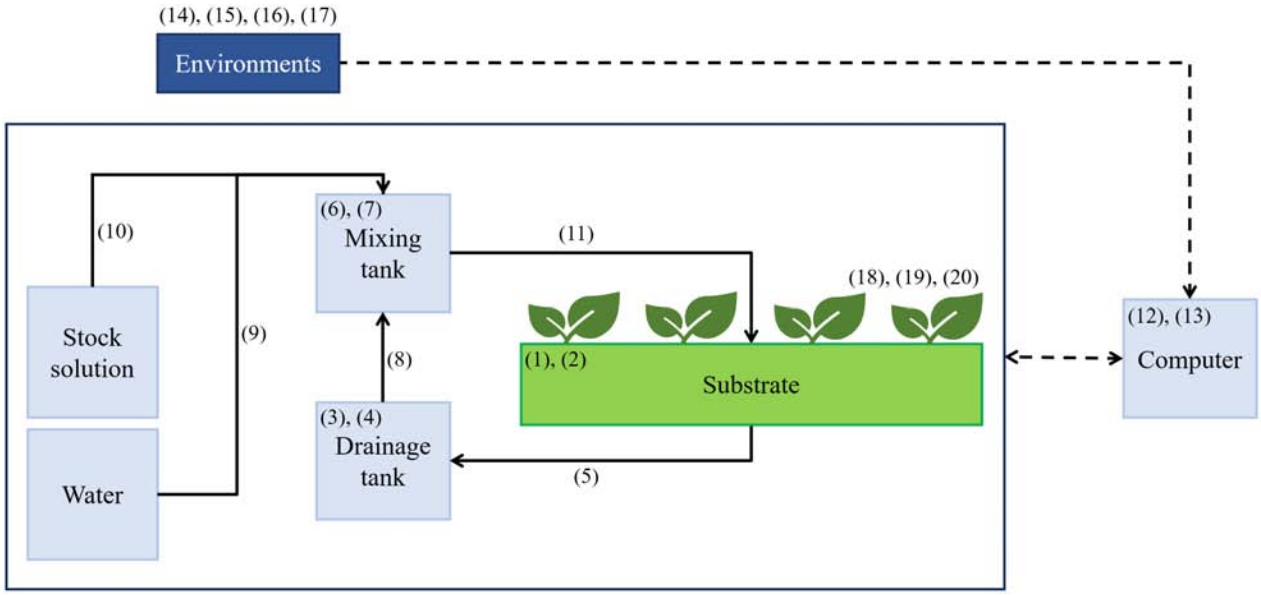

FIGURE 1 | A diagram of a closed-loop soilless culture system and measured data of nutrient solutions and growth environments. Refer to **Table 1** for the measured data (1–20).

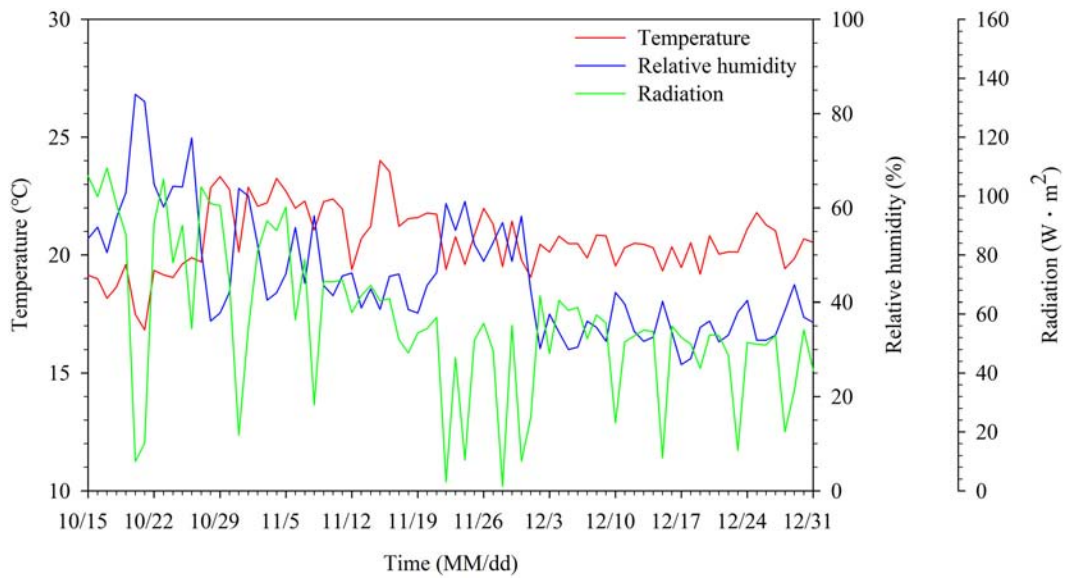

FIGURE 2 | Daily averages of temperature, relative humidity, and radiation in the greenhouse from 15 October to 31 December. Zeros were excluded when radiation was averaged.

Mean values for every hour were used. A total of 1,416 data points was used for this study.

Recurrent Neural Network

Recurrent neural network algorithms deal with chronological data with a returning cycle. Long short-term memory (LSTM), an RNN algorithm, can solve the vanishing gradient problem of RNN (Hochreiter and Schmidhuber, 1997). This means that LSTM remembers the data of a long previous sequence. The core of LSTM algorithm is a cell with several gates (**Figure 3**). LSTM accepts previous data with addition operation, so vanishing gradient or exploding gradient problem is not occurred. Therefore, LSTM can analyze long time data than simple RNN.

Long short-term memory cells can retain, save, and load information about previous data. LSTM receives current input and previous output simultaneously, and the received information is operated through the gates. Previous information

TABLE 1 | Ranges of measured input data in closed-loop soilless cultures.

(Number) Input data (unit)	Range
(1) Electrical conductivity (EC) of substrate (dS·m^{-1})	3.3–5.1
(2) Moisture content of substrate (%)	56.8–70.2
(3) EC of nutrient solutions in the drainage tank (dS·m^{-1})	3.5–6.0
(4) Volume of nutrient solutions in the drainage tank (L)	2.1–9.8
(5) Cumulative drainage volume per day (L)	0–25.3
(6) EC of nutrient solutions in the mixing tank (dS·m^{-1})	2.1–2.9
(7) Volume of nutrient solutions in the mixing tank (L)	3.2–6.9
(8) Mixing volume of drainage (L)	0–3.3
(9) Mixing volume of water (L)	0–3.9
(10) Mixing volume of stock solution (L)	0–0.1
(11) Cumulative irrigation volume per day (L)	0–50.8
(12) Preset radiation integral for irrigation control (J·cm^{-2})	8.8–100.0
(13) Target volume of nutrient solutions per irrigation event per dripper (mL)	110.0–220.0
(14) CO_2 concentration (μmol·mol^{-1})	312–574
(15) Light intensity (W·m^{-2})	0.0–293.3
(16) Temperature (°C)	16.5–33.8
(17) Relative humidity (%)	11.0–78.0
(18) Growth stage (day after transplanting, day)	99–176
(19) Plant height (cm)	115–181
(20) Number of nodes	18–31

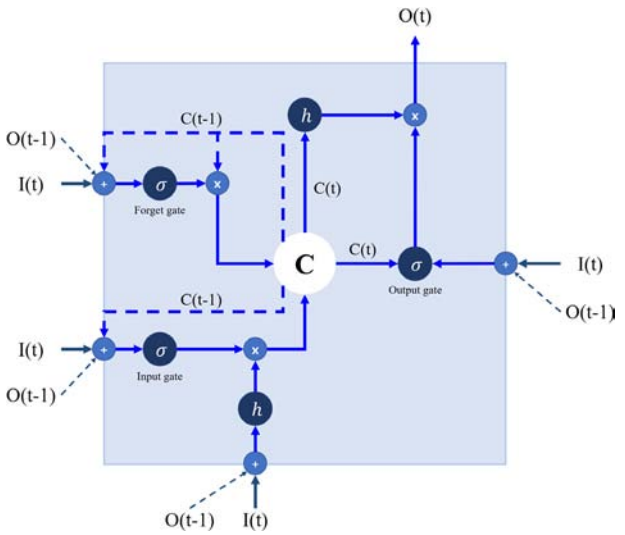

FIGURE 3 | A structure of a long short-term memory (LSTM). I, input vectors; O, output vectors; C, cell state; *h*, *tanh* for input and output activation function; σ, sigmoidal function for gate activation function; *t* and *t*–1, current and previous times, respectively. Refer to **Table 1** for the input (I) and output (O).

is saved as cell state, so sequenced data can be analyzed based on cell state. Gates are divided into three parts: input, forget, and output. The input gate determines how to select the data. The forget gate decides how much data should be forgotten and passes suitably forgotten previous data through a hyperbolic tangent function. The output gate combines cell state and input data and the combined output is sent to the next cell. The final output is printed when the predetermined time step is reached.

A modified LSTM algorithm called a gated recurrent unit (GRU) was developed (Cho et al., 2014). GRU has a similar structure to LSTM, except that it consists of update and reset gates. Since GRU has only two gates, it reduces computational complexity while retaining the advantages of LSTM. A specific RNN algorithm does not always yield the best prediction in all situations (Greff et al., 2015; Jozefowicz et al., 2015). Therefore, LSTM and GRU, the most well-known RNN algorithms, were compared. Similar to ordinary artificial neural networks (ANNs), RNN has hidden layers of perceptrons with activation function. In this study, input and output activation functions were set to hyperbolic tangent function, and gate activation function was set to sigmoidal function. The number of perceptrons and layers were variously combined to determine the optimal neural network structure.

Long short-term memory was adjusted to receive previous changes in environmental data and to predict the next hourly changes in substrate EC. The time step of LSTM was set every 6 h from 6 to 72 h and the output length set every 1 h from 1 to 24 h. The maximum time settings of output were used when comparing RNN structures. Then, input data were excluded one by one to determine which environment factors affect the change in root-zone EC.

To train the RNNs, the AdamOptimizer was used (Kingma and Ba, 2014). The hyperparameters for the LSTM and AdamOptimizer were set to commonly used values (**Table 2**). The GRU has the same hyperparameters as the AdamOptimizer, but forget bias does not need to be set. In the optimization process, neural networks are optimized to minimize cost (Rumelhart et al., 1988). In this study, mean square error (MSE) was used as a cost. Empirically, regressions based on ANNs usually use MSE instead of root-mean-square error (RMSE) as a cost for reducing computation (Esfe et al., 2016; Wang F. et al., 2017). The coefficient of determination (R^2) was used for training and test accuracy. RMSE was also used for verifying model robustness. TensorFlow (v. 1.2.1, Python Deep Learning Library, Google, Menlo Park, CA, United States) was used for the experiments.

TABLE 2 | Hyperparameters for recurrent neural network (RNN) and AdamOptimizer.

Parameter	Value	Description
Learning rate	0.001	Learning rate used by the AdamOptimizer
β_1	0.9	Exponential mass decay rate for the momentum estimates
β_2	0.999	Exponential velocity decay rate for the momentum estimates
E	1e−0.8	A constant for numerical stability
Forget bias*	1.0	Probability of forgetting information in the previous dataset
Time step	2–24	Number of datasets that the LSTM will see at one time

**Forget bias was used only for long short-term memory (LSTM).*

Data Preprocessing

Since RNNs use tangent functions and sigmoid functions internally, input data had to be normalized from 0 to 1 to improve training efficiency. In this study, RNN algorithms had the maximum time step of 72 h in environmental changes (input) and maximum output length of 24 h in EC changes (output). Both were trained after being combined into a single dataset. Data from 15 October to 24 December were randomly divided into training and validation datasets, and the rest of the data from December 25–31 were used to test the trained RNNs. Among the total datasets, 900 were used for training, 396 for validation, and 120 for test.

RESULTS

Accuracy of the Trained Models

Among all RNN structures, an LSTM of a single layer with 64 perceptrons showed the highest accuracy (**Table 3**). Although the RMSE of all structures ranged from 0.08 to 0.09, the single-layered LSTM showed the highest test accuracy with R^2 = 0.72. Multi-layers did not improve the accuracy of RNN models. Regardless of the number of layers, LSTM showed the higher accuracy than GRU. For the same training condition, which had multi-inputs and -outputs, conventional algorithms such as ARIMA model, multivariate regression, or multi-layer perceptrons could not be trained. With the validation datasets, R^2 was 0.92 and RMSE was 0.07 with the LSTM (**Figure 4A**), which was much higher than the test accuracy with R^2 = 0.72 and RMSE = 0.08 (**Figure 4B**). Because each 24-long output was a result of one calculation, the average of each predicted and measured values was compared.

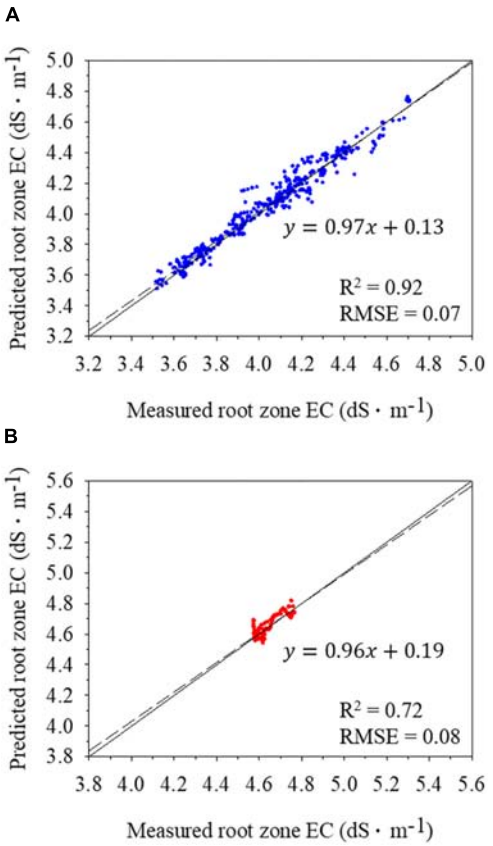

FIGURE 4 | Comparisons of predicted and measured root-zone electrical conductivities (ECs) of nutrient solutions using validation **(A)** and test **(B)** datasets. Solid and dashed lines represent 1:1 line and regression line, respectively. The average of each 24-long output was presented.

Optimization of Model Parameters

The accuracy of LSTM tended to increase with extension of time step and reduction of output length. The accuracy of LSTM was highest when the time step was set to maximum. R^2 for the test datasets was no less than 0.65 when the time step was longer than 12 h (**Figure 5A**). The time step longer than 24 h did not improve the accuracy. Meanwhile, R^2 was lowest when output length was 24 h, but all R^2s were no less than 0.72 (**Figure 5B**).

Among the input data, the EC of nutrient solutions in the drainage tank and cumulative irrigation volume per day most affected the substrate EC (**Table 4**). Both inputs reduced the test accuracy by 0.05. Substrate EC was the least influential factor in accuracy because the accuracy was rarely lowered even without substrate EC. For all inputs, the average value of reduction was 0.0295.

TABLE 3 | Test accuracies and root mean square errors (RMSEs) of trained recurrent neural network (RNN) algorithms.

Type of RNN	Test accuracy (R^2)	Test RMSE
Long short-term memory (LSTM)	0.72	0.08
Gated recurrent unit (GRU)	0.68	0.09
Multi-layered LSTM	0.70	0.08
Multi-layered GRU	0.68	0.09

Chronological Comparisons of Prediction

Trained LSTM detected the tendency and predicted the changes in EC, although there is little deviation between predicted and measured values (**Figure 6**). Prediction results followed the fluctuation of root-zone EC, even though variations from actual values occurred. The prediction of future 24-h EC showed different RMSEs (**Figure 7**). In particular, the first 3-h prediction showed lower RMSEs than the total validation RMSE. The RMSE tended to be higher in the data before 12-h, which was the beginning of the forecast. Especially, the RMSEs were lower in the first 3 h and became higher for 4–8 h. However, there was no large gap by time.

DISCUSSION

In this study, RNN showed the test R^2 of 0.72, indicating that RNN had a possibility of predicting future tendency of EC

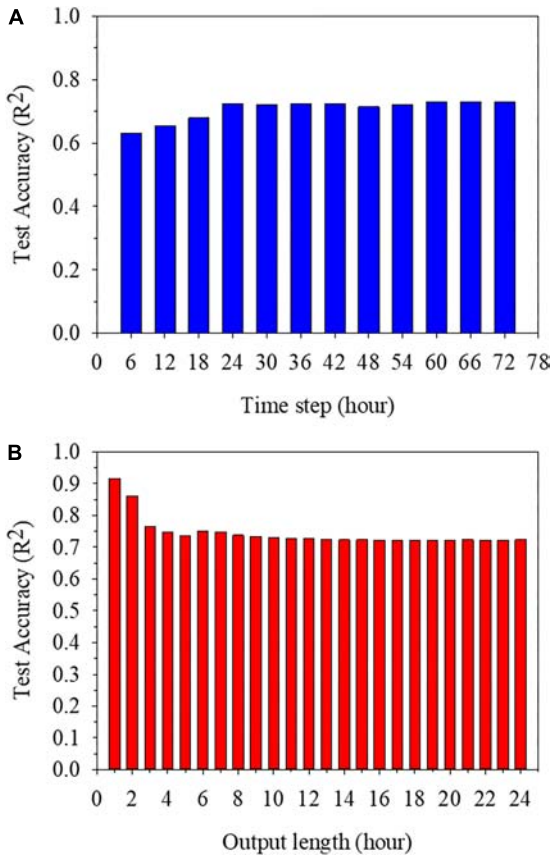

FIGURE 5 | Test accuracies of trained long short-term memory (LSTM) algorithms at different time steps **(A)** and output lengths **(B)**.

TABLE 4 | Test accuracies of the long short-term memory (LSTM) after excluding input data.

Excluded data	Test accuracy (R^2)	Excluded data	Test accuracy (R^2)
(1)[z]	0.72	(11)	0.67
(2)	0.70	(12)	0.69
(3)	0.67	(13)	0.68
(4)	0.69	(14)	0.70
(5)	0.69	(15)	0.68
(6)	0.68	(16)	0.69
(7)	0.69	(17)	0.68
(8)	0.68	(18)	0.70
(9)	0.69	(19)	0.70
(10)	0.70	(20)	0.71

[z] *Refer to* ***Table 1*** *for the excluded data number.*

changes (**Figure 4**). The trained RNNs with relatively shallow layers showed better accuracies in this study (**Table 3**). Recently, neural networks have deep structure in general (Ioffe and Szegedy, 2015; Silver et al., 2016) and RNNs have a very deep structure over time and therefore do not require fully connected multilayers in most cases (Jozefowicz et al., 2015). If the number of layers is the same, LSTM has more complex structure than GRU and has more parameters (Chung et al., 2014), resulting in higher accuracy. However, in case that the number of parameters should be small due to computational limitation, GRU can be used because the accuracy is not much different. Although ARIMA model is an algorithm to analyze chronological data, it could not predict future substrate EC. Since ARIMA model uses only target factor changes as input, it seems that the change in substrate EC itself did not show a definite periodicity. On the other hand, RNN can use other environmental factors as input, so it can correlate environmental changes with root-zone EC changes. Moreover, RNN has a unique structure and deals with huge sizes of input and output, so it is difficult to compare RNN with conventional algorithms or models.

Considering the accuracies in recent deep learning applications, the test R^2 of 0.72 in this study is not high and would be due to the relatively short estimation period (**Figure 4**). The period was a fraction of the cropping season and the data used for prediction was from 99 days after transplanting. Therefore, the earlier age of the plants could not be used because it was out of the trained ranges. However, deep learning algorithms can be more accurate when tested with big data of long periods to generalize to all possible conditions (Lopez et al., 2001). Other agricultural studies using deep learning have been conducted with big datasets with long collecting periods to cover almost all possibilities, such as seasonal influences (Trejo-Perea et al., 2009; Wang H. et al., 2017). Therefore, all datasets of other periods could improve model robustness. Adding more environmental and plant growth data to input elements can also increase the accuracy. Virtual conditions via simulation could be helpful for training the neural network (Beltramo et al., 2016). Moreover, if data are collected by similar methods used in this study, the trained LSTM can be applied to other periods or other plants using transfer learning (Gao et al., 2014; Shin et al., 2016).

EC interacts with crops and ambient environments continuously, so previous environments are related with EC changes. Therefore, the accuracy was improved with increasing time step, which represents the length of previous information (**Figure 5A**). However, since the nutrient solution was closed and controlled, the previous information more than 24 h did not affect the prediction accuracy. Therefore, 24-h time step is an appropriate input length. Meanwhile, the accuracy was deteriorated with increasing output lengths due to the increase in computation (**Figure 5B**). Obviously, the accuracy was better because the values to be predicted were reduced when the output length was shorter. However, output lengths that are too short cannot be used to help control nutrient solutions through EC forecasting. EC should be predictable from sunrise, at least when transpiration begins because transpiration and water have a significant interaction (Kramer, 1937; Greenwood and Beresford, 1979). Therefore, the maximum output length should be selected to predict the hourly changing tendency of EC in a day by using changes in environmental factors from the morning and previous day.

Through excluding input elements, it was found that the drainage nutrient solutions were highly related with the substrate EC (**Table 4**). However, the accuracy was not changed even if the substrate EC was eliminated from the input elements. LSTM uses the cell state to transmit the information of previous output

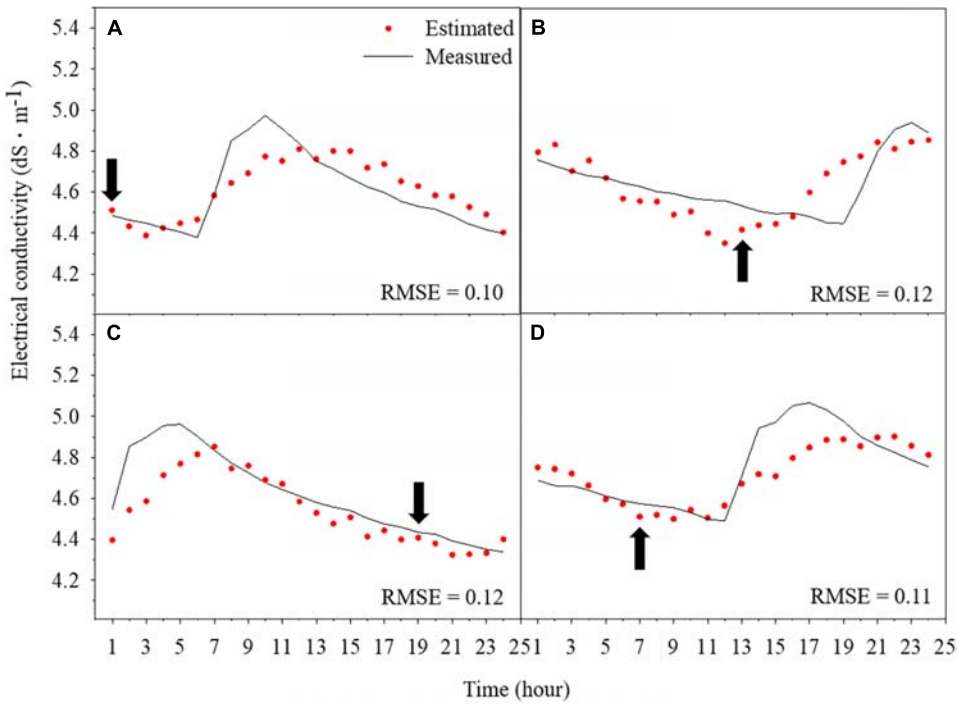

FIGURE 6 | Chronological comparisons of predicted root-zone electrical conductivity (EC) via trained long short-term memory (LSTM) and measured data from 0:00 to 23:00 on December 25 **(A)**, from 6:00 on December 26 to 5:00 on December 27 **(B)**, from 12:00 on December 27 to 11:00 on December 28 **(C)**, and from 18:00 on December 29 to 17:00 on December 30 **(D)**. Arrows represent the point of 00:00.

(Greff et al., 2015; Heffernan et al., 2017). The information about the substrate EC, which is the output, could be included in the cell state. However, the accuracy was reduced by excluding water-related environments in **Table 1**. The changes in EC are also affected by water content (Rhoades et al., 1976; Medrano et al., 2005). Therefore, water-related data were important to predict the substrate EC. However, it can be said that all input factors were appropriate because the accuracy does not collapse by exclusion of certain factors.

The difference between accuracies resulted from generalizing the entire range of data (**Figure 6**). Predicting EC changes during the day was difficult because the plants disturbed the water and nutrient environments by transpiration. Underestimated or overestimated predictions between about 6 and 12 a.m. could be resulted from the increasing transpiration. In addition, transpiration significantly affects the uptake of nutrients, which is related with the change in root-zone EC and varies with growth stage (Van Noordwijk, 1990; Baille et al., 1994; Le Bot et al., 1998). Therefore, variation of the root-zone EC can be larger depending on growth stage even when the drainage rate is controlled (Massa et al., 2011; Shin and Son, 2016). In the study, the data were acquired in the latter part of the cultivation (**Table 1**). Since the crops were sufficiently grown, a relatively large change in the substrate EC was observed. Therefore, the trained LSTM showed a low test accuracy, but it was acceptable performance.

The RMSE showed that the trained LSTM was able to predict the entire range with even accuracy (**Figure 7**). Due to the nature of LSTM, which is a black box modeling, it is impossible

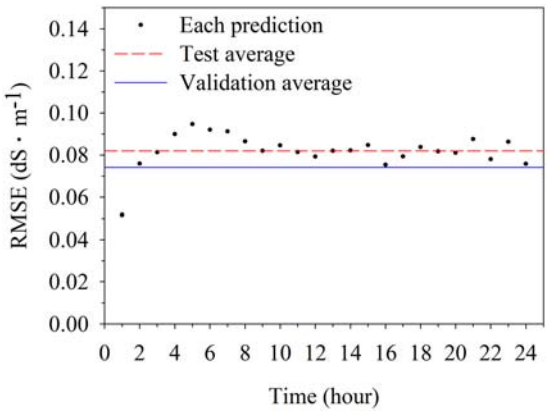

FIGURE 7 | Root mean square errors (RMSEs) of electrical conductivity (EC) of nutrient solutions. RMSEs separately calculated based on each prediction for 1 h were compared with total validation and test RMSEs.

to understand exactly what affected the RMSEs. It would not be the effect of EC change at a specific time slot because the model predicted the substrate EC at 10-min intervals. Further studies about RNN structure are needed to reveal the reason of slight differences in accuracy. However, the principle of EC-based nutrient control is maintaining the EC of nutrient solutions at a set point (Ahn et al., 2010). Therefore, predicting whether the EC will increase or decrease in the future can help with sophisticated

nutrient control. Because nutrient solution control depends on a contemporary EC in current soilless cultures (Neto et al., 2014; Kinoshita et al., 2016), predicted 3-h EC might improve the accuracy of nutrient control. In addition, since the RMSE did not change much near the test accuracy of 0.08 after the 9 h, it can be said that stable forecasts during a day are possible.

Comprehensively, LSTM showed acceptable accuracies in predicting substrate EC. In addition, it is known that EC and pH can be predicted together using ANNs (Ferentinos and Albright, 2002). In this study, the pH data were not used for model training, but the pH of nutrient solution could be predicted using the LSTM. Therefore, if pH and EC can be predicted together, growers could be able to cope with rapid changes in nutrient concentration caused by environmental changes. Furthermore, LSTM, which is effective in analyzing chronological data, could predict plant environments influenced by the accumulations of previous situations, such as plant growth and ion concentration of nutrient solutions.

CONCLUSION

Prediction models used in this study were based on a deep learning algorithm, RNN. Among the most popular RNN algorithms, a single-layered LSTM showed the highest test accuracy (R^2 = 0.72). The trained LSTM could be applied to control nutrient solutions in closed-loop soilless cultures based on prediction of future EC. Therefore, the algorithm could make planned management of nutrient solutions possible, reducing resource wastes. Prediction accuracy could be higher with additional data. Deep learning algorithms could be more accurate with additional data, so other environmental factors or plant growth data could improve model robustness. In particular, the LSTM can be extended to predict various factors which are influenced by the accumulations of previous situations. Further research on long-period control using LSTM is required.

AUTHOR CONTRIBUTIONS

TM constructed the artificial neural network, analyzed the root-zone EC, and wrote the manuscript. TIA developed the open-loop soilless culture system and measured the environment and growth data. JES designed and supervised the experiment and wrote the manuscript.

FUNDING

This research was supported by the MSIT (Ministry of Science and ICT), Korea, under the ITRC (Information Technology Research Center) support program (IITP-2017-2013-0-00877) supervised by the IITP (Institute for Information and Communications Technology Promotion).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AC and the handling editor declared their shared affiliation.

frontiers in Plant Science

MINI REVIEW
published: 22 August 2018
doi: 10.3389/fpls.2018.01254

Enhancing Quality of Fresh Vegetables Through Salinity Eustress and Biofortification Applications Facilitated by Soilless Cultivation

Youssef Rouphael[1] and Marios C. Kyriacou[2]**

[1] Department of Agricultural Sciences, University of Naples Federico II, Naples, Italy, [2] Department of Vegetable Crops, Agricultural Research Institute, Nicosia, Cyprus

Closed soilless cultivation systems (SCS) support high productivity and optimized year-round production of standardized quality. Efficiency and precision in modulating nutrient solution composition, in addition to controlling temperature, light, and atmospheric composition, renders protected SCS instrumental for augmenting organoleptic and bioactive components of quality. Effective application of eustress (positive stress), such as moderate salinity or nutritional stress, can elicit tailored plant responses involving the activation of physiological and molecular mechanisms and the strategic accumulation of bioactive compounds necessary for adaptation to suboptimal environments. For instance, it has been demonstrated that the application of salinity eustress increases non-structural carbohydrates and health-promoting phytochemicals such as lycopene, β-carotene, vitamin C, and the overall phenolic content of tomato fruits. Salinity eustress can also reduce the concentration of anti-nutrient compounds such as nitrate due to antagonism between nitrate and chloride for the same anion channel. Furthermore, SCS can be instrumental for the biofortification of vegetables with micronutrients essential or beneficial to human health, such as iodine, iron, selenium, silicon, and zinc. Accurate control of microelement concentrations and constant exposure of roots to the fortified nutrient solution without soil interaction can maximize their uptake, translocation, and accumulation in the edible plant parts; however, biofortification remains highly dependent on microelement forms and concentrations present in the nutrient solution, the time of application and the accumulation capacity of the selected species. The present article provides an updated overview and future perspective on scientific advances in SCS aimed at enhancing the sensory and bioactive value of vegetables.

Keywords: anti-nutrients, chemical eustressor, functional quality, floating system, micronutrients, mild salt stress, nutrient solution management, stress response

OPEN ACCESS

Edited by:
Dimitrios Savvas, Agricultural University of Athens, Greece

Reviewed by:
Giancarlo Fascella, Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria (CREA), Italy
Smiljana Goreta Ban, Institute of Agriculture and Tourism, Croatia

***Correspondence:**
Youssef Rouphael
youssef.rouphael@unina.it
Marios C. Kyriacou
m.kyriacou@ari.gov.cy

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *25 May 2018*
Accepted: *07 August 2018*
Published: *22 August 2018*

Citation:
Rouphael Y and Kyriacou MC (2018) Enhancing Quality of Fresh Vegetables Through Salinity Eustress and Biofortification Applications Facilitated by Soilless Cultivation. Front. Plant Sci. 9:1254. doi: 10.3389/fpls.2018.01254

SOILLESS MEANS FOR IMPROVING SENSORY AND FUNCTIONAL QUALITY OF VEGETABLES

The productivity of agricultural production systems is unprecedentedly challenged by projections for global population increase, by climate change and by shortage of the fundamental natural resources of water and arable land. In the case of vegetable crops, which contribute significant nutritive and bioactive value to the human diet, maximal productivity is attained under controlled

environments where production may expand vertically and temperature, light, nutrient supply, and atmospheric composition are controlled (Gruda, 2005, 2009). In particular, the technological advancement of closed soilless (hydroponic) cultivation systems (SCS; e.g., nutrient film, floating, and pot- and sacs-systems) based on recirculating nutrient solutions has maximized productivity per unit area and notably in terms of water use efficiency, by maximizing root contact with the nutrient source while minimizing evaporation and nutrient runoff (Treftz and Omaye, 2016). Despite the high capital investment and technological proficiency required for managing soilless systems, their expansion is propelled by the efficacy of optimized year-round production and standardized product quality irrespective of locality.

Besides the pressing issue of global food security, demand for high quality horticultural products is also on the rise, driven by the growing interest of society in fresh products of high organoleptic, nutritional, and functional quality. The quality of fresh horticultural commodities has been recently defined as "*a dynamic composite of their physicochemical properties and evolving consumer perception, which embraces organoleptic, nutritional and bioactive components*" (Kyriacou and Rouphael, 2018). Extrinsic characteristics of product quality are strongly influenced by socioeconomic and marketing factors which formulate consumer perception and generate quality prototypes. Despite the continued growth of the hydroponic industry consumers at large hold a negative bias toward SCS products which they consider artificial, less tasty, and of lower nutritional quality (Schnitzler and Gruda, 2002), just as organically grown fruits and vegetables are generally hailed as healthier and safer (Orsini et al., 2016). Nevertheless, it is apparent that key secondary metabolites which form the basis of functional quality in horticultural products can be modulated by appropriate management of SCS components. Exposure to biotic and abiotic stress underlies the superior nutritional quality often observed in organically grown products, since stress response entails the activation of physiological and molecular mechanisms necessary for adaptation to suboptimal environments, such as the biosynthesis of secondary metabolites (e.g., ascorbate, tocopherols, carotenoids, and glucosinolates; Orsini et al., 2016). Soilless systems can facilitate the precise application of an eustress (positive stress), such as moderate salinity or nutritional stress, through precise management of the concentration and composition (cationic and anionic proportions or single ions) of the nutrient solution, and thus may constitute a practical and effective means for improving the nutritional value of vegetables and for reducing the accumulation of anti-nutrient compounds, such as nitrates (Colla et al., 2018; Rouphael and Kyriacou, 2018; Rouphael et al., 2018a,b). Soilless culture can also be instrumental in the biofortification of edible plant portions with essential and/or beneficial micronutrients to human health. Biofortification with essential or beneficial micronutrients may constitute an effective means for supplying the human diet with iodine (I), selenium (Se), zinc (Zn), and silicon (Si) (White and Broadley, 2005). The present article provides an updated overview and future perspective on scientific advances in soilless cultivation aimed at enhancing the sensory and bioactive quality of vegetables through nutrient solution management and applications aimed at biofortification.

SALINITY EUSTRESS AND MACRONUTRIENT MANAGEMENT FOR ENHANCING NUTRITIONAL QUALITY OF HYDROPONICALLY GROWN VEGETABLES

Excessive concentration of sodium chloride (NaCl) in irrigation water and agricultural soils disturbs physiological processes in vegetable crops, leading to stunted growth and yield decline (Rouphael et al., 2017, 2018b). However, recent scientific reviews have indicated that vegetable crops may exhibit tailored responses to the application of eustress, such as mild to moderate salinity, as a result of stress-induced reshuffling of plant metabolism and strategic accumulation of bioactive compounds against suboptimal conditions (Kyriacou and Rouphael, 2018). Vegetable crops can synthesize a broad range of secondary metabolites to counteract oxidative damage and to scavenge reactive oxygen species (ROS) elicited by environmental stressors (Orsini et al., 2016). These health-promoting phytochemicals, abundant in stressed plants, can enrich the functional quality of fresh vegetables to the benefit of human diet (Khanam et al., 2012; Kyriacou and Rouphael, 2018).

Multiple studies have reported increase in the bioactive content of vegetables triggered by mild to moderate NaCl concentrations in the nutrient solution (Tzortzakis, 2009, 2010; Rouphael et al., 2018a,b). Although under soil conditions this technique for improving product quality poses a high risk of plant overstress (Hidaka et al., 2008), soilless culture may be an effective tool for modulating secondary metabolites without curbing growth and yield, through proper management of the nutrient solution's composition (Schwarz et al., 2009; Tomasi et al., 2015). Several studies have demonstrated that NaCl in the nutrient solution raises the levels of sugars, organic acids, and amino acids in several vegetable crops, like tomato (Zushi and Matsuzoe, 2015; Moya et al., 2017), pepper (Marin et al., 2009), melon (Rouphael et al., 2012b), watermelon (Colla et al., 2006), eggplant (Savvas and Lenz, 1996), lettuce (Sakamoto et al., 2014), and cauliflower (Giuffrida et al., 2017) thereby improving their organoleptic quality. The salt-induced osmoregulatory mechanism in hydroponically grown vegetables involves the biosynthesis of specific osmolytes (sugars, minerals, and amino acids such as proline and GABA) believed to function as osmoprotectants by counterbalancing the increase in vacuolar osmotic potential caused by the toxic accumulation of sodium and chloride ions (Hasegawa et al., 2000).

The application of salinity eustress may also affect health-promoting phytochemicals. For instance, increasing soilless nutrient solution electrical conductivity (EC) from 3 to 6.5 dS m^{-1} (Krauss et al., 2006), from 2.4 to 4.5 dS m^{-1} (Wu et al., 2004), and from 2.2 to 4.5 dS m^{-1}

(Moya et al., 2017) increased the lycopene, β-carotene, vitamin C, and total phenolic content of tomato. Giuffrida et al. (2017), showed that the functional quality of hydroponically grown cauliflower in response to moderate salt stress may depend on interactive variables, such as duration of exposure and plant phenological stage at the time of exposure (e.g., salinity stress applied constantly throughout the cultivation cycle or at the onset of flowering), with neoglucobrassicin concentration found two-fold higher in cauliflower heads supplied with saline nutrient solution (4 dS m^{-1}) compared to the non-saline (EC 2 dS m^{-1}) control treatment. Beneficial effects of mild to moderate salinity on nutritional and bioactive value was also reported for hydroponically grown leafy greens (Kim et al., 2008; Colla et al., 2013; Klados and Tzortzakis, 2014; Bonasia et al., 2017; Ntatsi et al., 2017; Petropoulos et al., 2017). For instance, Petropoulos and co-workers reported that increasing the EC from 1.8 to 6.0 dS m^{-1} increased ascorbic acid as well as α-tocopherol levels in spiny chicory. Similarly, Colla et al. (2013) and Bonasia et al. (2017) showed that raising the EC from 2.5 to 3.5 dS m^{-1} increased antioxidant compounds in wild rocket, and from 2.0 to 5.8 dS m^{-1} it improved the antioxidant activity, chlorogenic acid, cynarin, and luteolin levels in leaves of artichoke and cardoon grown in a floating system. However, response to NaCl is cultivar-dependent and the choice of the cultivar is critical for achieving the desired effects (Borghesi et al., 2011; Dominguez-Perles et al., 2011; Colla et al., 2013). Several workers have reported negative effects or no significant effects in response to NaCl application. Increasing the nutrient solution EC above 4.4 dS m^{-1} decreased lycopene and β-carotene content in tomato (De Pascale et al., 2001). Similarly, Petersen et al. (1998) and Bonasia et al. (2017) observed a decrease of ascorbic acid in tomato and wild rocket at EC 9.0 and 4.5 dS m^{-1}, respectively. Presumably the antioxidant system of salt-stressed plants does not effectively support ROS scavenging after the stress threshold for maintaining growth is exceeded (Rouphael et al., 2018a,b), whereas leaf area reduction in salt-sensitive cultivars can also modify fruit temperature and halt the synthesis of bioactive compounds (Dorais et al., 2008). Finally, salinity eustress can reduce nitrate accumulation in SCS leafy vegetables due to antagonism between nitrate and chloride for the same anion channel (Rubinigg et al., 2003). Vegetable nitrate remains of high interest to regulators due to possible effects on human health, while it also imparts vegetables a bitter taste (Colla et al., 2018). Borgognone et al. (2016) reported that minimizing nitrate supply in floating raft culture by partial substitution of calcium nitrate with calcium chloride increased phenols and flavonoids and lowered nitrates in cardoon leaves without affecting yield.

Although most published articles concerning the positive effects of nutrient solution EC on nutritional, organoleptic, and functional quality of soilless vegetables were based on greenhouse experiments in which NaCl was the predominant salt, several studies have shed light on the effects of salinity induced by macronutrients. For instance, Fallovo et al. (2009) determined the effects of macronutrient solution concentration (2, 18, 34, 50, or 66 mequiv L^{-1}, corresponding to an EC of 0.3, 1.2, 2.0, 2.8, and 3.6 dS m^{-1}, respectively) during the spring and summer seasons on leafy lettuce (*Lactuca sativa* L. var. *acephala*) grown in a floating system. The authors reported a linear decrease in qualitative characteristics (glucose, fructose, proteins, total carbohydrates, and starch contents) in response to an increase in the nutrient solution concentration from 2 to 66 mequiv L^{-1}. Similarly, Rouphael et al. (2012a) showed that raising the macronutrient solution concentration from 4 to 68 mequiv L^{-1} in floating raft culture increased biomass production but deteriorated leaf functional quality in both cardoon and artichoke by decreasing key polyphenols such as caffeic acid, chlorogenic acid, cynarin, and luteolin. Moreover, the management of the cationic proportions (K/Ca/Mg) in the nutrient solution facilitated by soilless culture has been also demonstrated as an effective tool for enhancing nutritional quality of fruit vegetables (Fanasca et al., 2006a). A high proportion of K in the nutrient solution caused a significant increase in tomato soluble solids and lycopene contents irrespective of cultivar ('Corfu' or 'Lunarossa' – standard or high-pigment cultivar), whereas high concentration of Mg improved the hydrophilic antioxidants (caffeic acid) and the total antioxidant capacity of the high-pigment 'Lunarossa' hybrid (Fanasca et al., 2006b). Synthesis and accumulation of the abovementioned antioxidant compounds in response to high Mg supply might relate to the increased activity of key enzymes such as glutamine synthetase that regulate ammonia assimilation and detoxification of plant tissues (Marschner, 2012).

SOILLESS BIOFORTIFICATION OF VEGETABLES WITH ESSENTIAL AND BENEFICIAL MICRONUTRIENTS

Biofortification of vegetables with essential and non-essential beneficial micronutrients caters to the demand for healthier diet and the need to address human micronutrient deficiency, known as "*hidden hunger*" (White and Broadley, 2005; Carvalho and Vasconcelos, 2013). However, the window between biofortification and toxicity effect is often quite narrow. Applications aiming at the accumulation of health-supporting micronutrients must be adjusted to avoid detrimental effects on plant growth (Rouphael et al., 2018a). Moreover, biofortification may depend upon several interacting factors, such as genotype, chemical form, application rate, and environmental and growing conditions (Tomasi et al., 2015).

Selenium and iodine have been particularly investigated since they are beneficial though non-essential microelements for human health. Uptake is higher when supplied in SCS nutrient solution where Se and I concentrations can be accurately controlled, as opposed to side-dressing of soil crops or foliar applications (Wiesner-Reinhold et al., 2017). Constant exposure of the root system to fortified nutrient solution and absence of micronutrient–soil interaction make SCS more efficient, thus maximizing uptake, translocation, and accumulation of these elements in the edible parts (Wiesner-Reinhold et al., 2017). However, micronutrient accumulation is highly dependent on the elemental concentration in the soilless solution, the time

of application and the accumulation capacity of the selected species. For instance, Signore et al. (2018) reported that iodine biofortification of carrots at 50 mg L^{-1} through the hydroponic solution reached cumulative levels toxic on the plants compared to foliar applications both under open-field and greenhouse conditions at the same rate. On the other hand, low rates (0.5–1.5 mg L^{-1}) of selenium in the nutrient solution increased Se concentration in several horticultural commodities such as spinach, lettuce, and basil without inducing toxic effects (Zhu et al., 2004; Malorgio et al., 2009; Ramos et al., 2011; Ferrarese et al., 2012; Puccinelli et al., 2017). Zhu et al. (2003), reported that biofortification with I in solution culture is easily feasible both with iodide (I^-) and iodate (IO_3^-), as the application of I at rates from 0.13 to 12.7 mg L^{-1} effectively fortified spinach with iodine. Similarly, Blasco et al. (2008) showed that the most appropriate rate of I^- in lettuce soilless culture is 5.1 mg L^{-1} or lower, whereas IO_3^- concentration of 1.3 to 30.5 mg L^{-1} achieved foliar accumulation of I without detriment to yield. Kiferle et al. (2013); Li et al. (2017), and Smoleń et al. (2018) indicated that also fruit vegetables and tuber crop such as tomato, pepper, and potato grown in soilless culture can be targeted for I and/or Se biofortification. Another important factor influencing Se and I accumulation in vegetables is their chemical forms. Vegetable species exposed to selenate (O_4Se^{-2}) rather than selenite (O_3Se^{-2}) and to I^- rather to IO_3^- can accumulate more Se and I in leaf or fruit tissues. This is because selenate is taken up actively by the more efficient sulfate transporter compared to the passive phosphate transporter used for taking up selenite (Wiesner-Reinhold et al., 2017). Uptake of I^- was much higher than IO_3^- since the latter form should be reduced to I^- before uptake, thus reducing bioavailability to vegetable crops (Blasco et al., 2008).

According to White and Broadley (2005), concentrations of 0.1–0.7 mg Zn g^{-1} dry weight can be achieved in leafy vegetables with no detriment to yield, making Zn biofortification of leafy greens a potential tool for enhancing dietary Zn intake (White et al., 2018). Adequate Zn addition in the nutrient solution (5.2–6.5 mg L^{-1}) allowed biofortification of *Brassica oleracea* coupled with significant synthesis and accumulation of amino acids (Arg, Asp, Glu, Gln, Hys, Lys, Phe, and Trp) while maintaining optimal growth (Barrameda-Medina et al., 2017). In addition, the production of Fe-enriched leafy vegetables such as lettuce using hydroponics is feasible, since the increase of Fe concentration in the nutrient solution 6 h before harvest resulted in significant increase of foliar Fe content without affecting yield (Inoue et al., 2000). Moreover, effective Si biofortification of soilless crops of basil, chicory, mizuna, purslane, Swiss chard, and tatsoi was demonstrated with SiO_2 supplementation of the nutrient solution at 50–100 mg L^{-1} with no detrimental effect on crop productivity (D'Imperio et al., 2016).

CONCLUSION AND THE CHALLENGES AHEAD

The demand for global food security under increasing biotic and abiotic pressures exacerbated by climate change makes protected cultivation of vegetable crops an inevitable necessity. Technological progress in the management of SCS drives productivity and mitigates high initial infrastructural costs. Moreover, flexibility and precision in modulating nutrient solution composition, in addition to controlling temperature, light, and atmospheric composition, renders SCS systems instrumental in targeting organoleptic and bioactive components of quality thus addressing demand for improved vegetable quality. The effective application of eustress, such as mild to moderate salinity or nutritional stress, can elicit targeted plant responses through the activation of physiological and molecular mechanisms and the strategic accumulation of bioactive compounds necessary for adaptation to suboptimal environments. Salinity eustress has been demonstrated to augment organoleptic components of quality such as soluble carbohydrates, and health-promoting phytochemicals such as lycopene, β-carotene, vitamin C, and polyphenols in vegetables; moreover, it may curb anti-nutrients such as nitrate owing to nitrate-chloride antagonism for uptake. Understanding the molecular and physiological mechanisms elicited by controlled plant eustress and those facilitating micronutrient uptake in interaction with genotype and environmental conditions will usher horticultural science into the era of tailoring superior sensory and functional quality vegetables.

Furthermore, SCS can facilitate the effective biofortification of vegetables with micronutrients essential or beneficial to human health, such as iodine, iron, selenium, silicon, and zinc. Accurate control of microelement concentrations and constant exposure of roots to the fortified nutrient solution without soil interaction can maximize their uptake, translocation, and accumulation in the edible plant parts. Biofortification remains, however, highly dependent on microelement forms and concentrations present in the nutrient solution, the duration of targeted applications, the developmental stage of plants, and the accumulation capacity of the selected species. These potentially interacting factors pose future challenges for research before SCS biofortification applications become effective tools for addressing nutrient deficiencies in human diet.

AUTHOR CONTRIBUTIONS

YR and MK had the original idea of eustress and biofortification of soilless vegetables and were both involved in writing the article.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 24 August 2018
doi: 10.3389/fpls.2018.01244

Impact of Cultivar and Grafting on Nutrient and Water Uptake by Sweet Pepper (*Capsicum annuum* L.) Grown Hydroponically Under Mediterranean Climatic Conditions

Andreas Ropokis[1], Georgia Ntatsi[1,2], Constantinos Kittas[3], Nikolaos Katsoulas[3] and Dimitrios Savvas[1]**

[1] *Laboratory of Vegetable Production, Department of Crop Science, Agricultural University of Athens, Athens, Greece,* [2] *Institute of Plant Breeding and Genetic Resources, Hellenic Agricultural Organization – ELGO DEMETER, Thessaloniki, Greece,* [3] *Laboratory of Agricultural Constructions and Environmental Control, Department of Agriculture Crop Production and Rural Environment, School of Agricultural Sciences, University of Thessaly, Volos, Greece*

OPEN ACCESS

Edited by:
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***Correspondence:**
Nikolaos Katsoulas
nkatsoul@uth.gr
Dimitrios Savvas
dsavvas@aua.gr

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *30 April 2018*
Accepted: *06 August 2018*
Published: *24 August 2018*

Citation:
Ropokis A, Ntatsi G, Kittas C, Katsoulas N and Savvas D (2018) Impact of Cultivar and Grafting on Nutrient and Water Uptake by Sweet Pepper (Capsicum annuum L.) Grown Hydroponically Under Mediterranean Climatic Conditions. Front. Plant Sci. 9:1244. doi: 10.3389/fpls.2018.01244

In closed-cycle hydroponic systems (CHS), nutrients and water should be delivered to the plants at identical ratios to those they are removed via plant uptake, to avoid their depletion or accumulation in the root zone. For a particular plant species and developmental stage, the nutrient to water uptake ratios, henceforth termed "uptake concentrations" (UC), remain relatively constant over time under similar climatic conditions. Thus, the nutrient to water uptake ratios can be used as nutrient concentrations in the nutrient solution (NS) supplied to CHS to compensate for nutrient and water uptake by plants. In the present study, mean UC of macro- and micronutrients were determined during five developmental stages in different pepper cultivars grown in a closed hydroponic system by measuring the water uptake and the nutrient removal from the recirculating NS. The experiment was conducted in a heated glasshouse located in Athens Mediterranean environment and the tested cultivars were 'Orangery,' 'Bellisa,' 'Sondela,' 'Sammy,' self-grafted and 'Sammy' grafted onto the commercial rootstock 'RS10' (*Capsicum annuum*). 'Sondela' exhibited significantly higher NO_3^-, Mg^{2+}, Ca^{2+} and B UC, while Bellisa exhibited higher K UC in comparison with all other cultivars. The UC of all nutrients were similar in the grafted and the non-grafted 'Sammy' plants, which indicates that this *Capsicum annum* rootstock does not modify the uptake of nutrients and water by the scion. The UC of macronutrients estimated in the present study (mmol L^{-1}) ranged from 2.4 to 3.7 for Ca, 1.0 to 1.5 for Mg, 6.2 to 9.0 for K, 11.7 to 13.7 for N, and 0.7 to 1.1 for P. The UC of N, K, Ca, and Mg were appreciably higher than the corresponding values found in Dutch tomato glasshouse, while that of P was similar in both locations during the vegetative stage and higher in the present study thereafter. The UC of Fe, Zn and B tended to decrease with time, while that of Mn increased initially and subsequently decreased slightly during the reproductive developmental stage.

Keywords: soilless culture, rootstock, calcium, iron, magnesium, bell pepper, scion

INTRODUCTION

In crops grown in closed hydroponic systems (CHS), the net volume of supplied water is essentially equal to that removed via transpiration, if the whole amount of collected drainage solution (DS) is consistently recycled. Furthermore, the input ratio between the mass of a nutrient and the volume of water in a CHS is equal to the concentration of this nutrient in the nutrient solution (NS) supplied to the plants to compensate for nutrient and water uptake by plants. This NS, which is mixed with the DS to be recycled, is commonly termed "nutrient solution for closed systems" (NSCS) (de Kreij et al., 1999). To avoid depletion or accumulation of nutrients in the root zone of a crop grown in a CHS, their concentrations in the NSCS should be equal to the corresponding nutrient to water uptake ratios by the plants, henceforth termed "uptake concentrations" (UC). For a particular plant species and developmental stage, the UC exhibit an appreciable stability over time under similar climatic conditions (Savvas and Lenz, 1995; Sonneveld and Voogt, 2009; Tzerakis et al., 2013). Thus, if the mean UC of all essential nutrients supplied via NS to a particular crop species are known, an appropriate NS composition can be established for the NSCS to be supplied to this crop species (Neocleous and Savvas, 2015).

Standard recommendations for macro- and micro-nutrient levels in NSCS for peppers are mostly based on research carried out in the Netherlands (de Kreij et al., 1999; Sonneveld and Voogt, 2009). However, standard recommendations about the composition of a NSCS for a particular plant species should be based on experimental data originating from similar climatic zones. Indeed, previous research showed that, in crops grown hydroponically under hot and dry climatic conditions such as those prevailing in the Mediterranean basin, the UC may be substantially different than those observed in north-European greenhouses (Neocleous and Savvas, 2015; Savvas et al., 2017). Furthermore, different cultivars, or different rootstocks in the case of grafted plants, may have an impact on nutrient and water uptake, thereby modifying the UC observed in crops of self-rooted plants (Savvas et al., 2010; Rouphael et al., 2016). Indeed, the uptake of nutrients by grafted plants may be influenced not only by the shoot but also by rootstock genotype (Savvas et al., 2017).

To date, in the international scientific literature there is a lack of data about UC arising from experiments with sweet pepper cultivated under hot and dry climatic conditions. Taking this gap of knowledge into consideration, the present study was designed to estimate mean UC for most macro- and micronutrients in a pepper crop grown in a Mediterranean environment, and compare them with similar data arising from north-European climatic conditions. Furthermore, given the high variability of commercial pepper varieties (Tsaballa et al., 2015; Silvar and García-González, 2016), as well as the increasing use of grafted seedlings to establish greenhouse crops of pepper (Penella and Calatayud, 2018), UC were established for four different cultivar types of pepper, one of which was either self-grafted or grafted onto a commercial rootstock. The anticipated data can be used to optimize the composition of NSCS supplied to pepper crops grown in CHS under climatic conditions characterized by mild winters, and dry and hot summers.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The experiment was conducted in a glasshouse at the Agricultural University of Athens (AUA). Four pepper cultivars (*Capsicum annuum* L.) were grown in recirculating NS according to the principles of the Nutrient Film Technique (NFT). Two cultivars of the bell type (Orangery and Sondela) and two of the elongated form (Bellisa and Sammy), one of which (Sammy) was either self-grafted or grafted on a rootstock RS10 (*Capsicum annuum* L.) were used in the experiment. The pepper cultivars chosen for this experiment were the most economically important types of sweet pepper (Bell or elongated form). Grafting was performed only in one sweet pepper cultivar 'Sammy' due to the fact that this cultivar was the most commonly grafted cultivar in commercial basis. The rootstock 'RS10' was chosen as being the most commonly used in commercial basis and due to the high compatibility with the tested scion. The three non-grafted cultivars and the two versions of 'Sammy' i.e., grafted and non-grafted, constituted five experimental treatments. On January 16, 2014, i.e., 2 months after sowing, the seedlings were at the stage of four leaves and had 16 cm mean height, were transferred into 20 closed-loop hydroponic circuits (experimental plots), which were supplied with NS. Each circuit comprised one channel, 3.0 m in length, 0.015 m in width, and 0.03 m in height, which accommodated nine plants. The plant density was 2.5 plants per m^2. All plants were supplied with a standard NS (de Kreij et al., 1999). Each treatment was replicated four times and thus 20 experimental hydroponic circuits were used. In each experimental unit, each of the 20 experimental units consisted of an individual supply tank, a pump, and irrigation pipes, thereby formed a closed circuit in which a NS was constantly recirculating according to the principles of the NFT. Climatic data during the experiment are given in **Table 1**.

The NS was automatically pumped at a rate of 0.4 m^3 h^{-1} to each channel while the total volume of the NS that was recirculating in each experimental unit amounted to 3 L per plant. In each unit, the nutrient and water uptake were compensated by automatically supplying a replenishment NS from an individual tank using a floater to maintain a constant NS level in the supply tank. The NS consumed by the plants was counted daily by recording the level difference in the tank filled with replenishment NS. The pH in the recirculating NS was adjusted once per day to 5.6 by adding appropriate amounts of nitric acid to the supply tank based on the actual pH level which was measured using a portable pH-meter. All channels were covered with black–white polyethylene sheets to avoid water evaporation. The total volume of recirculating NS in each experimental unit (including both the solution contained in the supply tank and that flowing in the gullies) amounted to 70 L.

The nutrient concentrations in the NS named as Starter Solution were in all treatments as follows: 6.0 mM K^+, 6.5 mM Ca^{2+}, 2.0 mM Mg^{2+}, 0.5 mM NH_4^+, 15.6 mM NO_3^-, 1.2 mM

TABLE 1 | Monthly averages of temperature (°C) and relative humidity (%) inside the experimental greenhouse.

		January	February	March	April	May	June	July
Temperature (°C)	Average	21.5	22.5	23.8	27.7	29.3	31.3	34.5
	Min	19.0	19.0	19.2	19.3	19.0	19.5	19.5
	Max	23.4	27.0	32.0	34.0	35.5	37.4	39.4
Relative humidity (%)	Average	72.1	71.6	70.6	68.7	62.5	59.1	52.4
	Min	66.3	63.4	58.4	56.8	48.5	44.4	38.8
	Max	74.4	73.6	72.3	70.6	67.8	63.8	56.6

$H_2PO_4^-$, 6.5 mM SO_4^{2-}, 15.0 μM Fe, 10.0 μM Mn, 7.0 μM Zn, 0.8 μM Cu, 50.0 μM B, and 0.5 μM Mo. The EC and pH in the above NS were 2.6 dS m^{-1} and 5.6, respectively. After transplanting, the nutrients and water that were absorbed by the plants were replenished daily by supplying replenishment NS with different nutrient concentrations than in the initial NS. The composition of replenishment NS was as follows: Vegetative stage: 5.3 mM K^+, 3.15 mM Ca^{2+}, 1.3 mM Mg^{2+}, 1.4 mM NH_4^+, 11.6 mM NO_3^-, 1.1 mM $H_2PO_4^-$, 1.2 mM SO_4^{2-}, 15.0 μM Fe, 10.0 μM Mn, 4.0 μM Zn, 0.8 μM Cu, 30.0 μM B and 0.5 μM Mo; Reproductive stage: 6.0 mM K^+, 2.7 mM Ca^{2+}, 1.1 mM Mg^{2+}, 0.8 mM NH_4^+, 10.6 mM NO_3^-, 1.1 mM $H_2PO_4^-$, 1.1 mM SO_4^{2-}, 15.0 μM Fe, 10.0 μM Mn, 0.7 μM Cu, 5.0 μM Zn, 25.0 μM B, and 0.5 μM Mo. The pH in the recirculating NS was daily adjusted to 5.6–5.7 by adding appropriate amounts of 1 N HNO_3 stock solution based on the actual pH level which was measured using a portable pH-meter.

Nutrient Uptake Calculations

Samples of recirculating NS were selected on a 4-weekly basis from all experimental units to determine the actual Ca, Mg, K, P, B, NO_3, Fe, Mn, and Zn concentrations. The concentrations of Ca, Mg, Fe, Mn, and Zn in both the NSs and the aqueous extracts of plant tissues were measured using an atomic absorption spectrophotometer (Perkin Elmer 1100A, Perkin Elmer, Waltham, MA, United States) while K was determined by flame photometry (Sherwood Model 410, Cambridge, United Kingdom). The NO_3^-, P and B concentrations in NS samples were measured by UV/VIS spectroscopy at 540, 880, and 420 nm, respectively.

Nutrient to water uptake ratios (uptake concentrations) for K, Mg, Ca, and Fe were estimated based on the removal of nutrients from the NS. In particular, the mean uptake concentration of the x micronutrient (C_{xu} in μmol L^{-1}, where x = Ca, Mg, K, P, B, NO_3, Fe, Mn, and Zn) was determined for four time intervals using the following mass balance equation:

$$Cxu = \frac{Vr\,(Cxbi - Cxei) + VuiCxa}{Vui}$$

where V_r (L) denotes the total volume of the recirculating NS in each experimental unit (70 L in the present study), V_{ui} (L) denotes the total volume of NS that was taken up by the plants in each experimental unit during the i time interval (i = 1…5), C_{xbi} and C_{xei} (μmol L^{-1}) denote the concentrations of the x nutrient in the recirculating NS on the first and the last day of the i time interval (i = 1…5), and C_{xa} (μmol L^{-1}) denotes the concentration of the x nutrient in the replenishment NS used in each treatment.

Statistical Analysis

All data were statistically analyzed by applying ANOVA using the PlotIT3.2® software package, version 3.2 for Windows (Scientific Programming Enterprises, Haslett, MI, United States). Data are presented in graphs as means ± SE.

RESULTS

As shown in **Figure 1**, 'Bellisa' and 'Sondela' consumed about 15% less water than 'Orangery' and 'Sammy' self-grafted, and about 20% less than Sammy grafted onto the commercial rootstock RS10. These differences reflected commensurate differences in the leaf area and the vegetative plant biomass (data not shown). However, as shown in **Table 2**, the differences in water consumption and vegetative plant biomass had no significant impact on the total fruit production, although 'Orangery' rendered a slightly higher yield than the other cultivars. In contrast to the total fruit yield, the number of fruit per plant and the mean fruit weight were strongly influenced by the genotype. In particular, 'Orangery' and 'Sondela' produced much less fruit than 'Sammy' but the weight of fruit from the latter cultivar was much smaller. As a result, the total yield was similar in all cultivars without any significant differences between them. On the other hand, hetero-grafting of 'Sammy' onto the commercial rootstock RS10 had no impact on the total fruit yield, the number of fruit per plant or the mean fruit weight. Due to the similar yield performance and the significantly lower cumulative water consumption in comparison to 'Sammy' and 'Belisa,' 'Sondela' exhibited a significantly higher water use efficiency (WUE) than these two cultivars (**Table 2**). However, the WUE of 'Orangery' was as high as that of 'Sondela.' It is worth to mention that grafting onto the commercial *Capsicum annuum* rootstock 'RS10' did not improve the WUE of 'Sammy.'

The Ca concentration in the recirculating NS was appreciably reduced in the time between the start of anthesis and the start of harvesting (**Figure 2C**), because the uptake of Ca per L of water was maximized (**Figure 2D**), irrespective of the cultivar. The increased Ca uptake during this developmental stage resulted in higher leaf Ca concentrations at commencement of harvesting in comparison with the vegetative growth stage (**Figure 2A**). After commencement of harvesting, the Ca UC tended to decrease gradually (**Figure 2D**), and this resulted in increased Ca levels

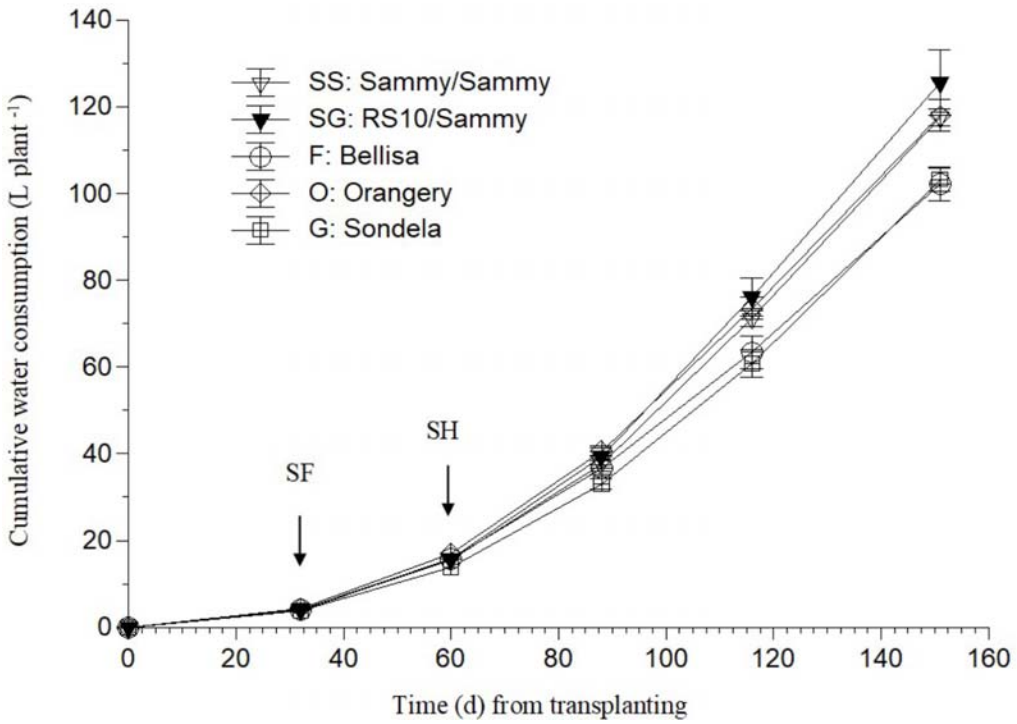

FIGURE 1 | Evolution of the cumulative water consumption (CWC) in a pepper crop grown in a closed hydroponic system as influenced by four different pepper cultivars ('Orangery,' 'Bellisa,' 'Sondela,' 'Sammy') and grafting ('Sammy' self-grafted or grafted onto the commercial rootstock 'RS10'). Vertical bars indicate ± standard errors of means. SF, start of flowering; SH, start of harvesting.

TABLE 2 | Influence of four cultivated varieties.

Grafting combination (rootstock/scion)	WUE (g L^{-1})	TFW/plant (kg)	TFN/plant (No)	MFW/plant (g)
'Sammy'/'Sammy'	28.3 b	3.36	46.00 a	72.94 b
'RS10'/'Sammy'	24.9 c	3.14	48.25 a	65.16 b
'Bellisa'	29.0 b	3.05	38.75 b	78.74 b
'Orangery'	33.1 a	3.97	26.25 cd	151.13 a
'Sondela'	33.3 a	3.44	23.33 d	147.31 a
Statistical significance				
Grafting combination	*	ns	**	***

*'Orangery' (O), 'Bellisa' (F), 'Sondela' (G), and 'Sammy' self-grafted (SS) or 'Sammy' grafted onto a commercial rootstock 'RS10' (SG), grown in closed hydroponic systems, on total fruit weigh (TFW) per plant, total fruit number (TFN) per plant, and average marketable fruit weigh (MFW) per plant. ns, *, **, and *** not significant or significant at $p \leq 0.05$, 0.01, and 0.001, respectively. Mean (n = 3) followed by different letters within the same column indicate significant differences for each factor according to the Duncan's multiple range test.*

in the recirculating NS, although the leaf Ca concentrations did not decrease. The fruit Ca concentrations increased during the late cultivation period, regardless of the root and shoot genotype (**Figure 2B**).

The Mg concentration in the recirculating NS tended to decrease slightly up to the start of harvesting but thereafter it showed an increasing tendency which was very weak in 'Sondela' and stronger in 'Sammy,' regardless of the root genotype (**Figure 3C**). On the other hand, the highest leaf Mg concentrations (**Figure 3A**) and Mg UC (**Figure 3D**) were measured in 'Sondela' throughout the cropping period. The leaf Mg concentrations were significantly higher at the fully vegetative stage (immediately after planting) than at later developmental stages, regardless of the root and shoot genotype. After commencement of flowering, both the leaf Mg concentrations and the Mg UC (**Figure 3D**) remained more or less constant during the whole cropping period. The fruit Mg concentrations tended to decrease up to day 120 from treatment initiation but this tendency was reversed at the late growing period. 'Sondela' exhibited consistently higher fruit Mg concentrations after commencement of harvesting in comparison with the other cultivars, while grafting had no impact on the fruit Mg level (**Figure 3B**).

The K concentration in the recirculating NS tended to increase slightly after the start of flowering and strongly after commencement of harvesting and up to 85 days after planting (**Figure 4C**). This increase in the recirculating NS was combined with a decrease in the leaf K concentration (**Figure 4A**) and the K UC (**Figure 4D**). Nevertheless, thereafter, both the leaf K concentration and the K UC increased again to similar levels with those measured in the interval between commencement of flowering and harvesting. Bellisa exhibited consistently higher UC than the other cultivars throughout the cropping period and this resulted in lower K concentrations in the recirculating NS and higher leaf K concentrations after the start of flowering.

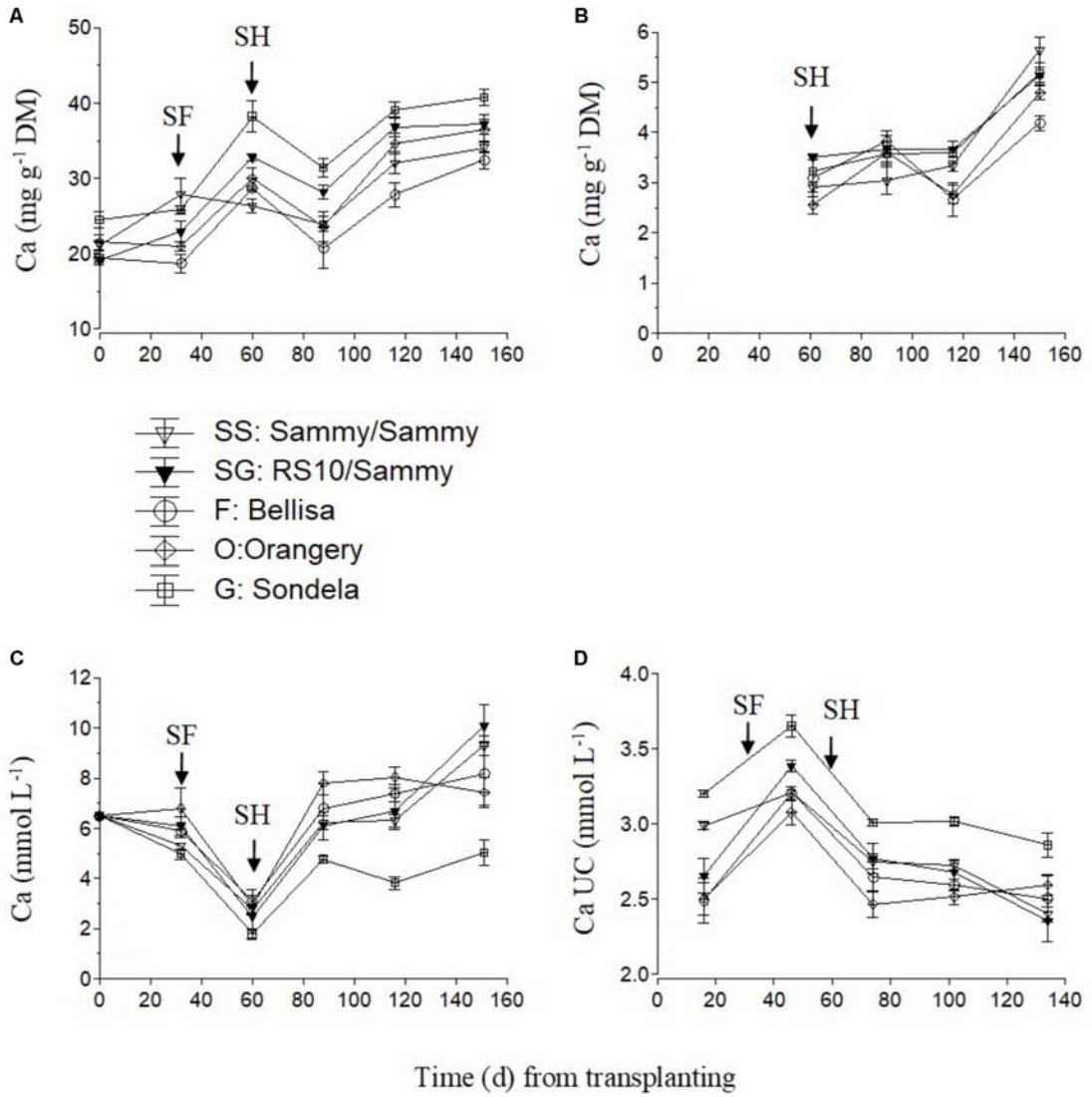

FIGURE 2 | Impact of different pepper cultivars ('Orangery,' 'Bellisa,' 'Sondela,' 'Sammy') and grafting ('Sammy' self-grafted or grafted onto the commercial rootstock 'RS10') on: (i) concentrations of Ca in the dry mass (DM) of young leaves **(A)** and fruit **(B)**, (ii) concentrations of Ca in the recirculating nutrient solution **(C)**, and (iii) apparent Ca uptake concentrations (UC), i.e., mmol of Ca uptake per L of water uptake **(D)**. Vertical bars indicate ± standard errors of means. SF, start of flowering; SH, start of harvesting.

In contrast, 'Orangery' exhibited lower K UC after the start of harvesting but the differences were not always significant. The fruit K concentrations tended to increase slightly after commencement of harvesting and up to 85 days after treatment initiation, with the exception of 'Sondela' and 'Bellisa' which exhibited lower and higher fruit K concentrations, respectively, than the other cultivars, although this was not consistent during the whole cropping period (**Figure 4B**).

The total nitrogen (NO_3-N and NH_4-N) concentrations in the recirculating NS solution (**Figure 5C**) and the leaves of pepper (**Figure 5A**) showed a slight decreasing tendency during the cropping period, while the total-N UC followed an increasing course up to the commencement of harvesting and shortly thereafter (**Figure 5B**). However, the total-N UC remained constant during the harvesting period. The self-grafted 'Sammy' plants tended to take up less N per L of water after the start of flowering and at the very early stage of harvesting but thereafter the N UC were not influenced by the pepper cultivar. The fruit N concentrations were not measured due to a technical failure which resulted in impairment of the samples.

The P concentration in the recirculating NS showed an increasing tendency in the interval from planting up to the start of flowering in self-grafted 'Sammy,' 'Orangery,' and 'Sondela' (**Figure 6C**) which was accompanied by a commensurate decrease in the P UC at the same time interval (**Figure 6D**). In contrast, the P UC and the P levels in the recirculating NS of 'Sammy' grafted onto RS10 and 'Belisa' did not change by the time of flowering commencement in comparison to the initial

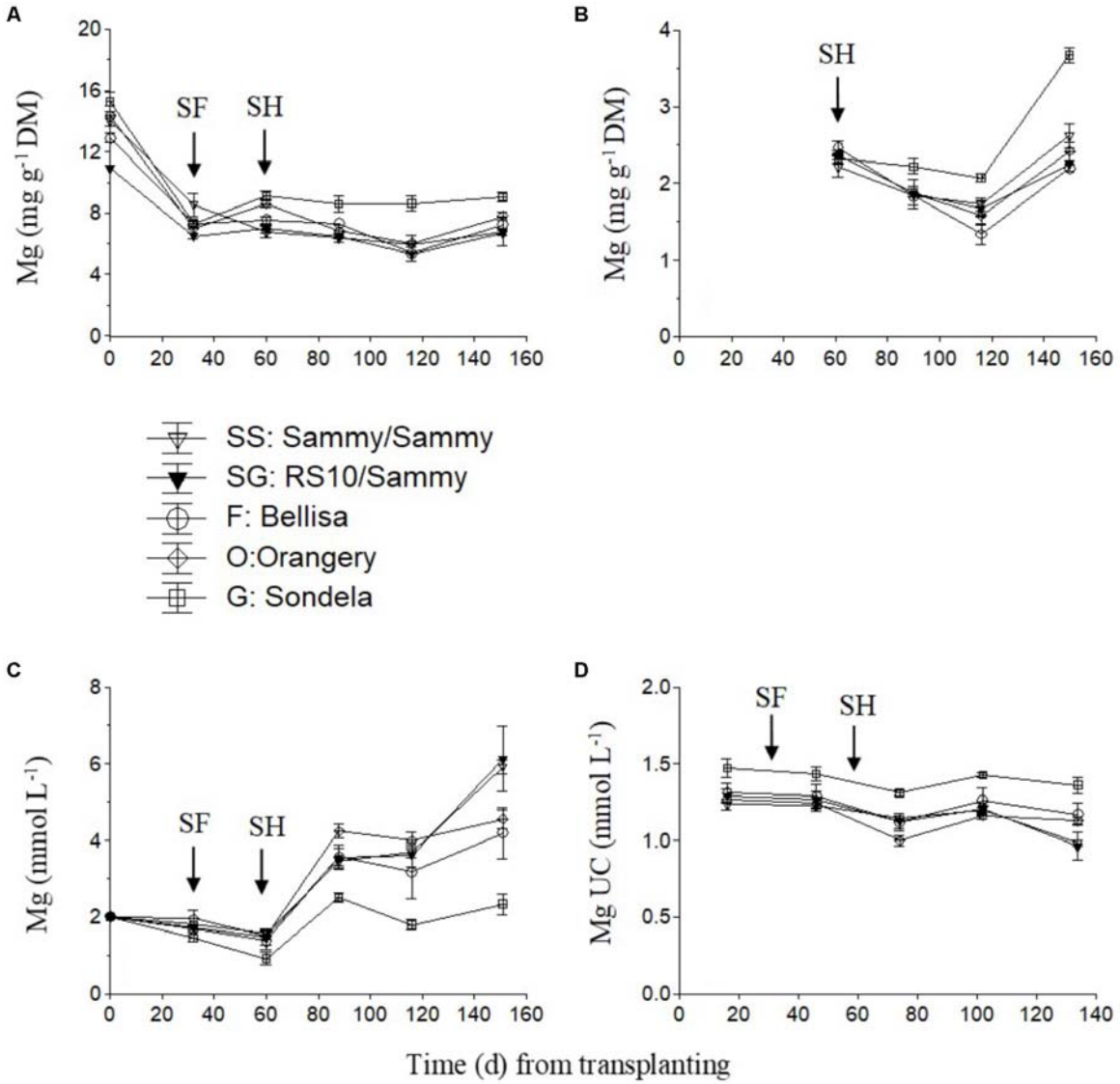

FIGURE 3 | Impact of different pepper cultivars ('Orangery,' 'Bellisa,' 'Sondela,' 'Sammy') and grafting ('Sammy' self-grafted or grafted onto the commercial rootstock 'RS10') on: (i) concentrations of Mg in the dry mass (DM) of young leaves **(A)** and fruit **(B)**, (ii) concentrations of Mg in the recirculating nutrient solution **(C)**, and (iii) apparent Mg uptake concentrations (UC), i.e., mmol of Mg uptake per L of water uptake **(D)**. Vertical bars indicate ± standard errors of means. SF, start of flowering; SH, start of harvesting.

vegetative growth stage. After the start of flowering, both the P concentration in the recirculating NS and the P UC remained more or less constant up to crop termination. Nevertheless, the leaf P concentration showed a consistent tendency to decrease after planting in all cultivars, which lasted up to the start of flowering (**Figure 6A**). The fruit P concentrations did not differ significantly between the different experimental treatments while it exhibited no consistent tendency during the cropping period (**Figure 6B**).

For micronutrients, only the UC are shown (**Figure 7**). The UC of Mn and Zn were highest during the vegetative growth stage and decreased slightly but constantly after commencement of harvesting. No consistent differences in UC between the different cultivars were found, although 'Orangery' showed a lower UC than the other cultivars during the vegetative stage (**Figure 7A**) and 'Sondela' showed a higher UC than the other cultivars (**Figure 7B**) in the interval between the start of flowering and the start of harvesting. The Fe UC also decreased constantly during the cropping period from 19.5 to 16 μmol L^{-1} on average, without any significant differences between the tested cultivars (**Figure 7C**). The B UC decreased from 27.6 μmol L^{-1} on average during the vegetative growth stage to about 25 μmol L^{-1} after the start of harvesting and remained roughly constant to these levels during the reproductive stage (**Figure 7D**). So significant differences between treatments could be found during the vegetative stage of growth, while during the late reproductive

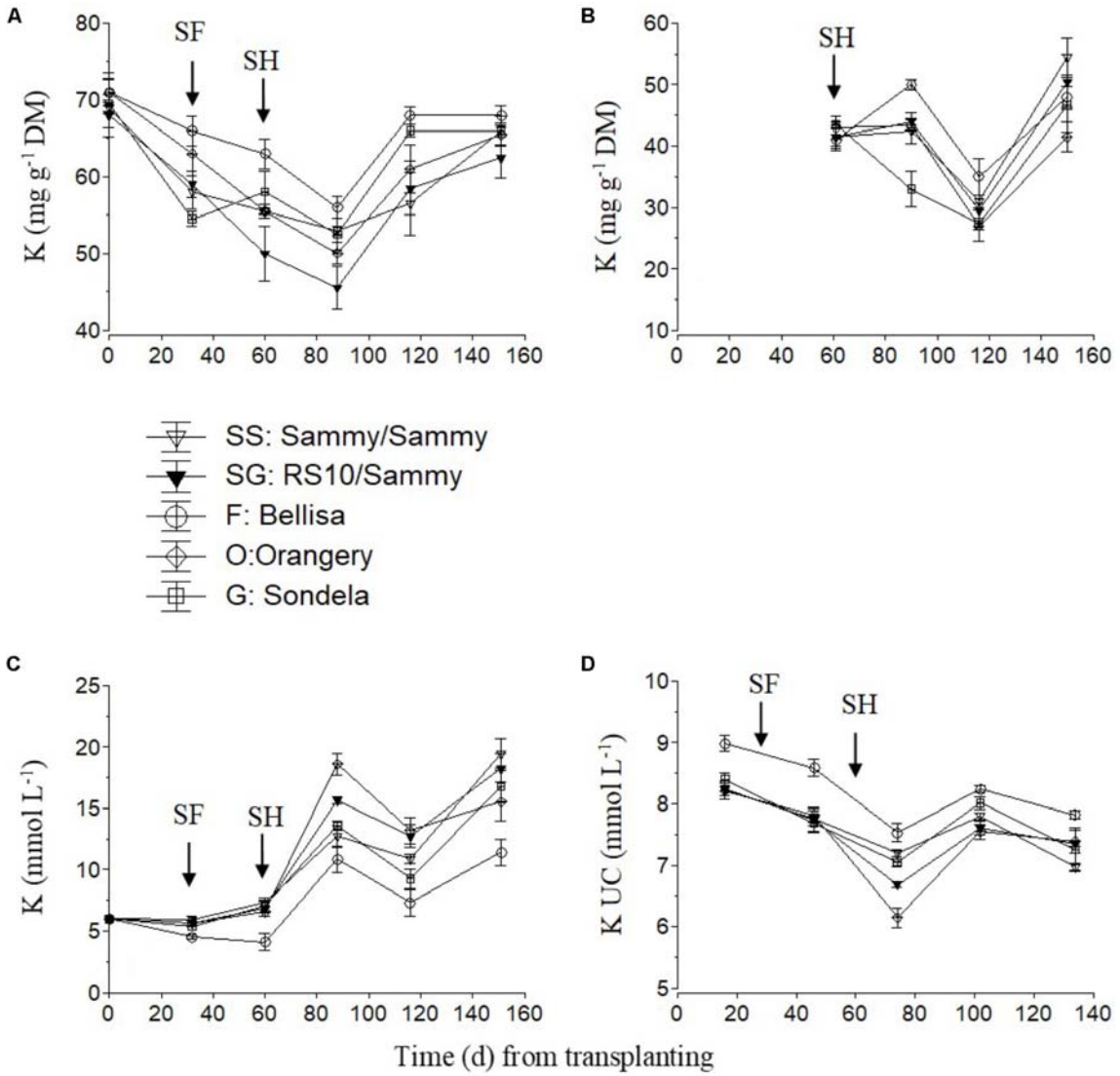

FIGURE 4 | Impact of different pepper cultivars ('Orangery,' 'Bellisa,' 'Sondela,' 'Sammy') and grafting ('Sammy' self-grafted or grafted onto the commercial rootstock 'RS10') on: (i) concentrations of K in the dry mass (DM) of young leaves **(A)** and fruit **(B)**, (ii) concentrations of K in the recirculating nutrient solution **(C)**, and (iii) apparent K uptake concentrations (UC), i.e., mmol of K uptake per L of water uptake **(D)**. Vertical bars indicate ± standard errors of means. SF, start of flowering; SH, start of harvesting.

stage (after the first 3 months from planting) the B UC of 'Sondela' was significantly higher than those of the other tested cultivars. Grafting of 'Sammy' onto the rootstock RS10 had no significant impact on the UC of micronutrients.

DISCUSSION

The present study showed that the developmental stage of the pepper plants has a strong impact on the nutrient to water uptake ratios (UC). For most nutrients, i.e., K, Ca, Mg, and all micronutrients studied, the UC tended to decrease with time and only the UC of total-N and P increased slightly after the initial vegetative stage. By definition, the UC (nutrient to water uptake ratios) depend on two variables, i.e., the rates of nutrient uptake and the rates of water uptake, which are independent to each other, since the plant metabolism is physiologically not related to transpiration (Taiz and Zeiger, 2002). Thus, any difference in the UC at different plant developmental stages or between different cultivars may be due to commensurate differences either in the nutrient uptake rates, or in the water uptake rates, or in both. As the plants are growing up, the leaf area increases and this results in increased rates of whole plant transpiration (Taiz and Zeiger, 2002). On the other hand, an increased leaf area results in increased rates of whole plant photosynthesis (Koyama and Kikuzawa, 2009), thereby raising the nutrient demand and

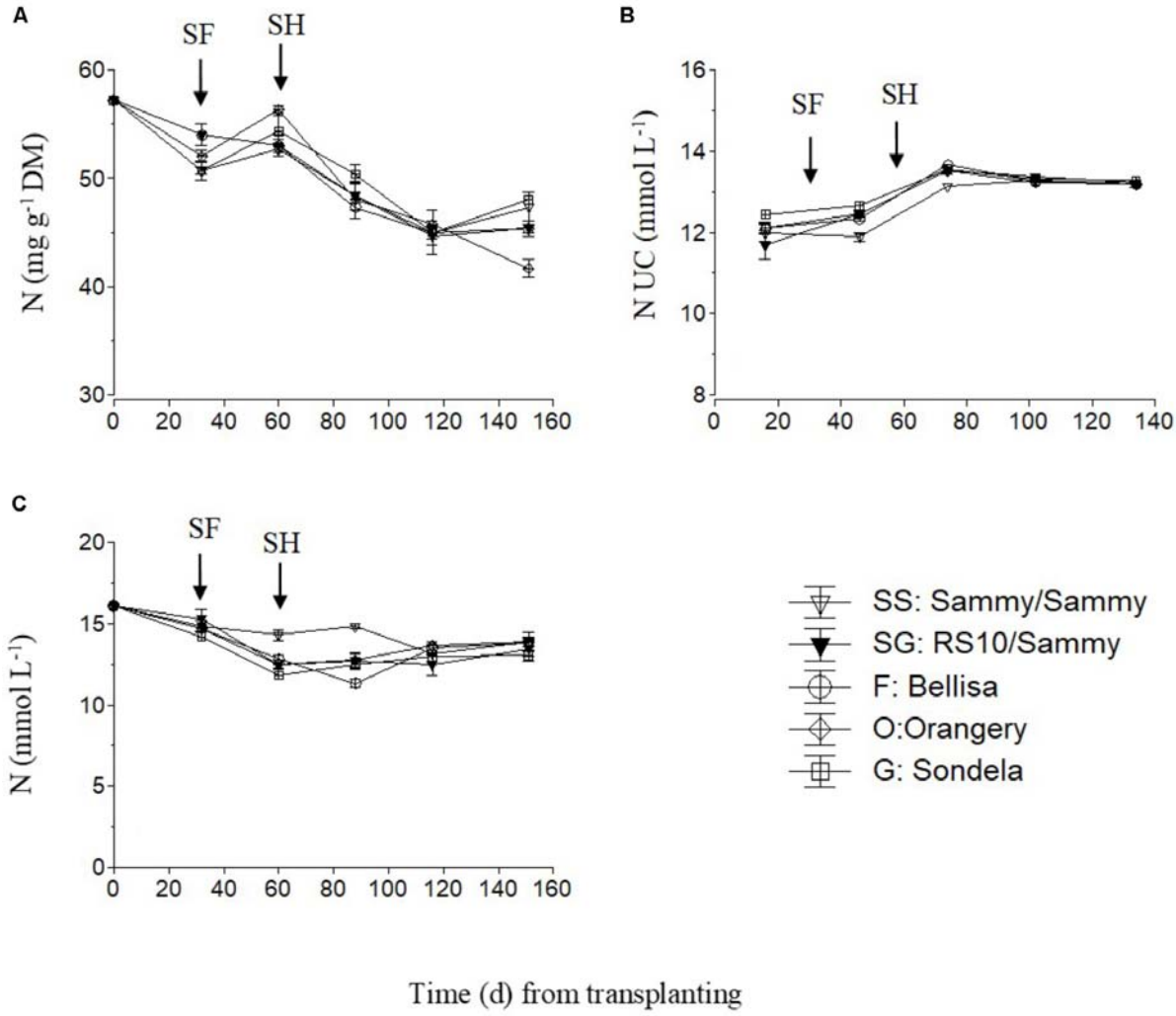

FIGURE 5 | Impact of different pepper cultivars ('Orangery,' 'Bellisa,' 'Sondela,' 'Sammy') and grafting ('Sammy' self-grafted or grafted onto the commercial rootstock 'RS10') on: (i) concentrations of total-N in the dry mass (DM) of young leaves **(A)**, (ii) apparent total-N uptake concentrations (UC), i.e., mmol of total-N uptake per L of water uptake **(B)**, and (iii) concentrations of total-N in the recirculating nutrient solution **(C)**. Vertical bars indicate ± standard errors of means. SF, start of flowering; SH, start of harvesting.

concomitantly the nutrient uptake rates. As a result, although the nutrient uptake and the water uptake are independent processes, the UC do not change dramatically during the cropping period, as has been shown in several studies (Savvas and Lenz, 1995; Sonneveld and Voogt, 2009). However, morphological changes at different plant developmental stages may differently affect the nutrient uptake rates than the water uptake rates. Thus, at the early plant developmental stages, all or most leaves are young, while at later developmental stages, when the plants enter the reproductive phase, a considerable part of the foliage consist of older leaves (Paltridge and Denholm, 1974). The rates of transpiration are much less affected by the age of the leaves than the rates of net photosynthesis. Consequently, as the age of the plants increases, the water uptake rates increase more than the nutrient uptake rates and thus the UC decrease, although in absolute terms the nutrient demand increases. The tendency of the UC for most nutrients (except of N and P) to decrease with time in the present study seems to result mainly from a stronger increase in water uptake rates with time than in the nutrient uptake rates.

The exceptions formed by the UC of N and P, which increased slightly as the plants entered the reproductive phase, may indicate a stronger increase in the N and P demand during the reproductive stage than the increase in the whole plant transpiration. An increase in the P demand by pepper as the plants pass from the vegetative to the reproductive stage can be ascribed to the appreciably higher P concentrations in the fruit than in the leaves of pepper. Indeed, as reported by Silber et al. (2005), the P concentrations in the leaves of pepper ranged from 2.1 to 3.7 mg g^{-1}, depending on irrigation frequency and P application rate, while the corresponding values in the fruit ranged from 6.2 to 7.5 mg g^{-1}. Similar differences in the P

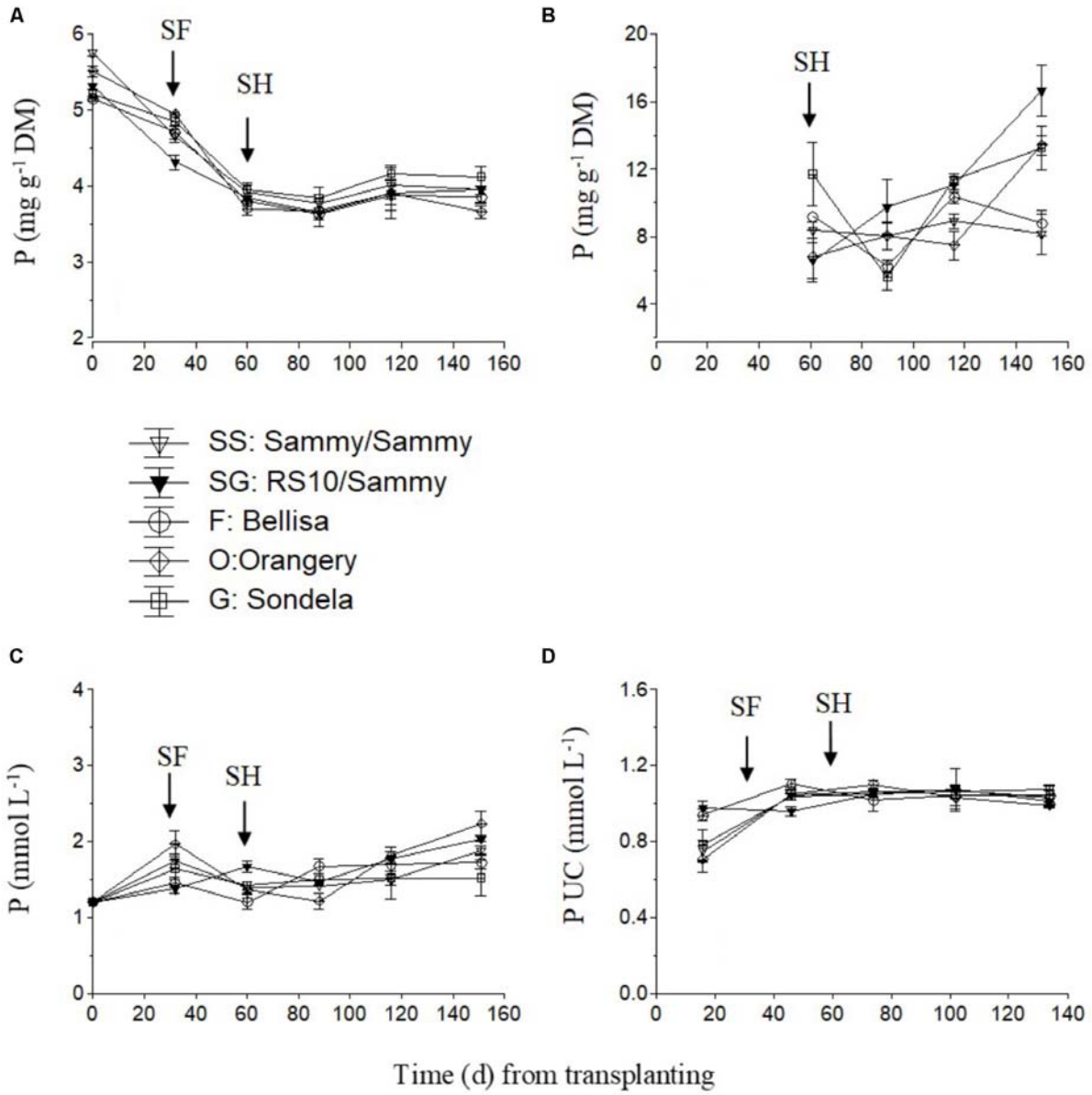

FIGURE 6 | Impact of different pepper cultivars ('Orangery,' 'Bellisa,' 'Sondela,' 'Sammy') and grafting ('Sammy' self-grafted or grafted onto the commercial rootstock 'RS10') on: (i) concentrations of P in the dry mass (DM) of young leaves **(A)** and fruit **(B)**, (ii) concentrations of P in the recirculating nutrient solution **(C)**, and (iii) apparent P uptake concentrations (UC), i.e., mmol of P uptake per L of water uptake **(D)**. Vertical bars indicate ± standard errors of means. SF, start of flowering; SH, start of harvesting.

concentration between leaves and fruit were found also in the present study (**Figure 6**). However, the nitrogen concentrations tend to be higher in the leaf than in the fruit (e.g., 48.6 vs. 37.7 mg g^{-1}, respectively, as reported by Bar-Tal et al., 2001). Hence, the slight increase in the N UC as the plants entered the reproductive stage in the present study cannot be ascribed to increased fruit N concentrations compared to those in leaves. An alternative explanation is a possible increase in the rates of denitrification as the crop was aging, which can be ascribed to the gradual increase in the air temperature, given that the crop was established on 16 January and the commencement of the reproductive stage coincided with the advent of the spring season. Nitrogen losses through denitrification in hydroponic NSs may be considerable, reaching levels up to 20% of the total supply, as has been shown by Daum and Schenk (1998). According to the method applied in the present study to estimate UC, denitrification losses are taken as apparent N uptake and, therefore, they tend to increase the estimated UC.

Since the UC represent uptake ratios between a nutrient and water, significant differences in the UC of any nutrient between cultivars originate from differences either in whole

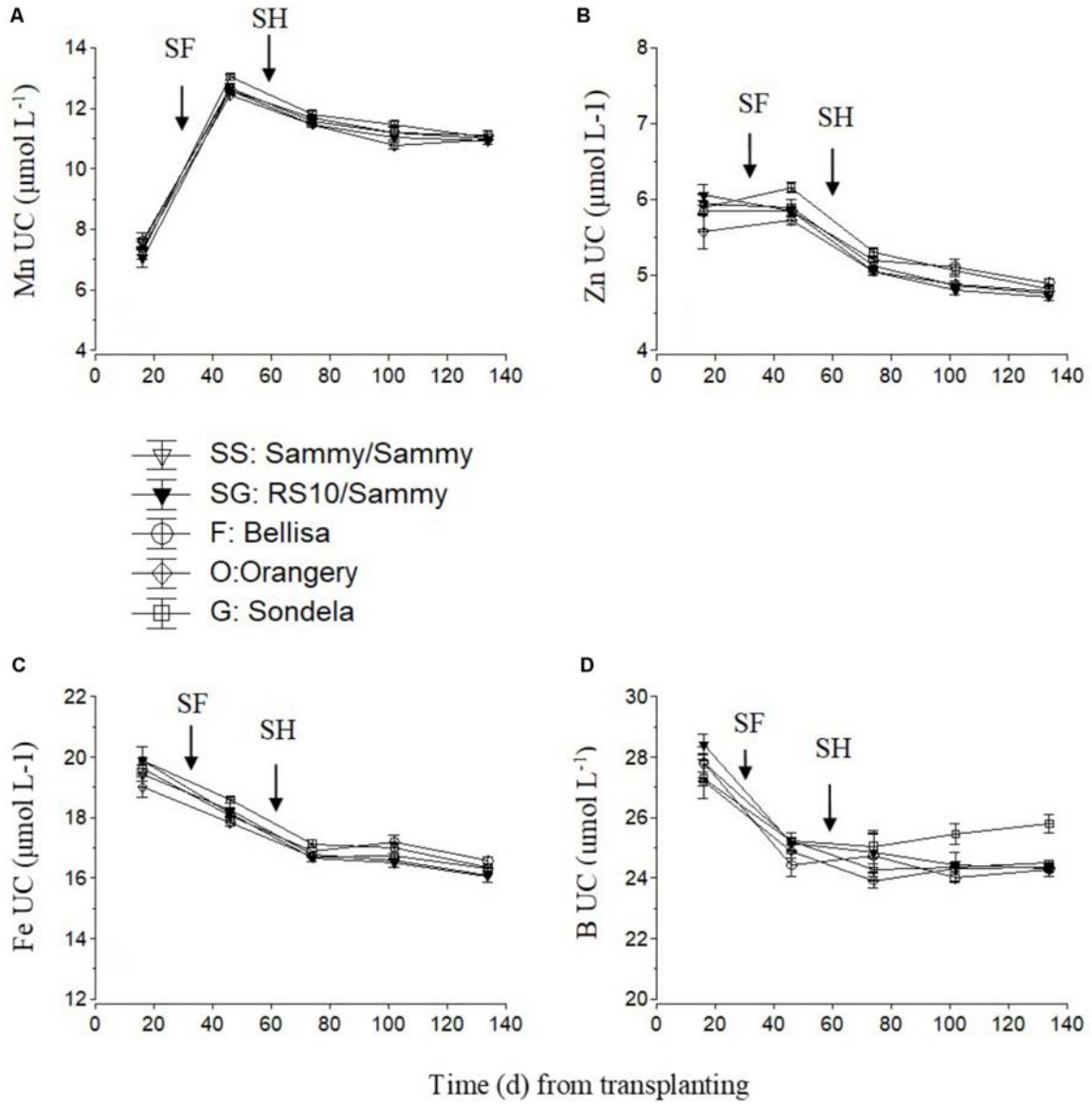

FIGURE 7 | Estimated apparent uptake concentration of micro-nutrient (UC), (i.e., mmol of micro-nutrient uptake per L of water uptake), as influenced by four cultivated varieties. i.e., 'Orangery' (O), 'Bellisa' (F), 'Sondela' (G), and 'Sammy' self-grafted (SS) or 'Sammy' grafted onto a commercial rootstock 'RS10' (SG). UC was calculated on the basis of **(A)** manganese (Mn), **(B)** zinc (Zn), **(C)** iron (Fe), and **(D)** boron **(B)** and water removal from the recirculating nutrient solution. Vertical bars indicate ± standard errors of means. SF, start of flowering; SH, start of harvesting.

plant transpiration owing to leaf area differences, or in the nutrient demand, or in a combination of them. Furthermore, any differences in the nutrient demand between cultivars are mainly due to commensurate differences either in tissue nutrient concentrations or in the fruit biomass. The significantly higher Ca and Mg UC by Sondela and K UC by Bellisa in comparison with those recorded in all other genotypes, which were consistent during the whole cropping period, are partly ascribed to lower whole plant transpiration (**Figure 1**). 'Sondela' and 'Bellisa' are cultivars grown for the production of red pepper fruits, which need longer time to ripen than those harvested green, as is the case with 'Sammy.' It is well known that the delay in fruit harvesting, which increases the fruit load, retards the vegetative growth (Engels et al., 2012), thereby reducing the leaf area per plan and concomitantly the whole plant transpiration. Nevertheless, the selective increase of only the UC of Ca and Mg in Sondela and K in Bellisa suggests that not only the whole plant transpiration but also the nutrient requirements are different between different cultivars. Indeed, while the whole plant transpiration was lower in both 'Sondela' and 'Bellisa' in comparison to the other genotypes, the leaf Ca and Mg concentrations were higher only in 'Sondela' during the reproductive stage of development. Hence, the factor that ultimately imposed higher Ca and Mg

UC in 'Sondela' than in the other genotypes was the higher concentration of Ca in the leaves, and Mg in both leaves and fruit of 'Sondela' (**Figures 2B**, **3B**). The reduced Ca and Mg concentrations in the NS that was recirculating in circuits accommodating 'Sondela' are in agreement with the higher Ca and Mg UC found for this cultivar. The same applies also for K in 'Bellisa.' The significantly higher K UC in 'Belisa' were also due to a combination of higher whole plant transpiration and higher leaf K concentrations as indicated by **Figures 1** and **4A**.

The UC of total-N and P do not seem to be influenced by the genotype of the cultivar. Finally, the UC of the micronutrients Fe, Mn, Zn, and B did not show a consistent influence of the genotype, although 'Sondela' exhibited significantly higher Zn and B UC than the other cultivars at certain developmental stages. Sondela exhibited higher boron UC only during the reproductive developmental stage, which points to an effect of the fruit load on boron uptake rather than a transpiration-related effect on B UC.

An appreciable body of related investigations has indicated that grafting may decrease the uptake of some nutrients while increasing the uptake efficiency for some other nutrients depending mainly on the rootstock genotype (Ruiz et al., 1997; Rouphael et al., 2008; Savvas et al., 2009; Colla et al., 2010). Therefore, grafting has been suggested as a means to limit nutrient and heavy metal toxicity, or to increase fertilizer use efficiency and prevent nutrient deficiencies in marginally fertile soils (Savvas et al., 2013; Rouphael et al., 2016). Many rootstocks used to graft vegetables are wild genotypes of the same species as the scion, relatives, or hybrids of them, which are characterized by more vigorous root systems than those of highly productive cultivars (Huang et al., 2010; Pico et al., 2017). However, the pepper rootstock RS10, which was used to graft 'Sammy' in the current research, belongs to the same species with the scion (*Capsicum annuum*) and not to any wild relative species of paper characterized by a vigorous root system. This is presumably the reason for the lack of any differences in leaf and fruit nutrient levels as well as in UC between self-grafted 'Sammy' plants and 'Sammy' plants grafted onto the rootstock RS10.

Comparing the UC found in the present study with those found by Voogt and Sonneveld (1997) in North European hydroponic greenhouses reveals that they are influenced by the contrasting climatic conditions. Indeed, the UC found by Voogt and Sonneveld (1997) in a pepper crop grown in a closed rockwool system in the Netherlands, averaged for the whole cropping period, were 2.2 mmol L^{-1} for Ca, 0.78 mmol L^{-1} for Mg, 4.6 mmol L^{-1} for K, 10.30 mmol L^{-1} for N, and 0.81 mmol L^{-1} for P. The corresponding UC found in the present study ranged from 2.4 to 3.7 mmol L^{-1} for Ca, 1.0 to 1.5 mmol L^{-1} for Mg, 6.1 to 9 mmol L^{-1} for K, 11.7 to 13.7 mmol L^{-1} for N, and 0.7 to 1.1 mmol L^{-1} for P. A comparison between the values found in the two different studies reveals that the UC of macronutrients found in the present study are clearly higher than those reported by Voogt and Sonneveld (1997). In a similar study (Savvas et al., 2017), tomato plants grown under Mediterranean climatic conditions had an increased need for N, P, K, Ca, Zn, and Cu and a decreased need for Fe, Mn, and B in comparison with the UC reported under North European climatic conditions (Sonneveld and Voogt, 2009). The differences in UC between greenhouses located in these two contrasting environments may originate from differences either in the mean light interception, as reported by Adams (1993), or in the method applied to determine the UC, as reported by Tzerakis et al. (2013), Neocleous and Savvas (2015), and Savvas et al. (2017). The method applied in the present study to determine the UC takes into consideration possible precipitation losses. Therefore, the obtained values should be correctly termed "apparent UC" (Adams, 2002). However, for a sufficient supply of nutrients to plants grown in CHS, it is essential to provide the amount of nutrients corresponding not only to net uptake but also to losses through precipitation or immobilization that may occur during the flow of the NS along the system. Therefore, the apparent nutrient UC determined in the present study constitute a sound basis to establish suitable NS compositions for pepper crops cultivated in CHS under Mediterranean climatic conditions.

CONCLUSION

The results of the present study indicated that different pepper cultivars may take up nutrients and water at different ratios under the same nutritional, irrigation, and climatic conditions, as indicated by the observed differences in the UC. More specifically, 'Sondela' exhibited the highest Mg and Ca UC throughout the cropping period and B UC during the reproductive stage in comparison with all other tested cultivars. Furthermore, 'Bellisa' exhibited significantly higher K UC throughout the cropping period in comparison with all other tested cultivars.

The tissue nutrient concentrations and the UC were similar in 'Sammy' self-grafted and 'Sammy' grafted onto the commercial rootstock 'RS10' (*Capsicum annuum*), which indicates that this *Capsicum annum* rootstock does not modify the uptake of nutrients and water by the scion. The developmental stage of the pepper plants had a strong impact on the UC of most nutrients and this is ascribed to changes in the mean physiological age of leaves and differences in fruits load. Based on the UC estimated in the present study, the NSs supplied to closed hydroponic crops of pepper should contain Ca, Mg, and B at higher concentrations than standard recommendations when the cultivated variety is 'Sondela' and K at higher concentrations when the cultivated variety is 'Bellisa.'

AUTHOR CONTRIBUTIONS

GN, CK, NK, and DS conceived and designed the experiments. AR and GN performed the experiments. AR, GN, NK and DS analyzed the data and wrote the paper. CK, NK, and DS reviewed the paper. All authors have read and approved the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 19 September 2018
doi: 10.3389/fpls.2018.01382

Production of Low-Potassium Content Melon Through Hydroponic Nutrient Management Using Perlite Substrate

*Md. Asaduzzaman[1,2], Md. Raihan Talukder[2,3], Hideyuki Tanaka[2], Makoto Ueno[2], Mikiko Kawaguchi[4], Shozo Yano[5], Takuya Ban[6] and Toshiki Asao[2]**

[1] Olericulture Division, Horticulture Research Centre, Bangladesh Agricultural Research Institute, Gazipur, Bangladesh, [2] Department of Agriculture, Faculty of Life and Environmental Science, Shimane University, Matsue, Japan, [3] Department of Environmental Science, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, Bangladesh, [4] Faculty of Home Economics, Otsuma Women's University, Tokyo, Japan, [5] Faculty of Medicine, Shimane University, Izumo, Japan, [6] Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Japan

Chronic kidney disease patients are restricted to foods with high potassium content but our daily diets including melon are rich in potassium. Therefore, we investigated the production of low-potassium melon through hydroponic nutrient management in soilless culture using perlite substrate during autumn season of 2012, 2014 and spring season of 2016. In the first study, melon plants were supplied with 50% standard 'Enshi' nutrient solution until first 2 weeks of culture. In 3rd and 4th week, amount of applied potassium was 50, 75, 100, and 125% of required potassium nitrate for each plant per week (based on our previous study). It was found that, melon plants grown with 50% of its required potassium nitrate produced fruits with about 53% low-potassium compared to control. In the following study, four cultivars viz. Panna, Miyabi shunjuukei, Miyabi akifuyu412, and Miyabi soushun banshun309 were evaluated for their relative suitability of low-potassium melon production. Results showed insignificant difference in fruit potassium content among the cultivars used. Source of potassium fertilizer as potassium nitrate and potassium sulfate and their restriction (from 1 or 2 weeks after anthesis) were also studied. There were no influences on fruit potassium content and yield due to sources of potassium fertilizer and restriction timings. In our previous studies, it was evident that potassium can be translocated from leaves to fruits at maturity when it was supplied nutrient without potassium. Thus, we also studied total number of leaves per plant (23, 24, 25, 26, and 27 leaves per plant). It was evident that fruit potassium, yield, and quality were not influenced significantly due to differences in number of leaves per plant. These studies showed that restriction of potassium nitrate in the culture solution from anthesis to harvest could produce melon fruits with low-potassium (>20%) content compared to potassium content of greenhouse grown melon (340 mg/100 g FW). Quality testing and clinical validation of low-potassium melon also showed positive responses compared to greenhouse grown melon.

Keywords: melon, potassium restriction, low-potassium melon, soilless culture, perlite substrate, chronic kidney disease

OPEN ACCESS

Edited by:
Nikos Tzortzakis, Cyprus University of Technology, Cyprus

Reviewed by:
Antonios Chrysargyris, Cyprus University of Technology, Cyprus
Youssef Rouphael, Università degli Studi di Napoli Federico II, Italy

****Correspondence:***
Toshiki Asao
asao@life.shimane-u.ac.jp

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *30 May 2018*
Accepted: *30 August 2018*
Published: *19 September 2018*

Citation:
Asaduzzaman M, Talukder MR, Tanaka H, Ueno M, Kawaguchi M, Yano S, Ban T and Asao T (2018) Production of Low-Potassium Content Melon Through Hydroponic Nutrient Management Using Perlite Substrate. Front. Plant Sci. 9:1382. doi: 10.3389/fpls.2018.01382

INTRODUCTION

Potassium plays important role in human body and maintains normal functioning of muscles, heart, and nerves through acid base equilibrium, enzymatic activation, and renal function (Russell, 2009; Crawford and Harris, 2011). It acts as the main electrolytes, largely accumulated within body cells and usually excreted through kidneys. However, patients with kidney dysfunctions can't excrete it completely and thus concentrated in blood outside cells. This increased levels of potassium inside human body cause hyperkalemia (Kes, 2001), which is a common life threating issue for the chronic kidney disease (CKD) patients (Jain et al., 2012). They are advised to avoid foods with higher potassium content. However, our daily dietary items including melon are rich in potassium (Weiner and Wingo, 1998). This food restriction becomes more severe in case of CKD patient taking dialysis. They are also suggested not to take raw vegetables with high potassium content. It is reported that before eating, excessive potassium in these vegetables can be partially removed by cutting into smaller pieces, boiled or soaked sufficiently in water (Burrowes and Ramer, 2008). Vegetables preparation following above methods may also result in loss of other nutrients and water soluble vitamins and minerals, and breakdown of desirable texture and taste as reported in lettuce (Yakushiji and Kagawa, 1975; Kimura and Itokawa, 1990). Therefore, production and supplementation of fruits and vegetables containing lower potassium would greatly improve dietary components of CKD patients.

Recently, hydroponic production technologies of low-potassium fruits and vegetables such as melon, strawberry, tomato, spinach, and lettuce have been developed in Japan (Ogawa et al., 2007; Asao, 2011; Mondal et al., 2016; Tsukagoshi et al., 2016). In general, quantitative management of nutrient solution was applied to restrict potassium nutrition without hampering plants normal growth and development. Plants were grown with standard nutrient solution during the early growth stage and then the culture solutions were replaced either by a non-potassium hydroponic fertilizer or supplied nutrient solution without potassium fertilizer at the later growth stage. Following this method potassium content of fruits and vegetables can be reduced without hampering normal growth and development of plants. This type of cultivation method is now commercially applied by Aizufujikako Co., Ltd. (Tokyo, Japan) to develop low-potassium content leaf lettuce. Hydroponic nutrient solution contains sufficient amount of essential nutrients and plant roots can uptake them luxuriously. If it is applied continuously, plants can uptake essential ions at very low concentrations. In a study, no adverse effect on growth, fruit yield, and fruit quality in tomato was reported when there is reduction of macronutrient concentrations to 50% of the control level (Siddiqi et al., 1998). High levels of potassium in the nutrient solution were found to increase fruit dry matter, total soluble solid content, and lycopene concentration of tomato (Fanasca et al., 2006) and strawberry (Ebrahimi et al., 2012; Rodas et al., 2013). Source of potassium fertilizer as potassium sulfate had influence on yield, and quality of passion fruit (Costa-Araujo et al., 2006) and strawberry (Khayyat et al., 2007) while potassium nitrate found to have no influence in pepper (Flores et al., 2004).

According to a dietitian, dialysis patients can eat melon if it contains 40% reduced potassium of a general melon (Standard Tables of Food Composition in Japan, 2011). In this regard, our research group aimed at developing low-potassium melon fruits with reduced potassium compared to general melon. In previous studies, we succeeded in reducing the potassium concentration in melon fruit (40% or more) by limiting the potassium nitrate concentration in the culture solution from the vegetative growth stage till harvest in container based hydroponics (Asao et al., 2013). When melon plants were grown with 1/4 potassium nitrate of standard nutrient solution, fruits potassium decreased to about 39% compared to control. In the following study, it was found that melon plants cultured with 1/16 and 0 levels of potassium nitrate produced fruits with 35 and 43% reduced potassium, respectively, compared to control. However, stable production of melon fruits with reduced potassium under large scale and using commercial soilless substrate would be useful and practical.

In solution culture, excessive potassium absorption and its accumulation in plant parts and remobilization to edible sink have been reported (Asao et al., 2013). Thus, it is difficult to find out the minimal potassium requirement to the plants. It was evident that, if potassium supply is restricted to zero then growth hampers, fruit quality decreases and fruit cracking occur. In this regards, use of suitable soilless media can provide ideal conditions of potassium absorption and increase both yield and quality crops. Rockwool has been widely used as growing media for horticultural crops because it has stable structure, high water holding capacity, and moderate porosity (Sonneveld, 1993; Smith, 1998; Raviv and Lieth, 2008; Asaduzzaman et al., 2012). However, this inorganic substrate is difficult to degrade, and waste often stockpiled resulting potential environmental risk (Cheng et al., 2011). Recently, perlite has emerged as an excellent growth medium for growing several horticultural crops including melon (Szmidt et al., 1988; Cantliffe et al., 2003; Hochmuth and Hochmuth, 2003; Fascella and Zizzo, 2005; Rodriguez et al., 2006). Its strong capillary attraction draws up solution from the bottom of substrate bag or container at the similar rate that the plants uptake water and nutrient leaving excess solution in the reservoir. Therefore, it enables optimum moisture conditions near root while in case of rockwool it is difficult to maintain optimum moisture levels because of its poor capillary action. In Florida, Holland, and United States, melons are being grown using 100% perlite substrate in bags or in containers. It can be recycled and reused for several years after cleaning and disinfecting properly (Hanna, 2005, 2006, 2010). Therefore, we used perlite substrate in potassium nutrient management studies for producing low-potassium melon.

We investigated four independent studies aiming stable production of low-potassium melons and their dietary supplementation to CKD patients. In this regard, several nutrient and cultural management practices were investigated to develop sustainable low-potassium melon production technology. Melon plants were grown in perlite substrate supplied with several percentages of required potassium during vegetative growth period. Effect of varietal differences, timing

of potassium restriction, and number of remaining leaves after pinching on growth, fruit quality, and potassium content of melons were studied.

MATERIALS AND METHODS

Melon Cultivar

Five cultivars of melon (*Cucumis melo* L. cv. Panna, Miyabi shunjuukei, Miyabi akifuyu412, Miyabi soushun banshun309 and Miyabi natsu206) were used for this study. These netted melons were grown in greenhouse following standing culture supported with jute ropes. One fruit per plant was maintained throughout the studies. The seeds of these melon cultivars were collected from Takii & Co., Ltd., Kyoto, and Yokohama Uki Co., Ltd., Tokyo, Japan. The cultivars have excellent sweetness with either green or orange flesh.

Enshi Nutrient Solution

Melon plants were grown in soilless culture using perlite bags with 50% 'Enshi' nutrient solution which is generally recommended for melon cultivation in Japan with an electrical conductivity (EC) of 1.32 dS/m and pH of 6.93 (Hori, 1966; **Supplementary Table S1**). In this present study, we have reduced the amount of potassium nitrate keeping other nutrients constant to produce low-potassium melon fruit in soilless culture. The slightly higher pH (6.93) of this nutrient solution, it is assumed to be contributed by the higher pH of tap water (pH 7.93) of laboratory that might lead to higher pH range of the nutrient solution used. It is mentionable that source of tap water in our research facility is the mountain nearby. The inherent nutritional composition of tap water used was also not so great but calcium, magnesium, and nitrate-nitrogen content were little higher (**Supplementary Table S2**). In our previous cultures and also in this present experiment, we did not find any nutritional deficiency in melon culture with pH value of 6.93.

Experimental Conditions

Four independent experiments were carried out in 100 m^2 glasshouses and 100 m^2 plastic house of Experimental Research Center for Biological Resources Science, Shimane University in hydroponics and soilless culture using perlite substrate. In the greenhouse, we used the only the central portion with plant density of 60 plants while in the plastic house 175 plants were accommodated in five different rows. The studies were conducted during the autumn season 2012, 2014 and spring season of 2016. The study area is generally characterized by a moderate weather condition. During the culture period of 2012, 2014, and 2016 the mean day/night temperatures were 26.9/20.0, 26.0/19.6, 25.1/19.4°C, respectively (**Supplementary Figure S1**).

Experiment I: Quantitative Management of Nutrient Solution of Melon Using Perlite Substrate

Seeds of melon cv. Panna were sown in cell trays with vermiculite. Germinated seedlings with high vigor and uniform in size were transplanted into plastic container with 50% 'Enshi' nutrient solution for nursery. After 1 week at five to seven leave stage, one seedling was planted in one plastic container filled with 30 L perlite substrate (**Supplementary Figure S2A**). For the first 2 weeks, melon plants were supplied with 50% standard 'Enshi' nutrient solution. In 3rd and 4th weeks, standard nutrient solutions were supplied in four splits viz. 50, 75, 100, and 125% of required potassium nitrate per plant (**Supplementary Table S3**). In our previous study, we calculated the amount of potassium nitrate required for one plant per week in hydroponic culture of melon (Asao et al., 2013). Female flowers of 11–14 nodes were kept for fruit development and others were removed. At these nodes, female flowers of secondary branches on first collateral node were pollinated and the branches were punched leaving the second node, and main shoot tips were punched at 25th node. After pollination, melon plants were supplied with standard nutrient solution without potassium nitrate. Melon plants grown with 50% standard nutrient solution in perlite and also in hydroponics were used as control.

Experiment II: Varietal Difference in Low-Potassium Melon Cultivation

Seeds of four melon cultivars viz. Panna, Miyabi shunjuukei, Miyabi akifuyu412, Miyabi soushun banshun309 were sown in cell tray with vermiculite on July 24, 2014. After germination, seedlings were transferred into plastic container with 50% 'Enshi' nutrient solution for nursery on August 31. Then similar size and vigor seedlings were transferred to plastic bag filled with 10 L perlite on August 11 (**Supplementary Figure S2B**). One plant was planted per bag for each variety having three replications and five bags in each replication. The plants were supplied with 50% standard nutrient solution. On the bottom of the perlite bag, a hole was made for discharging the surplus supplied nutrient solution. Potassium nitrate in the culture solution was supplied according to the growth of plants and the total supply amount became 30940 ml until 4 weeks then potassium nitrite supply was stopped (**Supplementary Table S4**). Pollination started on September 2 and female flowers of 11–14 nodes were kept for fruit development and others were removed. At these nodes, female flowers of secondary branches on first collateral node were pollinated and the branches were punched leaving the second node, and main shoot tips were pinched at 25th node on September 15. The cultivar "Panna" was harvested on October 29 and "Miyabi" cultivars were harvested on November 3.

Experiment III: Effects of Timing of Potassium Fertilizer and Potassium Deficiency on Melon Growth and Fruit Quality

Seeds of melon cv. Miyabi natsu206 were sown in cell trays with vermiculite on April 4, 2016. After germination, the similar sized seedlings with good growth were selected and transferred to plastic pots with perlite for raising seedlings as nursery on April 19. Then selected healthy plants were planted into plastic bag filled with 10 L of perlite on May 3. After planting at 5–6 leaf stage, the plants were supported with rope to standing upright with

iron wire (**Supplementary Figure S2C**). All the lateral shoots were removed except 12–14 nodes for flowering. Both male and female flowers booming started on June 3 and pollination of the flowers from 12 to 14 was done manually. After 7–10 days of pollination when size of the fruit was about the size of Ping-Pong balls, fruit thinning was performed leaving only one fruit per plant on June 13. In order to study the potassium content per fruit, we maintained one fruit per plant for all the cultures. At the same time, main shoot tips were punched at the 25th node. There were two timings of potassium fertilizer restriction for this experiment viz. 1 week after anthesis (June 11) and 2 weeks after anthesis (June 18). There were two sources of potassium fertilizer such as potassium nitrate and potassium sulfate. Melon plants were supplied with 50% standard nutrient solution from seedling planting to plant growth at 15th leaf stage. From May 28, plants were supplied with 75% nutrient solution (**Supplementary Table S5**).

Experiment IV: Influence of Different Number of Remaining Leaves on the Growth and Fruit Quality of Melons After Punching

Seeds of melon cv. Miyabi natsu206 were sown in cell trays with vermiculite on March, 2016. After germination, the seedlings of the same size with good growth were selected. On March 17, the seedlings were transferred to the seedling raising pot with perlite. A hole was drilled in a culture bag filled with 10 L of perlite and seedlings were planted on April 11. After transplanting, plants at 5–6 leaf stage were supported by jute rope to iron wire (**Supplementary Figure S2C**). All the lateral shoots were removed except 12–14 nodes for flowering. Both male and female flowers booming started on May 19 and pollination of the flowers from 12 to 14 was done manually. After 7–10 days of pollination when the size of the fruit was about the size of Ping-Pong balls, fruit thinning was performed leaving only one fruit per plant. At the same time, main shoot tips were pinched at the nodes leaving 23–27 leaves on June 3. Potassium nitrate was used as the potassium fertilizer for this culture. The timing of limiting potassium nitrate was 1 week after anthesis on May 16. From transplanting to the 17th leaf stage, melon plants were supplied with 50% standard nutrient solution. From May 14, the plants were supplied with 75% standard nutrient solution (**Supplementary Table S6**).

Analysis of Melon Fruit Qualities

Melon fruits were harvested and stored for 4 days (cv. Panna) and 7 days (cv. Miyabi) at room temperature for maturation. After maturation, melon fruits were cut into small pieces, mixed by juicer (Zojirushi BM-RS08-GA, Zojirushi Corporation, China) and then mixed juice was used for analyzing soluble solids, titratable citric acidity, and ascorbic acid. Soluble solid contents in melon sample were measured using a pocket digital refractometer (PAL-1, Atago Ltd., Tokyo, Japan) and repeated measurements were conducted after washing the prism with distilled water and also rinsed with the test juice. For determination of titratable acidity, 2 ml of melon juice were poured into a conical flask with 8 ml of distilled water and then 2 drops of phenolphthalein were added. At measurement, pH was adjusted to 8.2 using 0.1 N (w/v) NaOH. The quantity of NaOH (ml) required, and the amount for appropriate acidity was converted to citric acidity (%). Ascorbic acid contents in melon samples were determined following 2,4-dinitrophenylhydrazine (DNP) colorimetry. In 50 ml glass test tube, 0.5 ml of melon juice were taken and then 0.5 ml of 10% meta-phosphoric acid solution, 1 ml of distilled water, 1 ml of 0.03% 2,6-dichlorophenol–indophenol (DCP), 2 ml of thiourea, and 1 ml of DNP were added. The samples were then incubated for 3 h at 37°C in water bath (BW400, Yamato Scientific Co., Ltd., Japan). After incubation 5 ml of 85% H_2SO_4 were added keeping the test tubes cold water with ice bag. After cooling for about 30 min, ascorbic acid content was measured at 520 nm by Spectrophotometer (U-2900, Hitachi High Technologies Corporation, Tokyo, Japan).

Determination of Potassium and Other Mineral Content in Melon Fruit

The concentration of potassium, calcium, magnesium, iron, and sodium in melon fruits was measured using polarized Zeeman Atomic Absorption Spectrophotometer (Z-2310, Hitachi High Technologies Corporation, Tokyo, Japan). After maturation, edible parts of melon fruits (about 10 g) were measured by analytical Balance, XS204DRV, Greifensee, Switzerland) and placed in a 250 ml plastic bottle that contains 200 ml of 1% HCl. Then the samples were shacked in a bio shaker (Bio-Shaker BR-43FL, Japan) for 30 min at 150 rpm for complete extraction of mineral nutrients. Fruit samples were then filtrated (Advantec Grade No. 131) and analyzed for mineral contents.

Determination of Potassium and Other Mineral Content in Melon Plant Parts

Melon plant parts were divided into leaves, stem, and roots (only for hydroponic culture) and dried in a constant temperature oven (DKN 812, Yamato Scientific Co. Ltd., Japan) at 80°C for at least 72 h. Then plant parts were ground with a mixer machine (National MX-X53, Japan). Powdered samples (0.5 g) were mixed with 8 ml of HNO_3 and digested using microwave sample preparation system (ETHOS1, Milestone S.r.l, Bergamo, Italy). The digested samples were measured up to 50 ml of volumetric flask with distilled water and then filtered through qualitative filter paper (Grade no. 131). The filtered sample solutions were analyzed for potassium, calcium, magnesium, iron, and sodium by Zeeman Atomic Absorption Spectrophotometer.

Statistical Analysis

Analysis of variance was performed to test for significant effects of different potassium nitrate levels in the nutrient solution, different variety, potassium fertilizers and time of restriction, and number of remaining leaves after pinching on the plant growth variables, fresh weight per fruit and qualities and mineral nutrients in plant parts of melon in all four studies. Mean separations were performed by Tukey–Kramer test (Statcel 2

Software, OMS publication, Tokorozawa, Saitama, Japan) at $P < 0.05$.

RESULTS AND DISCUSSION

Potassium is an essential mineral nutrient and it has major physiological role in normal growth and development of plants (Taiz and Zeiger, 1998; Schachtman and Liu, 1999; Mengel, 2007; Szczerba et al., 2009). Thus, its requirement and uptake by plant roots are also high (Tisdale and Nelson, 1975; Mäser et al., 2002; Britto and Kronzucker, 2008; Szczerba et al., 2009). Hydroponic nutrients contain sufficient amount of all essential minerals for plants luxurious uptake. However, plants can uptake essential nutrients even at very low concentrations from a standard concentration (Hoagland and Arnon, 1950; Hori, 1966). Therefore, higher concentrations of nutrients are either not used by plants or their uptake does not influence higher production (Zheng et al., 2005; Rouphael et al., 2008). In this study, we limited the potassium fertilizer in hydroponic culture solution and investigate its impact on the fruit potassium content of melon. Four independent melon cultures were carried out in both hydroponics and soilless culture during both spring and autumn seasons from 2012 to 2016. In hydroponic culture technique, nutrient management is easy, simple and accurate. Nutrient concentration and composition of the culture medium can be modified during any growth stages of plant. In our studies, we measured growth parameters, dry matter partitioning and yield of melon at different potassium management strategies. Mineral nutrient content including potassium was also investigated to understand the source-sink relations due to potassium restriction in the culture solution.

Quantitative Management of Nutrient Solution of Melon Using Perlite Substrate

In our previous study, we determined the amount of weekly potassium absorption per melon plant in order to apply potassium nutrition in a quantitative manner (Asao et al., 2013). It was found that potassium requirement was great until 4th weeks of planting and then the demand decreased sharply. The absorption of potassium per plant was 11, 20, 33, 76, and 69 ppm in 1st, 2nd, 3rd, 4th, and 5th week of vegetative growth, respectively. In addition, plants grown in standard nutrient solution absorbed a higher amount of potassium whereas plants grown in nutrient solution with decreased potassium levels absorbed potassium proportionate to the applied amount in culture solution. The above study concludes that potassium requirement of melon plants remain greater at vegetative growth stage before anthesis and then decreases gradually and during fruit development absorption turns to minimum. As the tap water used for this study contains low concentration of potassium (about 1.0 ppm), even if we grow plant in solution lacking potassium fertilizer, still it can absorb potassium at minimum levels throughout the growing period (**Supplementary Table S2**). Based on these results we applied potassium at different percentages of absorbed potassium per plant in a week in order to investigate the lowest limit of potassium requirement.

Plant Growth and Chlorophyll Content

Plant growth variable such as stem length, dry weight of leaves and stem, and photosynthetic parameters were significantly influenced by the potassium nitrate management in perlite substrate and hydroponics culture (**Table 1**). In general, stem length was found shorter in perlite culture compared to hydroponics. In hydroponics, plants absorbed mineral nutrient luxuriously leading larger plant body. On the other hand in perlite culture, plant roots move toward the required nutrients. Stem lengths from 1–11 nodes (S1) and 12–25 nodes (S2) were significantly similar in all levels of potassium nitrate applied. Dry weights of leaves and stem were significantly higher in plants grown in hydroponics than in perlite culture. Plants grown in perlite using different levels of potassium nitrate produced significantly similar dry weight except for leaves from 12–25 nodes (L2) in 50- and 75% potassium application per plant. Relatively higher chlorophyll content was recorded in leaves in melon plants grown in hydroponics compared to perlite culture in both leaf positions (leaves at 15 and 20 nodes). In case of perlite culture, significantly lower leaf chlorophyll content was found in plant supplied with 50% of potassium nitrate. Dry matter production in plants grown in perlite was decreased to about 30–50% compared to plants grown in hydroponics. Similarly, it was reported that limited supply of potassium in the culture solution can inhibit growth and performance of tomato plants (Kanai et al., 2007).

Fruit Yield and Quality

It is reported that adequate amount of potassium is necessary for improving yields and fruit qualities such as fruit size and color, soluble solids and ascorbic acid content, shelf life and shipping quality of several horticultural crops (Geraldson, 1985; Lester et al., 2005, 2006, 2007; Kanai et al., 2007). In the present study, comparatively higher fruit yield was recorded in plant grown in hydroponics with 50% standard nutrient solution than perlite culture with 50% standard nutrient solution or its limited levels (**Table 1**). This yield reduction is mainly due to system differences of hydroponic solution culture and soilless culture using perlite substrate. It is evident from the results that fruit yield did not differ in the reduced potassium levels (50–125% of required potassium) and standard potassium nutrition. Fruit yield is supposed to decrease under potassium restricted cultivation. However, in our previous study fruit yield also not decreased due to potassium restriction in hydroponic solution (Asao et al., 2013). This indicates that the reduced levels of potassium fertilizer in the nutrient solution can still maintain potassium sufficiency for melon in hydroponics. Fruit qualities such as soluble solids and citric acid content did not differ significantly among the nutrient solutions applied either in hydroponics or in perlite culture. Plant cultivation under potassium limited condition needs special cultural practices. Simple reduction of potassium in the culture medium may not reduce the potassium in the fruits or edible parts. In this regards, the quantitative management of potassium in the nutrient solution during vegetative growth and restricted supply in the reproductive stage may result in fruits with lower potassium content. In the present study, potassium content in

TABLE 1 | Effect of limited supply of potassium in nutrient solution on the growth, chlorophyll content, yield and fruit qualities in leaves of melon grown in soilless culture using perlite substrate.

KNO_3 supply	Length (cm)		Dry weight (g)				SPAD		Fresh weight/fruit (g)	Soluble solids content (%)	Titratable citric acidity (%)
	S1	S2	L1	L2	S1	S2	L15	L20			
Hydroponics (50% std.)	103.7 a[y]	105.7 a	45.7 a	41.4 a	11.0 a	10.0 a	53.8 a	57.0 a	2410.4 a	14.7	0.20
Perlites (50% std.)	101.0 ab	98.5 ab	23.1 b	28.9 b	7.0 b	5.7 b	38.3 b	42.3 b	1750.4 b	15.5	0.19
50%[z]	98.8 ab	95.1 b	22.3 b	23.0 c	7.6 b	6.0 b	29.4 c	28.6 c	1647.3 b	14.0	0.15
75%	100.9 ab	98.9 ab	22.3 b	25.1 bc	7.1 b	5.8 b	34.4 bc	31.6 bc	1674.8 b	14.5	0.18
100%	95.4 b	97.0 b	22.7 b	27.4 b	7.7 b	6.3 b	33.4 bc	37.5 bc	1681.4 b	15.3	0.17
125%	101.2 ab	97.9 b	22.2 b	27.3 b	8.0 b	6.4 b	31.4 bc	35.0 bc	1724.4 b	15.3	0.17
Significance										ns	ns

[z]Percentage of potassium absorption per plant. [y]Means within a column followed by different letters are significantly different and "ns" indicates non-significant according to the Tukey's test at $P < 0.05$. L1, leaves from 1 to 11 nodes; L2, leaves from 12 to 25 nodes; S1, stem length from 1 to 11 nodes; S2, stem length from 12 to 25 leaves; L15, SPAD taken at leaf of 15 node; L20, SPAD taken at leaf of 20 node.

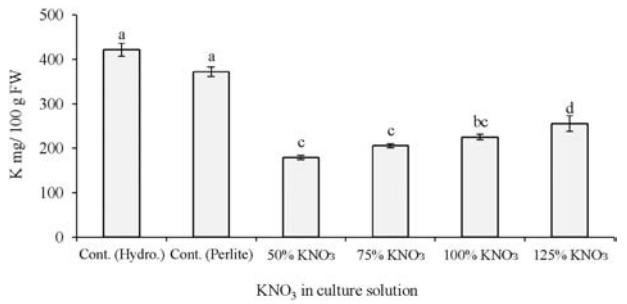

FIGURE 1 | Effect of limited supply of potassium fertilizer in the nutrient solution on the potassium content of melon fruits. Significant differences among treatments are indicated by different letters at $P < 0.05$. Error bars shows SE ($n = 11$). In perlite culture, 50–125% KNO_3 indicates percentage of potassium absorption per plant.

melon fruits was significantly influenced by the application of different types of nutrient solution in hydroponics and perlite culture (**Figure 1**). In general, plants grown with 50% standard nutrients showed higher fruit potassium in both culture systems. In case of perlite culture, plants grown with 50% of required potassium nitrate during 3 and 4th weeks produced fruits with the lowest potassium (53%) compared to perlite control. Under this potassium nitrate treatment, fruit potassium decreased to about 47% (179.4 mg/100 g FW) compared to potassium content in greenhouse melon (340 mg/100 g FW; Standard Tables of Food Composition in Japan, 2011). Similar results were found in our previous study, where about 39–43% fruit potassium was decreased due to the reduction in potassium nitrate fertilizer from 1/16th to 0 levels of standard hydroponic solution (Asao et al., 2013).

Fruit Mineral Nutrient Content

Potassium nitrate fertilization significantly affected the macro- and micronutrient content of fruits in both hydroponics and perlite culture (**Figure 2**). In general, calcium, magnesium, and iron concentration in hydroponic melon fruit was higher than the potassium nitrate levels in perlite culture (**Figures 2A–C**) while only sodium concentration showed the opposite results. Calcium concentration was similar in all the potassium nitrate levels. In perlite culture, magnesium concentration was decreased significantly in reduced potassium nitrate levels than 50% standard nutrient solution especially in 50 and 75%. Iron concentration was also showed a similar trend of decrease in reduced potassium nitrate levels than standard nutrient solution. Sodium concentration showed antagonistic trend than other minerals, especially potassium content in fruits (**Figure 1**) due to application of different types of nutrient solution (**Figure 2D**). Fruit sodium content was significantly decreased in hydroponics culture with 50% standard nutrient solution followed by perlite control. While in perlite culture, sodium content increase with the decrease of potassium nitrate concentrate in the culture solution and it was greater in 50% potassium nitrate level compared to other levels.

In the first experiment, melon plants grown in hydroponics and in perlite with reduced potassium nitrate affected the mineral nutrient content in the fruits. In general, the concentration of calcium, magnesium, and iron was decreased compared to control plants in hydroponics and perlite culture. This is due to the minimal supply of potassium nitrate in the perlite substrate. Sodium concentration in fruits showed clear antagonistic interaction with the potassium availability in the culture solution (**Figures 1**, **2D**). In case of limited supply of potassium nitrate, sodium concentration increase with the decrease of required potassium nitrate concentration in perlite culture. Compared to control plants in hydroponics, about 80 and 60% increased sodium were measured in fruits from plants cultured in perlite with 50% of required potassium and with standard nutrient solution, respectively. In other studies, potassium restriction in the culture solution showed significant increase in sodium and magnesium concentrations of leafy vegetables and tomato (Ogawa et al., 2012). Increased concentration of magnesium and sodium were reported to be compensated for the reduction of potassium. It was reported that availability of sodium can lead to up to 95% of the maximum yield at the critical level of potassium in field vegetable crops (Greenwood and Stone, 1998). In another study, it was found that decreased levels of potassium

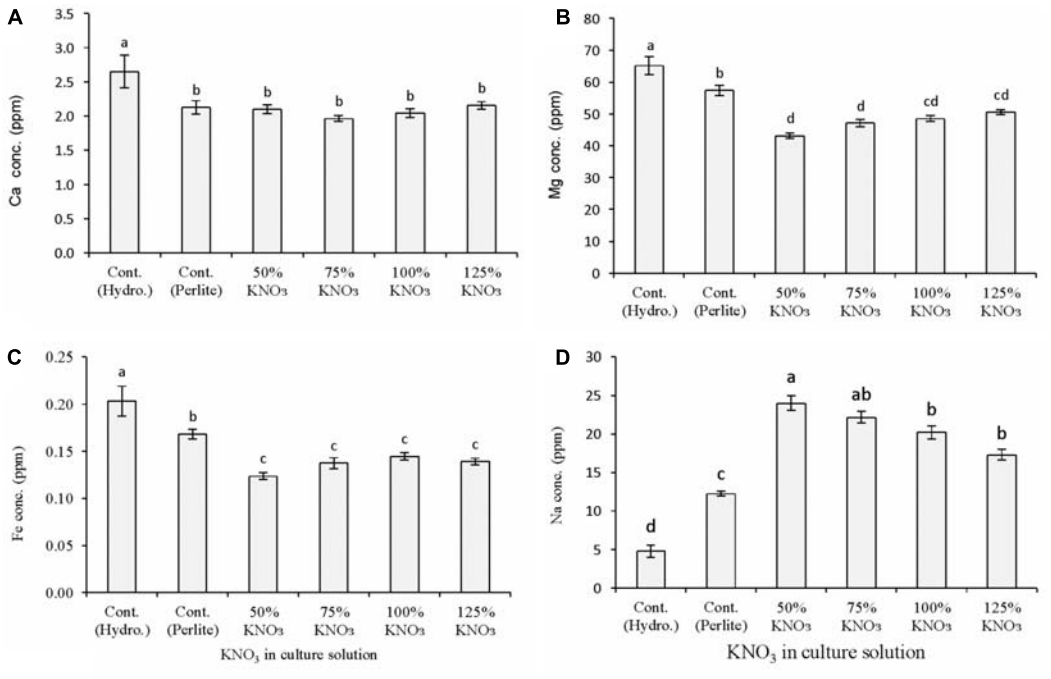

FIGURE 2 | Effect of limited supply of potassium fertilizer in the nutrient solution on the mineral nutrient content of melon fruits. Sub-figures **(A–D)** referring to Ca, Mg, Fe, and Na, respectively. Significant differences among treatments are indicated by different letters at $P < 0.05$. Error bars shows SE ($n = 11$). In perlite culture, 50–125% KNO_3 indicates percentage of potassium absorption per plant.

can increase sodium and magnesium content in tomato (Diem and Godbold, 1993; Pujos and Morard, 1997). Thus, supply of these two minerals should be considered in the studies dealing with potassium deficiency.

Mineral Nutrient Content in Plant Parts

Potassium nitrate restriction affected the micro- and macronutrient partitioning in leaves and stem of plants grown in hydroponics and perlite substrate (**Figures 3A–E**). Potassium content was mostly affected by fertilizer treatment. In leaves, potassium content decreased greatly (up to 64%) in limited potassium supply compared to plants grown with 50% standard nutrient solution in hydroponics and perlite substrate (**Figure 3A**). Both lower leaves (from 1 to 11 nodes) and upper leaves (12–25 nodes) showed a similar pattern of potassium content and it decreased with the decreased concentration of potassium nitrate in nutrient solution. In case of potassium content in stem, it was also decreased with the limited supply of potassium nitrate in the nutrient solution (**Figure 3A**). When 50% standard nutrient solution was supplied, stem potassium in plants grown in hydroponics was significantly higher than the plants grown in perlite substrate. Moreover, in perlite culture, potassium content was greatly reduced in S1 (81–88%) and S2 (83–90%) of plants grown with limited potassium nitrate (75–125%) compared to standard nutrient solution. It also revealed that potassium content in stem parts (S1 and S2) was significantly similar in limited potassium nitrate levels.

Accumulation of mineral nutrient was studied in parts of leaves and stem in order to reveal their accumulation and translocation under limited potassium condition. It was found that calcium and iron content was not decreased but was similar or higher in leaves and stems of plants grown in reduced potassium concentrations. In case of magnesium, an antagonistic interaction was observed in all parts (L1, L2, S1, and S2). Its concentration was increased in all levels of reduced potassium than that of control either in hydroponics or in perlite. Unlike fruit sodium content, its concentration in different parts of leaves and stem is increased significantly in plants supplied with reduced levels of potassium compared to hydroponics with standard nutrient solution. Antagonistic relations of potassium and sodium have been reported in faba bean and tomato as well (Cordovilla et al., 1995; Song and Fujiyama, 1996). It also reported that antagonistic interaction of sodium with calcium, potassium, and zinc showed adverse effects in plant growth (Shukla and Mukhi, 1979) on the other hand, it can replace osmotic function of potassium in plants and show position effect (Marschner, 1995; Subbarao et al., 2003; Ali et al., 2006; Kronzucker et al., 2008; Kronzucker and Britto, 2011).

Experiment II: Varietal Difference in Low-Potassium Melon Cultivation

Plant Growth

Plant growth variables and dry weights in plant parts significantly differed among cultivars grown under limited potassium nitrate cultivation using perlite substrate (**Table 2**). Plant height was

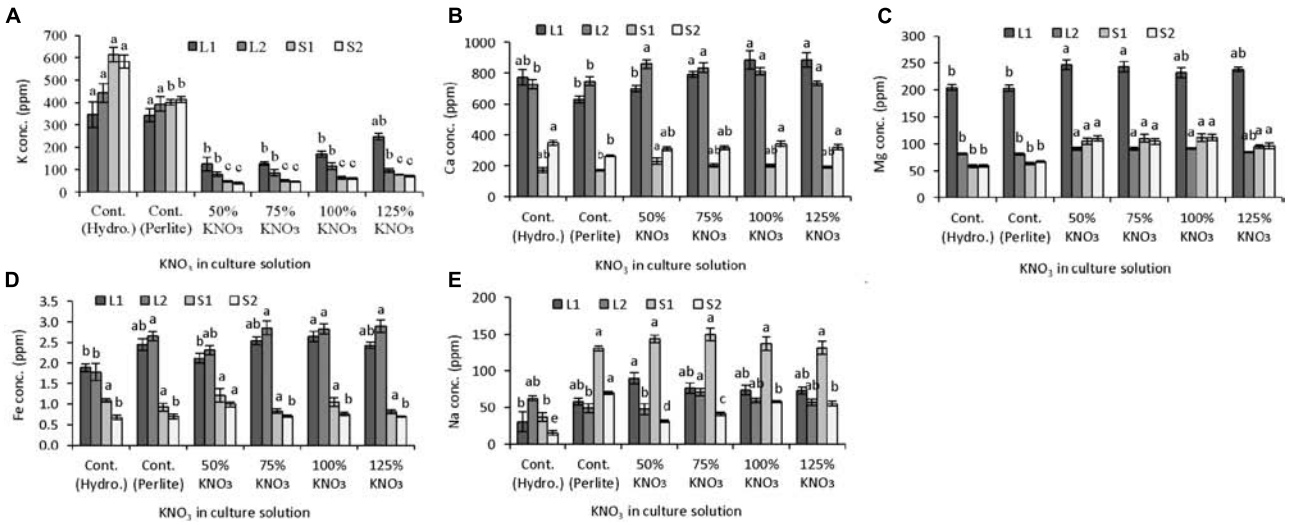

FIGURE 3 | Effect of limited supply of potassium fertilizer in the nutrient solution on the mineral nutrient content in plant parts of melon. Sub-figures **(A–E)** referring to K, Ca, Mg, Fe, and Na concentration in LI (1–11 nodes), L2 (12–25 nodes), SI (1–11 nodes), and S2 (12–25 nodes), respectively. Significant differences among treatments are indicated by different letters at $P < 0.05$. Error bars shows SE ($n = 11$). In perlite culture, 50–125% KNO_3 indicates percentage of potassium absorption per plant.

significantly shorter in cultivar "Panna" than three "Miyabi" cultivars. Among the "Miyabi" cultivars "Miyabi akifuyu412" produced the tallest plant. Although maximum leaf length was not varied, comparatively wider leaves were produced by "Miyabi" cultivars than "Panna." While three "Miyabi" cultivars produced significantly similar wider leaves. Dry weight of leaves under the fruiting nodes (L1) and also the above nodes (L2) were not significantly differed among the cultivars grown in limited potassium cultivation. In case of stem dry weight, all three "Miyabi" cultivars produced higher dry weight than "Panna." "Miyabi shunjuukei" produced lower S2 dry weight compared to other two "Miyabi" cultivars. In cultivation method of low-potassium leafy vegetables for dialysis patients, it was observed that plant growth in nutrient solution with lower potassium concentration had no significant influence on growth compared to the control plants (Ogawa, 2018).

Fruit Yield and Quality

Fruit yield under restricted potassium cultivation may vary due to growing seasons and environmental conditions. It has also been reported that potassium uptake depends on plant factors, including genetics (cultivars) and developmental stage such as vegetative versus reproductive stages (Rengel et al., 2008). Therefore, in the second study, we evaluated four melon cultivars for their potential mechanism of low-potassium fruit production. Results indicated that fruit yield and quality in four melon cultivars were influenced significantly under limited potassium nitrate fertilization in perlite culture (**Table 2**). The average fruit weight was significantly greater in "Miyabi akifuyu412" than other cultivars grown in this cultivation. The other two "Miyabi" cultivars including "Panna" produced significantly similar fruit weight. Soluble solid content was varied significantly among the cultivars and overall sweetness (about 10% soluble solids) was not so high compared to generally grown greenhouse melon (about 13% soluble solids). Tritratable citric acidity was lower in "Panna" compared to "Miyabi" cultivars while there were no significant differences among three "Miyabi" cultivars. Similar results were observed in case of ascorbic acid. Ascorbic acid content was found higher in three "Miyabi" cultivars than "Panna" under limited potassium nitrate application in perlite culture. In our low-potassium study with strawberry, we found that fruit qualities were affected by the strawberry cultivars in addition to reduced level of potassium nitrate (Mondal et al., 2016). Among the cultivars and potassium nitrate level, "Toyonoka" with 1/1 level of potassium nitrate produced higher ascorbic acid contents strawberry fruits while it was lower in other cultivars with 1/16 of potassium nitrate. Research results revealed that there were no differences in the other mineral contents between low-potassium lettuce and normal leaf lettuce, except for higher sodium and lower nitrate contents in low-potassium lettuce (Yoshida et al., 2014). Fruit cracking was observed and recorded in four melon cultivars grown under limited potassium nitrate cultivation (**Table 2**). Regarding the number of cracked fruit, comparatively fewer cracked fruits were recorded in "Miyabi akifuyu412" (2) followed "Miyabi soushun banshun309" (3). While greater number of cracked fruits were harvested from "Miyabi shunjuukei" (8) and "Panna" (7).

Fruit Mineral Content

In the second experiment, all mineral nutrients except magnesium were not varied significantly among the four cultivars under limited potassium nitrate supply from anthesis (**Figures 4A–E**). The cultivar "Panna" has lower magnesium content in fruits compared to three cultivars of "Miyabi" series. In "Panna," "Miyabi shunjuukei," and "Miyabi soushun banshun309," fruits potassium content was about

TABLE 2 | Growth parameters, yield and fruit quality of four melon cultivars grown in perlite substrate with limited potassium supply.

Cultivars	Plant height (cm)	Maximum leaf length (cm)	Maximum leaf width (cm)	Dry weight (g)				Fresh weight/ fruit (g)	Soluble solids (%)	Titratable citric acidity (%)	Ascorbic acid (ppm)	Cracked fruits/15 plants
				L1	L2	S1	S2					
Panna	117.6 c[z]	16.1	21.0 b	7.3	23.0	3.3 b	4.5 c	1142.6 b	10.0	0.4 b	309.9 b	7
Miyabi shunjuukei	158.1 b	15.3	23.1 ab	7.2	25.2	4.7 a	5.1 bc	1115.8 b	10.6	0.6 ab	490.3 a	8
Miyabi akifuyu412	188.9 a	15.9	27.1 a	7.1	24.2	5.0 a	6.0 a	1364.7 a	9.1	0.7 a	416.5 a	2
Miyabi soushun banshun309	165.4 b	15.1	22.9 ab	7.1	24.3	4.5 a	5.6 ab	1152.4 b	9.8	0.6 a	472.3 a	3
		ns		ns	ns				ns			

[z]*Means within a column followed by different letters are significantly different and "ns" indicates non-significant according to the Tukey's test at $P < 0.05$. L1, leaves from 1 to 10 nodes; L2, leaves from 11 to 25 nodes; S1, stem length from 1 to 10 nodes; S2, stem length from 11 to 25 leaves.*

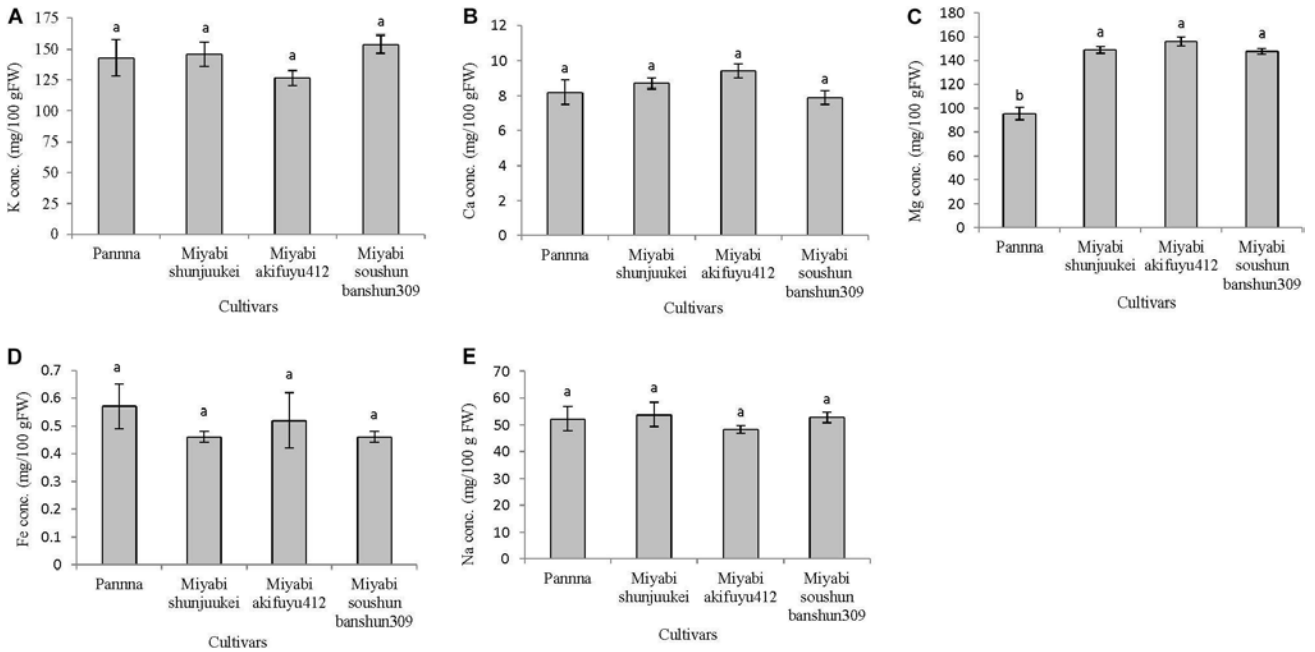

FIGURE 4 | Mineral nutrient content in fruits of four melon cultivars grown under limited potassium supply in nutrient solution. Sub-figures **(A–E)** referring to K, Ca, Mg, Fe, and Na concentration in fruits, respectively. Significant differences among treatments are indicated by different letters at $P < 0.05$. Error bars shows SE ($n = 10$).

143–154 mg/100 g FW which is about 55–58% reduction compared to potassium content in generally grown greenhouse melon (340 mg/100 g FW; Standard Tables of Food Composition in Japan, 2011), while in "Miyabi akifuyu412" this reduction was the highest (63%) (**Figure 4A**). It is evident that the difference in uptake in potassium in "Panna" and "Miyabi" mainly was due to difference in genetics or species difference and also at different developmental stages of the plant (Rengel et al., 2008). Calcium content was similar in all four melon cultivars grown and range from 7.9 to 9.4 mg/100 g FW (**Figure 4B**). Magnesium content was significantly reduced in "Panna" compared to three "Miyabi" cultivars under limited potassium nitrate supply (**Figure 4C**). About 1.5 times greater than "Panna" but similar magnesium content (about 151 mg/100 g FW) was measured in three "Miyabi" cultivars. Iron content was not differed due to the limited supply of potassium nitrate in nutrient solution in four melon cultivars and it was ranged from 0.46 to 0.57 mg/100 g FW (**Figure 4D**). There were no significant differences in sodium content in fruits of four cultivars and it ranges from 48.3 to 53.8 mg/100 g FW (**Figure 4E**).

Mineral Nutrient Content in Plant Parts

Potassium nitrate restriction in nutrient solution affected mineral nutrient content in leaves and stem of four melon cultivars (**Figures 5A–E**). In general, potassium content in stem was higher than leaves in all four cultivars (**Figure 5A**). In leaves either L1 or L2, potassium content did not differ significantly among the cultivars used. Compared to potassium content in plants grown with 50% standard nutrient solution (**Figure 3A**), about 3–23% potassium in L1 and 35–44% potassium in L2 were decreased in plants grown with limited potassium nitrate in four cultivars. Although potassium content in S1 was not different among the cultivars, its content was significantly affected in S2. In "Miyabi shunjuukei" potassium content of

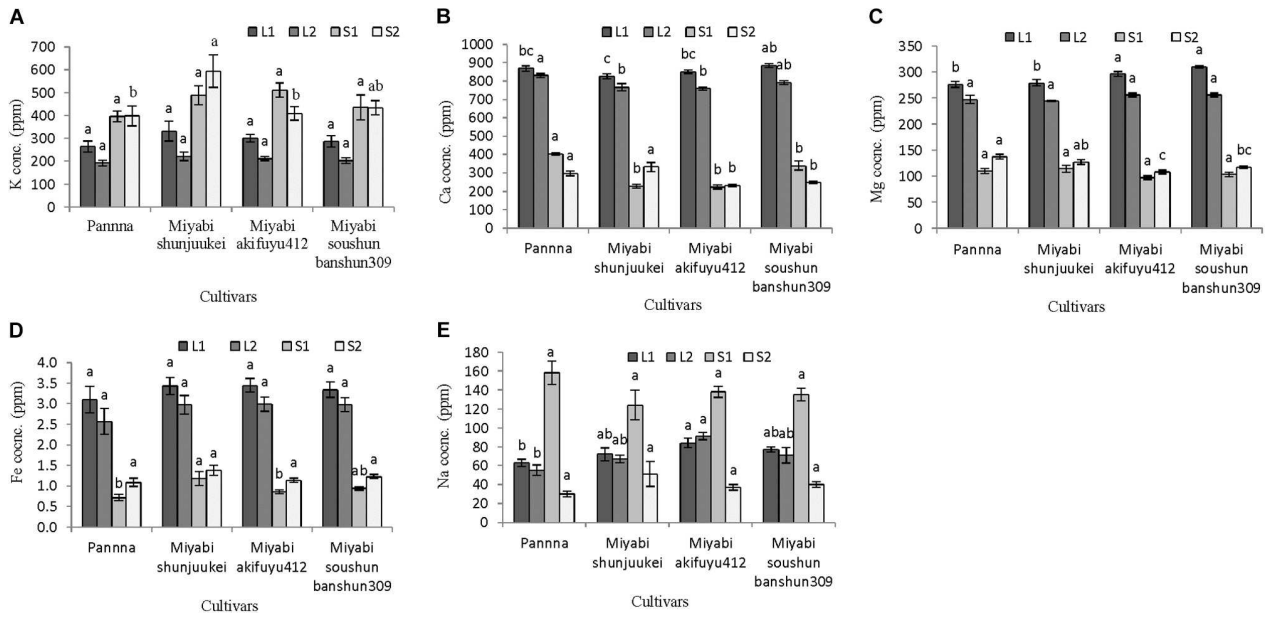

FIGURE 5 | Mineral content in different plant parts of four melon cultivars grown under limited potassium supply in nutrient solution. Sub-figures **(A–E)** referring to K, Ca, Mg, Fe, and Na concentration in Ll (1–10 nodes), L2 (11–25 nodes), S1 (1–10 nodes), and S2 (11–25 nodes), respectively. Significant differences among treatments are indicated by different letters at $P < 0.05$. Error bars shows SE ($n = 10$).

S1 part was significantly greater followed by "Miyabi soushun banshun309" and there was similar potassium content of S2 in other cultivars.

Calcium content showed opposite trend of potassium (**Figure 5B**), and in L1 of "Miyabi soushun banshun309" its content was highest compared to other cultivars while in L2, the same cultivar was followed by "Panna." In case of calcium content in S1, the cultivar "Panna" was higher than that of three "Miyabi" cultivars and among "Miyabi" cultivars it was not varied significantly. However, in S2 calcium content was also higher in "Panna" followed by "Miyabi shunjuukei" and other two cultivars showed similar content. In general, magnesium content also higher in leaves than stems and significantly differed among cultivars used (**Figure 5C**). In L1, magnesium content was higher in "Miyabi akifuyu412" and "Miyabi soushun banshun309" compared to other two cultivars while it was not affected in L2. On the other hand, magnesium content in S1 did not differ but it was found higher in "Panna" and "Miyabi shunjuukei." Iron content in leaves was much higher than in stem and it did not differ in L1, L2, and also S2 (**Figure 5D**). Its content in S1 was higher in "Miyabi shunjuukei" and "Miyabi soushun banshun309" compared to other two cultivars. Sodium content in different parts of leaves and stem were not varied in four cultivars used (**Figure 5E**). Interestingly, its content in S1 was about 2–5 times higher than S2. Potassium is known as the highly mobile mineral nutrient for plants. The above interaction might be due to the mechanism of cellular substitution of potassium by sodium related with osmotic balance and maintenance in cell vacuoles (Leigh, 2001; Amtmann et al., 2005; Ogawa and Yamauchi, 2006; Rengel et al., 2008).

TABLE 3 | Effect of different potassium fertilizer and potassium missing date on fruit weight, fruit potassium concentration and soluble solid content of melon cultivar grown in perlite bags.

Potassium fertilizer	Potassium missing date (month/day)[z]	Fresh weight/ fruit (g)	Potassium conc. (mg/100g FW)	Soluble solid content (%)
KNO_3	6/11	2027.6	256.6	11.5
	6/18	2091.6	258.0	10.8
K_2SO_4	6/11	1987.3	260.2	11.7
	6/18	2008.3	267.5	11.4
Analysis of variance	Potassium fertilizer	ns	ns	ns
	K missing date	ns	ns	*
	Interaction	ns	ns	*

[z]Potassium fertilizer supply was stopped 1 week after anther (6/11) and 2 weeks after anthesis (6/18). "ns" indicates non-significant and asterisk ("") indicates significant according to the Tukey's test at $P < 0.05$.*

Effects of Potassium Fertilizer Source and Time of Restriction on Melon Growth and Fruit Quality

Fruit Yield, Quality, and Potassium Content

In the third study, source of potassium fertilizer and time of restriction had no significant influence on the fruit yield and potassium concentration (**Table 3**). However, soluble solid was affected due to potassium nitrate restriction and its interaction with source of fertilizer. This phenomenon indicates that potassium restriction after 1 week of anthesis would result in low-potassium fruits production. In this study, fruit potassium content was not decreased greatly compared to first and second

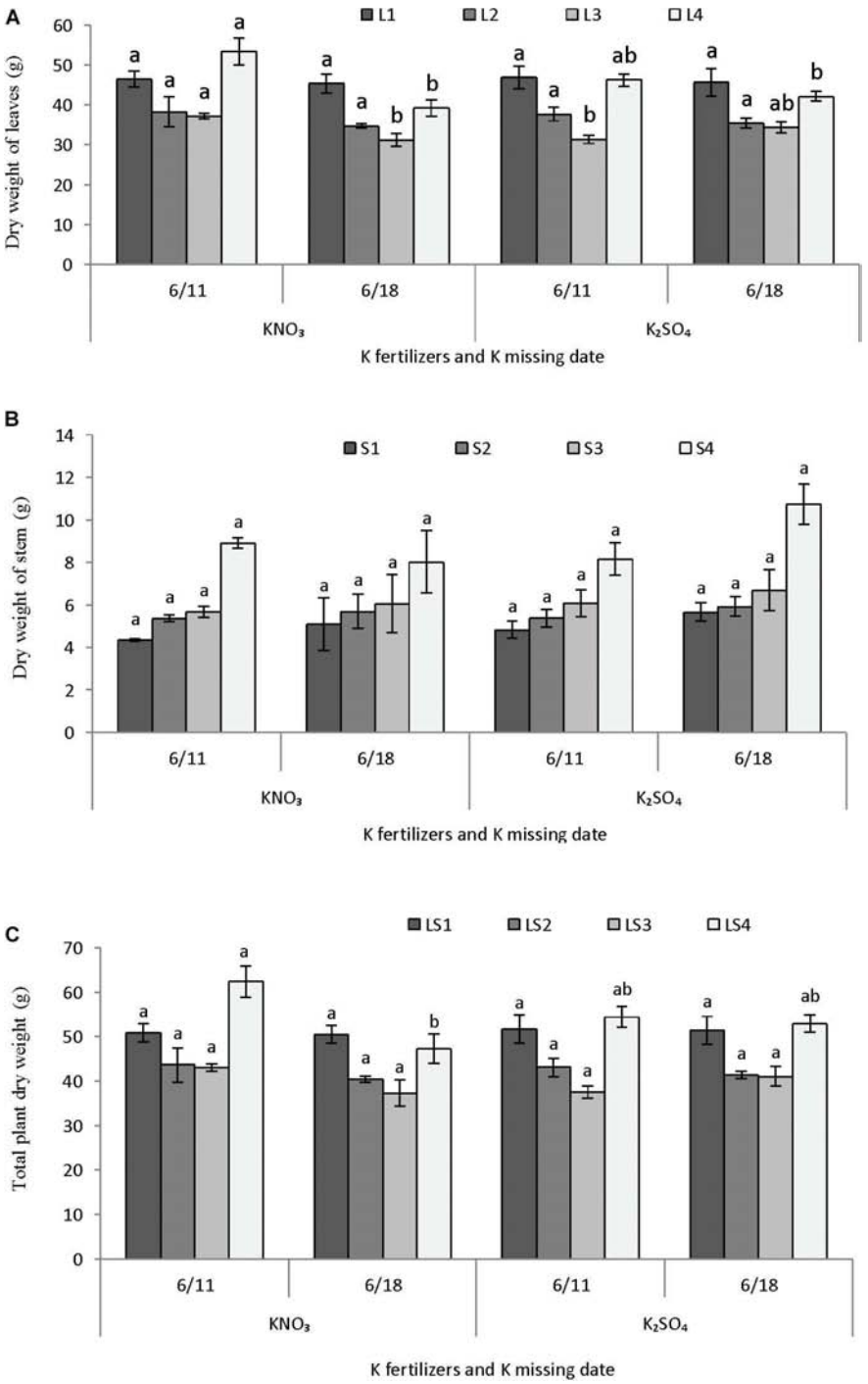

FIGURE 6 | Effect of different K fertilizers and K missing dates on the dry matter production of melon. Sub-figures **(A–C)** referring to dry weight of leaves [LI (1–6 nodes), L2 (7–12 nodes), L3 (13–18 nodes), and L4 (19–25 nodes)], stem [S1 (1–6 nodes), S2 (7–12 nodes), S3 (13–18 nodes), and S4 (19–25 nodes)], and total plant [LSI (1–6 nodes), LS2 (7–12 nodes), LS3 (13–18 nodes), and LS4 (19–25 nodes)], respectively. Significant differences among treatments are indicated by different letters at $P < 0.05$. Error bars shows SE ($n = 8$).

studies and it is evident that about 25% potassium decreased compared to potassium concentration of Standard Tables of Food Composition in Japan (2011). Review of available researches showed that potassium fertilization had positive effects on the melon fruit yield, qualities, sensory attributes, and bioactive compound for human health (Lester et al., 2010). It was mentioned that these effects will depend on the mode of fertilization and source of potassium. While other studies found

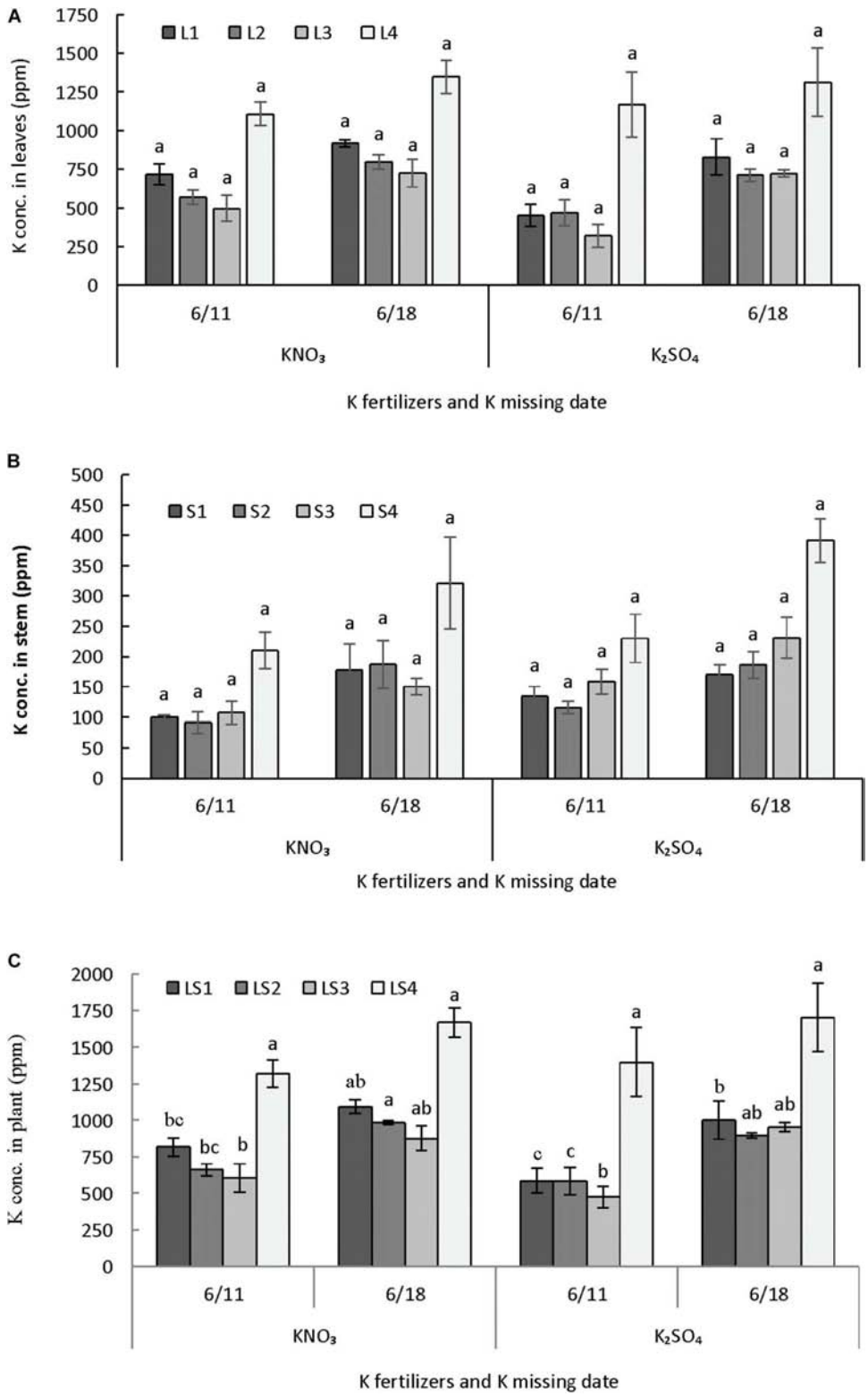

FIGURE 7 | Effect of different K fertilizers and K missing dates on K concentration in different plant parts of melon. Sub-figures **(A–C)** referring to K conc. leaves [L1 (1–6 nodes), L2 (7–12 nodes), L3 (13–18 nodes), and L4 (19–25 nodes)], stem [S1 (1–6 nodes), S2 (7–12 nodes), S3 (13–18 nodes), and S4 (19–25 nodes)] and total plant [LS1 (1–6 nodes), LS2 (7–12 nodes), LS3 (13–18 nodes), and LS4 (19–25 nodes)], respectively. Significant differences among treatments are indicated by different letters at $P < 0.05$. Error bars shows SE ($n = 8$).

that potassium fertilization may have little or no influence on fruit qualities of cucumber, bell pepper, strawberry, and watermelon (Hochmuth et al., 1994; Albregts et al., 1996; Locascio and Hochmuth, 2002; Perkins-Veazie et al., 2003; Umamaheswarappa and Krishnappa, 2004). Other studies showed that, potassium nitrate applied either in soil or as foliar during middle to late season, there were little or no improvements of marketable yield and nutritional qualities in muskmelon (Jifon and Lester, 2009). In our previous study, the measured fruit qualities of melon were unaffected except citric acid content when reduced levels of potassium nitrate applied in hydroponics (Asao et al., 2013).

Dry Matter Partitioning in Plant Parts

Potassium fertilizers and time potassium restriction showed significant influence on the dry matter partitioning of melon in perlite substrate (**Figures 6A–C**). Dry weight of lower leaves L1 (1–6 nodes) and L2 (7–12 nodes) were not differed due to supply of either potassium nitrate or potassium sulfate and also potassium restriction after 1 or 2 weeks of anthesis (**Figure 6A**). The upper leaves L3 (13–18 nodes) had greater dry weight in potassium nitrate supply and potassium restriction 1 week after anthesis, which is similar to potassium sulfate supply with potassium restriction 2 weeks after anthesis. The uppermost leaves L4 (19–25 nodes) had higher dry weights when potassium restricted after 1 week of anthesis and applied either potassium nitrate or potassium sulfate. Dry weights in four parts of stem did not differ significantly due application of either potassium nitrate or potassium sulfate and potassium restriction times after anthesis (**Figure 6B**). In general, dry weight of stem parts showed an increasing trend from lower to upper part (S1–S4). Total plant dry weight as four different parts of leaf and stem (LS1–LS3) except the upper part (LS4) was also not differed significantly due to application of potassium fertilizers or potassium restriction times after anthesis (**Figure 6C**). Dry weight of LS4 was significantly higher in potassium nitrate supply and potassium restriction after 1 week of anthesis, which similar in case of potassium sulfate supply with potassium restriction either 1 or 2 weeks after anthesis.

Potassium Content in Plant Parts

Potassium content in leaves and stem were not differed in parts due to application of potassium fertilizers and potassium restriction after anthesis but it was significantly different when leaves and stem considered together (**Figures 7A–C**). Potassium content was similar in leaves until 18 nodes (L1–L3) but it was higher in uppermost leaves (L4) (**Figure 7A**). Potassium content in stem followed the similar trend as it was observed in case of leaves (**Figure 7B**). Results revealed that potassium content in plant differed due to application of potassium nitrate or potassium sulfate and their restriction either 1 or 2 weeks after anthesis (**Figure 7C**). In lower portion of plant (LS1), potassium content was greater in plants grown with potassium restriction at 2 weeks after anthesis and supplied potassium nitrate fertilizer. In case of LS2 and LS3, potassium content was greater in plants with potassium restriction at 2 weeks after anthesis and application of either of the potassium fertilizers. LS4 was not affected by the application of two sources of potassium fertilizers and times of potassium restriction.

Influence of Different Number of Remaining Leaves on the Growth and Fruit Quality of Melons After Pinching

Fruit Yield, Quality, and Potassium Content

Number of leaves remains in the plant can contribute yield and qualities of melon fruits. In this study, number of leaves per plant (23–27) has no significant influence on the fruit yield, potassium concentration, and soluble solid content (**Table 4**). The average fruit yield ranges from 1224.3 to 1578.2 g in plants with 23–27 leaves. Number of leaves per plant also had no significant influence on potassium content in fruits. The lowest potassium content was measured in fruits from plants with 23 leaves, which is about 27% lower than generally grown greenhouse melon (Standard Tables of Food Composition in Japan, 2011). Number of leaves indicate the photosynthetic site, which contributes the soluble solid content of fruits. However, it was found that soluble solid content did not differ due to variation of leaves form 23 to 27. Potassium as plant nutrient is required in large quantities, and its excessive deficiency would result in decrease in fruit quality (size, sugar content, etc.) through reduction of stomatal conductance and CO_2 fixation (Cakmak, 2005). Thus, conversion of light energy to chemical energy impaired and ultimately phloem export of photosynthate from leaves to fruit decreased greatly. In this study, we examined whether low-potassium could be achieved without lowering fruit quality by adjusting the number of upper leaves (L4). It was shown that 23–25 leaves per melon plant are necessary for suitable fruit weight and optimum sugar content. The sugar content of fruits usually increased with the increase in number of remaining leaves, it increases the source of assimilate. Nishimura et al. (2008) recommended that at least 19 main leaves are necessary in order to maintain the quality of fruits in Earl's melon cultivation. The increase in the number of leaves not only increases the production of photosynthetic products but also greatly influences the distribution of inorganic components to each organ and leaf, and it has a big influence on the fruit sugar content. In this study, it was considered 12 leaves above

TABLE 4 | Influence of differences in the number of leaves per plant on fruit weight, potassium concentration, and soluble solid content in melon.

Leaves per plant[z]	Fresh weight/ fruit (g)	Potassium conc. (mg/100 g FW)	Soluble solid content (%)
23	1520.1	249.5	9.3
24	1578.2	276.5	9.9
25	1344.0	277.0	9.7
26	1224.3	301.9	9.9
27	1283.7	290.5	10.0
Significance	ns	ns	ns

[z]Detopping was done leaving 23, 24, 25, 26, and 27 leaves from the base. "ns" indicates non-significant according to the Tukey's test at $P < 0.05$.

TABLE 5 | Serum potassium and sodium levels, blood pressure, and pulse before and after eating low-potassium melon (Adopted from Talukder et al., 2016).

Eating low-potassium melon	Serum potassium (mEq/L)	Serum sodium (mEq/L)	Systolic BP (mmHg)	Diastolic BP (mmHg)	Pulse (/min)
Before	4.6 ± 0.4	136.6 ± 3.1	118.7 ± 11.8	70.1 ± 11.4	71.6 ± 13.5
After	4.6 ± 0.3	137.7 ± 1.9	119.4 ± 14.8	67.8 ± 9.6	71.7 ± 15.1
P-value	0.5	0.67	0.58	0.92	0.39

We served 100 g of low-potassium melon for dinner and measured serum potassium and sodium levels, blood pressure, and pulse in 9 CKD patients (estimated GFR <45 ml/min/1.73 m^2) with mean age of 69 in a hospital. No significant change was determined before and 1 day after eating low-potassium melon in addition to their usual diet.

the fruit and the number of remaining leaves was 24 and fruit was in the later branch at 12 nodes position. It was thought that although supply and uptake of potassium from the culture solution is low but translocation from leaves and stem would be higher. From the above point, the number of remaining leaves after pinching is 24 was considered suitable for low-potassium cultivation. In order to increase the sugar content we increased the nutrient solution concentration from 50 to 75% in the experiments I and II and in turn fruit potassium content decreased only about 20%. However, results indicated that sugar was not increased due to increase in the nutrient solution concentration during fruit development. Thus, it is evident that 50% nutrient solution would be suitable for low-potassium melon cultivation from seedling raising to harvesting. In the present study, we also reduce the amount of nutrient supplied to plant before 2 weeks of harvest. This was done to increase the potassium deficiency which could produce low-potassium content melon through potassium-deficient gradient and concentration stress.

Quality Testing and Clinical Validation of Low-Potassium Melon

Low-potassium melon fruits produced in the above studies were tested for their quality response by high school students and also by dieticians (**Table 5**). Melon fruits with different concentration of potassium and soluble solid content received different responses. The highest score was received by the melon fruits with 148% potassium concentration followed by 25% potassium concentration. However, participant's impression was that melon fruits with 148% potassium were bitter in taste while melon fruits with 25% potassium have less sweetness. Peoples generally like melon fruits with 51% potassium because it does not create stimulus inside the mouth. People with oral allergy syndrome like a comparatively low-potassium content melon.

We have tried to serve low-potassium melon for lunch or dinner in CKD patients to verify the safety (**Table 6**) and evaluate the effectiveness (**Figure 8**). We served 50 g of low-potassium melon and 50 g of normal melon blindly in 76 maintenance dialysis patients in their lunch box. After eating melon, they answered some questions regarding the aroma, taste, and feeling without any information about melon. Interestingly, they satisfied low-potassium melon at least as same as normal melon. Results were similar to those of healthy subjects (results not shown).

TABLE 6 | Response of high school student and managerial dieticians after eating low-potassium content melons.

Potassium conc. in melon (%)	Soluble solid content (%)	High school students[z]	Managerial dieticians[y]	Mean value
148	12.9	5.0[x]	5.0	5.0
102	13.6	3.0	2.5	2.7
74	13.5	1.3	1.5	1.4
51	13.2	1.7	2.1	1.9
25	11.1	4.0	4.0	4.0

[z]Figures are means of 9 high school students. [y]Figures are means of 11 managerial dieticians from Shimane University medical hospital. [x]Good taste score (high ∼ low) : 1 ∼ 5.

Dietary potassium for human comes largely from fruits and vegetables. It is necessary for the normal water balance between the cells and body fluids. Studies indicate that the average daily potassium intake is 2000–3900 mg (Kes, 2001; Pollock et al., 2005; Choi and Ha, 2013), which is too high for patients with kidney dysfunction to excrete. Thus, potassium intakes are limited to <1500 mg/day (Stage 5 patients) or 2000–2500 mg/day (Stages 3 and 4 patients) for the CKD patients with hyperkalemia (Pollock et al., 2005). On the other hand, most of our dietary items such as fruits including melon, fresh vegetables, seaweed, beans, and potatoes contain high potassium. Therefore, dietary management is an important aspect of improvement for dialysis patients and also provides clinical guidelines that recommend intake of micronutrients (Kopple, 2011). Dietary supplementation also reported to prevent hyperphosphatemia, hyperkalemia, hypertension, and water retention (Mailloux, 2000; Heerspink et al., 2009; Kalantar-Zadeh et al., 2009; Sherman and Mehta, 2009; Noori et al., 2010; Sanghavi et al., 2013).

Therefore, we validated our results on low-potassium content melon by supplementing in diet of the patients suffering from CKD admitted at Shimane University Hospital. We served both melon fruits with higher potassium concentration (482 mg/100 g FW), and also lower potassium concentration (183 mg/100 g FW), to compare responses from dialysis patients in regard of sweetness, taste, and texture. It was observed that the majority of the patients responded for melon fruits having half concentration of potassium in Standard Tables of Food Composition in Japan. They also wanted to recommend this low-potassium melon to other patients. In addition, many of them mentioned that

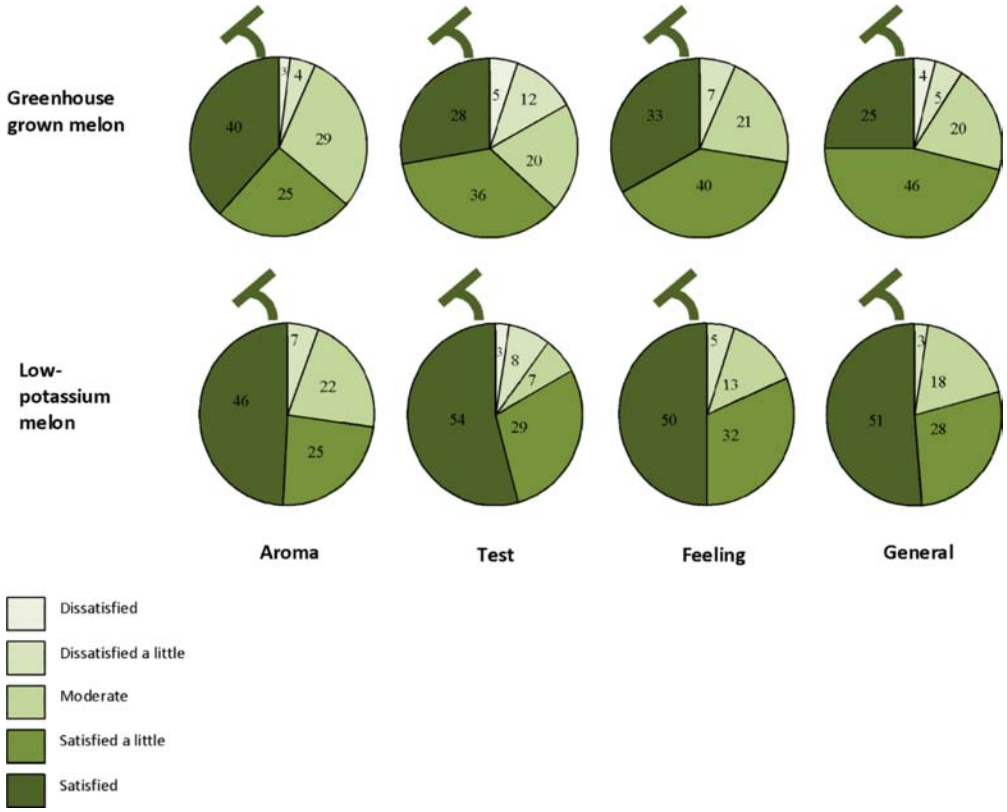

FIGURE 8 | Results from a questionnaire regarding low-potassium melon in 76 dialysis patients (Adopted from Talukder et al., 2016).

there was no tingling sensation of eating netted melon with low-potassium concentration. They also mentioned that it was delicious and easy to eat. Low-potassium melon, relieving the dietary restrictions of kidney disease patients, as there was no tingling sensation, everyone has a great feeling of eating such tasty melon.

We have also served low-potassium melon in the lunch or dinner menus of CKD patients to verify the safety and acceptability. We served 100 g of low-potassium melon for dinner and measured serum potassium and sodium levels, blood pressure, and pulse in 9 CKD patients (estimated GFR <45 ml/min/1.73 m^2) with mean age of 69 in a hospital. There were no significant change determined before and 1 day after eating low-potassium melon in addition to their usual diet (**Table 5**). In addition, we also evaluated the sensory attributes after eating low-potassium melon compared to normal melon form 76 dialysis patients (**Figure 8**). We served 50 g of low-potassium melon and 50 g of normal melon blindly in 76 maintenance dialysis patients in their lunch box. After eating melon, they answered some questions regarding the aroma, taste, and feeling without any information about melon. Interestingly, they satisfied by low-potassium melon at least as same as normal melon. Results were similar to those of healthy subjects. We observed positive responses in favor of eating low-potassium melon. Better results would be observed through continuous effort toward low-potassium melon production technique. Although the current results are in the experimental stage, in order to realize the dissemination of this low-potassium melon technology, we would consider the further restriction of potassium from the vegetative growth stage of melon plants. In a recent study, the low-potassium lettuce evaluated as lower in bitterness but higher in saltiness compared to normal lettuce (Yoshida et al., 2014). The overall preference score and higher preference score were significantly higher for low-potassium lettuce. Therefore, low-potassium lettuce might be useful for improving the diets and other varieties of food for CKD patients suffering from hyperkalemia.

CONCLUSION

In the first experiment, melon plants grown with reduced levels of potassium nitrate produced fruits with lower potassium content in perlite culture than in hydroponics. It was found that if 50% of required potassium nitrate supplied during 3rd and 4th weeks of after planting and then without potassium nutrition till harvest, fruit potassium decreased considerably (53%) compared to control. Under this quantitative potassium management, four cultivars were evaluated and there was no difference in fruit potassium content was evident but fruit potassium content was considerably lower than the general melon. In "Panna," "Miyabi

shunjuukei," and "Miyabi soushun banshun309," fruits potassium content was about 143–154 mg/100 g FW which is about 55–58% reduction compared to potassium content in generally grown greenhouse melon (340 mg/100 g FW), while in "Miyabi akifuyu412" this reduction was the highest (63%). In the third study, source of potassium fertilizer such as potassium nitrate and potassium sulfate and timing of potassium restriction form anthesis did not show any influence on the potassium content of melon. Through this study, compared to control at best 25% low-potassium in fruits was produced in plants grown with potassium nitrate and it restriction after 1 week of anthesis. Number of leaves per plant considered to be source of photosynthate and similarly source of potassium to be translocated during fruit development even under potassium restriction. However, our fourth study indicated that number of leaves remain in the plants (23–27) had no significant influence on low-potassium content of fruit and yield. However, if 23 leaves remain in the plant fruit potassium can be decreased to about 27%. Therefore, our results demonstrate that 50% standard nutrient supply during vegetative growth to anthesis and potassium nutrition restriction after 1 week of anthesis can produce low-potassium melon in perlite culture. Our further research will focus on the increasing efficiency of melon plants to sink the photosynthetic assimilate in fruits and increase quality especially soluble solids, antioxidants, phenols, carotenoids, and other human nutrition related qualities.

AUTHOR CONTRIBUTIONS

MA and TA designing and performing the experiments. MA and MT yield and growth measurement. MA, MT, and HT atomic absorption analysis of fruits and nutrient samples. HT, MU, MK, SY, TB, and TA quality testing and clinical validation. MA paper preparation. TA research coordination.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01382/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AC and handling Editor declared their shared affiliation at the time of the review.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 04 October 2018
doi: 10.3389/fpls.2018.01346

The Fight Against *Panax notoginseng* Root-Rot Disease Using Zingiberaceae Essential Oils as Potential Weapons

Yan-Jiao Yin[1†], Chuan-Jiao Chen[1†], Shi-Wei Guo[2], Ke-Ming Li[1,3], Yu-Nan Ma[1], Wu-Mei Sun[1,3], Fu-Rong Xu[1], Yong-Xian Cheng[3] and Xian Dong[1]**

[1] College of Pharmaceutical Sciences, Yunnan University of Traditional Chinese Medicine, Kunming, China, [2] College of Resources and Environmental Science, Nanjing Agricultural University, Nanjing, China, [3] Guangdong Key Laboratory for Genome Stability and Disease Prevention, School of Pharmaceutical Sciences, Shenzhen University Health Science Center, Shenzhen, China

OPEN ACCESS

Edited by:
Nikos Tzortzakis,
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Reviewed by:
Qi Chen,
Kunming University of Science and Technology, China
Antonios Chrysargyris,
Cyprus University of Technology, Cyprus

***Correspondence:**
Yong-Xian Cheng
yxcheng@szu.edu.cn
Xian Dong
dongxian_1655129@163.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: 21 May 2018
Accepted: 27 August 2018
Published: 04 October 2018

Citation:
Yin Y-J, Chen C-J, Guo S-W, Li K-M, Ma Y-N, Sun W-M, Xu F-R, Cheng Y-X and Dong X (2018) The Fight Against Panax notoginseng Root-Rot Disease Using Zingiberaceae Essential Oils as Potential Weapons. Front. Plant Sci. 9:1346. doi: 10.3389/fpls.2018.01346

The root of *Panax notoginseng* (*P. notoginseng*) is one of the most highly valuable medicinal herbs in China owing to its pronounced hemostatic and restorative properties. Despite this important fact, growing *P. notoginseng* is seriously limited by root-rot diseases. In studies aimed at developing a solution to this problem, environment-friendly essential oils (EOs) of five medicinal plants of the family Zingiberaceae were tested for their inhibitory effects on the growth of three main soil pathogens associated with the root-rot diseases of *P. notoginseng*. The results showed that the EOs of *Alpinia katsumadai* Hayata and *Zingiber officinale* Roscoe promote significant reductions in the mycelium growth of the pathogen *in vitro* at a concentration of 50 mg mL^{-1}, which is much higher than that needed (5 mg mL^{-1}) to reduce growth by the positive control, flutriafol. Furthermore, the chemical components of the two EOs were determined by using GC-MS analysis. Eucalyptol was found to account for more than 30% of the oils of the two plants, with the second major components being geranyl acetate and α-terpineol. These substances display different degrees of fungistasis *in vitro*. To further determine the effects of the EO of *Zingiber officinale (Z. officinale) in vivo*, soilless cultivation of *P. notoginseng* with pathogen inoculation was conducted in a greenhouse. Addition of the petroleum ether extract (approximately equal to EO) of *Z. officinale* to the culture matrix causes a large decrease in both the occurrence and severity of the *P. notoginseng* root-rot disease. The decreasing trend of net photosynthetic rate (P_n), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and transpiration rate (T_r) were all alleviated. In addition, the activities of catalase (CAT), peroxidase (POD), and the malondialdehyde (MDA) content were also largely reduced after pathogen infection, with the root activity being higher than that of the control. Taken together, the findings reveal that the EOs from plants might serve as promising sources of eco-friendly natural pesticides with less chemical resistance.

Keywords: *Panax notoginseng*, root-rot disease, Zingiberaceae, EOs, fungi

INTRODUCTION

Soilless cultivation is a method that employs a matrix or substrate only, instead of a natural soil for seedling cultivation and utilizes irrigation with nutrient solutions after planting. This new approach to cultivation was performed initially in China and has since then developed rapidly. In 2011, it was applied for the production of field crops, fruits, vegetables, and flowers over a total area of more than 3,000 hm^2. Soilless cultivation has great potential to be used for future modernization in agriculture (Wang Z. et al., 2013). The major advantage of this technique is that it uncouples plant growth from problems associated with the soil. Also, the regulation of plant nutrients via irrigation could manage the delivery of nutrients to plants.

At present, soil-borne diseases of medical plants have spread rapidly in many areas. Although rotation is the main method utilized to prevent these types of diseases, the narrow growth environment of medical plants prevents the implementation of this method because it increases planting costs and seriously affects the quality and yields of the products, both of which affect the development of the biopharmaceutical industry. Also, the use of a large number of pesticides, which are difficult to control, causes a vicious cycle of ecological flora imbalance and the deposition of pesticide residues and soil pollutants (Zhang et al., 2012). Searching for ways to prevent and control root-rot diseases has encountered a number of problems over the years.

Panax notoginseng is a perennial herb of the family Araliaceae, whose root is used as a medicinal herb in China. The recent pharmacological and clinical studies revealed that the root of *P. notoginseng* has significant pharmacological effects, especially on the prevention and treatment of cardiovascular and cerebrovascular diseases (Yao, 2002). However, *P. notoginseng* no longer exists in the wild state because of the environmental changes and large-scale uncontrolled excavation. Cultivation of *P. notoginseng* is mainly carried out in Wenshan, in the Yunnan province of China (Kan et al., 2016), where the yields and quality are the highest in the world (Wang Y. et al., 2013). Since its growth needs perennial shade and a warm, damp environment, intensive planting of *P. notoginseng* over large areas could easily lead to epidemic levels of pests and diseases.

Among the diseases impacting *P. notoginseng*, root-rot is the most common and difficult to control (Luo et al., 1997). Root-rot typically occurs in yearling *P. notoginseng* or in two-year-old *P. notoginseng* plants, where the disease is more severe. Pathogen infection is followed by wilting and yellowing of leaves and decaying of the underground parts. The average annual loss caused by root-rot is 5%∼20%, and it could increase up to 70% when the disease becomes more severe (Mao et al., 2013).

As a result of the impact described above, studies on *P. notoginseng* root-rot disease have increased significantly. In 1991, Cao and Qi (1991) proposed that *P. notoginseng* root-rot disease is caused by the specific mycorrhizal type, *Fusarium solani*. Later, Wang et al. (2015) suggested that *Fusarium oxysporum* E. F. Sm. and Swingle, and *Fusarium monilliforme* var. *intermedium* Neish and M. Legg are the the main pathogens responsible for this disease. Adding further complexity to the issue, a study by Miao et al. indicated that *Cylindrocarpon destructans* is an important pathogen of *P. notoginseng* root-rot disease (Miao et al., 2006). Due to the variety of proposals, the prevention and treatment of *P. notoginseng* root-rot disease has not been straightforward. In general, continuous cropping disorders of *P. notoginseng* can affect the diversity of soil microbial communities (Wang Y. et al., 2013; Li et al., 2017; Liu et al., 2018), and the soil microbial communities are influenced by multiple factors such as plant type, climate, soil properties, and agricultural practice (Li et al., 2017). Thus, it is possible for many sources of this soil-borne disease to exist.

In recent years, botanical pesticides have been a research topic of great interest because active components extracted from plants can inhibit bacteria and fungi. The results of efforts in this area have shown that the essential oils (EOs) of the plant family Zingiberaceae display strong inhibitory activities against some bacteria, including *Staphylococcus aureus* (Sivasothy et al., 2011) and fungi, such as *Aspergillus niger* (Sasidharan and Menon, 2010). Zingiberaceae is composed of about 53 genera and 1200 species, as exemplified by *Alpinia katsumadai* Hayata and *Alpinia oxyphylla* Miq. *Zingiber* which belong to *Alpinia*; *Zingiber officinale* Roscoe which belongs to *Zingiber*; *Kaempferia galanga* L., which belongs to *Kaempferia;* and *Curcuma Longa* L., which belongs to *Curcuma*. Many Zingiberaceae plant species are used as herbs and for flavoring. The main secondary metabolites of Zingiberaceae are polysaccharides, flavonoids, and EOs (Kress et al., 2002; Tushar et al., 2010). As safe substitutes for synthetic pesticides, the use of EOs will lead to a reduction in environmental pollutants and could play an important role in the prevention and control of root rot (Hashem et al., 2010). In the present study, we explored the soilless cultivation of *P. notoginseng*, and the antifungal properties of EOs from five kinds of Zingiberaceae plants species against three species of *P. notoginseng* root-rot pathogens were studied. The aim of the effort was to assess the effects of using EOs as biological pesticides in combination with the soilless cultivation method by measuring several *P. notoginseng*-related factors. In the investigation we specifically determined the effect on *P. notoginseng* plant growth, disease index, disease incidence, photosynthesis, and the activities of catalase (CAT), peroxidase (POD) and the malondialdehyde (MDA) content in order to evaluate how root-rot disease can be prevented without requiring treatments that result in environment pollution.

MATERIALS AND METHODS

Plant Cultivation

P. notoginseng seeds were seeded in a 1:2 mixture of quartz sand and roseite in a greenhouse. After germination, the seedlings were cultivated under 20°C with a relative humidity of 70 ± 10% and a photoperiod of 14 h during daytime. The seedlings were supplied with a full-strength Hoagland nutrient solution. The macronutrient composition of the Hoagland nutrient solution (in mg L^{-1}) includes 40 N (NH_4NO_3), 10 P (KH_2PO_4), 40 K (K_2SO_4 and KH_2PO_4), 57 Ca ($CaCl_2$), and 40 Mg ($MgSO_4$). The basal micronutrient composition (in mg L^{-1}) is 2.0 Fe (Fe-EDTA), 0.2 B (H_3BO_3), 0.5 Mn ($MnCl_2{\cdot}4H_2O$), 0.05 Mo [$(NH_4)6MO_7O_{24}{\cdot}4H_2O$], 0.01 Zn ($ZnSO_4{\cdot}7H_2O$), and 0.01 Cu

($CuSO_4 \cdot 5H_2O$) (Hoagland and Arnon, 1950). Dicyandiamide, a nitrification inhibitor, was added to prevent oxidation of ammonium. The pH and the EC of the nutrient solution used were 6.89 and 1005 μS cm^{-1}, respectively. The *P. notoginseng* seedlings were watered with Hoagland nutrient solution every 3 d during the cultivation.

Fungus Strains and Growth Conditions

A trial strain was isolated from the rotten root of *P. notoginseng* and identified as *F. oxysporum*, *F. solani,* and *C. destructans* by Sangon Biotech Co., Ltd (Shanghai, China). After activating 4 times on PDA medium, more vigorous strains were generated.

Sample Preparation

Five kinds of traditional Chinese medicinal materials were purchased from Yunnan Jinfa Pharmaceutical Limited Company (Kunming, Yunnan of China). Medicinal parts of *Kaempferia galanga* L., *Zingiber officinale* Roscoe, and *Curcuma Longa* L. were dry rhizoma. *Alpinia oxyphylla* Miq. was the dried ripe fruit. *Alpinia katsumadai* Hayata was dry seed. The EOs from five Zingiberaceae plants species, identified by Yong-Xian Cheng, were prepared by steam distillation for 7 h with 8-fold excess water (v w^{-1}). The EOs were collected and dried by using sodium sulfate and then stored at −20°C before being used.

Oxford Cup Experiment

A mycelium block was obtained with a 5 mm diameter hole punch and placed in the middle of the Petri dish. The four Oxford cups were then placed at the same distance around the mycelium block, where the distance between an Oxford cup and the middle of the Petri dish was 25 mm. Then, 200 μL of EOs were added to the Oxford cup. A solution of 10/1000 dimethyl sulfoxide (DMSO) and 1/1000 Tween 80 was used as a negative control, and 5 mg mL^{-1} flutriafol was used as a positive control. Each group was prepared in an identical manner four times. Finally, the culture dishes were placed in a microbiological incubator at 28°C. *F. oxysporum* and *F. solani* were cultured for 4 d and *C. destructans* was cultured for 9 d. Radial growth (RG) of the fungi was determined based on the average value of two perpendicular diameters (Pan et al., 2011). The growth inhibition rate was calculated using the following equation:

$$\text{Growth inhibition rate} = \frac{\text{RG of negative control} - \text{RG of treated Sample}}{\text{RG of negative control}} \times 100\%$$

IC_{50} Experiment

The experiments above led to the identification of EOs from *A. katsumadai* and *Z. officinale* as having an inhibition rate of more than 30%. The IC_{50} values of these EOs were determined using the method of Ikematsu et al. (2017). Each EO was dissolved in a solution of 10/1000 DMSO and 1/1000 Tween 80, and then diluted two-fold with the same solution to adjust the concentration to 1.17–600 mg mL^{-1}. A mixture formed from the filter-sterilized EOs (20 μL) and a quarter PDA growth medium without agar (150 μL) was added to cells of a 96-cell microtiter plate. The concentrations of conidial suspensions were adjusted to be 1×10^6 spores mL^{-1} for each fungus. Then, a standardized suspension of the fungus (30 μL) was added to each well. The mixture of 150 uL of the PDA medium without agar and 50 μL of 10/1000 DMSO and 1/1000 Tween 80 solution was used as a negative control. Hymexazol was used as a positive control. The plates, securely sealed with a polyester sealing film (VWR), were incubated in a fungal incubator at 28°C for 36 h, and the absorbance of each well was measured at 595 nm by an enzyme-labeled instrument (SkanIt RE 4.1).

GC-MS Analysis and Compound Identification of EOs From *A. Katsumadai* or *Z. Officinale*

Chemical compositions of EOs were analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). The GC apparatus used (Agilent Technology-provided equipment model) was equipped with an HP-5MS capillary column (30 m × 0.25 mm, film thickness of 0.25 μm). The oven temperature was initially set at 50°C for 2 min and then raised up to 130°C (at a rate of 5°C min^{-1}), subsequently by 4°C min^{-1} up to 190°C, and then by 20°C min^{-1} up to 220°C and held for 5 min. The electron ionization source was set at 70 eV. The detector and injector temperatures were set at 250°C and 230°C, respectively. Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1}. The scanned mass range was 30–550. The constituents of EOs were identified by comparing their retention time and mass spectra with those of the authentic samples contained in the NIST14 (National Institute of Standards and Technology-mass spectral) database to obtain the final assignments (Stein, 2005).

Antifungal Activities of Principal Components of EOs From *A. katsumadai* or *Z. officinale*

The above analysis showed that eucalyptol is the major component of the of the EOs from *A. katsumadai* and *Z. officinale,* accounting for more than 30%, and the second largest component of *A. katsumadai* is geranyl acetate and that of *Z. officinale* is α-terpineol. Oxford cup experiments were carried out with the three principal components. A solution of eucalyptol and geranyl acetate (W:W = 413.34 mg:186.66 mg) was prepared containing approximately the natural abundance amounts of the EOs in *A. katsumadai*. In the same way, eucalyptol and α-terpineol (W:W = 457.32 mg:142.68 mg) were mixed to obtain a solution of the EOs of *Z. officinale*. Flutriafol and hymexazol were used as positive controls and 10/1000 DMSO and 1/1000 Tween 80 mixture was used as a negative control. Eucalyptol (purity: 99%) was purchased from Shanghai Saen Chemical Technology Limited Company, while geranyl acetate (purity: ≥96%) and α-terpineol (purity: 96%) were purchased from Shanghai Yuanye Biotechnology Limited Company.

Preparation of Petroleum Ether Extract (Pee) From *Z. officinale*

Fresh *Z. officinale* (10.29 kg) was purchased from Shenzhen Vegetable Market and identified by Yong-Xian Cheng. The material was cut into slices and extracted with petroleum ether (60–90°C) under ultrasound at room temperature (3 × 30 L ×

1 h). The extract was concentrated under reduced pressure to afford a petroleum ether extract (8.35 g).

The Effect of the Pee From *Z. officinale* on the Incidence of *P. notoginseng in vivo*

A 1:2 ratio of sterilized quartz sand and roseite was used as the *P. notoginseng* culture medium. Various concentrations of PEE were mixed into the matrix (0 mg g^{-1}, 0.2 mg g^{-1}, and 0.4 mg g^{-1}). Healthy *P. notoginseng* seedlings were submerged in a sterilized or mixed conidial suspension containing 1×10^6 spores mL^{-1} of *F. oxysporum* for 2 h.

Four groups were set up as follows:

Negative Control (NC): healthy plants without the PEE and *F. oxysporum* infection;
Positive Control (PC): healthy plants without the PEE but with *F. oxysporum* infection;
0.2: healthy plants with 0.2 mg g^{-1} of the PEE and *F. oxysporum* infection;
0.4: healthy plants with 0.4 mg g^{-1} of the PEE and *F. oxysporum* infection.

Forty *P. notoginseng* plants were used in each group. After 30 d infection by the pathogen, the plants were graded for severity of wilt disease as 0 (not showing chlorosis), 1 (the stem is soft), 2 (the stem has fallen, but the leaf has not wilted), and 3 (plant wilting), and assigned a Disease index = $\sum$(rating × number of plants rated)/(total number of plants × highest rating) × 100; and a Disease incidence = (number of infected plants/total number of plants) × 100%.

Determination of Fresh Weight of Plant

The *P. notoginseng* plants were washed with tap water and then with distilled water, and finally dried with moisture absorbing paper. The whole plant was weighed to obtain the total fresh weight. The plants were divided into two parts from the stem base and each part was weighed.

Determination of Chlorophyll Content

After *F. oxysporum* infection for 30 d, the chlorophyll contents of plants were determined by using a SPAD-502 type chlorophyll meter, which gave SPAD values.

Determination of Photosynthetic Index

The P_n, g_s, C_i, and T_r of new fully expanded leaves of *P. notoginseng*, after *F. oxysporum* infection for 30d, were determined using a portable photosynthesis open system (model 6400; Li-COR, Lincoln, NE). The leaf temperature and relative humidity remained at 28°C and 50%, respectively, and the light flux intensity was 600 μmol photons m^{-1} s^{-1}. Data were recorded after equilibration (approximately 10 min) (Dong et al., 2018).

Determination of the Activity of POD and Cat

Using procedures developed earlier (Zhang et al., 2017) and also by our research group, we determined the activities of POD and CAT by using kits purchased from Beijing Solarbio Science (China). All measurements were performed according to the manufacturer's instructions.

Determination of the Content of MDA

Leaf pieces (0.2 g) were homogenized in 5 mL of 5% (w v^{-1}) trichloroacetic acid, and the homogenates were centrifuged at 3,000 g for 10 min. Portions (2 mL) of the supernatant was mixed with 2 mL of 0.67% (w v^{-1}) thiobarbituric acid. The mixtures were incubated in boiling water baths for 10 min, cooled to room temperature, and then centrifuged at 3,000 g for 30 min. The absorbances of the supernatants were measured at 450, 532, and 600 nm (Xie et al., 2015).

Determination of Root Activity

Root samples (0.5 g) were mixed thoroughly with a 0.4% TTC (2, 3, 5-Tripheyl Tetrazolium Chloride) solution and transferred to test tubes. The reaction was stopped by adding 2 mL of 1 M H_2SO_4 after incubation for 1.5 h at 37°C. The roots were ground with 4 mL ethyl acetate and the volume was increased to 10 mL before the absorbance was measured at 485 nm with ethyl acetate as a blank (Zhang and Chen, 2008) to obtain the Tetrazolium reduction intensity = Tetrazolium reduction (mg)/[Root weight (g)] [Time (h)]

Inhibitory Effects of Volatile and Non-volatile Components of the Pee From *Z. Officinale* on *F. Oxysporum*

The EOs (0.90 g) were extracted from the PEE (4.0 g) of *Z. officinale* by steam distillation with 100 mL of distilled water. The residual non-volatile portion was 0.60 g. The EOs and the non-volatile portions were respectively dissolved in solutions of 10/1000 DMSO and 1/1000 Tween 80 to obtain the final concentration of 50 mg mL^{-1}. The effects of different portions on pathogens were determined using the Oxford cup method described above.

Statistical Analysis

Statistical analysis was performed with an IBM SPSS Statistics 19.00 using one way ANOVA and Duncan (D) multiple comparisons test.

RESULTS

The Inhibition Effect of EOs From Five Medicinal Materials of Zingiberaceae

As shown in **Figures 1A,B**, five species of Zingiberaceae EOs display different degrees of inhibition against three fungi. The inhibition extents of *A. katsumadai* EO on *F. oxysporum*, *F. solani,* and *C. destructans* were 49.19, 41.10, and 56.32%, respectively. The inhibition by *A.katsumadai* EO against *C. destructans* was stronger than that by *Z. officinale* EO, whereas the inhibition by *Z. officinale* EO toward *F. oxysporum* was stronger than that by *A. katsumadai* EO. Specifically, the inhibition extent of *Z officinale* EO against *F. oxysporum* could reach 79.83% at a concentration of 50 mg mL^{-1}. The results clearly show that the inhibition by EOs from *A. katsumadai* and *Z. officinale* on the three fungi are stronger than those of the EOs

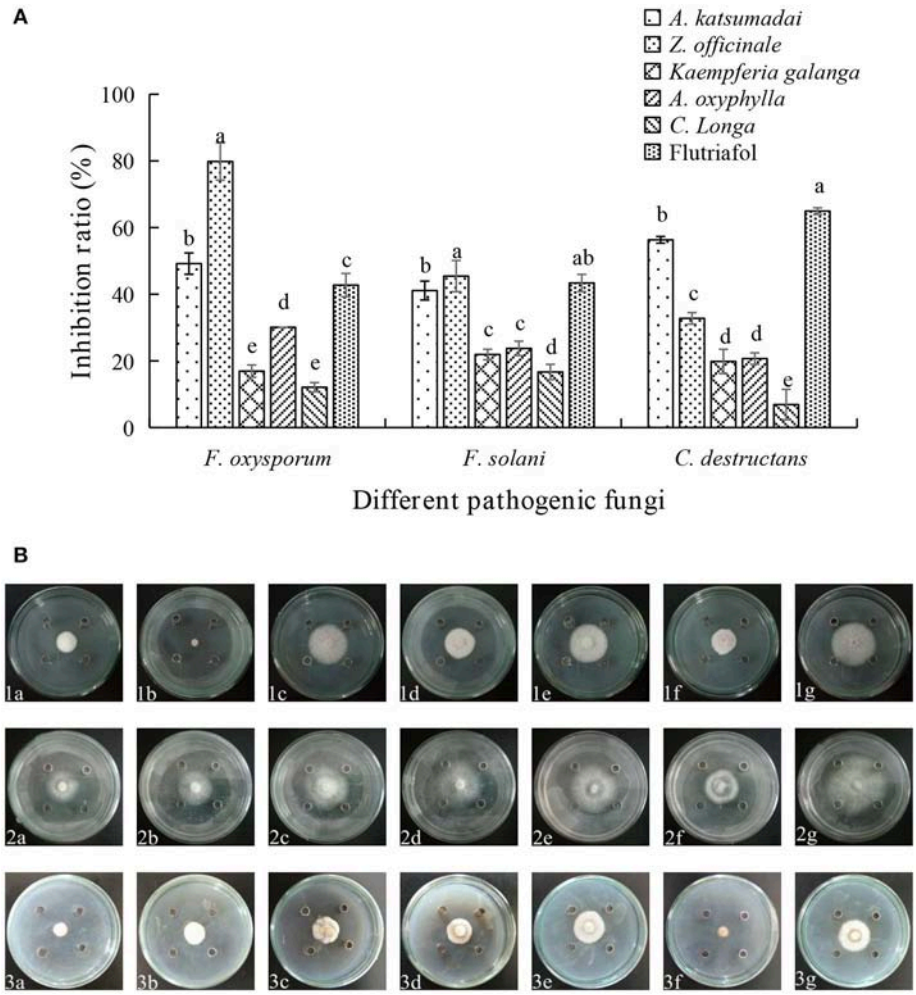

FIGURE 1 | (**A**) Effect of EOs from five Zingiberaceae plants on the growth of three kinds of root-rot fungi of *P. notoginseng*. **(B)** Inhibition of EOs from five Zingiberaceae plants on three species of fungi. The three species of fungi were (1) *F.oxysporum*, (2) *F. solani,* and (3) *C. destructans*. *Five* Zingiberaceae plants were (a) *A. katsumadai*, (b) *Z. officinale*, (c) *Kaempferia galanga*, (d) *A. oxyphylla*, and (e) *C. longa*; (f) Flutriafol as Positive Control, and (g) 10/1000 DMSO and 1/1000 Tween 80 mixture were the Negative Control. Each data point represents the mean ± SD of five replicates. Different letters represent significant differences ($P < 0.05$) among different treatments.

derived from the other three plants. Therefore, we chose these two EOs to proceed with the experiment.

Determination of IC_{50} Values

The inhibitory activities against three fungal strains correspond to IC_{50} values ranging from 16.65 to 109.96 mg mL^{-1}. As shown in **Table 1**, the EO from *A. katsumadai* could inhibit *F. oxysporum*, *F. solani,* and *C. destructans* with IC_{50} values of 21.86 mg mL^{-1}, 109.96 mg mL^{-1}, and 84.82 mg mL^{-1}, respectively. Similar effects are brought about by the EO from *Z. officinale* with an exception to its effect on *F. oxysporum*, which is more sensitive to the EO from *A. katsumadai*.

TABLE 1 | IC_{50} Determination of EOs from *A. katsumadai* or *Z. officinale* (mg mL^{-1}).

EOs	*F. oxysporum*	*F. solani*	*C. destructans*
A. katsumadai	21.86 ± 0.39	109.96 ± 1.26	84.82 ± 0.95
Z. officinale	79.04 ± 0.39	69.28 ± 3.44	94.34 ± 2.56
Hymexazol	22.09 ± 1.89	28.00 ± 1.29	16.65 ± 0.77

Analysis of EOs From *A. katsumadai* and *Z. officinale* by GC-MS

Chemical compositions of EOs from *A. katsumadai* and *Z. officinale* were determined by using GC-MS. It was found that the top five major compounds in EO of *A. katsumadai* are eucalyptol (30.03%), geranyl acetate (13.56%), geraniol (6.67%), (*E*)-2-decenyl acetate (6.42%), and α-phellandrene (4.84%). The principal compounds in the EO of *Z. officinale* are eucalyptol (35.33%), α-terpineol (11.02%), naphthalene,1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1methylethyl)-,(1α,4aβ,8aα)-(1α,4aβ,8aα)- (5.65%), camphene (4.11%), and α-farnesene (3.47%)

(**Tables S1, S2**). Among these substances, only eucalyptol is present in both plants and it is the most abundant component (**Figure 2**).

Antifungal Properties of Compounds From *A. katsumadai* and *Z. officinale*

Assessments of the antifungal activities of the principal components of the EOs showed that geranyl acetate inhibits *F. oxysporum*, *F. solani,* and *C. destructans* with the latter fungus being the most sensitive (53.24% inhibition). Eucalyptol is also active toward these fungi strains, but in this case, it appeared that *F. oxysporum* is the most sensitive with an inhibition rate of 50.88%. Interestingly, synergistic effects were observed to exist between eucalyptol and geranyl acetate, a mixture of which exhibits strong growth inhibition toward *F. oxysporum*, *F.solani,* and *C. destructans* with inhibition of 69.20, 35.03, and 50.00%, respectively (**Figures 3A,B**). By comparing the potency of α-terpineol and a mixture of it with eucalyptol, it was found that it displays comparable antifungal effects, thereby suggesting that all the three fungi strains are sensitive to α-terpineol with inhibition effects being 66.00, 62.18, and 85.25%, respectively. Finally, the antifungal potencies of α-terpineol against *F.solani* and *C. destructans* are much higher than that of the EO of *Z. officinale*. This observation suggests that α-terpineol is one of the major active components responsible for the antifungal properties of the EO of *Z. officinale*. Also, we found that this EO displays much stronger inhibition of *F. oxysporum* than does α-terpineol (**Figures 4A,B**).

In vivo Effects of the Petroleum Ether Extract (Pee) of *Z. officinale*

An *in vivo* experiment was conducted by using PEE to simulate the effects of the EOs. The growth of plants treated with 0.4 mg g^{-1} of PEE is significantly higher than those treated with 0.2 mg g^{-1} of PEE and the positive control (PC), and is similar to that of the negative control (NC) (**Figure 5A**). The fresh weight of the above-ground parts, under-ground parts, and the total fresh weight of 0.4 mg g^{-1} PEE treated plants are, respectively, 52.26, 28.78, and 44.20%, which are higher than the PC (**Figure 7A**). The results show that the disease index and incidence of *P. notoginseng* is reduced by treatment with PEE after *F. oxysporum* infection (**Figure 5B**). Upon infection of the pathogenic fungi, the chlorophyll content of the plants gradually decreases. When the amount of PEE added is 0.4 mg g^{-1}, the content of chlorophyll is 2.3 times higher than that of the PC and close to the NC (**Figure 5C**).

After treatment with PEE, the P_n, g_s, C_i, and T_r were increased significantly and are higher in the 0.4 mg g^{-1} PEE treated plant when compared to the PC. Compared with the PC, the extents of increase of P_n, g_s, C_i, and T_r in 0.4 mg g^{-1} PEE treated plants are 83.47, 87.50, 45.96, and 88.10%, respectively (**Figure 6**).

The malondialdehyde (MDA) content increases when the plant is diseased. Treatment with PEE at the concentration of 0.2 mg g^{-1} or 0.4 mg g^{-1}, causes a 5-fold decrease in the content of MDA, which is equivalent to the level in a healthy plant (**Figure 7B**). The root activity of the plant significantly decreases following pathogen infection but increases after treatment with PEE. Specifically, the root activity increases 7.0- or 11.4-fold when the plants are treated with 0.2 mg g^{-1} or 0.4 mg g^{-1} PEE, which is close to the value of the NC (**Figure 7C**). In addition, the activities of the enzymes catalase (CAT) and peroxidase (POD), which relate to the infection by pathogenic fungi, were determined. The results show that the activities of the two enzymes increase with the severity of the disease. The CAT activity of plants treated with PEE at doses of 0.2 mg g^{-1} and 0.4 mg g^{-1} was respectively reduced by 1.6 and 3.1 times compared with the PC, and the 0.4 mg g^{-1} treatment level was found to be close to that of the NC (**Figure 7D**). Moreover, PEE causes a reduction of POD activity in a dose-dependent manner. It was observed that a 4.1- or 7.3-fold reduction of the POD activity is promoted by treatment with 0.2 mg g^{-1} or 0.4 mg g^{-1} of PEE, respectively (**Figure 7E**).

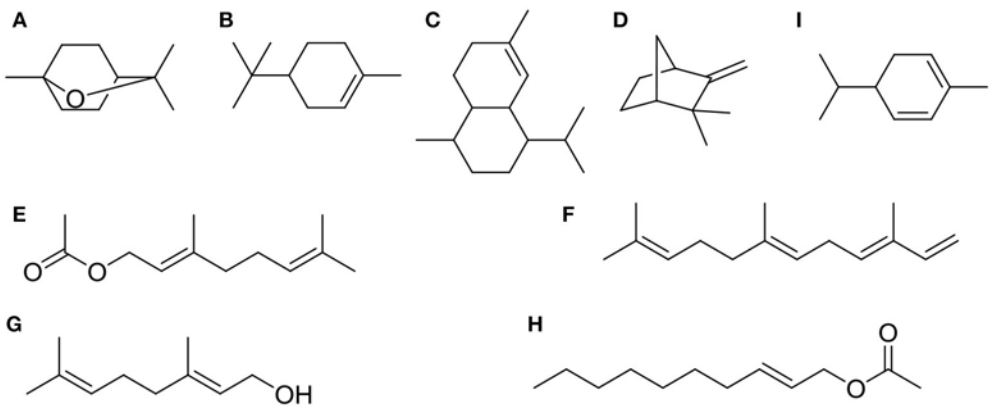

FIGURE 2 | The top five chemical structures of *A. katsumadai* and *Z. officinale* EOs through GC-MS analysis. **(A)** eucalyptol, **(B)** α-terpineol, **(C)** naphthalene,1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-,(1α,4aβ,8aα)-(1α,4aβ,8aα)-, **(D)** camphene, **(E)** α-farnesene, **(F)** geranyl acetate, **(G)** geraniol, **(H)** (*E*)-2-decenyl acetate, and **(I)** α-phellandrene.

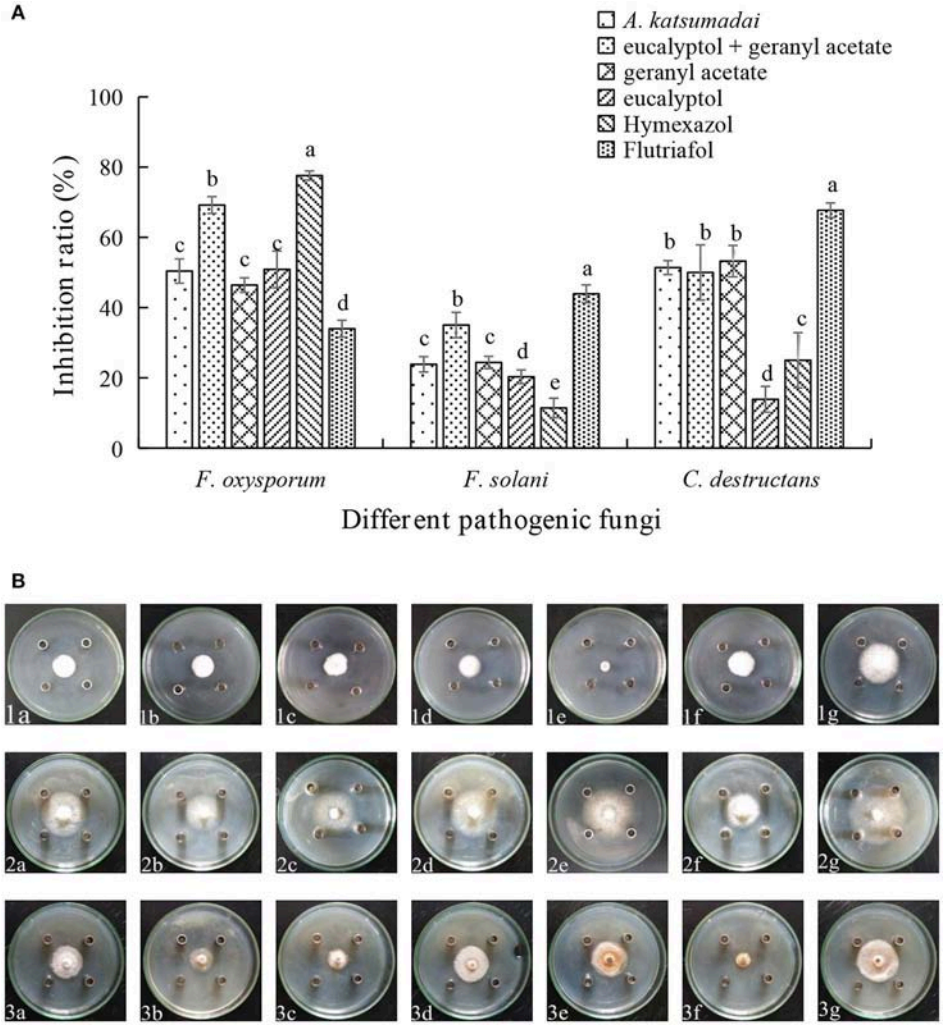

FIGURE 3 | (A) Inhibition ratio of principal compounds from *A. katsumadai* EO on *three* fungi. **(B)** Inhibition of principal compounds from *A. katsumadai* EO on three fungi. The three species of fungi were (1) *F.oxysporum*, (2) *F. solani,* and (3) *C. destructans.* Seven treatments were (a) *A. katsumadai* EO, (b) eucalyptol + geranyl acetate, (c) geranyl acetate, (d) eucalyptol, (e) Hymexazol, and (f) Flutriafol was the Positive Control and (g) 10/1000 DMSO and 1/1000 Tween 80 mixture was the Negative Control. Each data point represents the mean ± SD of five replicates. Different letters represent significant differences ($P < 0.05$) among different treatments.

Inhibition of Volatile and Non-volatile Components of Pee on *F. oxysporum*

To determine if the effects of the EOs are equivalent to those of PEE, an experiment was carried out to compare the antifungal potency of the volatile and non-volatile portions of the PEE. The results show that the volatile portion inhibits *F. oxysporum* with an inhibition extent of 70.97% but the non-volatile portion promotes only 3.23% inhibition, thereby supporting the rationale of using PEE as a substitute for EOs (**Table 2**, **Figure S1**).

DISCUSSION

In recent years and with the increased economic value of *P. notoginseng*, questions have been raised about the methods used to plant *P. notoginseng*. Problems related to diseases caused by intensive planting are becoming more serious, especially those associated with root rot. At present, many methods exist for controlling root-rot disease, such as chemical, physical, and biological treatments. The most commonly used control method is soil fumigation with a synthetic chemical. However, pesticides are banned in organic agriculture because the residues of these substances in foods have long-term health effects (Akoto et al., 2013). In addition, pesticides accumulate in the soil and exert adverse effects on the beneficial soil microflora (Ahemad and Khan, 2011).

The antibacterial mechanism of action of plant EOs involves several events including the destruction of cell membranes, exudation of cell contents, condensation of cytoplasm, and change of membrane osmotic pressure (Gustafson et al., 1998; Lambert et al., 2001; Devi et al., 2010). At present, few reports

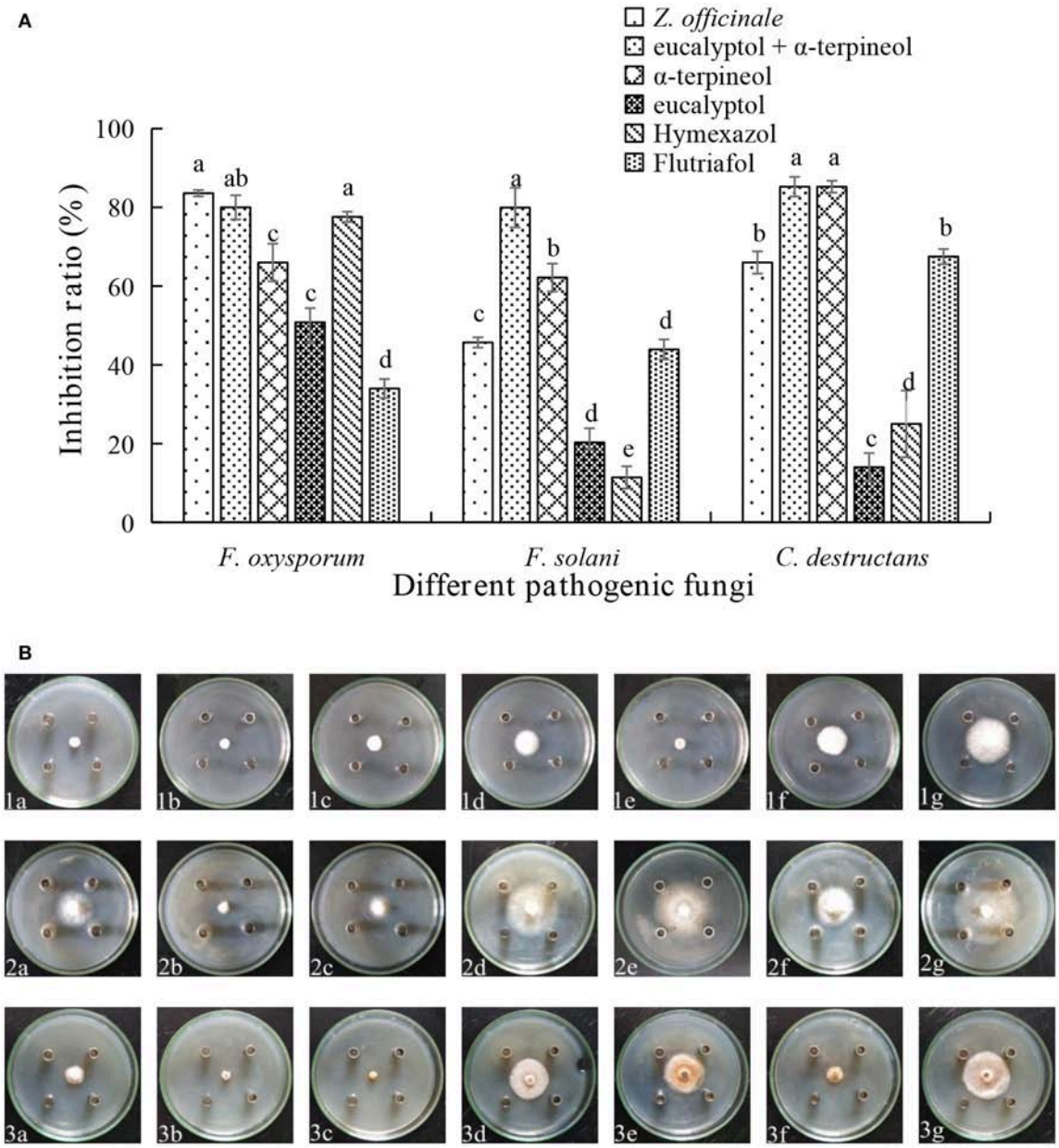

FIGURE 4 | (A) Inhibition ratio of principal compounds from *Z.officinale* EO on *three* fungi. **(B)** Inhibition of principal compounds from *Z.officinale* EO on three fungi. The three species of fungi were (1) *F.oxysporum*, (2) *F. solani,* and (3) *C. destructans.* Seven treatments were (a) *Z.officinale* EO, (b) eucalyptol + α-terpineol, (c) α-terpineol, (d) eucalyptol, (e) Hymexazol, and (f) Flutriafol was the Positive Control, and (g) 10/1000 DMSO and 1/1000 Tween 80 mixture was the Negative control. Each data point represents the mean ± SD of five replicates. Different letters represent significant differences ($P < 0.05$) among different treatments.

exist describing the bacteriostatic mechanism of the EOs of Zingiberaceae, but some contain the suggestion that lipophilic substances in the EOs of *Z. officinale* play a more important role in the bacteriostatic process. These substances can penetrate the fungi cell membrane, react with the enzymes on the membrane, destroy the enzymatic system of fungi, and further damage the function of their genetic material. In addition, the EOs can react with the proteins on the cell membranes, destroy phospholipid bimolecular layers and cell structures, make more EOs infiltrate into the cells, and eventually lead to the death of fungi (Farag et al., 1989; Sikkema et al., 1994; Abd EI-Baky and Baroty, 2008; Elbaroty et al., 2010).

The aim of the current study was to assess the use of environment-friendly EOs from five medicinal plants in the Zingiberaceae family by determining their inhibitory effects on the growth of three main soil-borne pathogens associated with the root-rot disease of *P. notoginseng*. The findings reveal that the EOs from *A. katsumadai* or *Z. officinale* significantly reduce

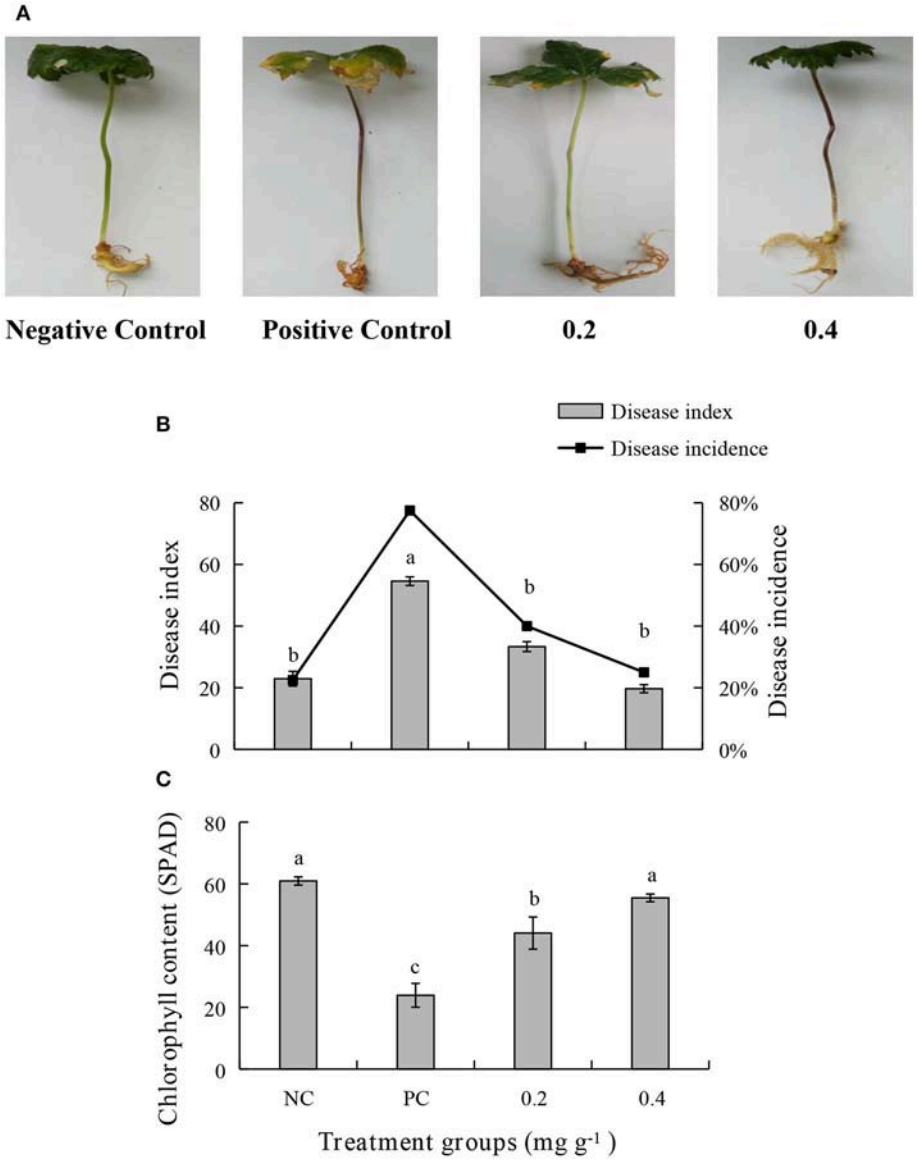

FIGURE 5 | Effects of different treatments on **(A)** symptoms of disease, **(B)** disease index, disease incidence, and **(C)** chlorophyll content of *P. notoginseng*. Plant disease index in different treatment groups. Negative Control (NC): healthy plants without Petroleum Ether Extract (PEE) and *F. oxysporum* infection; Positive Control (PC): healthy plants without PEE but with *F. oxysporum* infection; 0.2: plants with 0.2 mg g^{-1} PEE and *F. oxysporum* infection; 0.4: plants with 0.4 mg g^{-1} PEE and *F. oxysporum* infection. Each data point represents the mean ± SD of five replicates. Different letters represent significant differences ($P < 0.05$) among different treatments.

mycelium growth of the test pathogens *in vitro* (**Figures 1A,B**). This result is similar to the previously reported inhibitory effect of *Z. officinale* on *F. oxysporum* (Ginting et al., 2013). The EO of *A. katsumadai* has a similar or lower IC_{50} value in comparison to hymexazol toward the three tested fungi. The *A. katsumadai* EO was found to be the most effective for inhibition of *F. oxysporum* with an IC_{50} value of 21.86 mg mL^{-1} (**Table 1**). In addition, it has been reported that *Amomum tsao-ko* also displays an inhibitory effect on *F. oxysporum* (Sun et al., 2018).

GC-MS was employed to elucidate the chemical substances responsible for the antifungal properties of the EOs. In this study, 54 and 60 compounds were identified as being present in the EOs from *A. katsumadai* and *Z. officinale*, respectively (**Tables S1, S2**). The findings show that eucalyptol is present in both plants as the principal component, and that α-terpineol is the second major component. It was previously reported that the principal components of EO extracted from plants, among which eucalyptol is present to the extent of 30%, also have inhibitory effects on some fungi (Marei et al., 2012). In order to further determine if the abundant compounds of EOs are active, eucalyptol and α-terpineol were tested *in vitro* individually and as a mixture. It was found that the

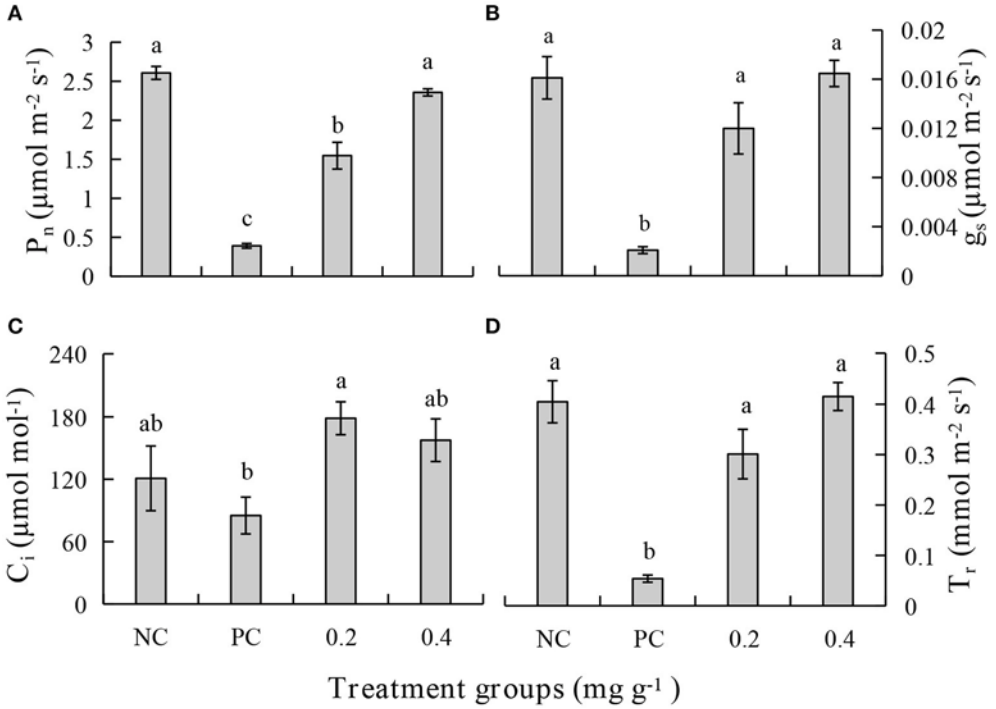

FIGURE 6 | Effects of different treatments on **(A)** photosynthetic rate (P_n), **(B)** stomatal conductance (g_s), **(C)** intercellular CO_2 concentration (C_i), and **(D)** transpiration rate (T_r) of *P. notoginseng*.

antifungal activities of mixtures of the principal compounds are greater than those of the individual compounds but less than the parent EO (**Figures 3A,B**, **4A,B**). Studies have shown that eucalyptus displays different degrees of inhibition against the four plant pathogenic fungi, *Rhizoctonia solani*, *Fusarium oxysporum*, *Penecillium digitatum,* and *Asperigallus niger* (Marei et al., 2012).

It had been reported that the EOs not only affect the normal functions of the cell membranes but they also perturb the stability of the lipid layer of the cell membranes. In earlier studies, eucalyptol and α-terpineol were identified as oxygenated monoterpenes components of *Z. officinale* (Kordali et al., 2016). The mechanisms of antifungal action of monoterpenes such as camphene, (*R*)-camphor, (*R*)-carvone, 1,8-cineole, and cuminaldehyde were not fully elucidated. However, the results of several studies led to the conclusion that these substances inhibit pectin methyl esterase, thereby promoting changes in the degrees of methyl esterification of pectins, which are major components of the fungi cell walls (Marei et al., 2012). It was speculated that PG enzymes might play an important role in the penetration of the plant root epidermis by *F.oxysporum* and upward expansion of the xylem (Beckman, 1987).

According to the GC-MS analysis carried out by Cai et al. (2008), the components and their relative amounts of the PEE from *Anoectochilus roxburghii* (Wall.) Lindl. are almost the same as those of its EOs. The method of EO and PEE extraction employed by us are the same as that used by Cai. A total of 72 components were characterized, accounting for 97.70% of the Eos, and69 components were identified, accounting for 95.40% of PEE. Moreover, the main components in both the EOs and PEE are aliphatic compounds. Also, the PEE extraction process is relatively simple and the yield is higher. Owing to this, we have conducted *in vivo* experiments using PEE instead of EO, which were aimed at determining the physiological indexes of *P. notoginseng*. The results show that the occurrence and severity of *P. notoginseng* root-rot disease is greatly decreased by adding PEE to the culture matrix (**Figures 5**–**7**).

In the current study, we observed that degradation of chlorophyll content after infection of *P. notoginseng* by *F. oxysporum* that finally leads to symptoms being displayed by the plant. The leaves of the above-ground part of the plant begin yellowing, and the whole plant wilts when the disease becomes more severe (**Figure 5A**). These observations are consistent with the previous conclusion that *F.oxysporum* infects the roots, stems, veins, and leaves through the xylem.

The bottom leaves of the host become chlorotic. This change then gradually reaches the top leaves, and finally the whole plant turns yellow, wilts, and then dies (Liang et al., 2014). During the infection process, some pathogenic toxins are secreted and these substances cause wilting and lodging of the *P. notoginseng* plants (Zhao et al., 2017). *F. oxysporum* infection causes the fresh weight of the entire above-ground and under-ground parts of the *P. notoginseng* plants to decrease significantly (**Figure 7A**). The findings are consistent with the previous results, which showed that the fresh weight of leaves, stems, and roots of *P. notoginseng* decrease significantly after *F. oxysporum* infection (Dong et al., 2018). The disease incidence of *P. notoginseng* plants, in the absence of PEE addition, is up to 77.5% (**Figure 5B**).

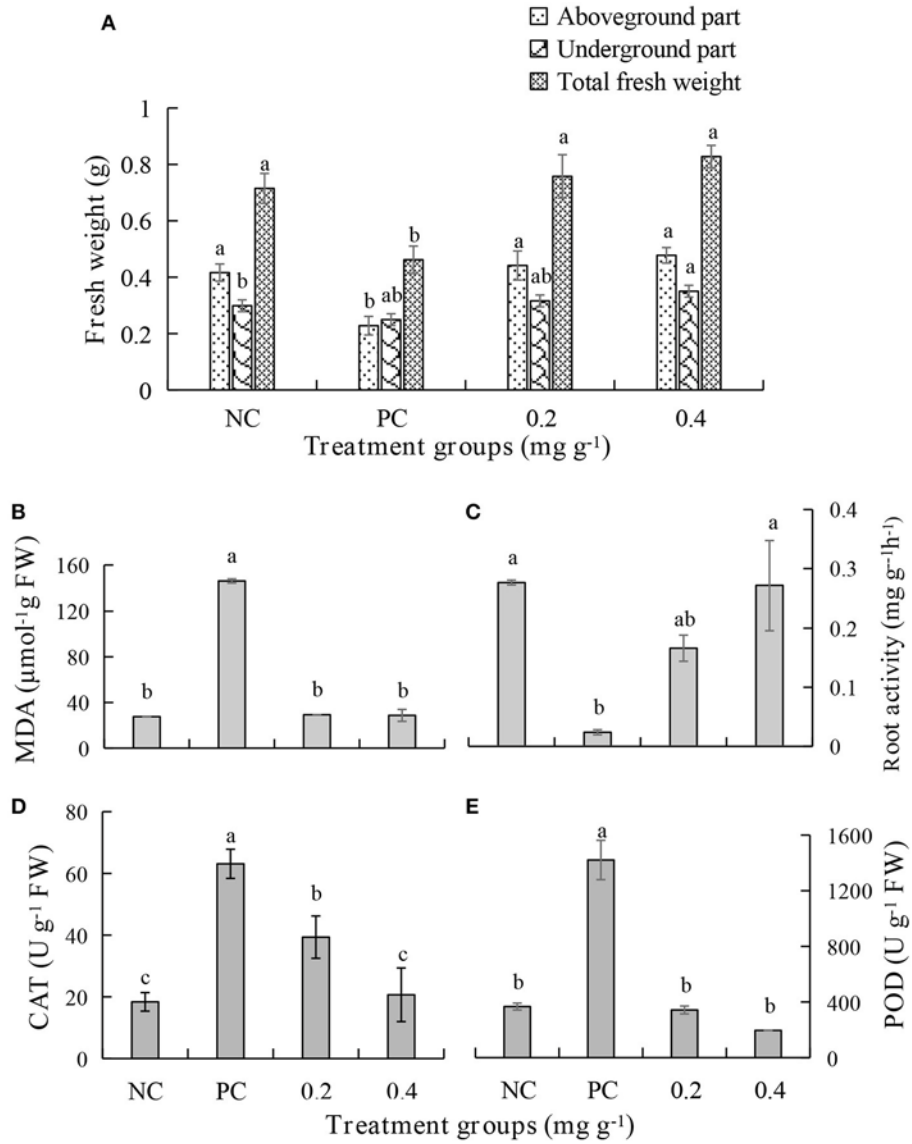

FIGURE 7 | Effects of different treatments on **(A)** fresh weight, **(B)** the malondialdehyde (MDA) content, **(C)** root activity, **(D)** the activities of catalase (CAT), and **(E)** peroxidase (POD) of *P. notoginseng*.

The results of the related studies have shown that *F. oxysporum* infection can significantly reduce photosynthesis occurring in *P. notoginseng* plants (Dong et al., 2018). Our study showed that P_n, g_s, C_i, and T_r in the PC treatment are significantly lower than those in the NC treatment (**Figure 6**). The decrease in P_n in infected leaves is a result of stomatal closure or disruption of metabolic pathways of photosynthetic products promoted by water stress caused by disease (Duniway and Slatyer, 1971; Lorenzini et al., 1997; Pinto et al., 2000). It is known that planting of xylem increases the resistance of the plant to water, which leads to water deficit in leaves, thereby decreasing photosynthesis and transpiration of leaves (Bowden et al., 1990; Lorenzini et al., 1997). It was reported that vascular wilt may be caused by disruption to photosynthesis, thylakoid electron transport, carbon reduction cycle, and CO_2 supply (Allen et al., 1998). In the current study, we found that the reduction of photosynthesis in plants infected with *F. oxysporum* is significantly alleviated by treatment with the PEE from *Z. officinale* (**Figure 6**). Photosynthesis provides carbohydrates for the growth of plants, and on being infected with *F. oxysporum*, the fresh weight of plants decreased significantly (**Figure 7A**). Malic acid and hydrogen peroxide are important intermediates in photorespiration (Wingler et al., 2000). Photorespiration is closely related to photosynthetic metabolism (Wingler et al., 1999) and plays an important role in biotic and abiotic stress. However, the relationship between the role of these intermediates in metabolism and disease resistance is unclear. Therefore, it is of great significance to clarify the function of photorespiration in the defense response of *P. notoginseng* against pathogen infection.

TABLE 2 | Inhibitory effects of volatile or non-volatile portion of PEE from *Z. officinale* on *F. oxysporum*.

Different treatment	*F. oxysporum*	
	Colony diameter (mm)	Inhibition ratio (%)
Volatile portion	9.00 ± 9.44^{c}	70.97 ± 3.95^{a}
Non-volatile portion	30.50 ± 1.08^{a}	3.23 ± 1.62^{c}
Flutriafol	18.88 ± 0.48^{b}	39.11 ± 1.54^{b}
Negative control	31.00 ± 0.82^{a}	0.00 ± 1.86^{c}

Different letters in the same column represent significant differences ($P < 0.05$) among different treatments.

Vascular wilt disease is a factor involved in pathogenic fungi and host defense response (Dan, 1990). For example, mycelium, toxin, and host defense responses caused by pathogenic fungi can block the plant vascular bundle tissue, thus reducing the water transport capacity and photosynthetic rate of the plant (Pivonia et al., 2002). However, the issue of plant water stress induced by wilt is still controversial (Lorenzini et al., 1997) and the physiological mechanism of the decrease in photosynthesis induced by wilting is not clear (Nogués et al., 2002). When the plant is infected by a pathogen, the disease or susceptibility of a plant to a disease depends on whether the plant can prevent growth and reproduction of the pathogen. The increased activity of resistance-related enzymes such as phenylalanine ammonia lyase (PAL) and polyphenol oxidase (PPO) are related to plant resistance (Dempsey and Klessig, 1995). It was found earlier that the POD and PPO activities increase significantly with the development of disease in the leaves of plants. Also, the deposition of phenols is an important defense mechanism for fighting pathogen infection, because it plays an important role in hypersensitivity and cell wall enhancement (Franke et al., 1998). Phenols are precursors of lignin and the synthesis of phytoprotectants (Yingsanga et al., 2008). Lignin is a widely distributed polymer, which enhances the ability of plants to resist degradation of pathogen enzymes and plays an important role in the defense response of vascular plants (Huang and Hartman, 1998). Thus, the increased activity of these three enzymes is closely related to cell injury, wound repair, and disease resistance (Préstamo and Manzano, 1993). In addition, root activity helps the plant roots avoiding the absorption of arsenic and other toxic substances to provide protection (Singh et al., 2007). In our studies, the root activity of the PC was found to be significantly lower than that of the NC (**Figure 7C**). The results of other studies indicate that the increase of root activity is related to the enhancement in the oxidation ability of POD (Tiwari et al., 2002). Also, studies have shown that POD, CAT, and SOD together comprise an antioxidant defense system *in vivo* (Chen, 2016). Importantly, we found that the levels of POD and CAT in the PC are significantly higher than in the NC (**Figures 7D,E**). MDA is a product of unsaturated lipid peroxidation in biofilms (Ciniglia et al., 2015) and, as a result, its quantity can directly reflect the degree of membrane lipid peroxidation (Draper and Hadley, 1990) and the amount of its accumulation determines the degree of damage to plants (Chowhan et al., 2013). In this light, we observed that after *F. oxysporum* infection the amount of MDA in the PC is significantly higher than in the NC (**Figure 7B**).

In summary, the findings arising in this study indicate that the EOs from Zingiberaceae or the volatile components of PEE have deleterious effects on *P. notoginseng* root rot. This observation suggests a possible alternative non-chemical pesticide approach for the continuous cropping *of P. notoginseng*. Last but not least, this study should pave the way for the use of Zingiberaceae EOs as effective ingredients during soilless production of *P. notoginseng,* which suppress pathogenic *P. notoginseng*-borne fungi.

AUTHOR CONTRIBUTIONS

Y-XC and XD designed the experiment, analyzed the data and wrote the paper. Y-JY, C-JC, K-ML, Y-NM, and W-MS performed the experiments. S-WG and F-RX commented on the manuscript. All authors have read and approved the manuscript.

FUNDING

This work was supported by the National Key Research and Development Program of China Research and Development of Comprehensive Technologies on Chemical Fertilizer and Pesticide Reduction and Synergism (2017YFD0201402), National Natural Science Foundation of China (81660626, 81273408), Yunnan Provincial Science and Technology Department-Applied Basic Research Joint Special Funds of Yunnan University of Traditional Chinese Medicine [2017FF116 (-014)], and Yunnan Applied basic Research Program-Youth Project (2015FD034).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01346/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 10 December 2018
doi: 10.3389/fpls.2018.01765

Effects of Salinity on Tagetes Growth, Physiology, and Shelf Life of Edible Flowers Stored in Passive Modified Atmosphere Packaging or Treated With Ethanol

Antonios Chrysargyris, Andreas Tzionis, Panayiota Xylia and Nikos Tzortzakis**

Department of Agricultural Sciences, Biotechnology and Food Science, Cyprus University of Technology, Limassol, Cyprus

Irrigation with saline water causes significant crop yield loss. However, short-term saline application might cause less negative effects on yield yet at the same time improve quality aspects of edible products. Tagetes (*Tagetes patula* L.) plants were subjected to salinity (0, 50, and 100 mM NaCl) and harvested flowers were stored up to 14 days in passive modified atmosphere packaging (with or without ethanol application). Salinity of 100 mM NaCl decreased plant biomass and plant size (i.e., height) and had a negative effect on physiological processes such as stomatal closure and chlorophylls content decrease. Salinity increased flower polyphenols, antioxidant activities, and total carotenoids but decreased anthocyanins, and greater impacts were found at salinity of 100 mM NaCl, providing higher antioxidant value of the edible flowers. Short-term saline exposure of tagetes plants activated metabolic processes and as a result there was an accumulation of minerals such as N, P, Na, and Zn on edible flowers. During storage, salinity maintained but ethanol application increased the flower CO_2 production. Ethanol application decreased the decay of flowers subjected to 100 mM NaCl. Flower weight losses and marketability accelerated at salinity of 100 mM NaCl after 14 days of storage. Tagetes flowers demonstrated induction in both non-enzymatic (i.e., proline content) and enzymatic mechanisms (catalase) to overcome stress caused by salinity during harvest stage and/or ethanol at storage. Our results have shown that short-term exposure to salinity and/or ethanol is able to achieve higher carotenoids and anthocyanins levels and these compounds can be considered as a new source of nutraceuticals.

Keywords: edible flowers, tagetes, *Tagetes patula*, antioxidant capacity, shelf-life, hydroponics, nutraceutical foods

OPEN ACCESS

Edited by:
Stefania De Pascale, Università degli Studi di Napoli Federico II, Italy

Reviewed by:
María Serrano, Universidad Miguel Hernández de Elche, Spain
Giacomo Cocetta, Università degli Studi di Milano, Italy

****Correspondence:***
Antonios Chrysargyris
a.chrysargyris@cut.ac.cy
Nikos Tzortzakis
nikolaos.tzortzakis@cut.ac.cy

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *13 October 2018*
Accepted: *14 November 2018*
Published: *10 December 2018*

Citation:
Chrysargyris A, Tzionis A, Xylia P and Tzortzakis N (2018) Effects of Salinity on Tagetes Growth, Physiology, and Shelf Life of Edible Flowers Stored in Passive Modified Atmosphere Packaging or Treated With Ethanol. Front. Plant Sci. 9:1765. doi: 10.3389/fpls.2018.01765

INTRODUCTION

High consumer preferences in fresh produce with increased popularity of edible flowers is resulting from their important properties for human health because of their abundance in bioactive and nutraceutical components, which offers further marketing opportunities (Mlcek and Rop, 2011). Among others, several plant species are used for disease treatment practices as well as natural additives in foods (Kaisoon et al., 2012; Rachuonyo et al., 2016). The nutritional value of edible

flowers is quite similar to the one of leafy vegetables in terms of proteins, fats, polysaccharides, minerals, and vitamins (Upadhyay, 2011) while their antioxidative properties are well appreciated as they are rich in carotenoids and flavonoids (Mato et al., 2000; Friedman et al., 2010).

Intensive cultivation of plants for the production of edible flowers will contribute to the market needs and consumers' expectations. However, less fertile fields, lack of knowledge about cultivation practices and/or transportation/storage parameters may limit the expansion of edible flowers market. Moreover, salinized land areas are expanding over time along the seaside areas, such as the Mediterranean basin. Salinity is one of the main abiotic factors that decrease crop yields and plant growth by causing hyperionic and hyperosmotic effects on soil solution around rhizosphere (Munns, 2002; Chrysargyris et al., 2018). This results in disturbance of water and minerals uptake by the roots and consequently decrease in yield and quality of the products. Fresh products of lower quality will reflect decrease in storage life of the fresh commodity. As a consequence, producers often have to cope with salinity due to the absence of good quality watering and they have to grow plants in soil or in soilless culture under saline conditions which is of challenge, and occasionally one-way direction. Plants grown in saline environment subjected to physiological and biochemical changes, manage the production of reactive oxygen species (ROS), by activating antioxidative mechanisms. To overcome oxidative stress, plants detoxify ROS by increasing the specific activity of antioxidant enzymes (superoxide dismutase-SOD, catalase-CAT, peroxidase-POX, glutathione peroxidase-GPX, glutathione *S*-transferases-GST, ascorbate peroxidase-APX, dehydroascorbate reductase-DHAR, glutathione reductase-GR, and monodehydroascorbate reductase-MDHAR) or producing non-enzymatic antioxidant molecules (ascorbate, glutathione, α-tocopherol, etc.) (Chrysargyris et al., 2018). The first line for ROS detoxification initiated by SOD increases in order to convert O_2^- to H_2O_2, and thereafter the H_2O_2 produced is scavenged by catalase and a variety of peroxidases (Tarchoune et al., 2010). Catalase dismutates H_2O_2 into H_2O and O_2, whereas POX decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants (Chrysargyris et al., 2018).

Edible flowers are present in culinary arts by adding flavor, freshness, color, exotic and spicy aroma, improving appearance and are increasingly favored in gourmet cuisine (Kelley et al., 2003). Diverse use of edible flowers is noticed in restaurants and catering services since they are used as garnishes and/or trimming to meals or additives in soups, fresh salads, sweets and savory dishes (Mlcek and Rop, 2011). On top of the fresh use of edible flowers, they can also be consumed dried in drink preparation, in ice cubes in cocktail making and canned in sugar (Mlcek and Rop, 2011).

Like fruit and vegetables, harvested flowers deteriorate quite fast and need to be cooled and stored quickly at chilled temperature (1–5°C) for 2–14 days (Kelley et al., 2003). However, edible flowers could be much more perishable compared to certain fruits. The flowers mainly are highly rotten and their storage duration has a substantial role in determining their retailing value. Fresh produce, including edible flowers, deteriorates with symptoms of browning, chlorophyll bleaching, tissue breakdown, off-flavors and decay. These changes are related to senescence whereas increased rates of respiration, water loss, enzyme activities and/or opportunistic microorganisms' infection are the key factors for tissue breakdown (Ragaert et al., 2007). The quality and storage duration of edible flowers are firmly associated with the preharvest culturing management used by the producers, including fertigation practices and saline levels. Additionally, postharvest preservation management used in packing houses also influences the quality and storage of fresh commodities, including edible flowers. Among others, ethanol application during postharvest preservation of fresh produce (Han et al., 2006; Tzortzakis and Economakis, 2007; Tzortzakis, 2010) and cut flower (Kaur and Mukherjee, 2013; Begri et al., 2014) has already been examined, while the ethanol application method usually includes dipping rather than vaporization (Begri et al., 2014). The effectiveness of ethanol is related to the increase of the vase life of carnation flowers by preventing the biosynthesis and action of ethylene (Heins and Blakely, 1992).

Tagetes patula, commonly known as tagetes/marigold, has various (orange, yellow, mixed) color flowers and bitterish, clove-like flavor (Mlcek and Rop, 2011). Tagetes species are widely known for their flavonoids and terpenes content (Munhoz et al., 2014). As a result, they possess antimicrobial (Gakuubi et al., 2016), insecticidal (Perich et al., 1995), larvicidal (Giarratana et al., 2017), and antioxidant (Fu and Mao, 2008) properties and are used in various countries as traditional medicines to treat colic, diarrhea, vomit, fever, skin diseases and hepatic disorders (Jain et al., 2012).

Not much information is known about the physiology, biochemistry, and postharvest performance of this species when grown in saline environments. The objectives of the present study were to examine (i) the effects of saline levels on tagetes growth, plant physiology and quality of edible flowers, (ii) the postharvest performance of edible flowers from plants grown in saline environments, (iii) the effects of ethanol-treated flowers during chilled storage, and (iv) the combined effect of salinity (in preharvest) and the ethanol application (in postharvest). As a result, this can achieve a better understanding of the responses of tagetes plants to salinity during growth and storage.

MATERIALS AND METHODS

Plant and Experimental Conditions

The present study took place at the greenhouse hydroponic infrastructure of Cyprus University of Technology during the autumn of 2017. Air temperature varied from 29 ± 2°C and 21 ± 2°C during day and night, respectively.

Tagetes (*T. patula* L.) seedlings were grown in nursery (T = 18.5–18.8°C; RH = 72–76%, Light:Dark = 16:8 h) for a 3-week period, fertigated with nutrient solution (20-20-20) of electrical conductivity (EC) 2.1 mS/cm for 1 week in order to

get plant growth uniformity at the stage of two-true leaves. Seedlings were transplanted in 400 L capacity tanks (40 plants per tank).

Experimental Set Up

Once tagetes plants were acclimated to the soilless culture environment, they were grown for 42 days to a complete nutrient solution (NS) in deep flow technique (DFT) system. The nutrient solution was refilled with the stock solution every week, to restock nutrients that might have been absorbed. The composition of the stock solution (1:100 v/v) in water was: NO_3^--N = 13.65, K = 7.05, PO_4-P = 1.29, Ca = 7.63, Mg = 2.81, SO_4^{-2}-S = 1.12, and Na = 1.92 mmol/L, respectively; and B = 30.00, Fe = 25.00, Mn = 10.23, Cu = 0.75, Zn = 4.00, and M*o* = 0.51 μmol/L, respectively. The optimal pH and EC of the NS were 5.9 and 2.0 mS/cm, respectively. The NS pH was recorded every other day and tailored accordingly (because of water alkalinity) using H_2SO_4 (5% v/v). Nutrient solution was oxygenated twice a day for 0.5 h by means of a pressure pump.

Thereafter, plants were exposed for additional 10 days at three saline (0, 50, and 100 mM NaCl) levels. Each salinity treatment was divided into two tanks and each tank supported four polyethylene trays of 10 plants capacity per tray. The half plants (40 out of 80 plants) were considered further as experimental for the present study. Each saline treatment consisted of four biological replications (10 plants/replication; 40 plants in total for each treatment) which were subjected to further measurements. The regular EC of the nutrient solution was 2.0 mS/cm for the control treatment (0 mM NaCl), 6.0 mS/cm for the salinity of 50 mM NaCl, and 10.0 mS/cm for the salinity of 100 mM NaCl, as it was kept constant during the salinity stress period (**Supplementary Figure S1**).

Plant Growth Parameters

One hundred and twenty plants of tagetes were used in the present study. After 6 weeks of plant growth under complete NS plus 10 days of saline short-time stress, four biological replications (each replication was a pool of three measurements in individual plants) for each treatment were studied in detail for plant growth measurements. Plant height, fresh and dry plant weight were measured for the aerial part of the plant (i.e., leaves and stems). The dry matter content was acquired by drying samples to constant weight using a thermo-ventilated oven at 65°C.

Physiological Parameters and Photosynthetic Pigment Content

Every 2 days for a period of 10 days of saline stress, maximum F_v/F_m photochemical quantum yields of PSII were determined with an OptiSci OS-30p Chlorophyll Fluorometer (Opti-Sciences) according to Chrysargyris et al. (2017). Chlorophylls (Chl a, Chl b, and total-Chl) content was determined at the end of the experiment based on the method previously described (Chrysargyris et al., 2017). Leaf stomatal conductance was measured on the 4th and 5th sun-exposed leaf from the top of the plant (three measurements per leaf) by using a ΔT-Porometer AP4 (Delta-T Devices-Cambridge, United Kingdom) in accordance with the manufacturer's instructions.

Plant Mineral Content

At the end of the experiment, leaf and flower minerals were determined as described in Chrysargyris et al. (2018). Sub samples (0.2–0.3 g) were digested using hydrochloric acid (2 N HCl). K and Na were determined by means of flame photometry (JENWAY, PEP-7 Jenway, Dunmow, United Kingdom), P was determined spectrophotometrically (Multiskan GO, Thermo Fischer Scientific, United States), Mg, Ca, Cu, Fe, and Zn, were determined by an atomic absorption spectrophotometer (PG Instruments AA500FG, Leicestershire, United Kingdom) and N with the help of the Kjeldahl method (BUCHI, Digest automat K-439 and Distillation Kjelflex K-360, Switzerland). Data was expressed in g/kg and mg/kg of dry weight for macro- and micronutrient, respectively.

Polyphenols and Antioxidant Activity of Leaves and Flowers

Leaves and flowers (0.5 g) polyphenols were extracted (Chrysargyris et al., 2016) with 10 mL of methanol (50% v/v) and supernatant was analyzed for total phenolics and total antioxidant activity by means of the 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) methods. Polyphenols content was determined using the Folin-Ciocalteu method at 755 nm according to Marinou et al. (2013) and the results were expressed as equivalents of gallic acid (Scharlau, Spain) per gram of fresh weight. The antioxidant capacity measurement using the DPPH, FRAP, and ABTS assays was performed as previously described (Wojdylo et al., 2007; Chrysargyris et al., 2017). The results for antioxidant activities were expressed in equivalents of trolox per gram of fresh weight.

Carotenoids and Anthocyanins Content of Flowers

Total carotenoid content was measured at 480 nm as described by Rafiq et al. (2008) with adjustments. Carotenoids were measured using the following equation: Carotenoids (μg) = 4 × A_{480} × volume (mL) (Pessarakli, 1997) and results were expressed as mg of carotenoids per gram of fresh weight.

Total anthocyanins were measured with the pH-differential method (Loizzo et al., 2015) with slight adjustments. In brief, 1 mL of the extract [500 mg in 15 mL methanol/dH_2O/HCl (70:29:1)] was mixed with (a) 3.5 mL of potassium chloride buffer (0.025 M, pH 1) or (b) 3.5 mL of sodium acetate buffer (0.025 M, pH 4.5). The absorbance of each solution was measured at 520 and 700 nm. The absorbance difference was calculated as follows: $A = [(A_{520} - A_{700})_{pH}1.0 - (A_{520} - A_{700})_{pH4.5}]$ and the anthocyanin content was calculated with the following equation: mg of cyanidin 3-glucoside equivalents/L = (absorbance × MW × dilution factor × 1000)/(ε × 1); using the molar absorptivity (ε)

and molecular weight (MW) of cyanidin 3-glucoside (ε = 26900; MW = 449.2). Results were expressed in mg of cyanidin 3-glucoside equivalents per gram of fresh weight.

Hydrogen Peroxide Content and Lipid Peroxidation of Flowers

Hydrogen peroxide (H_2O_2) content of flowers was determined according to the method of Loreto and Velikova (2001). Flower tissue (0.2 g) was ground in ice cold 0.1% trichloroacetic acid (TCA) and centrifuged at 15000 *g* for 15 min. Aliquot (0.5 mL) of the supernatant was mixed with 0.5 mL of 10 mM potassium-phosphate buffer (pH = 7.0) and 1 mL of 1 M potassium iodide. The absorbance of samples and standards was measured at 390 nm and results were expressed as μmol H_2O_2/g fresh weight.

Lipid peroxidation was assessed according to Azevedo-Neto et al. (2006) and measured in terms of malondialdehyde content (MDA). Flower tissue (0.2 g) was homogenized in 0.1% TCA and the extract was centrifuged at 15000 *g* for 10 min. The reaction mixture of 0.5 mL extract and 1.5 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA was incubated at 95°C for 25 min and then cooled on ice bath. The absorbance was determined at 532 nm. Results were expressed as nmol of MDA/g fresh weight.

Proline Content and Antioxidant Enzymes Activities of Flowers

Harvested flowers (four replicates/treatment) were homogenized in a 50 mM potassium-phosphate ice-cold extraction buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1% (w/v) polyvinylpyrrolidone (PVPP), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.05% Triton X-100 (pH = 7.0). Protein content was determined using bovine serum albumin as described previously (Chrysargyris et al., 2017).

Proline was determined according to acid-ninhydrin and toluene method at 520 nm (Khedr et al., 2003). Results were expressed in micrograms of proline per gram of fresh weight. Catalase (CAT) and superoxide dismutase (SOD) activity were assayed according to Jiang and Zhang (2002). CAT was assayed in a reaction mixture (1.5 mL) containing 50 mM K-phosphate buffer (pH 7.0), 10 mM H_2O_2 and an enzyme aliquot. The reduction of H_2O_2 was measured at 240 nm. The results were expressed in CAT units/mg of protein (1 unit = 1 mM of H_2O_2 reduction per min). SOD was assayed using a photochemical method; a reaction mixture (1.5 mL) containing 50 mM K-phosphate buffer (pH 7.5), 13 mM methionine, 75 μM nitro blue tetrazolium (NBT), 0.1 mM EDTA, 2 μM riboflavin and an enzyme aliquot. The reaction began by exposing the mixture to a light source of two 15 watt fluorescent lamps for 15 min and was stopped by placing the tubes in the dark. Absorbance was determined at 560 nm and activity was expressed in units/mg of protein. Peroxidase activity (POD) was determined as described by Tarchoune et al. (2012) following the increase in absorbance at 430 nm. Calculations were performed using the coefficient of extinction of 2.47 mM/cm. One POD unit was defined as the amount of enzyme to decompose 1 μmol of H_2O_2 per minute. Results were expressed in units/mg of protein. The activity of APX was determined according to Zhu et al. (2004) by the decrease in the absorbance of ascorbate at 290 nm. Results were expressed in units APX/mg of protein.

Edible Flower Processing and Shelf-Life Parameters

Postharvest Experimental Set Up for Flowers

Harvested plants with flowers were transferred to the laboratory within 30 min and were chosen on the basis of similar flower quality. In order to examine the impacts of salinity on flowers quality, a batch of flowers (5 flowers; ∼38.2 g total weight) was stored directly in polyethylene terephthalate (PET) plastic trays (1 L capacity) up to 14 days. Another batch of flowers was vaporized once with absolute ethanol (0.1% v/v) during a storage of 14 days. The flowers were placed in PET trays (∼4–5 flowers per tray) with snap-on lids, sealed with parafilm and cooled at 5°C, under passive modified atmospheric packaging (MAP). Moisturized (autoclaved dH_2O) filter paper was placed in each container to maintain high relative humidity during the storage period as described previously (Tzortzakis and Economakis, 2007).

The storage life of fresh produce that maintains not only appearance and safety but also nutritional value is reflected in good quality produce and consumer acceptance (Delaquis et al., 1999). For this reason, after 7 and 14 days of storage at 5°C, CO_2 concentration inside the plastic trays and quality attributes were recorded on four replications per treatment. Temperature (T°C) and relative humidity (RH %) of the refrigerated trays were monitored during storage period (T 5 ± 0.3°C and RH 87 ± 2%).

Determination of CO_2, Weight Loss, and Color of Flowers

Flower respiration was estimated by measuring CO_2 concentration of the packages using the Dual gas analyzer (International Control Analyzer Ltd., United Kingdom). Gaseous samples were drawn through septa with a syringe to prevent gas leakage from the packages. Flower weight loss was measured, as the weight of each container was registered before and after storage at 5°C for 7 and 14 days, and results were calculated in weight loss percentage.

The color of the flowers was evaluated with a colorimeter (Chroma meter CR400 Konica Minolta, Japan) where the L^* (lightness), a^* (green to red), and b^* (blue to yellow) values were recorded on day 0, 7, and 14 (three measurements per replicate/four replicates per treatment). The chroma value (C) was calculated with the following equations $C = (a^{*2}+b^{*2})^{1/2}$ (Bolin and Huxsoll, 1991).

Determination of Phenolics, Antioxidants, Proline, and Lipid Peroxidation of Flowers

Total phenolic content, carotenoids and anthocyanins content, the DPPH, FRAP, and ABTS scavenging activity, proline content, hydrogen peroxide production, lipid peroxidation, and antioxidant enzymes of flowers were determined as described above following 7 and 14 days of storage.

Determination of Decay and Marketability of Flowers

The decay severity was macroscopically evaluated after 14 d of storage with or without ethanol vapor at 5°C/90% RH. The degree of flower decay was rated (at 0.5 intervals) using a scale of 1–4, where 1-clean, no decay, 2-decay less than 25% of the surface, 3-moderate decay (25–50% decay), and 4-severe decay (>50% decay). Plant marketability was also assessed on a 9-point scale (1-low or poor and 9-high or very excellent).

Statistical Methods

Data was statistically analyzed with the use of IBM SPSS v.21 (IBM Corp., Armonk, NY, United States) software, subjected to analysis of variance (ANOVA), and was expressed by means ± SE ($n = 4$; each replicate consisted of three individual measurements from a pool of plants). Significant differences between mean values were determined using the Duncan Multiple Range Test at $P = 0.05$.

RESULTS

Growth and Physiological Parameters

Table 1 presents the effects of salinity concentration into the NS on the plant growth and physiology related parameters. Plant height and leaf stomatal conductance significantly ($P < 0.05$) decreased with salinity of 100 mM NaCl while no differences were found among control and low salinity levels (50 mM NaCl). Both fresh and dry weight of the plants decreased in salinity of 100 mM NaCl. Maximum quantum efficiency of PSII significantly decreased after 3 days of salinity (50 and 100 mM NaCl) stress, whereas it remained unaffected up to the 10th day of saline treatments (**Supplementary Figure S2**). Chlorophylls (Chl a and total Chls) decreased with the application of 100 mM NaCl (**Table 1**). No differences were found for leaf total phenolics (averaged in 18.77 mg GAE/g Fw) and antioxidant activity (averaged in 7.48, 4.46, and 2.29 mg trolox/g Fw for FRAP, DPPH, and ABTS, respectively) between control and saline plants (data not shown).

TABLE 1 | Effect of salinity levels (0, 50, and 100 mM NaCl) on tagetes plant height (cm), biomass fresh and dry weight (Fw, Dw; g/plant), leaf stomatal conductance (mmol/m^2/s), leaf Chlorophyll a (Chla; mg/g Fw), Chlorophyll b (Chlb; mg/g Fw), total Chlorophylls (total Chl; mg/g Fw) in plants grown hydroponically.

	0 mM NaCl	50 mM NaCl	100 mM NaCl
Plant height	35.00 ± 0.73a^Y	35.16 ± 1.42a	30.66 ± 1.43b
Biomass Fw	98.02 ± 8.31ab	122.75 ± 18.39a	80.96 ± 6.91b
Biomass Dw	11.23 ± 0.69ab	14.56 ± 2.04a	10.41 ± 0.76b
Stomatal conductance	412.50 ± 19.84a	396.66 ± 37.23a	184.33 ± 31.10b
Chlorophyll a	1.73 ± 0.03a	1.74 ± 0.05a	1.39 ± 0.02b
Chlorophyll b	0.77 ± 0.05a	0.61 ± 0.01b	0.42 ± 0.01c
Chlorophyll total	2.50 ± 0.07a	2.36 ± 0.05a	1.82 ± 0.01b

^{Y}Results are expressed as means ± SE (n = 4). Values in rows followed by the same letter are not significantly different, P ≤ 0.05.

Considering the flowers produced by tagetes plants, salinity treatment increased several flowers physiological parameters tested in this study (**Table 2**). Total phenolics and carotenoids as well as antioxidant activities (determined by FRAP, DPPH, and ABTS methods) increased with the application of salinity, especially at the high levels of 100 mM NaCl. The opposite was evident concerning the anthocyanins content as salinity decreased the anthocyanins up to 65%, compared with the control treatment (**Table 2**). No differences were found in flower Chroma, and *L*, *a**, and *b** color values (**Supplementary Table S1**).

Considering that H_2O_2 production indicates an induction of salinity stress, the greatest production was found in tagetes flowers derived from 100 mM NaCl-stressed plants, followed by the once subjected to salinity of 50 mM NaCl (**Table 3**). Salinity increased the production of H_2O_2 and proline content but decreased the APX activity. Low saline levels of 50 mM NaCl increased the activity of both CAT and POD while high salinity of 100 mM NaCl had similar levels to the control for both enzymes' activities. No differences were found concerning lipid peroxidation and SOD activities among treatments (**Table 3**).

Leaf and Flower Mineral Content

Mineral content in leaves and flowers is presented in **Table 4**, with effects of salinity to be mainly noticed on the flowers rather

TABLE 2 | Effect of salinity levels (0, 50, and 100 mM NaCl) on tagetes flowers total phenolics (μmol GAE/g Fw), antioxidant activity (FRAP, DPPH, ABTS: mg trolox/g Fw), carotenoids (mg/g Fw), and anthocyanins (mg cyn-3-glu/g Fw) in plants grown hydroponically.

	0 mM NaCl	50 mM NaCl	100 mM NaCl
Total phenols	50.59 ± 1.58b^Y	57.20 ± 0.66a	61.60 ± 2.92a
FRAP	40.90 ± 0.64b	53.00 ± 2.46a	47.84 ± 2.74a
DPPH	21.93 ± 0.44b	25.68 ± 1.60ab	28.40 ± 1.77a
ABTS	3.96 ± 0.21b	4.49 ± 0.11a	4.53 ± 0.13b
Carotenoids	0.155 ± 0.0127b	0.212 ± 0.0020a	0.187 ± 0.0107a
Anthocyanins	0.084 ± 0.0132a	0.029 ± 0.0054b	0.038 ± 0.0062b

^{Y}Results are expressed as means ± SE (n = 4). Values in rows followed by the same letter are not significantly different, P ≤ 0.05.

TABLE 3 | Effect of salinity levels (0, 50, and 100 mM NaCl) on tagetes flowers on the lipid peroxidation (MDA; nmol/g Fw), hydrogen peroxide production (H_2O_2; μmol/g Fw), proline content (μg/g Fw), and antioxidant enzymes activities (SOD, CAT, POD, APX in units/mg protein) in plants grown hydroponically.

	0 mM NaCl	50 mM NaCl	100 mM NaCl
H_2O_2	17.30 ± 0.07c^Y	18.63 ± 0.25b	23.30 ± 0.57a
MDA	51.26 ± 1.27a	58.79 ± 0.74a	58.34 ± 3.44a
Proline	0.287 ± 0.007c	1.312 ± 0.030b	1.997 ± 0.026a
SOD	14.02 ± 0.04a	15.72 ± 0.80a	15.09 ± 0.01a
CAT	16.27 ± 0.32b	19.45 ± 0.01a	16.72 ± 0.38b
POD	0.39 ± 0.03b	0.61 ± 0.07a	0.36 ± 0.02b
APX	3.79 ± 0.04a	2.23 ± 0.04b	1.43 ± 0.01c

^{Y}Results are expressed as means ± SE (n = 4). Values in rows followed by the same letter are not significantly different, P ≤ 0.05.

TABLE 4 | Effect of salinity levels (0, 50, and 100 mM NaCl) on tagetes leaves and flowers minerals in plants grown hydroponically.

Leaves	0 mM NaCl	50 mM NaCl	100 mM NaCl
N (g/kg)	33.45 ± 1.65a[Y]	33.94 ± 2.38a	29.32 ± 0.87a
K (g/kg)	21.55 ± 1.73a	23.48 ± 0.89a	23.76 ± 0.97a
Ca (g/kg)	4.75 ± 0.61b	11.87 ± 2.17a	13.91 ± 2.93a
P (g/kg)	6.15 ± 0.66a	6.89 ± 0.52a	4.95 ± 0.60a
Mg (g/kg)	4.14 ± 0.65a	3.40 ± 0.67a	4.06 ± 0.52a
Na (g/kg)	4.99 ± 0.45b	5.28 ± 0.51b	9.35 ± 1.12a
Fe (mg/kg)	180.55 ± 14.29a	104.30 ± 1.92b	108.90 ± 6.07b
Zn (mg/kg)	109.78 ± 3.13a	110.71 ± 4.96a	98.68 ± 3.38b
Cu (mg/kg)	38.69 ± 9.22a	46.09 ± 11.39a	47.99 ± 11.44a
Flowers	**0 mM NaCl**	**50 mM NaCl**	**100 mM NaCl**
N (g/kg)	15.81 ± 0.33c	17.26 ± 0.14b	19.22 ± 0.18a
K (g/kg)	11.77 ± 0.46a	11.44 ± 0.35a	10.98 ± 0.77a
Ca (g/kg)	4.96 ± 1.76b	12.33 ± 2.24a	3.73 ± 1.55b
P (g/kg)	4.14 ± 0.22b	4.77 ± 0.0.08a	5.03 ± 0.08a
Mg (g/kg)	0.54 ± 0.05a	0.47 ± 0.04a	0.27 ± 0.03b
Na (g/kg)	2.79 ± 0.03c	3.49 ± 0.13b	4.06 ± 0.08a
Fe (mg/kg)	106.16 ± 4.09a	71.03 ± 12.13b	50.54 ± 6.10b
Zn (mg/kg)	76.20 ± 6.81b	110.37 ± 2.54a	116.25 ± 2.24a
Cu (mg/kg)	22.63 ± 8.92a	27.74 ± 6.21a	26.92 ± 6.65a

[Y]*Results are expressed as means ± SE (n = 4). Values in rows followed by the same letter are not significantly different, P ≤ 0.05.*

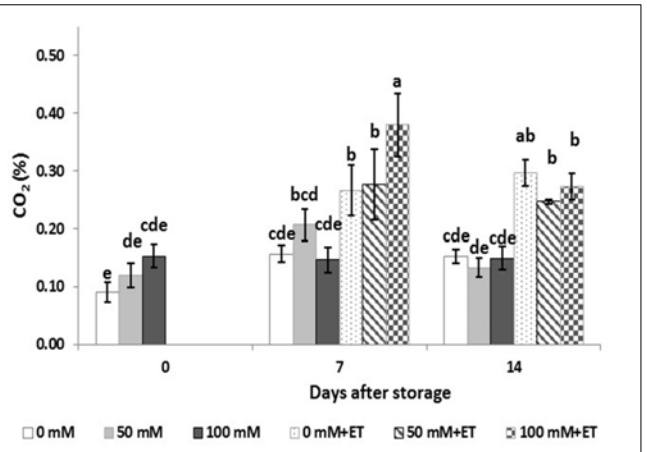

FIGURE 1 | Effect of salinity levels (0-50-100 mM NaCl) on greenhouse-grown tagetes flowers in modified atmosphere packaging (with or without vaporized ethanol-ET) on the respiration of treated flowers during 7 and 14 days of storage at 5°C. Significant differences ($P < 0.05$) among treatments are indicated by different letters. Error bars show SE ($n = 4$).

than the leaves. Therefore, in flowers, increased salinity levels resulted in increased ($P < 0.05$) Na and N content and decreased Fe content. Flowers accumulated more P and Zn when the tagetes plants were subjected to both salinity levels when compared to the control plants. Salinity of 100 mM NaCl decreased ($P < 0.05$) the Mg content in flowers. The levels of K and Cu did not vary among the treatments. Interestingly, salinity of 50 mM NaCl accumulated Ca in flowers almost three times compared to control treatment (non-saline treated plants) (**Table 4**).

In the case of tagetes leaves, salinity of 100 mM NaCl accumulated ($P < 0.05$) more Na but less Zn when compared to low (50 mM NaCl) salinity or control treatment (**Table 4**). Salinity in both low and high levels accumulated Ca in leaves but had lesser Fe content when compared with the non-saline treated plants. No differences were found for N, K, P, Mg, and Cu content among treatments.

Edible Flower Processing and Shelf-Life Parameters

The levels of CO_2 accumulated in the head space of saline and/or ethanol processed tagetes edible flowers, which is depending on the respiration rates, varied according to time of storage as well as the treatments applied (**Figure 1**). Tagetes flowers obtained by saline-treated plants immediately after harvest had no significant differences in CO_2 concentration inside the trays, despite the increase trend of CO_2 concentration as the salinity levels were increased. Following storage up to 14 days, salinity did not have any effect on CO_2 concentration. Interestingly, the vapor application of ethanol increased CO_2 concentration in trays derived by flowers respiration during storage, compared to untreated ethanol flowers. This increase of flowers respiration was greater in plants grown at 100 mM NaCl at 7 days compared to 50 mM NaCl and control treatments.

No differences were found on flower color indicated as Chroma (ranged from 83.44 to 94.79), L (ranged from 48.23 to 56.72), a^* (ranged from 35.99 to 43.13), and b^* (ranged from 71.86 to 86.11) color values on flowers during postharvest storage (**Supplementary Table S2**).

Weight loss significantly ($P < 0.05$) increased at salinity of 100 mM NaCl during storage (**Figure 2A**), as on days 7 and 14 weight loss of tagetes reached 12.48%, and 19.64%, respectively. Ethanol application increased water loss in both control and 50 mM saline-treated flowers (**Figure 2A**).

Flower marketability and decay after 14 days of storage are presented in **Figure 2B**. Therefore, the marketability of flowers decreased ($P < 0.05$) when plants were grown at high (100 mM NaCl) saline levels, while ET itself did not change the marketability of the edible flowers. Moderate decay up to 50% was found in 100 mM NaCl-treated flowers. Interestingly enough, ET application alleviated the induced decay found in 100 mM NaCl-treated flowers to levels similar to the relevant control treatment (0 mM NaCl+ET).

Total phenolics were reduced in salinity of 100 mM NaCl with ethanol compared to non-saline harvested flowers exposed to ET vapors (0 mM NaCl+ET), following 14 days of storage at 5°C (**Figure 3A**). Antioxidant activities decreased mainly due to the increased saline levels, storage duration and/or ET vapor as assayed by DPPH, FRAP, and ABTS methods (**Figures 3B–D**). The content of carotenoids increased due to saline- and ET-application as well as the storage period (7 days versus 14 days of storage) (**Figure 3E**). Thus, carotenoids increased with the application of low (50 mM NaCl) salinity compared to the control treatment following 7 days of storage. The highest anthocyanins content was found in

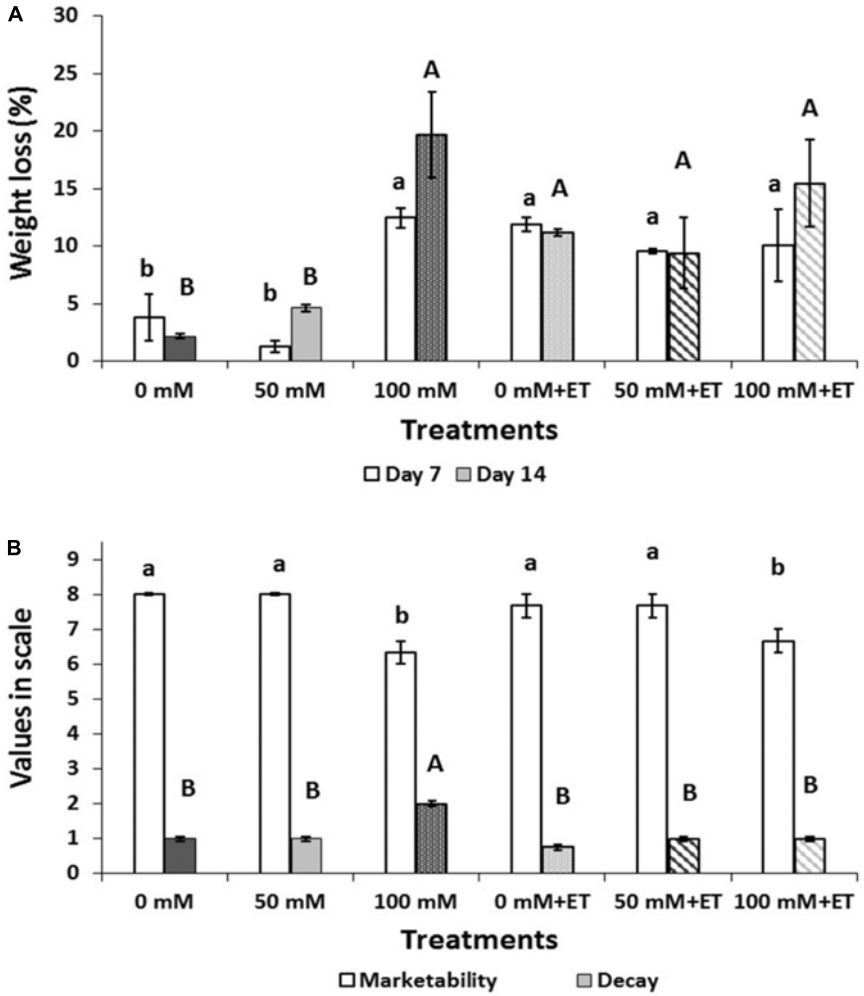

FIGURE 2 | Effect of salinity levels (0-50-100 mM NaCl) on greenhouse-grown tagetes flowers in modified atmosphere packaging (with or without vaporized ethanol-ET) on the **(A)** weight loss and **(B)** marketability (scale 1–9) and decay (scale 1–4) of treated flowers during 7 and 14 days of storage at 5°C. Significant differences ($P < 0.05$) among treatments are indicated by different letters. Error bars show SE ($n = 4$).

flowers treated with salinity of 100 mM NaCl and stored for 7 and 14 days (**Figure 3F**) while ET-vaporized flowers revealed low content of anthocyanins compared to the non-vaporized flowers.

Salinity of 100 mM NaCl increased lipid peroxidation as measured by MDA concentration after 7 days of storage while ET caused less increase in MDA (**Figure 4A**). After 14 days of storage, MDA decreased in salinity of 100 mM NaCl or in ET non-saline treated flowers. The production of H_2O_2 increased with the application of ET after 7 days of storage (**Figure 4B**). Indeed, ET combination with salinity (50 mM NaCl+ET and 100 mM NaCl+ET) revealed H_2O_2 decreases to similar levels as the 50 mM NaCl.

Following 7 days of storage, salinity of 100 mM NaCl increased the CAT activity and proline content (**Figures 4D,F**) while no differences were found in APX and POD activities (**Figures 4C,E**). At 14 days of storage, salinity of 100 mM NaCl decreased POD activity and increased proline content whereas APX and CAT activities remained at similar levels to the control treatment (non-saline flowers) (**Figures 4C–F**).

ET application, following 7 days of storage, decreased both APX and CAT but increased proline content (**Figures 4C,D,F**) and this effect persisted till the 14th day for CAT and proline. The application of ET in saline-treated flowers decreased POD and increased CAT and proline content after 7 days of storage while APX was reduced after 14 days of storage with the salinity+ET combination (**Figures 4C–F**). No differences were found in SOD activity among treatment and/or days of storage (data not shown).

DISCUSSION

The present study investigated the impacts of saline levels on plant growth and physiology, mineral content and storability of tagetes edible flowers. In addition, ethanol application was tested

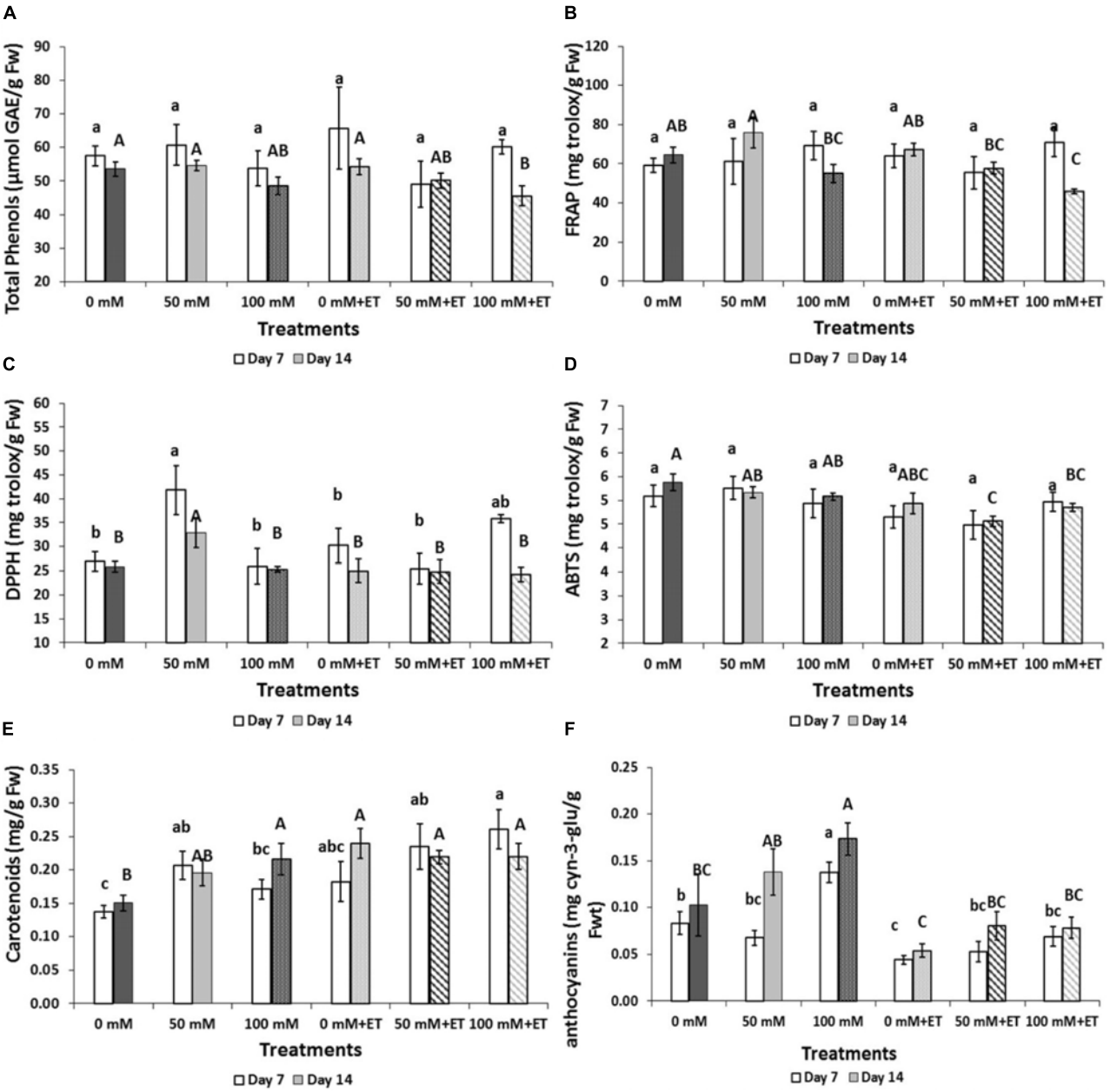

FIGURE 3 | Effect of salinity levels (0-50-100 mM NaCl) on greenhouse-grown tagetes flowers in modified atmosphere packaging (with or without vaporized ethanol-ET) during storage at 5°C on the content of total phenols, carotenoids, anthocyanins, and antioxidant activity. **(A)** Total phenols, **(B)** FRAP, **(C)** DPPH, **(D)** ABTS **(E)** carotenoids, and **(F)** anthocyanins. Significant differences ($P < 0.05$) among treatments are indicated by different small or capital letters for days 7 and 14, respectively. Error bars show SE ($n = 4$).

under passive modified atmospheric packaging as a well-known means of postharvest preservation of fresh produce (Tzortzakis and Economakis, 2007). Cut flowers are the reproductive organs of a plant and they are more sensitive to deterioration and tissue break down than the relevant plant vegetative parts, i.e., leaves and stems (Serek and Reid, 2000). Edible flowers are cut off the stem at the pedicel and, therefore, they are more sensitive and exposed to additional stress. However, edible flowers had previously received less attention than cut flowers and even lesser attention than fresh fruits and vegetables due to their low production and market interest (Kou et al., 2012).

Plants exposed to saline conditions often demonstrate a decrease in the ability to absorb water and minerals causing rapid reduction in growth and yield and inducing several metabolic changes (Hendawy and Khalid, 2005; Chrysargyris et al., 2018). Salt stress is complex and leads to the formation of ROS such as singlet oxygen (O_2^1), superoxide (O_2^-) and hydrogen peroxide (H_2O_2) with harmful effects on

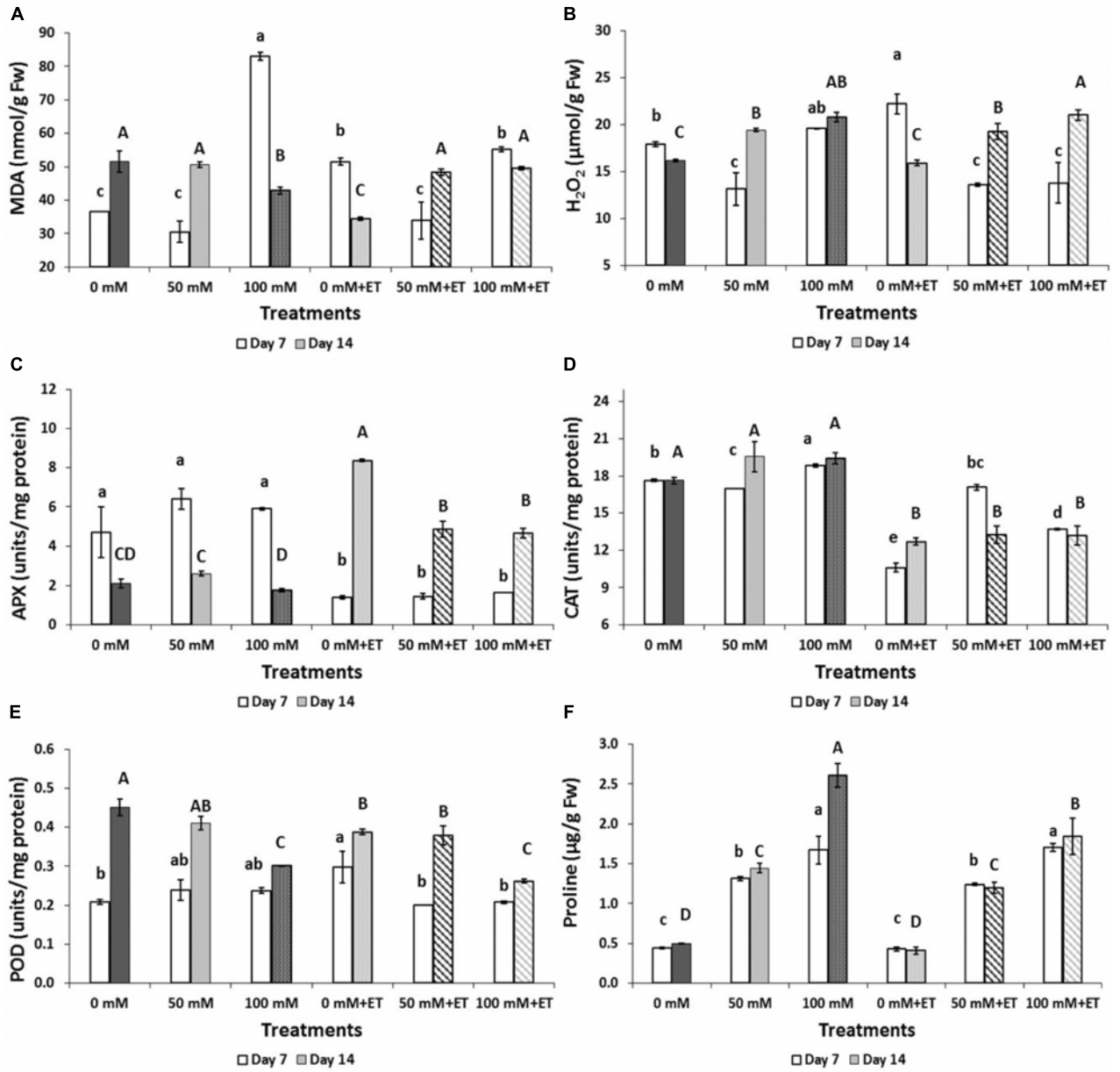

FIGURE 4 | Effect of salinity levels (0-50-100 mM NaCl) on greenhouse-grown tagetes flowers in modified atmosphere packaging (with or without vaporized ethanol-ET) during storage at 5°C on the flower damage index, proline, and antioxidant enzymes activities on greenhouse-grown tagetes. **(A)** Lipid peroxidation (MDA), **(B)** H_2O_2, **(C)** APX, **(D)** CAT, **(E)** POD, **(F)** proline. Significant differences ($P < 0.05$) among treatments are indicated by different small or capital letters for Day 7 and 14, respectively. Error bars show SE ($n = 4$).

biomolecules (lipids, proteins, nucleic acids) due to oxidative damage (Gill and Tuteja, 2010; Chrysargyris et al., 2018). In the current study, 100 mM NaCl significantly ($P < 0.05$) reduced plant biomass and plant size (i.e., height) and affected physiological processes negatively. Therefore, plants closed the leaf stomata to overcome salinity stress and at the same time salinity caused osmotic stress and water deficiency, which was determined by the stomatal conductance reductions. Stomatal conductance reduction is an adaptation plant mechanism to salinity stress and has been reported in *Plandago* spp. (Izadi-Darbandi and Mehdikhani, 2018) and *Calendula officinalis* (Khalid and Teixeira da Silva, 2010). The reduction in growth is also related to the decreased content of chlorophylls as observed at 100 mM NaCl application, which can be one of the factors for the photosynthetic rates decrease (Kiarostami et al., 2010). Plant growth and chlorophylls content reductions under saline conditions have been reported for several species (Tarchoune et al., 2010; Chondraki et al., 2012; Klados and Tzortzakis, 2014; Chrysargyris et al., 2018). Chaparzadeh et al. (2004) reported plant biomass reduction in *C. officinalis*

subjected to salinity of 100 mM NaCl on a long term basis while Villarino and Mattson (2011) observed such reductions at the *T. patula*. Tagetes plants subjected to saline conditions exhibited an F_v/F_m ratio higher than 0.80, indicating absence of severe stress (Bjorkman and Demmig, 1987), as this is related to the short-term exposure to salinity. Indeed, the application of short-term saline stress (10 days) did not change the Chroma and L, a^*, and b^* color values of the flowers. Therefore, visually flowers maintained their marketability value on chroma related parameters.

Plants cope with salinity induced stress by altering metabolic processes and stimulating the formation of phenolics and antioxidant activity to scavenge free radicals and ions chelators (Balasundram et al., 2006). Examining polyphenols and antioxidant activities in leaves and flowers leads to the conclusion that the effects of salinity could be clearly seen only on the latter (i.e., flowers). Therefore, flowers polyphenols and antioxidant activities (determined by three antioxidant assays of FRAP, DPPH and ABTS) as well as carotenoids increased with the application of salinity, with more pronounced impacts at salinity of 100 mM NaCl. This indicates higher nutraceutical value for the edible flowers when antioxidants and carotenoids increased in flowers subjected to salinity stress. Similar findings were found by Chaparzadeh et al. (2004) at *C. officinalis* flowers. Considering that plants change metabolic processes due to salinity stress, changes first take place at the vegetative part (i.e., leaves) and then are followed by changes at the reproductive organs (i.e., flowers). Thus, unchanged polyphenols and antioxidants in leaves possibly indicate that any inductions as a reaction of the plant to overcome saline stress took place before the 10th day. The decrease of anthocyanins in flowers subjected to salinity could be correlated with the decrease in plant growth/development as several metabolic processes were slowed down, other pigments accumulation or that anthocyanins have been already involved at the antioxidative mechanisms of the plant and their content had been exhausted. However, further studies requited to that direction, as the effect of salinity on anthocyanins accumulation is varied. Borghesi et al. (2011) for example, showed an opposite behavior in anthocyanins accumulation in two tomato genotypes when subjected to salinity.

Tagetes flowers were richer in potassium (averaged in 11.40 mg/kg dry weight) than in sodium (averaged in 3.45 mg/kg dry weight), which is quite useful for preventing cardiovascular diseases. Short-term saline exposure of tagetes plants activated metabolic processes such as accumulation of minerals (such as N, P, and Zn) on edible flowers which is of great importance to human health. Zinc deficiency causes impaired growth, immune dysfunction, increased morbidity and mortality as well as abnormal neuro-behavioral development (Mayer et al., 2008). Nitrogen and phosphorus have a substantial role in several metabolic processes since phospholipids, as a constitute of nucleic acid, are involved in protein synthesis, DNA, RNA, and ATP (Rouached et al., 2010). Consumption of 12 g of dry *T. patula* in salads, for example, can provide the 25% of the daily needs of potassium for adults (Rop et al., 2012).

Salinity and/or ET application causes stress which benefits the accumulation of ROS. Plants develop scavenging mechanisms against ROS detoxification by increasing the activity of antioxidant enzymes, such as SOD, APX, CAT, and glutathione reductase (GR) (Foyer and Noctor, 2011). In the present study, tagetes flowers demonstrated activation of both non-enzymatic (i.e., proline content) and enzymatic mechanisms (CAT) to overcome ROS detoxification. High accumulation of proline in plant tissue is an important adaptive mechanism of salt tolerance as proline is regarded as a source of energy, carbon and nitrogen for the recovering tissues, by acting as a compatible solute in osmotic adjustment and reduces membrane oxidative damage (Hossain et al., 2014). Proline acts as an osmolyte and reduces the osmotic potential, thus reducing toxic ion uptake (Chrysargyris et al., 2018). CAT is valuable for the elimination of hydrogen peroxide in peroxisomes by oxidases involved in β-oxidation of fatty acids, photorespiration and purine catabolism (Garg and Manchanda, 2009). The decreased enzymatic activities of APX and POD or the unaffected SOD activity is probably indicating that either enzymes were not involved in H_2O_2 scavenging or enzymes activities have been spent for H_2O_2 capture and ROS in general at an earlier stage. SOD is used for plant primary detoxification and is later followed by APX and POD (Chrysargyris et al., 2018).

Very few studies have examined the storage conditions of edible flowers and a gap of knowledge is evidenced on the postharvest preservation of edible flowers (Kou et al., 2012). The storage period of edible flowers is usually limited within a couple of days so high-tech storage and shipping conditions are required to extent their shelf life and simultaneously reduce the deteriorated products waste. Moreover, crop cultivation in controlled manner, as soilless culture, can yield produce of high quality and possible storability. Modified atmospheric packaging is commonly used for fresh produce quality maintenance, prolonging shelf life, and decreasing the microbial load of perishable commodities (Tzortzakis, 2010; Kou et al., 2012). Fresh produce stored on MAP conditions usually demonstrates an increase in carbon dioxide and a decrease in oxygen concentration and this actually retards the produce respiration process. In the present study ethanol enhanced the flower metabolic processes as it increased CO_2 production in tagetes flowers stored for 14 days, while salinity and/or storage period had no significant effects on it. Similarly, Kou et al. (2012) reported increased CO_2 accumulation in edible carnations and snapdragons following 7 days of storage. Ethanol prevents a rise in respiration rate and autocatalytic ethylene production/action (Asoda et al., 2009).

The increase in CO_2 production was followed by weight loss in flowers subjected to ET even from the 7 days of storage. Flower weight loss increased (up to two and eightfold) in 100 mM NaCl-treated plants following 7 and 14 days of storage, respectively. This affected the flower marketability as it was decreased ($P < 0.05$) when plants were grown at high (100 mM NaCl) saline levels while ET itself did not change the marketability of the flowers. Therefore, tagetes flowers did not benefit on marketability terms with the application of ET, as it was previous reported on fruits and vegetables, whereas ethanol can be used for fresh produce preservation (Pesis, 2005; Asoda et al., 2009). Even though ethanol did not improve flower marketability, ET application alleviated the induced decay found in 100 mM

NaCl-treated flowers to levels similar to the relevant control treatment (0 mM NaCl+ET). The antimicrobial properties of ethanol have been reported in several commodities (Karabulut et al., 2004; Tzortzakis, 2010). The increased decay observed at salinity of 100 mM NaCl-treated flowers may be related to the increased respiration metabolism and weight loss of the flowers but also to the high moisture content inside the tagetes packaging, as strong condensation was evidenced.

During storage, flowers maintained their color as several indicators (Chroma, a^*, b^*, L) remained at similar levels. Therefore, neither saline levels nor ET-vapors affected the color of tagetes flowers which established the maintenance of marketability, and the possible utilization of salinity and/or ET for improve/maintain the edible flowers quality.

The content of polyphenols decreased in flowers subjected to stress of salinity of 100 mM NaCl with ethanol after 14 days of storage at chilled temperature of 5°C whereas flower antioxidant activity (assayed by DPPH, FRAP, and ABTS) decreased mainly due to increased saline levels, storage duration and/or ET vapor. Thus, flowers were gradually deprived of their ability to detoxify ROS after 14 days of storage and alternative processes such as carotenoids and flavonoids accumulation might have taken place. The stress caused by saline- and ET-application resulted in increased content of carotenoids whereas higher anthocyanins content was found in flowers treated with salinity of 100 mM NaCl and stored for 7 and 14 days. This is of great importance, as stressed flowers by saline and/or ET were able to achieve higher carotenoids and anthocyanins levels and be a new source of nutraceutical foods. El Kereamy et al. (2002) reported that ethanol triggers gene expression leading to accumulation of anthocyanins during berry ripening, when ethanol (5% v/v) was applied on Cabernet Sauvignon grape fruit at veraison stage. The increased consumer and market demand for plant-based products with high antioxidant status may acknowledge edible flowers subjected to short-term salinity stress as products of added value which could prevent oxidative damage in human health. Fresh produce consumption with antioxidants could prevent chronic diseases such as type-2 diabetes, cancer, cardiovascular, and neurodegenerative disorders (Petrova et al., 2016).

CONCLUSION

Edible flowers are rich in flavonoids, phenolics, and terpenes, exhibit biocidal activities and possess beneficial properties for human health. Unfortunately, in spite of their agronomic potential, the concept of eating flowers is still viewed with doubt. Considering that salinity maintained the flower quality and storability, despite the reduction in growth and productivity of saline-treated plants, producers can consider the use of saline water for irrigation needs as a short-time stress for tagetes crops. Edible flowers subjected to saline conditions accumulated minerals such as N, P, Na, and Zn. Short-term exposure to saline condition and/or ethanol vapor application trigger flower metabolic process (both non-enzymatic (i.e., proline content) and enzymatic mechanisms (catalase) to overcome stress) and resulted higher carotenoids and anthocyanins levels during storage. It is the activation of several important metabolic processes with higher antioxidant status of the edible flowers that can be considered as a source of nutraceutical foods. Further efforts are needed to improve the postharvest preservation of the flowers when subjected to saline-stress conditions.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

AC, AT, and NT designed and performed the experiments and physiological measurements. AC, PX, and AT performed the postharvest measurements. AC and NT analyzed the data and critically discussed the data. NT and AC prepared the manuscript. NT coordinated the research.

ACKNOWLEDGMENTS

Thanks to Mrs. Maria Koutroumani (Ministry of Education, Research and Religious Affairs, Athens, Greece) for language editing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01765/full#supplementary-material

FIGURE S1 | Fluctuation of drainage pH and EC (mS/cm) under different salinity levels (0, 50, and 100 mM NaCl) in hydroponically grown tagetes plants.

FIGURE S2 | Effect of salinity levels (0, 50, and 100 mM NaCl) on tagetes maximum quantum efficiency of PSII (F_v/F_m) in plants grown hydroponically.

TABLE S1 | Effect of salinity levels (0, 50, and 100 mM NaCl) on tagetes flowers color values (L, a^*, b^*, Chroma) in plants grown hydroponically. ^{Y}Results are expressed as means±SE (n = 6). Values in rows followed by the same letter are not significantly different, $P \leq 0.05$.

TABLE S2 | Effect of salinity levels (0, 50, and 100 mM NaCl), ethanol application (no ethanol, with ethanol) and storage period (7 and 14 days) on water loss (%), color (L, a^*, b^* values), total phenolic content (mg GAE/g Fwt), antioxidants (mg trolox/g Fwt), carotenoids (mg/100 g Fw) and anthocyanins (mg cyn-3-glu/100 g Fw) on tagetes flowers during postharvest storage. ns, *, **, and *** indicate non-significant or significant differences at $P \leq$ 5%, 1%, and 0.1%, respectively, following two-way ANOVA.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 30 August 2019
doi: 10.3389/fpls.2019.01040

Functional Quality, Mineral Composition and Biomass Production in Hydroponic Spiny Chicory (*Cichorium spinosum* L.) Are Modulated Interactively by Ecotype, Salinity and Nitrogen Supply

Martina Chatzigianni[1,2], *Georgia Ntatsi*[1,3]*, *Maria Theodorou*[2], *Aristidis Stamatakis*[2], *Ioannis Livieratos*[2], *Youssef Rouphael*[4] and *Dimitrios Savvas*[1]*

[1] Department of Crop Science, Laboratory of Vegetable Crops, Agricultural University of Athens, Athens, Greece, [2] Department of Sustainable Agriculture, Laboratory of Soil Science and Plant Diagnostics, Mediterranean Agronomic Institute of Chania, Chania, Greece, [3] Institute of Plant Breeding and Genetic Resources ELGO-DEMETER, Thessaloniki, Greece, [4] Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy

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***Correspondence:**
Georgia Ntatsi
ntatsi@aua.gr
Dimitrios Savvas
dsavvas@aua.gr

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: 23 October 2018
Accepted: 26 July 2019
Published: 30 August 2019

Citation:
Chatzigianni M, Ntatsi G, Theodorou M, Stamatakis A, Livieratos I, Rouphael Y and Savvas D (2019) Functional Quality, Mineral Composition and Biomass Production in Hydroponic Spiny Chicory (Cichorium spinosum L.) Are Modulated Interactively by Ecotype, Salinity and Nitrogen Supply. Front. Plant Sci. 10:1040. doi: 10.3389/fpls.2019.01040

The hydroponic cultivation of spiny chicory (*Cichorium spinosum* L.), also known as stamnagathi, allows the development of year-round production. In the current study, two contrasting stamnagathi ecotypes originating from a montane and a coastal-marine habitat were supplied with nutrient solution containing 4 or 16 mM total-N in combination with 0.3, 20, or 40 mM NaCl. The primary aim of the experiment was to provide insight into salinity tolerance and nutrient needs in the two ecotypes, thereby contributing to breeding of more resilient cultivars to salinity and nutrient stress. Nutritional qualities of the stamnagathi genotypes were also tested. The coastal-marine ecotype was more salt tolerant in terms of fresh shoot biomass production and contained significantly more water and macro- and micro-nutrients in the shoot per dry weight unit. The root Na^+ concentration was markedly lower in the coastal-marine compared to the montane ecotype. The leaf Na^+ concentration was similar in both ecotypes at external NaCl concentrations up to 20 mM, but significantly higher in the montane compared to the coastal-marine ecotype at 40 mM NaCl. However, the leaf Cl^- concentration was consistently higher in the coastal-marine than in the montane ecotype within each salinity level. The marine ecotype also exhibited significantly less total phenols, carotenoids, flavonoids, and chlorophyll compared to the montane ecotype across all treatments. Integrating all findings, it appears that at moderate salinity levels (20 mM), the higher salt tolerance of the coastal-marine ecotype is associated with mechanisms mitigating Na^+ and Cl^- toxicity within the leaf tissues, such as salt dilution imposed through increased leaf succulence. Nevertheless, at high external NaCl levels, Na^+ exclusion may also contribute to enhanced salt tolerance of stamnagathi. Both ecotypes exhibited a high N-use efficiency, as their shoot biomass was not restricted when the total-N supply varied from 16 to 4 mM. The leaf organic-N was not influenced by salinity, while the interaction ecotype × N-supply-level was insignificant, indicating that

the mechanisms involved in the salt tolerance difference between the two ecotypes was not linked with N-acquisition or -assimilation within the plant. The current results indicate that both ecotypes are promising germplasm resources for future breeding programs.

Keywords: bioactive molecules, closed soilless system, landraces, macro-minerals, nitrate, salinity eustress, stamnagathi

INTRODUCTION

Consumer perception of the capacities of fresh functional plant-based foods to support human health and longevity has increased, especially during the last two decades (Kyriacou et al., 2016). These changes in consumer behavior have fueled the critical reassessment of the fresh-fruit and -vegetable qualities as part of the 'personalised nutrition' concept, as defined in a recent paper as '*a dynamic composite of physicochemical properties and evolving consumer perception, which embraces organoleptic, nutritional and bioactive components*' (Kyriacou and Rouphael, 2018). Accordingly, crop growers, food nutritionists, extension specialists, as well as scientists are seeking to identify vegetable crops that may be produced inexpensively for the fresh-food markets and that present high nutraceutical (and organoleptic) properties (Slavin and Lloyd, 2012).

Towards that end, spiny chicory (*Cichorium spinosum* L.), a dwarf perennial species within the *Asteraceae* family, which is also known in Greek language as *stamnagathi*, is gaining popularity in many parts of Greece (Crete) and throughout the Mediterranean basin (Cyprus, Libya, Malta, Sicily, Spain, and Turkey) as a functional culinary trend (Brieudes et al., 2016). In addition to its unique taste, consumption of *C. spinosum* has increased to its high levels of health-promoting phytochemical compounds, such as vitamins (C, E, and K1), phenolic acids (chicoric and 5-O-caffeoylquinic), total glutathione, proteins, fatty acids, carotenoids (β-carotene and lutein), and minerals (Zeghichi et al., 2003; Vardavas et al., 2006; Petropoulos et al., 2016; Petropoulos et al., 2017). The commodity commands very high prices and is ever increasingly demanded by fresh vegetable markets. Therefore, the greenhouse cultivation of *C. spinosum* has the immediate potential for expansion (Ntatsi et al., 2017a). However, in many areas of the Mediterranean region, vegetable farmers are forced to use poor quality water (i.e., highly saline), and especially in coastal-marine regions where leafy vegetables are grown under protected cultivation. Excessive concentration of sodium chloride (NaCl) in irrigation water and/or soil induces osmotic as well as ionic stresses, which lead to several morphological, anatomical, physiological, and metabolomic changes (Munns, 2005; Colla et al., 2013a; Ntatsi et al., 2017a; Rouphael et al., 2017a; Rouphael et al., 2017b; Rouphael et al., 2018a). In particular, the excessive Na^+ and Cl^- concentrations in the root zone are harmful to most vegetable crops, as they can cause pigment (chlorophyll and carotenoids) degradation (Lucini et al., 2015), hamper the macro- and micronutrient uptake, translocation, and assimilation (Grattan and Grieve, 1998), and limit net CO_2 assimilation (Colla et al., 2013b). As a result, the plants exhibit stunted growth and yield is reduced significantly (Lucini et al., 2016; Rouphael et al., 2017a). Although salinity generally reduces the crop productivity, in many cases mild to moderate salt stress also known as *eustress* can trigger the biosynthesis and accumulation of bioactive secondary compounds (carotenoids, phenolic compounds, organosulfuric compounds, polyamines, *etc.*), as demonstrated for several vegetable crops grown under protected cultivation (Rouphael et al., 2012, Rouphael et al., 2017a; Rouphael et al., 2017b; Rouphael et al., 2018b). However, the accumulation or degradation of specific organic molecules and secondary metabolites depends on several interacting factors such as plant species or ecotype (cultivar), and the period and magnitude of exposure, as well as the agronomic management options (Rouphael and Kyriacou, 2018).

Moreover, leafy vegetables grown commercially, particularly those cultivated in hydroponics, are focused on maximizing yield through intensification of fertilizer use and especially nitrates, the most important source of nitrogen (N), which boosts productivity (Borgognone et al., 2013). Minimizing N supply, while maintaining yield and nutritional qualities and avoiding negative environmental impacts, is of special importance to growers and presents a major sustainability challenge to the vegetable industry (Colla et al., 2010; Colla et al., 2011; Borgognone et al., 2016). In addition, several authors (Stefanelli et al., 2010; Becker et al., 2015; Borgognone et al., 2016) have demonstrated that low N-availability can increase the concentration of health promoting bioactives such as phenolic acids and flavonoids, as shown for several diverse forms of leafy vegetable crops destined for fresh consumption as well as for the food-processing industries.

Despite the increasing economic importance of *C. spinosum* as a new niche product, there is a lack of information in the scientific literature concerning its response to salt stress. One relevant study is that of Klados and Tzortzakis (2014) who found that increasing the NaCl concentration in the supplied nutrient solution to 40 mmol L^{-1} reduced the plant biomass production but increased the concentration of total phenolics, bitterness, and sourness of leaves. In another study, Petropoulos et al. (2017) found that increasing the electrical conductivity to 6 and 8 dS m^{-1} in the root zone of *C. spinosum* increased the leaf protein content on fresh weight basis, and the antioxidant activity, but had no impact on the concentration of phenolic compounds. Nevertheless, nothing is known regarding how levels of these phytochemicals vary in response to N-fertilizer dose, NaCl concentration, or their interaction.

Considering this background, a three-factorial experiment was designed to study the effects of N level and NaCl concentration in the supplied nutrient solution on growth,

mineral composition, and nutritional attributes (phenolics, carotenoids, antioxidant activity, and flavonoids) of two contrasting stamnagathi ecotypes originating either from a coastal-marine or from a montane habitat. The primary aim of this experiment was to provide some insight into the mechanisms underlying salinity tolerance of these two contrasting ecotypes, as well as into their possible links to the level of N supply, thereby contributing to breeding of more resilient cultivars to salinity and nutrient stress. An additional objective of the present study was to test the hypothesis that moderate levels of combined salinity and N-shortage stress improve some nutritional quality characteristics of stamnagathi without compromising fresh biomass production, which is of economic interest for growers.

MATERIALS AND METHODS

Growth Conditions and Plant Material

Seeds of stamnagathi (*Cichorium spinosum* L.) were harvested from wild plants originating from two different areas of Crete: a coastal zone (Stavros, North-East Chania Crete, 35°59′17.79″N and 24°09′79.74″E) and a montane site (Tavri at Omalos on the mountain Lefka Ori in Chania, 1200 m altitude, 35°29′30.77″N, 24°15′78.44″E). The collection of the stamnagathi seeds took place on 23rd and 25th of September 2014 for the montane and coastal-marine ecotypes, respectively. The seeds were germinated as described below, and the resultant seedlings were cultivated in an unheated glasshouse during the 2015 winter–spring growing season at the Mediterranean Agronomic Institute of Chania (MAICh) Crete, Greece (35°29′40.45″N and 24° 02′57.42″E). The glasshouse layout of the test material comprised five double-rows of each ecotype, plus one additional row at each outer-border that served as a guard-row. The length and width of each double-row were 10 and 0.2 m, respectively. The plants were grown under natural light conditions. The average day/night temperatures (± standard deviations) inside the glasshouse were 19.34 ± 2.15/12.64 ± 1.68°C in February, 21.39 ± 2.03/14.47 ± 1.73°C in March, 23.65 ± 2.05/16.03 ± 2.04°C in April, and 25.69 ± 0.84/17.83 ± 1.39°C in May (from the 1st to the 18th).

Experimental Design, Crop, and Nutrient Solution Management

On 4th of December 2014, the seeds of the two ecotypes were first sown in trays, with one seed being placed into one of the 84 holes (per tray). Each hole was filled with a peat:perlite (3:1 [v/v]) mixture. The pH of the growing medium was 6 and no NPK fertilizers were added, as the peat was enriched with nutrients. The macronutrient content of peat was as follows: Nitrogen, phosphorus, potassium, and magnesium were 100, 115, 125, and 100 mg L^{-1}, respectively, while all essential trace elements were included (Klassman Plug Mix extra plus). For that reason, the seedlings were irrigated with water regularly as required, while all treatments were applied after transplanting. On 30th of January 2015, 2 months after sowing, the seedling-plugs were removed from the tray and transplanted into perlite packed in bags (Perloflor Hydro 1, Athens, Greece). The particle size of the perlite granules ranged from 0.5 to 2.5 mm. After transplanting, the plants were fertigated using different nutrient solutions in each treatment as described below, and the drainage water was not reused. Each perlite bag (33 L) was 1 m in length, 24 cm in width, and 16 cm in height and accommodated four equally spaced plants. The space between plants within rows was 20 cm, with 48 plants per row. To allow free drainage of excess nutrient solution, two holes were made at the bottom of each bag.

Twelve treatments were derived from a factorial combination of three NaCl concentrations (0.3, 20, or 40 mM), two levels of -N concentrations (4 or 16 mM), and two stamnagathi ecotypes from either a montane (M) or a coastal (C) site. The treatments were arranged in a randomised complete-block design with four replicates per treatment. As a total, 48 experimental units (plots) with 12 plants (3 bags × 4 plants per bag) in each plot (n = 576 plants) were established. The salinity and N treatments were initiated directly after transplanting.

The macronutrient concentrations in the different nutrient solution treatments are presented in **Table 1**. The micronutrient concentrations were identical in all treatments, as follows: 15.0 μM Fe, 8.0 μM Mn, 6.0 μM Zn, 0.7 μM Cu, 30.0 μM B, and 0.5 μM Mo. The decrease of the NO_3-N level from 16 to 4 mM in the low NO_3-supply treatments was compensated for by an equivalent increase of the SO_4^{2-} and Cl^- concentrations,

TABLE 1 | The electrical conductivity (EC), pH, and the concentrations of K^+, Ca^{2+}, Mg^{2+}, Na^+, Cl-, NO_3^-, $H_2PO_4^-$, and SO_4^{2-} in the six different nutrient solution treatments that were applied to the plants by combining two levels of total-N supply (4 or 16 mmol L^{-1}, denoted as 4TN and 16TN, respectively) and three different salinity levels (0.3, 20, and 40 mM, respectively) in the nutrient solution.

Salinity	**0.3**	**20**	**40**	**0.3**	**20**	**40**
Total-N level		**4 mmol L^{-1}**			**16 mmol L^{-1}**	
EC (dS m^{-1})	2.10	4.10	6.14	2.10	4.10	6.14
pH	5.60	5.60	5.60	5.60	5.60	5.60
K^+ (mmol L^{-1})	7.50	7.50	7.50	7.50	7.50	7.50
Ca^{2+} (mmol L^{-1})	4.40	4.40	4.40	4.40	4.40	4.40
Mg^{2+} (mmol L^{-1})	1.50	1.50	1.50	1.50	1.50	1.50
Na^+ (mmol L^{-1})	0.3	20.00	40.00	0.3	20.00	40.00
Cl^- (mmol L^{-1})	6.37	26.07	46.07	0.30	20.00	40.00
NO_3^- (mmol L^{-1})	3.00	3.00	3.00	15.00	15.00	15.00
H_2PO_4 (mmol L^{-1})	1.20	1.20	1.20	1.20	1.20	1.20
SO_4^{2-} (mmol L^{-1})	4.81	4.81	4.81	1.84	1.84	1.84

thereby maintaining the same total nutrient anion and cation concentrations in both NO_3-supply levels. Thus, the only differences in EC between treatments were those imposed by the addition of NaCl. The pH of the nutrient solution supplied to the plants was 5.6 in all treatments, while the EC was 2.10, 4.10, and 6.14 dS m^{-1}, corresponding to 0.3 mM (non-salt control), 20 mM, or 40 mM NaCl, respectively. Nutrient solution was delivered to the plants through a drip-irrigation system using pumps connected to an electronic timer. Each plant was supplied with nutrient solution from an individual emitter having a flow rate of 2 L h^{-1}. The irrigation frequency was adjusted according to the integral of solar radiation intensity aiming to result in a drainage fraction of 30%. This resulted in two to four irrigation applications per day (140–280 ml per plant) to each experimental unit. Moreover, the pH and EC of the drainage solution were monitored during the growing cycle by collecting samples three times per week. During the whole experiment, no spraying for disease or insect control, and no heating was applied.

Plant Material

The shoots of the plants were harvested on 03/31/2015, i.e., 60 days after transplanting (DAT). The edible shoots were harvested when the majority of non-salinized plants treated with 16 mM were considered commercially ripe according to local cultivation practices (rosette diameter between 20 and 27 cm, number of leaves >30). Harvesting entailed cutting the main stem of each plant at about 1 cm above the growing medium using a sharp knife. After the first production cycle, the roots with the basal part of the stem were left to sprout again for a second production cycle. The second harvest took place on 05/18/2015 (108 DAT). At the second harvest, both the shoots and root of the plants were collected. At both production cycles, the harvested plants from each experimental unit were pooled into a single sample, placed immediately in plastic bags, and transported to the laboratory for processing and further analysis.

Ionomic Analyses

Leaf samples originating from 12 plants per experimental unit were collected at the first harvest, i.e., 60 days after transplanting, and divided into two sub-samples. The first sub-sample was dried and milled to determine the leaf N, P, K^+, Ca^{2+}, Mg^{2+}, and S concentrations. The second sub-sample was stored immediately after harvest at −18°C and used later to determine total chlorophyll, total phenolics, flavonoids, and β-carotene concentrations, as well as total antioxidant activity. The plant samples collected at the second harvest (2nd production cycle) were separated into shoots and roots and used to determine the concentrations of N, P, K^+, Ca^{2+}, and Mg^{2+} in the roots, Cl^- in leaves, and Fe, Mn, Zn, Cu, and Na^+ in both leaves and roots. The Cl^- concentration in the root was not measured, because the samples were lost due to a technical failure. The samples used to determine tissue mineral concentrations were weighed and dried at 80°C for 72 h until they reached a constant weight. Subsequently, they were weighed again to determine the corresponding dry biomass as well as the dry matter percentage (and moisture content). The dried tissue samples (leaves and roots) were powdered using a blade-mill and passed through a 40-mesh sieve. Sub-samples (300 mg) of all dried plant tissue samples were used to determine the concentrations of the above-mentioned macro- and microelements. Organic-N was determined by applying the Kjeldahl method. Briefly, 250 mg of dry leaf and root tissues was digested in a Gerhardt apparatus after adding 10 ml of concentrated H_2SO_4 in the presence of a catalyst mixture (100 g K_2SO_4 + 16 g $CuSO_4$ + 1.5 g Se). The distillation was carried out using a Vapodest 40 (Gerhardt) after addition of 50 ml H_2O and 60 ml of 40% NaOH (Bremner, 1965). Phosphorus, K^+, Ca^{2+}, Mg^{2+}, Na^+, Mn, Fe, Zn, and Cu were determined in aqueous extracts obtained as follows. Powdered plant tissues placed on crucibles were dry-ashed at 450°C for 6 h, and the ash was dissolved using 10 ml of 2 N hydrochloric acid (HCl). Subsequently, the crucibles were transferred to a hot plate at 80°C for a few minutes, and the hydrochloric solution was filtered and transferred to 100-ml flasks, which were filled up with distilled water. The mineral concentrations in the obtained aqueous extracts were determined using an Inductively Coupled Plasma-Optical Emission Spectrometry Instrument (ICP-OES PSFO 2.0, Leeman Labs INC., USA). The ICP-OES operating conditions were as follows: nebulizer gas flow rates: 0.5 L min^{-1}; auxiliary gas flow: 0.5 L min^{-1}; plasma gas flow: 15 L min^{-1}; pump rate: 45 rpm, and ICP RF power: 1100 W. The S concentration was extracted from 250 mg samples with deionized water at 80°C in a shaking water bath for 10 min (ShakeTemp SW22, Julabo, Seelbach, Germany). The resulting solution was filtered, diluted, and analyzed by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA). A conductivity detector with IonPac AG11-HC guard column and IonPac AS11-HC analytical column (Dionex Corporation) was used for the analysis of S. The determination of Cl^- in the plant-tissue extracts and nutrient solutions was performed by titration with $AgNO_3$ in the presence of K_2CrO_4 (Eaton et al., 1995).

Functional Quality Analyses

For the chlorophyll analysis, 5 g of fresh leaf tissue was collected at the first harvest, i.e., on 03/31/2015, from three plants per replicate. Chlorophyll was extracted by grinding the tissue with a mortar and pestle using ammoniacal acetone. The resulting extracts were centrifuged at 3,000 × g for 15 min. The chlorophyll contents were determined by UV-Vis spectrophotometry (Specord 250, Jena, Germany). The absorbance of the solution was measured at 645, 652, and 663 nm for chlorophyll-a, chlorophyll-b, and total chlorophyll contents, respectively. Formulae and extinction coefficients used for the determination of chlorophyll contents were described by Lichtenhaler and Wellburn (1983).

The total phenolic content in methanolic extracts was determined using the Folin-Ciocalteau procedure (Singleton et al., 1999) with gallic acid as a standard. One gram of fresh tissue from two plants per replicate was extracted with 50% methanol (1:1 methanol:dH_2O) using mortar and pestle. The mixture was put in falcon tubes filled up to 10 ml, followed by sonication for 15 min. Then, the samples were centrifugated

for 15 min at 4°C and 5000 rpm. A 100 μL aliquot of the supernatant was combined with 500 μL of Folin-Ciocalteau's reagent (Sigma Aldrich Inc, St Louis, MO, USA) and 400 μL of 7.5% sodium carbonate/water (w/v). Absorption was measured after 30 min at 765 nm using a UV–vis spectrophotometer, and the result was expressed as mg gallic acid (Sigma Aldrich Inc., St Louis, MO, USA) per 100 g dry weight. Briefly, 1 g of raw plant material was weighted and transferred in a mortar with 4 ml of the extraction buffer (50 ml methanol 80% + 1.37 ml HCl 37%). Afterwards, the falcons were transferred on an orbital shaker at 200 rpm at room temperature and, after 2 h, were centrifuged for 15 min at 5000 rpm. The supernatant (Solution A) was transferred to a new falcon. Folin-Ciocalteau preparation: 1 ml of Folin-Ciocalteau + 9 ml dH_2O. In the Solution A (300 μL), 3 × 750 μL of Folin-Ciocalteau was added (Solution B), vortexed for a few seconds, and then incubated for 5 min. In Solution B, 3 × 750 μL of Na_2CO_3 was added and then stored in a dark place at room temperature for 90 min. Absorbance of the mixture, blue in color, was determined at 765 nm using water as blank.

The aluminium chloride colorimetric method was used to measure the total flavonoids content of the stamnagathi extracts as described by Zhishen et al. (1999). Briefly, 1 g of fresh tissue from two plants per replicate was extracted and 1 ml aliquot of appropriately diluted sample or standard solutions of catechin (20, 40, 60, 80, and 100 mg L^{-1}) was added to a 10 ml volumetric flask containing 4 ml H_2O. At time-zero, 0.3 ml 5% $NaNO_2$ was added to the flask. After 5 min, 0.3 ml 10% $AlCl_3$ was added. At 6 min, 2 ml 1 M NaOH was added to the mixture. Immediately, the reaction flask was diluted to volume with the addition of 2.4 ml of H_2O and thoroughly mixed. Absorbance of the mixture, pink in color, was determined at 510 nm using water as blank. Total flavonoids of leaves were expressed on a fresh weight basis as mg 100 g^{-1} catechin equivalents (CE).

The total antioxidant activity was determined according to the DPPH (2,2-diphenyl-L-picrylhydrazyl) radical scavenging spectrophotometric assay as reported by Choi et al. (2002). For this analysis, 10 ml of methanol (80%) was mixed in a falcon tube with 1 g of fresh tissue obtained from two plants per replicate and transferred on an orbital shaker for 2 h at 200 rpm. The fresh tissue was collected from a leaf originating from the middle of the rosette, taking care to be of the same physiological age in all samples. Subsequently, the falcons were centrifuged for 15 min at 5000 rpm and the supernatant was transferred to a new 50 ml falcon. For the preparation of the DPPH solution, 2.36 mg DPPH was diluted in 100 ml MeOH. Afterwards, 25 μL of supernatant and 975 μL DPPH solution were vortexed and stored in a dark place for 30 min (t = 30). Absorbance of the mixture was recorded at 515 nm, using methanol as blank (t = 0).

Finally, carotenoids (β-carotene) were determined according to the Nagata and Yamashita (1992) method. In specific, 1 g of fresh cut leaves from two plants per replicate was homogenized with acetone:hexane 4:6 [v:v] using a mortar and a pestle. The mixture was placed in 50 ml falcons, filled up to the final volume of 16 ml with acetone:hexane mixture, shaken vigorously, and stored overnight until the two phases were separated. An aliquot was taken from the upper solution for measurement of optical density at 663, 645, 505, and 453 nm in spectrophotometer, using acetone:hexane mixture as blank. Carotenoids were calculated according to the following equation: $\beta\text{-carotene} = 0.216*A_{663} - 1.22*A_{645} - 0.304*A_{505} + 0.452*A_{453}$ and expressed as mg 100 ml^{-1} of extract.

Statistical Analysis

Experimental data were analysed by applying three-way ANOVA to assess main effects [Ecotype (E), Total-N, and Salinity], three first-order interactions (E × total-N, E × Salinity, Total-N × Salinity), and one second-order interaction (E × total-N × Salinity). Multiple comparisons of means were performed by applying Duncan's Multiple Range Test at a confidence level of 0.05 after performing three-way ANOVA. All statistical analyses were carried out using the STATISTICA software package, version 9.0 for Windows (StatSoft Inc., Tulsa, USA). The normality was respected for all parameters, and no data transformation was required for any parameter.

RESULTS

Biomass and Yield Production

The shoot fresh weight (FW) of both stamnagathi ecotypes was not influenced by the level of total-N in the supplied nutrient solution, and no interactions between E × N, N × S, and E × N × S were observed. (**Table 2**). However, an interaction between the ecotype and salinity was observed. In particular, the shoot FW was similar in both ecotypes under non-saline conditions, but was significantly higher in the coastal-marine than in the montane ecotype at moderate (20 mM) and high (40 mM) NaCl-salinity. Furthermore, the shoot DW and the dry matter content (DMC) were significantly higher in the montane ecotype in comparison with the coastal-marine ecotype (**Table 2**). For both ecotypes, the shoot dry weight (DW) was restricted by the increase of salinity from 0.3 to 40 mM, while at 20 mM NaCl the reduction in shoot DW was insignificant. However, the N level applied in the nutrient solution had no significant impact on shoot DW (**Table 2**). Salinity and total-N supply level had no impact on the shoot DMC of stamnagathi.

Although significant interactions were observed, the leaf of the montane ecotype exhibited higher chlorophyll a and b and total chlorophyll concentrations compared to the coastal-marine ecotype (**Table 2**). A significant E × S interaction was observed for chlorophyll a and total chlorophyll. In the montane ecotype, these parameters increased under both saline conditions (20 and 40 mM), while no difference was observed for the coastal-marine ecotype. Regardless of the ecotype, the leaf chlorophyll a and total chlorophyll were significantly higher when plants were grown under high N supply level combined with high salinity (20 and 40 mM) when compared with low-N treatments. At moderate and high salinity (20 and 40 mM NaCl), the leaf chlorophyll b in leaves of the montane ecotype was significantly increased by the higher total N supply level, compared with the other treatments.

TABLE 2 | Impact of seed origin (montane or coastal-marine ecotype), total nitrogen concentration (4 or16 mmol L^{-1}, denoted as 4-N and 16-N, respectively), and salinity (0.3, 20, and 40 mM, respectively) in the nutrient solution supplied to hydroponically grown stamnagathi on shoot fresh weight (FW), shoot dry weight (DW), shoot dry matter content, chlorophyll a (Chl a), chlorophyll b (Chl b), and total chlorophyll (Tot chl).

Sources of variation		Shoot FW (g/plant) 1st growing cycle	Shoot DW (g/plant) 1st growing cycle	Dry matter content (%) 1st growing cycle	Chl a (mg g^{-1} FW) 1st growing cycle	Chl b (mg g^{-1} FW) 1st growing cycle	Tot chl (mg g^{-1} FW) 1st growing cycle
Ecotype	Montane	24.7	3.03	12.00	1.26	0.45	1.78
	Coastal	29.9	2.65	8.28	0.90	0.30	1.26
N level (N)	4 mM	27.7	2.71	10.15	1.03	0.35	1.45
	16 mM	26.8	2.97	10.14	1.12	0.40	1.59
Salinity	0.3 mM	27.4	3.14^{a}	10.22	1.04$^{b'}$	0.36	1.45$^{b'}$
	20 mM	27.9	2.81^{ab}	10.11	1.10$^{a'}$	0.38	1.56^{a}
	40 mM	26.7	2.57^{b}	10.11	1.09$^{ab'}$	0.38	1.56$^{a'}$
				Interactions			
E × N	Montane 4-N	25.4	2.88	11.84	1.22	0.43	1.71
	Montane 16-N	23.9	3.18	12.16	1.30	0.47	1.85
	Coastal 4-N	30.0	2.55	8.45	0.85	0.28	1.19
	Coastal 16-N	29.7	2.75	8.11	0.94	0.32	1.32
E × S	Montane 0.3 NaCl	26.8 ^{bc}	3.57	12.08	1.16^{b}	0.42	1.63^{b}
	Montane 20 NaCl	24.2 ^{cd}	2.90	12.02	1.34^{a}	0.47	1.89^{a}
	Montane 40 NaCl	23.1 ^{d}	2.63	11.91	1.28^{a}	0.47	1.83^{a}
	Coastal 0.3 NaCl	27.9 ^{b}	2.71	8.34	0.91^{c}	0.31	1.26^{c}
	Coastal 20 NaCl	31.5 ^{a}	2.72	8.20	0.97^{c}	0.29	1.23^{c}
	Coastal 40 NaCl	30.2 ^{ab}	2.52	8.30	0.90^{c}	0.30	1.28^{c}
N × S	4 N – 0.3 NaCl	28.9	2.87	10.24	1.03^{C}	0.36	1.44^{B}
	4 N – 20 NaCl	27.0	2.82	10.10	1.07^{BC}	0.35	1.49^{B}
	4 N – 40 NaCl	27.3	2.44	10.10	0.99^{C}	0.35	1.42^{B}
	16 N – 0.3 NaCl	25.8	3.41	10.19	1.04^{C}	0.36	1.45^{B}
	16 N – 20 NaCl	28.7	2.81	10.12	1.13^{AB}	0.41	1.63^{A}
	16 N – 40 NaCl	26.0	2.69	10.11	1.19^{A}	0.42	1.69^{A}
E × N × S	Montane 4 N – 0.3 NaCl	28.7	3.18	11.80	1.18	0.43^{b}	1.66
	Montane 4 N – 20 NaCl	24.2	2.90	11.97	1.30	0.42^{b}	1.81
	Montane 4 N – 40 NaCl	23.4	2.57	11.74	1.17	0.43^{b}	1.66
	Montane 16 N – 0.3 NaCl	24.8	3.96	12.36	1.13	0.40^{b}	1.60
	Montane 16 N – 20 NaCl	24.2	2.91	12.06	1.37	0.51^{a}	1.96
	Montane 16 N – 40 NaCl	22.8	2.68	12.07	1.40	0.52^{a}	2.00
	Coastal 4 N – 0.3 NaCl	29.0	2.56	8.67	0.88	0.29^{cd}	1.22
	Coastal 4 N – 20 NaCl	29.8	2.74	8.23	0.84	0.27^{d}	1.16
	Coastal 4 N – 40 NaCl	31.2	2.35	8.46	0.82	0.27^{d}	1.18
	Coastal 16 N – 0.3 NaCl	26.7	2.85	8.01	0.94	0.33^{c}	1.29
	Coastal 16 N – 20 NaCl	33.2	2.70	8.17	0.90	0.31^{cd}	1.30
	Coastal 16 N – 40 NaCl	29.1	2.69	8.14	0.99	0.33^{c}	1.37
P values	Ecotype (E)	0.0004	0.0295	0.0000	0.0000	0.0000	0.0000
	N level (N)	0.6941	0.1501	0.5817	0.0004	0.0000	0.0001
	Salinity (S)	0.2090	0.0426	0.7572	0.0452	0.1825	0.0084
	E × N	0.5904	0.7559	0.6176	0.6812	0.8195	0.9026
	E × S	0.0186	0.1628	0.9861	0.0017	0.0074	0.0031
	N × S	0.3366	0.4204	0.6457	0.0042	0.0105	0.0071
	E × N × S	0.6789	0.6992	0.6510	0.3677	0.0433	0.2385

For each parameter, means for each salinity level (n = 4) within columns followed by different capital, lower-case, and tones letters are significantly different according to the Duncan's multiple range test.

Mineral Composition and Partitioning

The increase of the external NaCl concentration to 20 mM raised the Cl^- concentration in the leaves of both stamnagathi ecotypes, while a further increase to 40 mM increased the leaf Cl^- only in the montane ecotype (**Table 3**). However, increasing the N concentration in the nutrient solution from 4 to 16 mM significantly reduced the leaf Cl^- concentration in the leaves of both ecotypes. Moreover, the leaf Cl^- concentration in the coastal-marine ecotype was always significantly higher than that in the montane ecotype within each NaCl and total-N level. The leaf Na^+ concentration (**Table 3**) increased as the NaCl concentration in the supplied nutrient solution was raised from 0 to 40 mM in both ecotypes. However, at the highest NaCl salinity level (40 mM), the leaf Na^+ concentration increased to higher levels in the montane than in the coastal-marine ecotype.

The leaf K^+ was significantly higher in the coastal-marine ecotype than in the montane ecotype when compared within each combination of high salinity with low and high N concentrations, as shown by the significant E × N × S interaction. However, the level of salinity that reduced the leaf K^+ was depending on both the ecotype and the total-N supply

TABLE 3 | Impact of seed origin (montane or coastal-marine ecotype), total nitrogen concentration (4 or16 mmol L^{-1}, denoted as 4-N and 16-N, respectively), and salinity (0.3, 20, and 40 mM, respectively) in the nutrient solution supplied to hydroponically grown stamnagathi on leaf Cl, Na, K, Ca, Mg, P, organic N, and sulfates.

Sources of variation		Leaf Cl (mg g^{-1} DW) 1st growing cycle	Leaf Na (mg g^{-1} DW) 1st growing cycle	Leaf K (mg g^{-1} DW) 1st growing cycle	Leaf Ca (mg g^{-1} DW) 1st growing cycle	Leaf Mg (mg g^{-1} DW) 1st growing cycle	Leaf P (mg g^{-1} DW) 1st growing cycle	Leaf organic N (mg g^{-1} DW) 1st growing cycle	Leaf sulfates (mg g^{-1} DW) 1st growing cycle
Ecotype	Montane	25.6	12.6	38.4	9.2	1.70	8.07	38.3	3.00
	Coastal	38.4	11.6	52.8	15.3	2.48	7.87	34.2	3.17
N level (N)	4 mM	40.5	12.1	43.8	11.2	2.00	7.88	35.6	4.62
	16 mM	24.5	12.1	47.6	13.3	2.18	8.05	36.9	1.55
Salinity	0.3 mM	16.2	3.2^{C}	53.0	13.7	2.38^{a}	7.28^{b}	36.8	3.34^{A}
	20 mM	35.0	14.5^{B}	46.2	12.7	2.08^{b}	7.67^{b}	35.5	2.91^{B}
	40 mM	46.3	18.6^{A}	37.8	10.3	1.82^{c}	8.94^{a}	36.5	3.01^{AB}
				Interactions					
E × N	Montane 4-N	31.3^{B}	13.2	38.2	8.5	1.63	8.07	37.9	4.42
	Montane 16-N	20.0^{C}	13.5	38.5	9.8	1.76	8.06	38.8	1.57
	Coastal 4-N	47.8^{A}	11.9	49.2	13.9	2.37	7.69	33.3	4.81
	Coastal 16-N	29.0^{B}	11.7	52.4	16.7	2.57	8.04	35.1	1.53
E × S	Montane 0.3 NaCl	11.9^{e}	2.9^{d}	47.3	10.6	1.95	7.30	37.3^{a}	3.18
	Montane 20 NaCl	26.1^{c}	15.5^{c}	37.2	9.3	1.64	7.72	38.7^{a}	2.89
	Montane 40 NaCl	38.8^{b}	21.5^{a}	30.5	7.6	1.51	9.19	39.0^{a}	2.92
	Coastal 0.3 NaCl	20.5^{d}	2.9^{d}	58.4	16.9	2.81	7.25	36.3^{ab}	3.49
	Coastal 20 NaCl	43.8^{a}	14.8^{c}	49.1	16.1	2.47	7.66	32.3^{c}	2.93
	Coastal 40 NaCl	50.9^{a}	17.7^{b}	44.9	13.1	2.12	8.69	33.9^{bc}	3.09
N × S	4 N – 0.3 NaCl	23.1	2.9	51.1	12.2	2.37	7.25	34.5^{B}	5.15^{a}
	4 N – 20 NaCl	40.5	15.2	43.0	11.0	1.86	7.99	34.7^{B}	4.28^{c}
	4 N – 40 NaCl	57.8	19.6	37.0	10.5	1.78	8.40	37.5^{AB}	4.42^{b}
	16 N – 0.3 NaCl	9.3	3.0	54.6	15.3	2.39	7.30	39.1^{A}	1.52^{d}
	16 N – 20 NaCl	29.4	15.1	43.3	14.4	2.25	7.38	36.3^{AB}	1.54^{d}
	16 N – 40 NaCl	34.8	19.6	38.4	10.1	1.85	9.48	35.4^{B}	1.59^{d}
E × N × S	Montane 4 N – 0.3 NaCl	18.0	2.8	48.7^{bc}	9.9^{e}	1.97	7.79	34.2	4.79
	Montane 4 N – 20 NaCl	28.2	15.3	33.8^{e}	8.0^{f}	1.48	7.86	37.6	4.22
	Montane 4 N – 40 NaCl	47.2	21.5	32.1^{e}	7.6^{f}	1.46	8.57	41.8	4.25
	Montane 16 N – 0.3 NaCl	5.7	3.1	45.9^{cd}	11.2^{de}	1.94	6.81	40.4	1.56
	Montane 16 N – 20 NaCl	24.0	15.6	40.6^{d}	10.6^{e}	1.81	7.57	39.8	1.56
	Montane 16 N – 40 NaCl	30.3	21.6	28.9^{e}	7.6^{f}	1.55	9.80	36.2	1.59
	Coastal 4 N – 0.3 NaCl	28.1	3.1	53.4^{b}	14.4^{b}	2.77	6.71	34.8	5.50
	Coastal 4 N – 20 NaCl	52.8	15.1	52.3^{b}	14.0^{bc}	2.25	8.12	31.8	4.34
	Coastal 4 N – 40 NaCl	62.4	17.8	41.9^{d}	13.6^{bc}	2.09	8.23	33.2	4.59
	Coastal 16 N – 0.3 NaCl	12.9	2.9	63.4^{a}	19.4^{a}	2.85	7.78	37.8	1.48
	Coastal 16 N – 20 NaCl	34.7	14.5	58.0^{a}	18.2^{a}	2.70	7.19	32.8	1.51
	Coastal 16 N – 40 NaCl	39.3	17.6	48.0^{bc}	12.6^{cd}	2.16	9.15	34.6	1.59
P values	Ecotype (E)	0.0027	0.0386	0.0000	0.0000	0.0000	0.2927	0.0000	0.0788
	N level (N)	0.0000	0.9155	0.1121	0.0000	0.0290	0.3519	0.1241	0.0000
	Salinity (S)	0.0000	0.0000	0.0000	0.0000	0.0000	0.0029	0.4254	0.0456
	E × N	0.0168	0.3158	0.1810	0.0187	0.6192	0.1497	0.6397	0.0506
	E × S	0.0157	0.0090	0.4106	0.2178	0.3288	0.7593	0.0391	0.7172
	N × S	0.1316	0.9514	0.4676	0.0000	0.1050	0.8327	0.0128	0.0403
	E × N × S	0.4351	0.9794	0.0000	0.0092	0.9127	0.3192	0.0512	0.6479

For each parameter, means for each salinity level (n = 4) within columns followed by different capital and lower-case letters are significantly different according to the Duncan's multiple range test.

level (no difference between both ecotypes was observed under low NaCl and low N). In particular, in plants of the montane ecotype exposed to low N-supply, the leaf K^+ was significantly reduced by exposure to 20 mM NaCl, while exposure to 40 mM did not further reduce the leaf K^+. On the other hand, under high N-supply, no difference was observed for both ecotypes exposed to 20 mM NaCl, while leaf K^+ decrease was observed at 40 mM NaCl.

A significant E × N × S interaction was observed for the leaf Ca^{2+} concentration. The leaf Ca^{2+} was significantly higher in the coastal-marine ecotype than in the montane ecotype, when compared within each combination of salinity and total-N supply, except for coastal ecotype exposure to 16N-40 NaCl, which was similar to montane 16N-0.3 NaCl. High N supply (16 mM) decreased significantly the leaf Ca^{2+} at both ecotypes when exposed to high salinity (40 mM NaCl). However, at the high level of total-N supply,

the leaf Ca^{2+} was significantly reduced only by exposure to 40 mM NaCl, while 20 mM NaCl had no significant impact on the leaf Ca^{2+} concentration. At the low level of total-N supply, the leaf Ca^{2+} was significantly reduced by exposure to 20 mM NaCl without any additional reduction when salinity was further increased to 40 mM NaCl in the montane ecotype, while it was not influenced by salinity in the coastal-marine ecotype.

The leaf Mg^{2+} was significantly higher in the coastal-marine ecotype than in the montane ecotype, regardless of salinity and total-N supply level. Furthermore, the high total-N supply level increased slightly but significantly the leaf Mg^{2+} concentration without any significant interaction with ecotype and salinity. Increasing the salinity from 0.3 to 20 mM NaCl in the supplied nutrient solution decreased significantly the leaf Mg^{2+}, while a further increase of salinity to 40 mM NaCl reduced the leaf Mg^{2+} to even lower levels, without any interaction with the ecotype and the level of N-supply.

The increase of salinity from 0.3 to 40 mM NaCl of the supplied nutrient solution increased leaf phosphorus (P) concentration significantly regardless of the ecotype. At the lower total N supply level, exposure of stamnagathi to NaCl salinity reduced the leaf SO_4^{2-} concentration regardless of the ecotype, while for the high N supply level no significant differences were found. For both ecotypes, increasing N-supply decreased leaf SO_4^{-2} concentration. Under salinity conditions, the leaf organic N content was significantly greater in the montane ecotype compared to that measured in the coastal-marine ecotype and for material harvested from both production cycles (data shown only for the first production cycle), while no significant difference was observed at 0.3 mM NaCl (**Table 3**). A significant interaction between total-N and salinity indicated that under non-saline conditions a high total-N supply (16 mM) resulted in higher leaf organic-N levels compared to low N supply (4 mM).

The leaf Fe and Mn concentrations in the coastal-marine ecotype were significantly higher than in the montane ecotype, although significant interactions were observed for Mn (**Supplementary Table 2**). Salinity and N supply increased the leaf Fe concentration regardless of ecotype. The leaf Mn concentration of the montane ecotype was increased when the total N increased from 4 to 16 mM. The highest leaf Mn concentration was observed when plants were grown under no salinity combined with high total N level, compared to the other treatments. Similarly, an increase in the total N level from 4 to 16 mM increased the leaf Zn concentration of the montane ecotype, while the inverse was observed in the coastal-marine ecotype. The leaf Mn and Zn concentrations in the coastal-marine ecotype were reduced under moderate and high salinity conditions, while no differences were observed in the montane ecotype. However, salinity decreased the leaf Zn concentration under low total N, while under high total N, the leaf Zn was increased by salinity at 20 mM NaCl and decreased at 40 mM NaCl. Salinity increased the leaf Cu in the coastal-marine ecotype at both N supply levels, while in the montane ecotype salinity had no impact on leaf Cu.

The root Na^+ concentration increased as the NaCl concentration in the supplied nutrient solution was raised from 0 to 40 mM, but the increase was depending on both the ecotype and the N-supply level as indicated by the significant ecotype × N-supply × salinity interaction (**Supplementary Table 1**). The root Na^+ concentration was significantly higher in the montane than in the coastal-marine ecotype at all salinity levels but the differences were larger at the moderate (20 mM) and the high (40 mM) salinity levels.

The root K^+ and Ca^{2+} concentrations were significantly higher in the montane ecotype compared to those measured in the coastal-marine ecotype, regardless of salinity and total-N supply level (**Supplementary Table 1).** The level of total-N supply had no significant impact on the root K^+ and Ca^{2+} concentrations. Increasing the salinity from 0.3 to 20 or 40 mM NaCl in the supplied nutrient solution resulted in a significant decrease of the root K^+ and Ca^{2+}. The root Mg^{2+} concentration was not influenced by either of the treatments tested in the current study. The root P concentration was significantly higher in the montane population compared to that originating from a coastal-marine habitat. The organic-N concentration was significantly higher in the roots of the montane ecotype compared to the coastal-marine ecotype under non-saline conditions, but this difference disappeared when the plants of both ecotypes were exposed to 20 or 40 mM NaCl. The level of total-N supply and salinity had no consistent impact on the organic-N concentration in the roots of stamnagathi.

Root Fe, Zn, and Cu concentrations of montane ecotype were reduced under high salinity conditions regardless of the total N level applied. The root Fe, Mn, Zn, and Cu concentration in the coastal-marine ecotype increased by increasing the salinity from 0.3 to 40 mM NaCl under low N concentration, while the inverse was the case under high total N concentrations, except for Mn. Root Mn of the montane ecotype was not affected by the salinity level when the N supply was low (**Supplementary Table 2**).

Bioactive Content and Radical Scavenging Activity

The leaves of the montane ecotype contained significantly more total phenols (61%), carotenoids (42%), and flavonoids (28%) than the coastal-marine ecotype, although significant interactions were observed **(Table 4)**. Furthermore, the montane ecotype exhibited a significantly higher antioxidant activity than the coastal-marine ecotype under low salinity. Salinity increased significantly the total phenolics concentration in the leaves of both ecotypes, regardless of the N-supply level **(Table 4)**. However, in the coastal-marine ecotype, the increase of total N supply had no impact on total phenolics, while it reduced its content for the montane ecotype.

For the montane ecotype, the flavonoids level was reduced by increasing the salinity from 0.3 to 40 mM NaCl under both N concentrations, while flavonoid levels of the coastal-marine ecotype were reduced only under the low N levels. However, the antioxidant activity of the montane ecotype was not affected either by the salinity level or by the total N level. On the other hand, elevated levels of salinity increased the antioxidant activity of the coastal-marine ecotype under both N-supply levels.

TABLE 4 | Impact of seed origin (montane or coastal-marine ecotype), total nitrogen concentration (4 or 16 mmol L^{-1}, denoted as 4-N and 16-N, respectively), and salinity (0.3, 20, and 40 mM, respectively) in the nutrient solution supplied to hydroponically grown stamnagathi on total phenolics, flavonoids, carotenoids, and antioxidant activity.

Sources of variation		Total phenolics (mg GAE 100 g^{-1} FW) 1st growing cycle	Flavonoids (mg catechin 100 g^{-1} FW) 1st growing cycle	Carotenoids (mg 100 ml^{-1} extraction) 1st growing cycle	Antioxidant activity (mg Trolox 100 g^{-1} FW) 1st growing cycle
Ecotype	Montane	164.4	67.1	1.28	35.1
	Coastal	104.1	52.3	0.88	31.4
N level (N)	4 mM	136.8	58.7	1.09	33.2
	16 mM	131.7	60.7	1.07	33.4
Salinity	0.3 mM	114.9^{B}	63.8	1.10^{ab}	30.7
	20 mM	136.9^{A}	57.9	1.17^{a}	34.6
	40 mM	150.9^{A}	57.4	0.98^{b}	34.5
		Interactions			
E × N	Montane 4-N	173.0^{a}	64.8	1.28	34.7
	Montane 16-N	155.7^{b}	69.3	1.27	35.5
	Coastal 4-N	100.6^{c}	52.6	0.90	31.6
	Coastal 16-N	107.6^{c}	52.0	0.86	31.2
E × S	Montane 0.3 NaCl	135.8	72.8	1.30	34.7
	Montane 20 NaCl	170.5	64.6	1.42	35.2
	Montane 40 NaCl	186.7	63.9	1.12	35.4
	Coastal 0.3 NaCl	94.0	54.8	0.89	26.7
	Coastal 20 NaCl	103.3	51.2	0.91	33.9
	Coastal 40 NaCl	115.1	50.9	0.85	33.7
N × S	4 N – 0.3 NaCl	112.1	60.9	1.12	31.6
	4 N – 20 NaCl	139.3	59.3	1.20	33.9
	4 N – 40 NaCl	159.1	56.0	0.96	34.0
	16 N – 0.3 NaCl	117.8	66.8	1.07	29.8
	16 N – 20 NaCl	134.5	56.5	1.13	35.2
	16 N – 40 NaCl	142.7	58.8	1.00	35.0
E × N × S	Montane 4 N – 0.3 NaCl	143.3	65.6^{bc}	1.37	34.4^{abc}
	Montane 4 N – 20 NaCl	183.6	67.0^{b}	1.42	34.5^{abc}
	Montane 4 N – 40 NaCl	192.0	61.9^{de}	1.05	35.2^{ab}
	Montane 16 N – 0.3 NaCl	128.3	80.0^{a}	1.22	35.0^{abc}
	Montane 16 N – 20 NaCl	157.4	62.1^{cd}	1.42	36.0^{a}
	Montane 16 N – 40 NaCl	181.5	65.8^{bc}	1.18	35.6^{a}
	Coastal 4 N – 0.3 NaCl	80.8	56.1^{ef}	0.86	28.7^{d}
	Coastal 4 N – 20 NaCl	94.9	51.6^{fg}	0.98	33.3^{bc}
	Coastal 4 N – 40 NaCl	126.2	50.0^{g}	0.87	32.9^{c}
	Coastal 16 N – 0.3 NaCl	107.3	53.5^{fg}	0.92	24.7^{e}
	Coastal 16 N – 20 NaCl	111.6	50.8^{g}	0.83	34.4^{abc}
	Coastal 16 N – 40 NaCl	104.0	51.7^{fg}	0.82	34.4^{abc}
P values	Ecotype (E)	0.0000	0.0000	0.0000	0.0000
	N level (N)	0.3657	0.0126	0.4206	0.6054
	Salinity (S)	0.0000	0.0000	0.0343	0.0000
	E × N	0.0377	0.0024	0.8617	0.0927
	E × S	0.0869	0.0640	0.3194	0.0000
	N × S	0.3080	0.0007	0.5275	0.0052
	E × N × S	0.0975	0.0001	0.4595	0.0132

For each parameter, means for each salinity level (n = 4) within columns followed by different capital and lower-case letters are significantly different according to the Duncan's multiple range test.

DISCUSSION

The present study has revealed that stamnagathi is appreciably tolerant plant to salinity, and that different ecotypes exhibit different degrees of salt tolerance, which may depend also on the level of salt normally experienced under the natural conditions to which they have adapted. Indeed, NaCl levels up to 40 mM did not reduce the shoot FW in the coastal-marine ecotype, while the shoot FW of the montane ecotype was significantly reduced at salinity level of 40 mM. Klados and Tzortzakis (2014), Ntatsi et al. (2017a), and Petropoulos et al. (2017) also reported on a high tolerance of stamnagathi to salinity. In specific, Ntatsi et al. (2017a) showed that the montane ecotype of stamnagathi is tolerant to 40 and 80 mmol L^{-1} NaCl or isosmotic solutions of $CaCl_2$, Na_2SO_4, and KCl. Using metabolomics analysis, Ntatsi et al. (2017a) showed that a major mechanism conferring high salt tolerance to stamnagathi is efficient osmoprotection due mainly to increased levels of γ-aminobutyric acid (GABA), glutamate, pyroglutamate, L-proline, and sucrose. In the study of Ntatsi and co-workers (2017a) the salt tolerance of stamnagathi was not associated with reduced transport of Na^+ and Cl^- ions to the photosynthetically active leaves. Combining these considerations and the metabolomics analysis, led to the conclusion that osmoprotection and/or efficient compartmentation of the toxic

salt ions to the vacuoles is the major mechanism by which saline-tolerant genotypes of stamnagathi may combat salinity. Controlled salt inclusion combined with efficient intracellular compartmentation of the salt ions to the vacuoles appears as the most efficient strategy deployed by salt tolerant plant species to sustain plant growth rates in saline environments (Shabala and Cuin, 2007; Munns and Gilliham, 2015; Liang et al., 2018). As a rule, plants relying on salt exclusion to combat salinity, which retain more Na^+ in the roots while transporting less Na^+ and/or Cl^- to the photosynthetically active leaves, are vulnerable to salinity (Shabala and Cuin, 2007; Wu, 2018). In the current study, the coastal-marine ecotype, which exhibited a higher salt tolerance than the montane ecotype, contained appreciably more Cl^- (33% on average) than the montane ecotype within each NaCl-salinity level, which shows that salt tolerance in stamnagathi is not associated with Cl^- exclusion. However, compared to the montane ecotype, the coastal-marine ecotype also contained less Na^+ in the roots (48% on average) at both salinity levels and less Na^+ in the leaves (8% on average) at the highest salinity level (40 mM NaCl), which indicates that efficient Na^+ exclusion may play also a complementary role in the salt tolerance of this ecotype.

Under salinity conditions, salt tolerant plant species can decrease their leaf water potential by compartmentalizing the toxic Na^+ and Cl^- salts to the vacuoles and synthesizing compatible organic solutes in the cytosol to avoid cell dehydration (Shabala, 2013; Bassil and Blumwald, 2014). As a result, a sufficiently high-water potential gradient between leaf cells and the external solution can be maintained, despite the salinity-induced decrease in the external water potential (Shabala, 2013; Mansour and Ali, 2017). However, many compatible organic solutes contributing to osmoregulation between the cytosol and the vacuole bear fixed negatively charged sites, i.e., carboxylic anions, which are electrochemically balanced by K^+ (Munns and Gilliham, 2015). Furthermore, Mg-ATP and Mg-PP_i provide energy to proton pumps transporting actively H^+ into the vacuoles, which are subsequently excreted back to the cytosol through Na^+/H^+ antiporters, thereby facilitating Na^+ sequestration to vacuoles (Roy et al., 2014). In agreement with this consideration, the K^+ and Mg^{2+} concentrations in the leaves of the coastal-marine ecotype, which exhibited a higher tolerance to salinity, were significantly higher (27% and 31% respectively) than in leaves of the montane ecotype within each salinity and total-N supply level. Calcium contributes mainly to stabilization of the cell walls and membranes, while its concentration in the cytosol is low (Cabañero et al., 2006; Kader and Lindberg, 2010). However, calcium is associated with cell membrane protection against disruption caused by increased external NaCl salinity (Rengel, 1992; Tuna et al., 2007). Furthermore, Ca^{2+} and other divalent cations (Mg^{2+}, Zn^{2+}) may prevent K^+ leakage in response to salinity, thus contributing to maintenance of a higher K^+/Na^+ ratio in the cytoplasm, and concomitantly to higher salt tolerance (Shabala and Cuin, 2007). Consequently, the higher leaf Ca^{2+} concentration in the coastal-marine ecotype within each salinity and total-N supply level is in-line with its higher salt tolerance compared to that of the montane ecotype.

The higher organic-N concentrations in the leaves of the montane ecotype when the plants were exposed to NaCl-salinity may be associated with the lower leaf Cl^- concentrations in this ecotype. Indeed, Cl^- acts antagonistically to the NO_3^- uptake, and plants exposed to NaCl salinity show generally lower leaf NO_3^- concentrations (Munns and Termaat, 1986; Knight, et al. 1992; Grattan and Grieve, 1998). A higher NO_3^- availability in the leaves of the montane ecotype owing to lower leaf Cl^- levels resulted presumably in higher rates of NO_3^- assimilation into organic N compounds.

Salinity has been reported to either increase, restrict, or have no impact on tissue phosphorus concentrations. Salinity-induced increases in the tissue P concentrations have been observed mainly in experiments with plants grown hydroponically (Grattan and Grieve, 1998). The P concentrations in nutrient solutions applied to hydroponic crops are far higher than those typically found in the soil solution. As suggested by Nieman and Clark (1976), P concentrations that are optimal under non-saline conditions may impose a higher P uptake to levels resulting even to toxic tissue P concentrations in crops grown hydroponically under saline conditions. Nevertheless, in the present study, the increase of tissue P at 40 mM NaCl due to salinity did not result in any visible symptoms of P-toxicity.

Our results on plant biomass production of stamnagathi at both total-N supply levels corroborate the finding of Chatzigianni et al. (2018). With respect to the interactions of the total-N supply with salinity treatment, our results showed that the NaCl salinity had no impact on leaf sulfates when the supply of total-N was 16 mM, but reduced the SO_4^{2-} uptake when the total-N supply was lowered to 4 mM. It is well known that sulfates (SO_4^{2-}) compete with both nitrates and chlorides in uptake sites (Kinjo and Pratt, 1971; Marschner, 2012). Thus, it seems that, in the present study, the high NO_3^- concentration suppressed any antagonistic effect of Cl^- on SO_4^{2-} uptake, while at low total-N, which entailed low NO_3^- levels in the root zone, increasing levels of Cl^- restricted the uptake of SO_4^{2-}. Another salinity × total-N interaction was that the high N supply resulted in higher leaf organic-N levels only in the absence of NaCl salinity. Presumably, this is because under saline conditions the restriction of NO_3-uptake by Cl^- (Grattan and Grieve, 1998) counteracted the enhanced NO_3-supply in the high-N treatment.

In contrast to the leaf Ca^{2+}, the root Ca^{2+} concentration was significantly higher in the montane ecotype compared to that measured in the coastal-marine ecotype. A likely explanation for this finding is that the greater root Ca^{2+} concentration is linked with the higher root Na^+ concentrations for the montane ecotype. It is well established that Ca^{2+} enhances membrane selectivity to ion uptake and transport through the plasma membrane and the tonoplast (Hepler, 2005). Thus, the greater root Na^+ concentrations in the coastal-marine ecotype may have stimulated Ca^{2+} accumulation to the root cells aiming to a more-efficient compartmentation of root Na^+ to the vacuoles of the root xylem parenchyma.

Of the four metallic micronutrients, only Fe exhibited a consistent tendency to increase its concentration in the leaf tissues with increasing salinity, regardless of the stamnagathi ecotype and the level of total-N supply. Increased tissue Fe

concentrations with increasing salinity have been reported also by other investigators and for several plant species, including tomato, soybean, and squash (Mass et al., 1972), strawberry (Turhan and Eris, 2005), and zucchini squash (Víllora et al., 2000). However, in other studies dealing with different plant species, salinity had no effect on tissue-Fe level for lettuce (Lazof and Bernstein, 1999) and reduced it in sunflower (Sanchez-Raya and Delgado, 1996).

The impact of salinity on the concentrations of the other three metallic micronutrients was dependent upon the N-supply level and also stamnagathi ecotype. Mass et al. (1972), Víllora et al. (2000), Tunçturk et al. (2008), and Weisany et al. (2014) also found that the impact of salinity on the leaf and root concentrations of Mn and Zn in tomato, soybean, and squash was also dependent on the plant species and cultivar. These results indicate a link to the specific genotype, which dictates the mode and extent of salt tolerance deployed by each species, ecotype, and even cultivar. This is presumably the case with Cu, given that in the present study salinity raised considerably the Cu levels in leaves of the coastal-marine ecotype, which is more tolerant to salinity, while it had no impact (at 4 mM total-N) or imposed only a slight Cu increase (at 16 mM total-N) in the montane ecotype. All metallic macronutrients are constituents of antioxidant enzymes and/or other metabolites (Marschner, 2012). Thus, the strong differences in the leaf Fe, Mn, Zn, and Cu concentrations between the coastal-marine and the montane ecotype may be associated with commensurate differences in metabolic functions. The parameters measured in the research reported here do not allow for a clear understanding of the different mechanisms, which appear to underpin the distinction of metabolic functions for the two ecotypes tested. Further research is needed to determine the metabolic profiles of the two ecotypes, which could be linked with the results of the mineral composition presented here.

Soilless culture systems are known to facilitate the precise application of *eustress* (positive stress) such as mild to moderate NaCl and nutritional (e.g., N) stress, through a precise management of the NS concentration, thus triggering the biosynthesis of secondary metabolites (phenols, flavonoids, carotenoids) necessary for adaptation to suboptimal environments (Rouphael and Kyriacou, 2018; Rouphael et al., 2018c). These antioxidant molecules are of high importance to human health and longevity, and thus, they indirectly attribute an extra value (compared to non-stressed crops) to the basic nutritional characteristics of leafy and fruit vegetables (Orsini et al., 2016).

Phenolic compounds act as co-substrates, decomposing H_2O_2 through oxidation by peroxidase, thereby contributing to ROS scavenging and thus to enhanced tolerance to oxidative stress (Sudhakar, et al., 2001). The slight increase of total phenolics as the salt level increased indicates that this large group of secondary metabolites contains antioxidant compounds that may well contribute to salt tolerance in stamnagathi. Lim et al. (2012) found that the exposure of buckwheat (*Fagopyrum esculentum* M.) sprouts to salinity enhanced the total phenolics content due mainly to an increase in the concentration of four compounds, particularly isoorientin, orientin, rutin, and vitexin. In contrast to the total phenolics, carotenoids and flavonoids (a sub-group of phenolics) did not increase when stamnagathi was exposed to salinity, which indicates that these antioxidant compounds are not linked with salt tolerance mechanisms in stamnagathi. In contrast to stamnagathi, other plant species exhibited an increase in both carotenoids and flavonoids under salt stress conditions (Lim et al., 2012; Wang et al., 2018). There were significantly greater concentrations of total phenolics, carotenoids, and flavonoids in the montane compared with the coastal-marine ecotype, irrespective of salinity exposure and level. Nevertheless, the coastal-marine ecotype was more efficient than the montane ecotype in increasing its antioxidant activity when the plants were facing salt-stress, and this may be associated with the naturally higher salt tolerance capacity of the coastal-marine ecotype. The higher concentrations of total phenolics carotenoids and flavonoids in the montane ecotype may be associated with enhanced ability of this ecotype to combat other stresses beyond salinity (e.g., low temperature stress). Indeed, recently published results have shown that the degree of low-temperature tolerance in grapevine varieties was positively related with their total phenolics content (Król et al., 2015).

In the present study, the leaf chlorophyll content was influenced more by ecotype than by salinity or the level of N supply. Petropoulos et al. (2016) also reported different leaf chlorophyll concentrations between different ecotypes of *C. spinosum*. The higher leaf chlorophyll concentrations in the less salt tolerant montane ecotype, and similar leaf chlorophyll concentrations at all salinity levels in the coastal-marine ecotype, show that the biosynthesis of this pigment plays a key role in stamnagathi salt tolerance. Thus, the higher leaf chlorophyll concentrations of the montane ecotype may be related to higher tolerance to another abiotic stress. Given that during its evolution the montane ecotype was faced with much lower temperatures during the winter than the coastal-marine ecotype, the higher chlorophyll concentration in this ecotype may be an adaptation aiming to enhance its tolerance to cold. Indeed, as reported by Sanghera et al. (2011) and Li et al. (2018), higher leaf chlorophyll concentrations in plants are associated with higher cold tolerance.

The higher dry matter content in the montane ecotype may also be related to a higher cold tolerance for this ecotype compared to that originating from a coastal location. Indeed, a higher tolerance of plants to sub-optimal temperatures is associated with a higher dry matter percentage originating mainly from increased starch accumulation (Venema et al., 1999; Król et al., 2015; Ntatsi et al., 2017b). On the other hand, a low dry matter content in leaves may be a consequence of increased leaf succulence, which is an adaptive morphological characteristic of plants to salinity (Greenway and Munns, 1980; Shabala, 2013). Thus, an additional explanation for the lower dry matter content in the coastal-marine ecotype is that these plants tend to develop leaves that are more succulent as an adaptation to salinity, which agrees with its higher salt tolerance compared to that of the montane ecotype.

CONCLUSION

A general conclusion derived from this study is that the coastal-marine ecotype is superior in terms of salt tolerance compared to the montane ecotype and can be used as a good source of germplasm in breeding programs aiming to develop stamnagathi cultivars, which are resilient to salinity stress. On the other hand, the montane ecotype is superior in terms of nutritional value, and it presents leaves with better texture (more crispy) due to the appreciably higher dry matter content of their edible shoots, and it contains more total phenols, flavonoids, and carotenoids. Also, under non-saline conditions, the montane ecotype has a higher antioxidant capacity. An additional trait of the montane ecotype, which advocates for a higher nutritional quality compared to the coastal-marine ecotype, is its lower nitrate concentration in the edible shoot (Chatzigianni et al., 2018), especially when the N supply is not low.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: DS and IL. Performed the experiments and the analyses: MC, MT, AS, IL, GN, and DS. Analyzed the data: MC, GN, YR, MT, and DS. Wrote the paper: MC, GN, YR, and DS. Reviewed the paper: GN, IL, YR, and DS. All authors have read and approved the manuscript.

FUNDING

During the 3 years of experiments, candidate PhD student MC was supported by a scholarship from Bodossakis Foundation.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Pete Iannetta from the James Hutton Institute who critically read the manuscript and improved the quality of the English in the text.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01040/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 18 December 2019
doi: 10.3389/fpls.2019.01494

Iodine Accumulation and Tolerance in Sweet Basil (*Ocimum basilicum* L.) With Green or Purple Leaves Grown in Floating System Technique

Luca Incrocci[1], Giulia Carmassi[1], Rita Maggini[1], Caterina Poli[1], Djamshed Saidov[2], Chiara Tamburini[1], Claudia Kiferle[3], Pierdomenico Perata[3] and Alberto Pardossi[1]*

[1] Department of Agriculture, Food and Environment, University of Pisa, Pisa, Italy, [2] Samarkand Agricultural Institute, Samarkand, Uzbekistan, [3] Plantlab, Institute of Life Sciences, Sant'Anna School of Advanced Studies, Pisa, Italy

OPEN ACCESS

Edited by:
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Reviewed by:
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***Correspondence:**
Luca Incrocci
luca.incrocci@unipi.it

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *06 January 2019*
Accepted: *28 October 2019*
Published: *18 December 2019*

Citation:
Incrocci L, Carmassi G, Maggini R, Poli C, Saidov D, Tamburini C, Kiferle C, Perata P and Pardossi A (2019) Iodine Accumulation and Tolerance in Sweet Basil (Ocimum basilicum L.) With Green or Purple Leaves Grown in Floating System Technique. Front. Plant Sci. 10:1494. doi: 10.3389/fpls.2019.01494

Iodine deficiency is a serious world-wide public health problem, as it is responsible for mental retardation and other diseases. The use of iodine-biofortified vegetables represents a strategic alternative to iodine enriched salt for people with a low sodium diet. However, at high concentrations iodine can be toxic to plants. Therefore, research on plant iodine toxicity is fundamental for the development of appropriate biofortification protocols. In this work, we compared two cultivars of sweet basil (*Ocimum basilicum* L.) with different iodine tolerance: "Tigullio," less tolerant, with green leaves, and "Red Rubin," more tolerant and with purple leaves. Four greenhouse hydroponic experiments were conducted in spring and in summer with different concentrations of iodine in the nutrient solution (0.1, 10, 50, 100, and 200 μM), supplied as potassium iodide (KI) or potassium iodate (KIO_3). Plant growth was not affected either by 10 μM KI or by 100 μM KIO_3, while KI concentrations higher than 50 μM significantly reduced leaf area, total plant dry matter and plant height. The severity of symptoms increased with time depending on the cultivar and the form of iodine applied. Growth inhibition by toxic iodine concentrations was more severe in "Tigullio" than in "Red Rubin," and KI was much more phytotoxic than KIO_3. Leaf iodine concentration increased with the iodine concentration in the nutrient solution in both varieties, while the total antioxidant power was generally higher in the purple variety. In both basil cultivars, a strong negative correlation was found between the photosynthesis and the leaf iodine content, with significant differences between the regression lines for "Tigullio" and "Red Rubin." In conclusion, the greater tolerance to iodine of the "Red Rubin" variety was associated with the ability to withstand higher concentrations of iodine in leaf tissues, rather than to a reduced accumulation of this element in the leaves. The high phenolic content of "Red Rubin" could contribute to the iodine tolerance of this purple cultivar.

Keywords: anthocyanic variant, iodine toxicity, leaf antioxidant capacity, plant mineral nutrition, leaf gas exchanges, hydroponic system

INTRODUCTION

Iodine deficiency is a serious world-wide public health problem, as it is responsible for important diseases such as cretinism and goiter. With the exception of sea-derived food, most foods have generally a low iodine content ranging from 30 to 100 μg I kg^{-1} fresh weight, mainly depending on the site of production (Zimmermann, 2017). The adequate Recommended Daily Allowance (RDA) for iodine is 90–120, 150, and 290 μg per day respectively for children, adults, and pregnant or breastfeeding women (EFTSA NDA Panel, 2014).

Since the 1920s, the main strategy to contrast low iodine intake has been the systematic iodination of table salt, which has considerably reduced the incidence of iodine deficiency disorders (Zimmermann, 2017). Anyway, currently 30–38% of the world's population remains with insufficient iodine intake and is at risk for iodine deficiency (White and Broadley, 2009). Salt iodination alone is insufficient to satisfy the total requirement of iodine for human health, because iodine could be lost from table salt by volatilization (Mottiar and Altosaar, 2011). Moreover, a large part of the population at risk of cardiovascular diseases cannot use iodized table salt (Kiferle et al, 2013). The World Health Organization (2007) has promoted the assumption of iodine through the consumption of seafood and biofortified food such as vegetables, since the iodine contained in these foods can be easily assimilated by humans up to 99% of its amount (Weng et al, 2008).

Although iodine is a not-essential element for plants, it is currently considered beneficial. Plant can uptake iodine by the root or by the stem or leaves through the stomata and/or the cuticular waxes with high degree of unsaturation that are susceptible to iodine addition (Tschiersch et al., 2009). Iodine could reach the aerial plant organs either in dissolved form (like sea aerosol or spray) or as a gas (I_2 and/or CH_3I).

Many studies showed that the plant response to iodine application is variable according to the chemical form used (iodide, I^-; iodate, IO_3^-; iodoacetate, ICH_2COO^-), the concentration applied, and the growing system (Medrano-Macías et al., 2016; Duborská et al., 2018). Iodine toxicity is reported in the order $I_2(aq) > I^- > IO_3^-$ (Mackowiak and Grossl, 1999; Dai et al., 2009). The assessment of iodine toxicity becomes more complicated in the case of cultivation in soil, due to the different stability and interaction of each chemical species with biotic and abiotic soil components.

Unfortunately, relevant information on the uptake mechanism and phytotoxic effects of I^- and IO_3^- in higher plants is sparse. In contrast, significant research has been devoted to iodine biofortification, as confirmed by the numerous studies on the application of I^- and/or IO_3^- to various crop species. According to a review by Medrano-Macías et al. (2016), most of the work has been conducted on vegetable crops (more than 12 different species), such as lettuce (Smoleń et al., 2014a), spinach (Zhu et al., 2003; Smoleń and Sady, 2012), tomato (Caffagni et al., 2011; Kiferle et al., 2013; Smoleń et al., 2015b), and on cereals such as rice (Kato et al., 2013), wheat (Mao et al., 2014), and barley (Duborská et al., 2018).

To the best of our knowledge, no study on either iodine toxicity or biofortication of sweet basil has been published. Sweet basil (*Ocimum basilicum* L.) is widely cultivated and mostly used for food preparation, especially in Italy and Mediterranean regions (Makri and Kintzios, 2008), and in recent years also purple-leafed variants of sweet basil have been increasingly used, especially for ornamental purposes (Makri and Kintzios, 2008; Pardossi et al, 2015). In particular, the cultivar "Red Rubin" has been extensively characterized for its higher tolerance to boron toxicity compared with green-leafed cultivars (Landi et al., 2013; Landi et al., 2014; Pardossi et al., 2015). Boron toxicity induces leaf chlorosis and necrosis, which reduce photosynthesis and dry matter accumulation (Landi et al., 2013), and both these symptoms are quite similar to those produced by iodine toxicity.

Starting from the above evidences, the aim of the present work was to assess the tolerance to iodine toxicity by two cultivars of sweet basil grown in hydroponic system: "Red Rubin" a purple-leafed variety with high concentration of anthocyanins in the leaf epidermis and the typical green leafed "Tigullio."

Further aims of this work were to model the relationship between the concentration of I^- or IO_3^- in the nutrient solution or in the leaves and the growth inhibition of sweet basil, and to investigate whether the tolerance to iodine toxicity was associated with: i) a reduced iodine uptake and accumulation; ii) an interaction between iodine concentration and the uptake of other nutrients; iii) a reduction of the photosynthetic activity.

MATERIALS AND METHODS

Plant Material and Growing Conditions

Four different experiments were conducted in a glasshouse in Pisa (Tuscany, central-western Italy), with seedlings of two cultivars of sweet basil, "Tigullio" and "Red Rubin." Seeds were purchased from Franchi Sementi (Milano, Italy). The seedlings were grown in hydroponic culture (floating system) in late spring or in early summer (see **Table 1** for details). Inside the greenhouse, plants were grown under natural light.

The seeds of basil were sown using trays with rockwool plugs (Grodan Plug®, Grodan Rockwool B.V., Roermond, The Netherlands). After sowing, the trays were maintained five days in a germination chamber with a constant temperature of 25°C and 75% relative humidity. Afterwards, they were moved to the greenhouse for the correct development of the plantlets, which were transferred in the hydroponic system at the stage of four visible true-leaves. In Experiments 1, 2, 3, each hydroponic system consisted of a polystyrene tray floating in a 50-L plastic tank (each tank hosted 16 plants, 8 "Tigullio" plus 8 "Red Rubin"), while 3-liter plastic pots were used in *Experiment 4*, each one hosting 4 plants of the same variety. Planting density was approximately 96 plants m^{-2} of ground area. In all experiments, the nutrient solutions were prepared using tap water and appropriate amounts of salts of analytical grade (Carlo Erba Reagents, Milano, Italy) to avoid iodine contamination. The tap water contained 4.0 mM HCO_3-, 4.5 mM Cl, 0.25 mM S–H_2SO_4, 2.7 mM Ca, 0.8 mM Mg, 2.0 mM Na and negligible concentrations of other nutritive ions, including iodine (0.02 ± 0.01 μM I). The nutrient solution had a pH and an electrical conductivity (EC) of 5.5 and 2.4 dS m^{-1}, respectively, and contained the following concentrations of macroelements and trace elements:

TABLE 1 | Basic information of the greenhouse experiments conducted in 2015 and 2016 on of two cultivars ("Tigullio" and "Red Rubin") of sweet basil (*Ocimum basilicum* L.) grown hydroponically with different concentrations and form of iodine in the nutrient solution.

Parameter	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Growing season	June 2015	July 2015	May 2016	May 2016
Hydroponic system	50-L tank	50-L tank	50-L tank	3-L pot
Plants per tank or pot	16	16	16	4
Plant age at transplanting (days from sowing)	18	14	14	14
Plant age at the onset of iodine treatment (days from sowing)	24	21	21	21
Treatment duration (days)	21	16	14	12
Iodine concentrations under investigation				
KI (μM)	10, 100	10, 50,100, 200	50, 100, 200	100, 200
KIO_3 (μM)	10, 100	–	100, 200, 400	100, 200
Average air temperature (C°)	29.4	32.4	24.1	24.3
Daily average global radiation (MJ m^{-2} day^{-1})	12.6	13.3	10.1	10.4
Determination				
Plant growth	√	√	√	√
Mineral element tissue content	–	–	√	–
Iodine toxicity modelling	–	√	–	–
Iodine tissue content	–	√	√	√
Chlorophylls and carotenoids content	–	√	√	√
Water uptake	–	–	–	√
CO_2 assimilation (A)	–	–	–	√

10.0 mM N–NO_3-; 1.0 mM P–H_3PO_4; 3.8 mM S–H_2SO_4, 8.5 mM K; 4.5 mM Ca; 2.0 mM Mg; 2.0 mM Na; 4.5 mM Cl; 40.0 μM Fe; 25.0 μM B; 10.0 μM Mn; 10.0 μM Zn; 3.0 μM Cu; 1 μM Mo. The tested iodine concentrations ranged from 0.10 ± 0.03 μM (control) and 200 μM (**Table 1**). In each hydroponic system, the large volume of the nutrient solution limited the variations of pH (5.5–6.0), EC (2.3–2.5 dS m^{-1}) and ion concentrations, which were checked in all treatments every 2–3 days and remained within 3–5% the initial values throughout the experiments (data not shown).

Experimental Design

The treatments were defined by a combination of two factors: the concentration of iodine in the nutrient solution and the cultivar ("Tigullio" and "Red Rubin"). In all experiments, the treatments were arranged in a totally randomized design with three replicates, each consisting of a tank or a pot.

The experimental treatments were differentiated one week after transplanting in the hydroponic system by adding potassium iodide (KI) or potassium iodate (KIO_3) of high purity (purity > 99%; Duchefa Biochemie B.V., Haarlem, The Netherlands) directly to the nutrient solution.

Four experiments were carried out with both basil genotypes.

In *Experiment 1* we investigated the effect of iodine source (KI or KIO_3) and concentration (0.1, 10 and 100 μM) on plant growth. The experiment was conducted in summer 2015 and lasted 21 days.

In *Experiment 2* KI was tested at the following concentrations: 0.1, 10, 50, 100, and 200 μM. The experiment was conducted in summer 2015 and lasted 16 days.

In *Experiment 3* we studied the effect of the iodine source (KI or KIO_3) and its concentration in the nutrient solution on plant growth and leaf nutrient content. The following concentrations were used: 0.1, 50, 100, 200 μM for KI, and 100, 200, 400 μM for KIO_3. The experiment was conducted in spring 2016 and lasted 14 days.

In *Experiment 4* we assessed the effect of 100 and 200 μM KI or KIO_3 on gas exchange at the two leaves of the 2nd node. The antioxidant capacity and the contents of chlorophylls, carotenoids and total phenols were also assessed in the same leaves, along with the photosynthetic activity (through leaf gas exchange measurements). The experiment was carried out for 14 days in spring 2016.

Determinations and Measurements

Dry Matter and Mineral Ion Determinations in Plant Tissue

The tissues were dried for three days in a 70°C oven to constant mass. After the determination of total dry matter (DW), they were ground in a laboratory mill to a powder. For mineral content determinations, 200 mg oven dried ground samples were wet digested in a mixture of nitric and perchloric acids (HNO_3:$HClO_4$ 5:2 v/v) at 230°C for 1 h. Atomic absorption spectrometry (Varian Spectra AA240 FS, Australia), was used to quantify K, Ca, Mg, Na, Fe, Mn, Cu, and Zn, while spectrophotometry was employed for P determination using the molybdenum blue method (Olsen and Sommers, 1982). Nitrogen was determined by the micro-Kjeldahl procedure (Kacar, 1972); nitrate content was measured by spectrophotometry using the salicylic–sulphuric acid method (Cataldo et al., 1975) after extraction of 100 mg dry samples with 20 ml water for 1 h. The same methods were used to analyze the nutrient solutions.

Iodine Determination in Plant Tissue and Nutrient Solution

The leaf iodine concentration was determined on samples taken from the whole leaf biomass (Experiments 2 and 3) or from leaves of the 2nd node (*Experiment 4*).

The concentration of total iodine was determined by inductively coupled plasma mass spectrometry (ICP-MS)

both in the nutrient solutions and in plant tissues (200 mg powdered dry matter), according to the official method for iodine determination in foodstuffs (European Standard BS EN 15111:2007), which consisted in an alkaline extraction (90°C for 3 h) with 25% tetramethylammonium hydroxide (TMAH). The samples were filtered and analysed by ICP-MS using tellurium as internal standard. The analyses were carried out by a private laboratory (Pontlab, Pontedera, Pisa, Italy).

Chlorophyll and Carotenoids Determination in Leaves

Five disks (12 mm diameter; approximately 0.5 g fresh weight, FW) were sampled from 2nd node leaves, placed in 10-ml test tubes and soaked with 5 ml methanol. The tubes were sonicated 15 min in ice bath four times and stored overnight at −20°C. After the separation of the supernatant, the extraction of the disks was repeated with 5 ml fresh methanol. The two supernatant aliquots were pooled and, after proper dilution (1:5 or 1:10) with methanol, the absorbance of the extracts was read at 665.2, 652.4, and 470 nm. The concentrations of the pigments (μg ml^{-1}) were calculated according to Lichtentahler and Buschmann (2001):

$$[\text{chlorophyll } \mathbf{a}] = 16.72 \times A(665.2) - 9.16 \times A(652.4) \quad (1)$$

$$[\text{chlorophyll } \mathbf{b}] = 34.09 \times A(652.4) - 15.28 \times A(665.2) \quad (2)$$

$$[\text{carotenoids}] = \{1000 \times A(470) - 1.63 \times [\text{chlorophyll } \mathbf{a}] - 104.96 * [\text{chlorophyll } \mathbf{b}]\} / 221 \quad (3)$$

where A (665.2), A (652.4), and A (470) represent the absorbance at the wavelength (expressed in nm) reported in parentheses. The content of total chlorophylls and carotenoids in the tissues was expressed as μg g^{-1} FW.

Total Phenolic Content and Antioxidant Capacity

Preparation of Plant Leaf Extracts

The determinations of total phenolic content and antioxidant capacity were performed using the leaves at the 2nd node. The analyses were carried out in quadruplicate in methanolic extracts as follows: 0.15 g of leaf tissues were homogenized in a mortar with 5 ml of 70% methanol (v/v) and extracted overnight at 4°C in the dark, under continuous agitation. After centrifugation (5 min, 10,000 rpm at RT) the clear supernatant was collected and used for the subsequent analyses.

Total Phenols

The concentration of total phenols was determined using the Folin-Ciocalteu reagent, as reported by Kang and Saltveit (2002). Briefly, 125 μl of leaf methanolic extracts were diluted in distilled water (1:4), mixed with the Folin-Ciocalteau reagent to a final volume of 750 μl and vortexed. After 5 min, 1.25 ml of a 7% (w/v) sodium carbonate solution and 1 ml distilled water were added to the mix, which was left to stand at room temperature in the dark for 90 min. The spectrophotometric readings were carried out at 765 nm and the results were expressed in terms of gallic acid equivalents, on the basis of a standard calibration curve.

Antioxidant Capacity

It was evaluated by using the DPPH and the FRAP assays (Dudonné et al., 2009). For DPPH analyses, different volumes of the extract (from 5 to 50 μl) were diluted in 70% methanol and added to a fixed volume (335 μl) of a freshly prepared DPPH radical solution (0.25 mM 1,1-diphenyl-2-picrylhydrazyl in 70% methanol) to reach the final volume of 1 ml; the mixture was vortexed and left to stand at room temperature for 30 min in the dark. The absorbance of the resulting solution was read at 517 nm. A blank solution (control) was prepared by mixing 70% methanol with DPPH solution, and the scavenging activity was determined by the following equation:

$$\%\ \text{scavenging activity} = 100 \times (A_{control} - A_{sample}) / A_{control}, \quad (4)$$

where A is the absorbance at 517 nm.

The tissue antioxidant capacity was expressed as inhibitory concentration (IC_{50} value), which indicates the sample concentration that is required to scavenge 50% of DPPH radical. The IC_{50} value was calculated by plotting the scavenging activity against the concentrations of the samples.

The FRAP assay was carried out mixing 2.0 ml acetate buffer (0.25 M, pH 3.6), 900 μL freshly prepared FRAP reagent (2 mM ferric chloride and 1 mM 2,4,6-tris(2-pyridyl)-s-triazine in acetate buffer), 100 μL plant methanolic extract. A calibration curve was prepared with ferrous ammonium sulfate standard solutions, containing 0–1,000 μM Fe(II). Absorbance was recorded at 593 nm.

Plant Water Uptake Measurement

In *Experiment 4*, four basil plants were cultivated in 3-liter pots, and each pot was weighted every 2–3 days without the plants and their floating tray support. After weight determination, the nutrient solution was completely replaced with fresh nutrient solution.

Gaseous Exchange Determinations

A CIRAS-2 portable photosynthesis system (PP Systems, Amesbury, MA, USA) was used for the determination of gas exchanges at 500 μmol m^{-2} s^{-1} of Photosynthetic Active Radiation (PAR) intensity, 450 ppm CO_2 concentration, 65% relative humidity and 27°C leaf temperature, which was close to the greenhouse temperature during the measurements. In particular, CO_2 assimilation (A), transpiration, leaf stomatal conductance (Gs) and intercellular CO_2 concentration (Ci) were determined by real-time measurements, which were performed at the opposite leaves of the 2nd node. Three replicates were analysed for each treatment.

Modelling and Statistics

Modelling Growth Response to Iodine Level

The relationship between the nominal iodine concentration in the root zone versus total plant dry biomass or stem length was determined in *Experiment 2* for both cultivars, using the Maas and Hoffman model for crop response to salinity (Maas and Hoffman, 1977). According to this model, the relative growth (DW) of root, stem, leaf or whole plant or the relative stem height (Y^*), expressed as the percentage of maximum growth or stem height (*Ymax*), is plotted as a function of the KI concentration in the nutrient solution (X). For X below a critical threshold value (t) no significant difference is observed in Y^* (plateau region); thereafter, by increasing X, Y^* decreases according to a linear function with slope s (linear region):

$$Y^* = 100 - s \cdot (X - t) \quad (5)$$

Data of root, stem, leaf and whole plant DW and plant height measured at the end of *Experiment 2* were computed using the procedure described by Magán et al. (2008) to evaluate *Ymax*, t and s. For each basil genotype, one-way analysis of variance (ANOVA) was conducted with KI as the source of variation, to select the treatments with the highest statistically similar values of root, stem leaf or whole plant DW or stem length. The corresponding average value (*Ymax*) was used to evaluate Y^* at each KI level. To determine the values of s and t, a series of linear regressions was performed to fit the pool of data in the linear region. The regression line with the highest determination coefficient (R^2), whose slope provided the value of s, allowed to evaluate t as the KI concentration at which Y^* assumed the value *Ymax*.

Statistical Analysis

In all the experiments the data were subjected to two-way ANOVA, with the cultivar and the iodine treatment as the sources of variation. Mean values were separated according to the Duncan's test, at $P < 0.05$. The program Statgraphics Plus 5.1 (StatPoint, Inc., Herndon, VA, USA) was used to perform the ANOVA and linear regression. A representative run is reported for each experiment, which was repeated two times with similar results.

RESULTS

Iodine Phytotoxicity Symptoms

In all the experiments, the symptoms of iodine toxicity appeared within three days of exposure as a reduction of stem elongation, leaf expansion and root development, and within one week as chlorotic interveinal yellow patches of the older leaves (**Figure 1**). The

FIGURE 1 | Symptoms of iodine toxicity in sweet basil (*Ocimum basilicum* L., cultivar "Tigullio") grown hydroponically for 14 days from 9th to 23rd May 2016 (*Experiment 3*) with 200 μM KI in the nutrient solution. **(A** and **B)** general chlorosis and yellow interveinal patches three days after the onset of iodine treatment. **(C)** after one week of iodine treatment brown necrotic spots of basal leaves after seven days. **(D** and **E)**: severe leaf chlorosis and necrosis, and leaf drop after 14 days.

severity of symptoms increased with time, depending on the form and concentration of the iodine applied as well as on the cultivar. In KI-treated plants (**Figure 1**), early phytotoxicity symptoms appeared already after a few days as shorten new developed nodes, discolorations and chlorotic areas in the leaves. After 2 weeks iodine treatment, the plants showed serious phytotoxicity symptoms on all the leaves and few bottom leaves had also dropped.

The symptoms were less severe in "Red Rubin" than in "Tigullio" and when the plants were treated with KIO_3 than with KI at the same concentrations (**Figure 2**).

Experiment 1

The green-leaved cultivar "Tigullio" grew faster than "Red Rubin" and after 21 days the total plant dry weight of "Tigullio" plants was approximately three times higher than the dry weight of "Red Rubin" plants.

Neither 10 nor 100 μM KIO3 concentrations affected plant growth in both cultivars. On the contrary, 100 μM KI in the nutrient solution significantly reduced the dry weight of roots, leaves and stems, respectively, by 22, 67, and 80% in "Tigullio," and by 17, 22, and 23% in "Red Rubin." When the plants were exposed to 100 μM

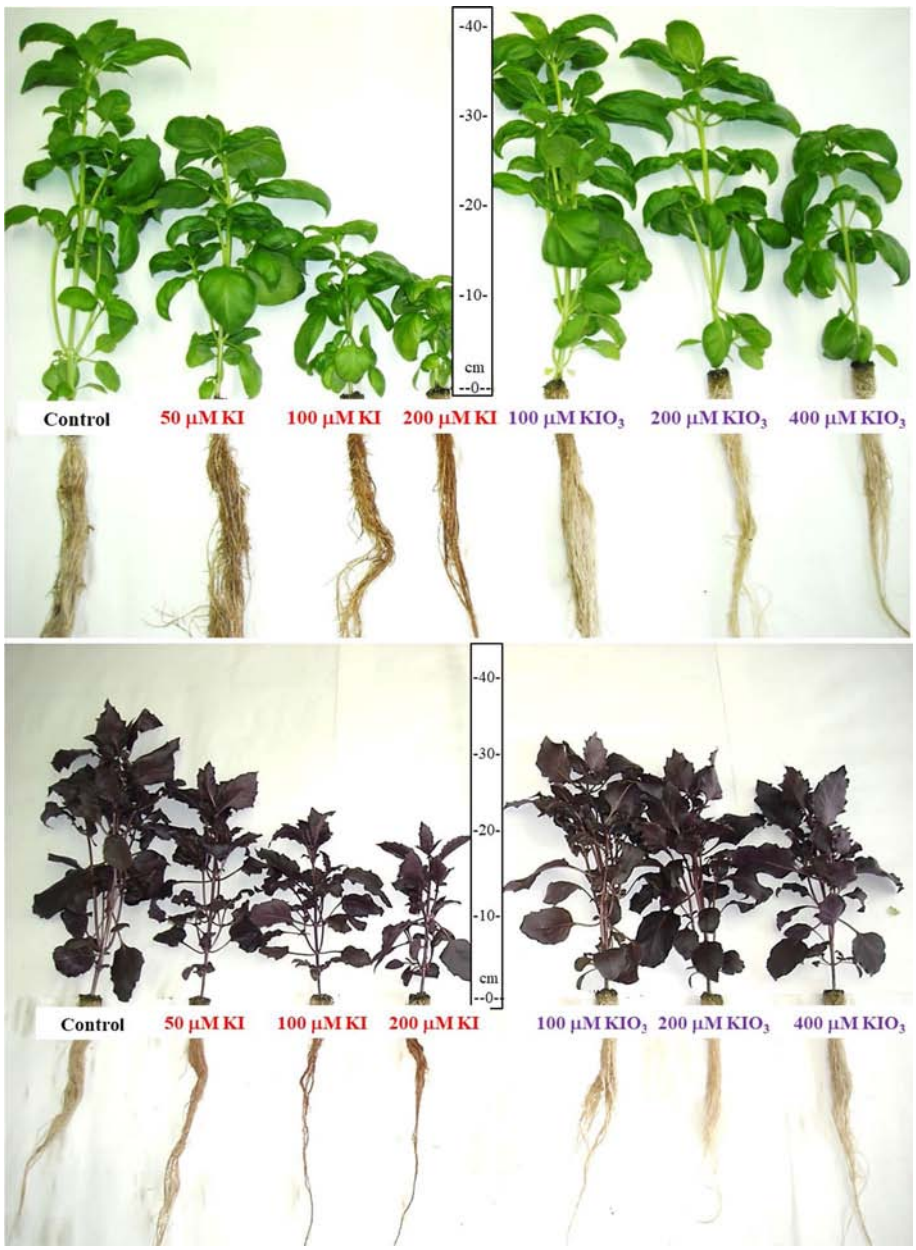

FIGURE 2 | Effect of increasing concentrations of potassium iodide (KI; control = 0.1 μM) or potassium iodate (KIO_3) in the nutrient solution on the growth of two cultivars ("Tigullio," top and "Red Rubin," bottom) of sweet basil (*Ocimum basilicum* L.). For each cultivar and each iodine concentration, both the aerial part and the root system of the plants at the end of the experiment are shown. Plants were grown in floating system for 14 days, from 9th to 23rd May 2016 (*Experiment 3*).

KI, plant height and leaf area were reduced, respectively, by 64 and 68% in "Tigullio" and by 33 and 13% in "Red Rubin" (**Table SM1**).

Experiment 2

After 16 days treatment, a reduction in plant growth was observed in both basil cultivars at KI concentrations higher than 10 μM; however, growth inhibition was less severe in "Red Rubin" than in "Tigullio" (**Table 2**). For example, compared to the control, 200 μM KI concentration reduced total plant dry matter and plant height, respectively, by 42 and 46% in "Red Rubin," and both by 73% in "Tigullio". In both cultivars, leaf iodine content increased with increasing KI concentration in the nutrient solution (**Table 2**). Iodine accumulation in leaf tissues was more pronounced in "Red Rubin" than in "Tigullio": at 200 μM KI concentration, leaf iodine contents were 6,955 ± 915 in "Red Rubin" and 5,225 ± 380 mg kg^{-1} DW in "Tigullio."

The Maas-Hoffman equations for relative plant height or dry biomass against the KI concentration in the nutrient solution are reported in **Table 3** and **Figure SM1**. In "Tigullio" the threshold iodine concentrations (*t*) for leaf DW, total plant DW and plant height were within 17.8 μM and 12.4 μM (on average, *t* was 16.0 μM), while the slope *s* was similar for the three growing parameters under consideration (on average, *s* was −0.42% μM^{-1}). Compared with "Tigullio," "Red Rubin" showed a higher *t* (on average, it was 29.8 μM) and a lower absolute value of *s* (−0.24% μM^{-1}, on average).

When plant growth response was analysed against leaf iodine concentrations, *t* and *s* were, respectively, 272 mg kg^{-1} DW and −0.015% mg^{-1} kg DW in "Tigullio," and 667 mg kg^{-1} DW and −0.005% mg^{-1} kg DW in "Red Rubin" (**Table 3**).

Experiment 3

The main goal of this experiment was to determine if the growth inhibition of sweet basil due to toxic iodine concentrations in the nutrient solution was associated with a reduced uptake of other nutrients. Different concentrations of KI (50, 100, and 200 μM) or KIO_3 (100, 200, and 400 μM) were tested in order to induce different levels of growth inhibition.

As found in *Experiments 1* and *2*, "Red Rubin" showed a higher iodine tolerance than "Tigullio" and KI was more toxic than KIO_3 (Supplementary Material, **Table SM2**). The growth reduction induced by 400 μM KIO_3 was similar to the effect produced by the 50 μM KI treatment (**Figure 2**; Supplementary Material, **Table SM2**).

TABLE 2 | Effect of different concentrations of potassium iodide (KI; control = 0.1 μM) in the nutrient solution on plant height, root, stem, leaves and total dry weight (DW), and leaf iodine content of two cultivars ("Tigullio" and "Red Rubin") of sweet basil (*Ocimum basilicum L.*).Each value is the mean (± standard deviation, SD) of three replicates. Plants were grown hydroponically under greenhouse conditions for 16 days from 29th June to 14th July 2015 (Experiment 2).

Cultivar	Treatment	Plant height (cm $plant^{-1}$)	Root DW (g $plant^{-1}$)	Stem DW (g $plant^{-1}$)	Leaf DW (g $plant^{-1}$)	Total DW (g $plant^{-1}$)	Leaf iodine content (mg kg^{-1})
"Tigullio"	Control	41.5 ± 2.0 a	1.05 ± 0.06 a	3.05 ± 0.35 a	5.43 ± 0.32 a	9.53 ± 0.62 a	23 ± 5g
	10 μM KI	40.7 ± 0.7 a	1.00 ± 0.07 a	3.17 ± 0.23 a	5.51 ± 0.46 a	9.68 ± 0.64 a	295 ± 48 f
	50 μM KI	35.4 ± 1.4 b	0.89 ± 0.07 b	2.27 ± 0.22 b	4.55 ± 0.21 b	7.71 ± 0.42 b	1423 ± 155 e
	100 μM KI	21.2 ± 1,1 e	0.61 ± 0.08 c	1.15 ± 0.15 d	2.87 ± 0.17 ef	4.63 ± 0.38 e	3093 ± 311 d
	200 μM KI	11.3 ± 0.9 g	0.49 ± 0.05 d	0.37 ± 0.08 f	1.69 ± 0.21 g	2.54 ± 0.31 g	5225 ± 380 b
"Red Rubin"	Control	31.1 ± 1.9 c	0.49 ± 0.03 d	1.79 ± 0.18 c	3.45 ± 0.09 cd	5.73 ± 0.30 c	25 ± 6g
	10 μM KI	29.6 ± 0.6 cd	0.46 ± 0.03 de	1.66 ± 0.24 c	3.62 ± 0.16 c	5.74 ± 0.24 c	420 ± 29 f
	50 μM KI	27.8 ± 0.2 d	0.40 ± 0.04 e	1.23 ± 0.09 d	3.39 ± 0.14 d	5.02 ± 0.08 d	1645 ± 121 e
	100 μM KI	21.3 ± 1.2 e	0.39 ± 0.05 e	1.06 ± 0.16 e	3.04 ± 0.09 e	4.49 ± 0.18 e	3865 ± 404 c
	200 μM KI	16.1 ± 1.1 f	0.31 ± 0.04 f	0.47 ± 0.10 f	2.53 ± 0.07 f	3.31 ± 0.19 f	6955 ± 915 a
Analysis of variance							
Cultivar		***	***	***	***	***	***
Treatment		***	***	***	***	***	***
Cultivar×Treatment		***	***	***	***	***	***

*Significance: *** P < 0.001. Values followed by the same letter within a column are not significantly different (P < 0.05; Duncan's test).*

TABLE 3 | Maas-Hoffman equation for relative (Y*, %) plant height and relative leaf and total dry weight against the concentration of potassium iodide (X = 0.1, 10, 50, 100 and 200 μM) in the nutrient solution or leaf iodine content (mg kg^{-1} DW) of two cultivars ("Tigullio" and "Red Rubin") of sweet basil (*Ocimum basilicum* L.). Plants were grown hydroponically under greenhouse conditions for 16 days from 29th June to 14th July 2015 (*Experiment 2*).

Parameter	Maas-Hoffman equation	R^2	Maas-Hoffman equation	R^2
	Tigullio		Red Rubin	
Nutrient solution iodine concentration (μM)				
Plant height	Y* = 100–0.43 (X–17.8)	0.87	Y* = 100–0.30 (X–26.7)	0.85
Leaves	Y* = 100–0.40 (X–12.4)	0.84	Y* = 100–0.18 (X–33.4)	0.92
Whole plant	Y* = 100–0.45 (X–17.8)	0.84	Y* = 100–0.24 (X–29.2)	0.81
Leaf iodine concentration (mg kg^{-1} DW)				
Leaves	Y* = 100–0.015 (X–272)	0.96	Y* = 100–0.005 (X–667)	0.92

TABLE 4 | Effect of different concentrations of potassium iodide (KI; control = 0.1 μM) or potassium iodate (KIO_3) in the nutrient solution on some leaf mineral content ($N–NO_3$, total N, P, K, Ca, Mg, Fe, Mn, Zn, and Cu) of two cultivars ("Tigullio" and "Red Rubin") of sweet basil (*Ocimum basilicum* L.). Each value is the mean (± SD) of three replicates. Plants were grown hydroponically under greenhouse conditions for 14 days from 9th to 23rd May 2016 (Experiment 3).

Treatment	NO_3^- (mg kg^{-1} FW)	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Zn (mg kg^{-1})	Cu (mg kg^{-1})
Tig. Control	1196 ± 85 e	4.23 ± 0.22 a	0.82 ± 0.09 a	5.72 ± 0.08 a	2.13 ± 0.12 d	0.43 ± 0.02	101 ± 15 c	83 ± 8	123 ± 10 b	13 ± 2
Tig. 50 μM KI	1229 ± 72 e	4.20 ± 0.16 a	0.73 ± 0.06 ab	5.54 ± 0.15 ab	2.28 ± 0.10 c	0.41 ± 0.02	108 ± 10 c	78 ± 10	81 ± 11 c	13 ± 1
Tig. 100 μM KI	1362 ± 99 de	3.92 ± 0.26 ab	0.65 ± 0.06 b	5.29 ± 0.51 b	2.13 ± 0.12cd	0.41 ± 0.03	114 ± 11 c	71 ± 10	63 ± 8 c	12 ± 2
Tig. 200 μM KI	1661 ± 65 bc	3.32 ± 0.15 b	0.60 ± 0.07 bc	4.67 ± 0.39 c	2.55 ± 0.28 b	0.42 ± 0.06	141± 21b	78 ± 7	86 ± 8 c	13 ± 1
Tig. 100 μM KIO_3	1329 ± 70 de	4.57 ± 0.17 a	0.84 ± 0.11 a	5.80 ± 0.40 a	2.57 ± 0.12 b	0.43 ± 0.03	135 ± 18 b	87 ± 10	139 ± 11 ab	15 ± 2
Tig. 200 μM KIO_3	1362 ± 76 de	4.51 ± 0.11 a	0.84 ± 0.07 a	5.85 ± 0.15 a	2.47 ± 0.11 b	0.39 ± 0.03	126 ± 13 b	91 ± 9	154 ± 12 a	14 ± 2
Tig. 400 μM KIO_3	1461 ± 83 d	3.97 ± 0.20 ab	0.86 ± 0.16 a	5.48 ± 0.31 ab	2.91 ± 0.19 a	0.42 ± 0.05	135 ± 12 b	90 ± 12	149 ± 10 a	14 ± 2
RR. Control	1627 ± 66 bc	4.27 ± 0.08 a	0.69 ± 0.10 b	5.50 ± 0.73 ab	2.04 ± 0.15 d	0.40 ± 0.03	110 ± 20 c	83 ± 9	108 ± 17 bc	16 ± 2
RR. 50 μM KI	1794 ± 100 ab	3.98 ± 0.20 ab	0.64 ± 0.05 b	5.12 ± 0.29 b	2.17 ± 0.24 cd	0.41 ± 0.02	159 ± 22 a	75 ± 10	83 ± 8 c	15 ± 1
RR. 100 μM KI	1827 ± 63 a	4.05 ± 0.07 a	0.58 ± 0.06 c	5.03 ± 0.18 bc	2.00 ± 0.12 d	0.39 ± 0.02	147 ± 25 ab	65 ± 9	58 ± 9 c	14 ± 1
RR. 200 μM KI	1860 ± 85 a	3.25 ± 1.17 b	0.57 ± 0.05 c	4.87 ± 0.35 c	2.16 ± 0.21 c	0.44 ± 0.04	135 ± 18 b	74 ± 11	68 ± 7 c	13 ± 1
RR. 100 μM KIO_3	1727 ± 66 b	4.52 ± 0.11 a	0.68 ± 0.09 b	5.04 ± 0.24 bc	2.27 ± 0.24 c	0.41 ± 0.03	159 ± 10 a	77 ± 12	138 ± 12 a	17 ± 2
RR. 200 μM KIO_3	1794 ± 78 ab	4.48 ± 0.10 a	0.69 ± 0.07 b	5.35 ± 0.15 ab	2.24 ± 0.13 c	0.39 ± 0.02	160 ± 15 a	83 ± 6	154 ± 13 a	16 ± 2
RR. 400 μM KIO_3	1845 ± 91 a	4.44 ± 0.08 a	0.67 ± 0.06 b	5.18 ± 0.38 b	2.89 ± 0.20 a	0.37 ± 0.04	128 ± 11 b	80 ± 11	160 ± 14 a	12 ± 1
Analysis of variance										
Cultivar	*	ns	***	ns	ns	ns	ns	ns	ns	ns
Treatment	**	***	***	**	*	ns	**	ns	**	ns
Cultivar×Treatment	ns	*	Ns	*	**	ns	ns	ns	ns	ns

*Significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. ns: not significant. Values followed by the same letter within a column are not significantly different ($P < 0.05$; Duncan's test).*

In the control, there was no important difference between "Tigullio" and "Red Rubin" as regards the leaf concentration of macronutrients and micronutrients, apart from a slightly lower content of P in "Red Rubin" (**Table 4**).

Iodine level did not influence the leaf concentrations of Mg (0.41% ± 0.04 mg kg^{-1} DW), Mn (77 ± 12 mg kg^{-1} DW) and Cu (14.1 ± 2.5 mg kg^{-1} DW). Compared with the control, the 200 μM KI treatment decreased the leaf content of total N, P, K, and Zn, and increased the leaf content of Ca and Fe in both basil cultivars (**Table 4**). Evident effects of KIO_3 on leaf nutrient content were observed only in the 400 μM treatment in both cultivars, and consisted in a significant increase in the leaf content of Ca, Fe and Zn, without any significant reduction in the content of other nutrients.

The addition of iodine to the nutrient solution did not have important effects of leaf nitrate content (expressed on a fresh weight basis). Compared to the control (1,196 ± 85 mg NO_3^- kg^{-1} FW) a significant increase in leaf nitrate content was observed only in "Tigullio" plants grown at 200 μM KI (1,661 ± 65 mg NO_3^- kg^{-1} FW). In general, "Red Rubin" accumulated more nitrates in leaf tissues than "Tigullio."

At the end of the experiment, in both the cultivars and for all the concentrations tested, KI produced a greater increase in iodine leaf content than the same concentration of KIO_3 (**Figure 3**). For example, a 14-days cultivation in a nutrient solution containing 200 μM KI or KIO_3 produced an iodine leaf content of 4,250 ± 460 or 687 ± 84 mg kg^{-1} DW in "Tigullio" and 5,433 ± 218 or 717 ± 68 mg kg^{-1} DW in "Red Rubin," respectively. A significant linear relationship between iodine leaf content and KI or KIO_3 concentration in the nutrient solution was found for both cultivars. However, for the KIO_3 treatments, the slopes and intercepts of the regression lines for "Tigullio" and 'Red Rubin' were not significantly different ($P < 0.05$); therefore, the data of both cultivars were pooled and a single regression line was obtained (**Figure 3**).

Experiment 4

In this experiment we investigated the relationship between CO_2 assimilation (A) and iodine content in plants of both cultivars grown with different concentrations of KI (50, 100 and 200 μM) or KIO_3 (100, 200 and 400 μM). The determinations were performed after 12 days of treatment on basal leaves (2nd node) that were already present at the start of the experiment.

Both 100 and 200 μM KI caused a significant decrease of dry biomass accumulation and leaf area, while only 200 μM KIO_3 induced a slight reduction of these parameters (data not shown) in agreement with the results of the previous experiments. For the sake of brevity, only the mean values of the total leaf area at the end of the experiment are here reported, for "Tigullio" and "Red Rubin": 906 ± 64 and 602 ± 55, 897 ± 50 and 564 ± 48, 824 ± 42 and 445 ± 37, 499 ± 51 and 426 ± 29, 376 ± 32 and 401 ± 21 cm^2 $plant^{-1}$ for control, 100 μM KIO_3, 200 μM KIO_3, 100 μM KI, and 200 μM KI treatments, respectively.

In the control, net photosynthesis (A) was approximately 50% lower in "Red Rubin" than in "Tigullio" (**Figure 4A**). In both basil cultivars, all tested KI and KIO_3 concentrations significantly

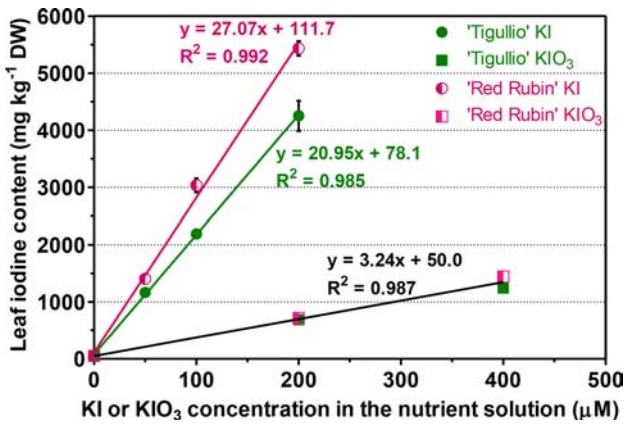

FIGURE 3 | Influence of different concentration of potassium iodide (KI; control = 0.1 µM) or potassium iodate (KIO_3) in the nutrient solution on the iodine concentration in the leaf tissues of two cultivars ("Tigullio" and "Red Rubin") of sweet basil (*Ocimum basilicum* L.). Each value is the mean (± SD) of three replicates. The linear regression lines for the KI treatments are reported along with the equations for both varieties. For the KIO_3 treatments, only the result of the regression analysis of the pooled data of both cultivars is reported, since the individual slopes and intercepts for each cultivar are not significantly different (*P* < *0.05*). Plants were grown hydroponically under greenhouse conditions for 14 days from 9th to 23rd May 2016 (*Experiment 3*).

decreased leaf photosynthesis (**Figure 4A**) while stomatal conductance (Gs) was significantly reduced only by KI (**Figure 4B**). In "Tigullio" plants, no significant effects of iodine were found on internal CO_2 concentrations (Ci; **Figure 4C**).

In general, the basal leaves of "Red Rubin" had a higher iodine content than those of "Tigullio" (**Figure 4D**). For example, at 200 µM KI leaf iodine content was 5,623 ± 995 mg kg^{-1} DW in "Tigullio" and 7,720 ± 346 mg kg^{-1} DW in "Red Rubin" (**Figure 4D**).

In both basil cultivars, a close negative correlation was found between A and iodine content as measured on the same leaves, with significant differences between "Tigullio" and "Red Rubin" as regards the intercept and the slope of the linear regression (**Figure 5**).

The content of carotenoids, total chlorophylls and phenols (gallic acid equivalents), and total antioxidant capacity were also determined in the same leaves sampled for leaf gas exchange measurements (**Figure 6**). In "Tigullio" a reduced content of total chlorophylls and carotenoids compared with the control (1.23 ± 0.04 and 0.26 ± 0.01 mg g^{-1} FW, respectively) was observed with 100 and 200 µM KI (on average, 0.96 ± 0.8 and 0.22 ± 0.02 mg g^{-1} FW, respectively), while no significant effect was observed in the leaf pigment concentrations of "Red Rubin" (1.13 ± 0.10 and 0.23 ± 0.02 mg g^{-1} FW, for total chlorophylls and carotenoids, respectively; **Figures 6A**, **B**). Moreover, the A reduction observed in "Tigullio" with the 200 µM KI treatment was associated to a significantly lower concentration of carotenoids and total chlorophylls (**Figures 6A**, **B**).

Both 100 and 200 µM KI treatments determined an increase in total phenols and antioxidant capacity on mature leaves (2nd node, **Figures 6C**, **D**). In both cultivars, the concentration of total phenols increased with the KI concentration in the nutrient solution and was not influenced by KIO_3. In the control, both

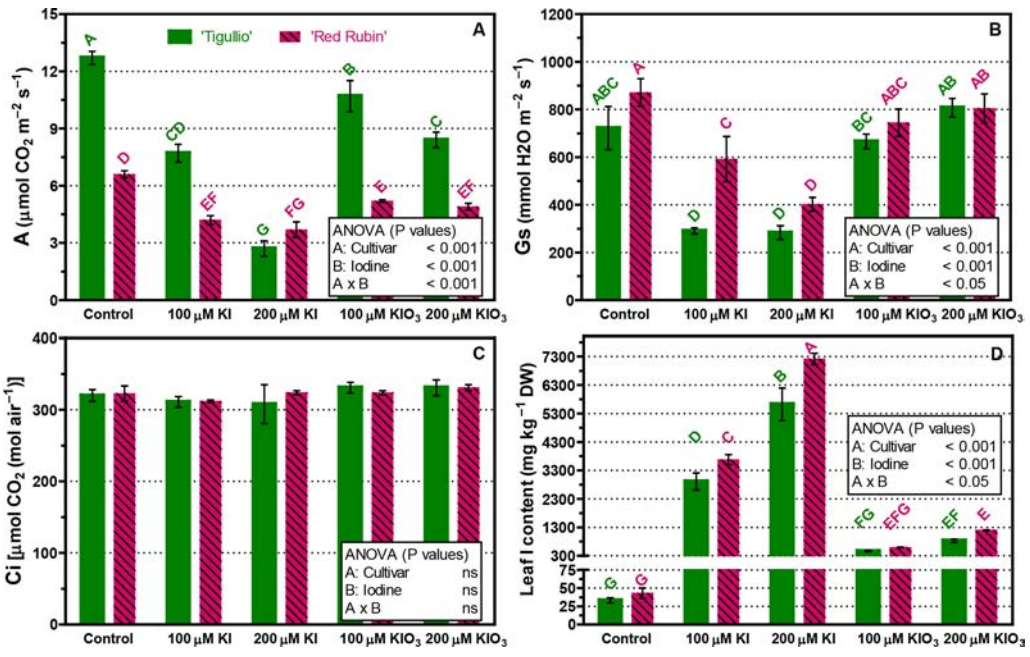

FIGURE 4 | Influence of different concentrations of potassium iodide (KI; control = 0.1 µM) or potassium iodate KIO_3 in the nutrient solution on net assimilation (A), stomatal conductance (Gs), internal CO_2 concentration (Ci) and iodine content of basal leaves of two cultivars ("Tigullio" and "Red Rubin") of sweet basil (*Ocimum basilicum* L.). The measurements were taken on the opposite leaves of the 2nd node, at the end of the experimental period. Each value is the mean of three replicates (± S.D.); bars with the same letter indicate values that are not significantly different (*P* < *0.05*). Plants were grown hydroponically under greenhouse conditions for 12 days from 9th to 21st May 2016 (*Experiment 4*).

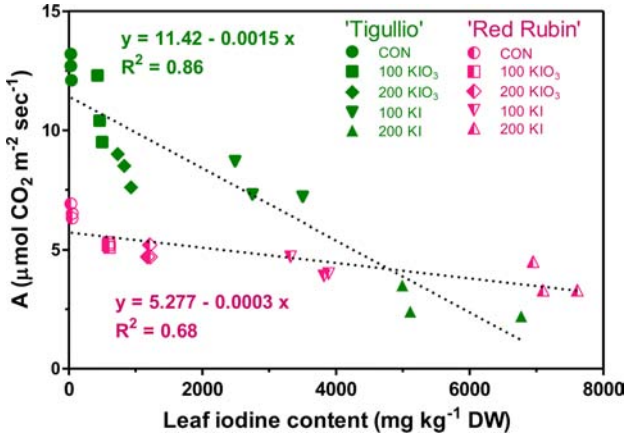

FIGURE 5 | Linear regression between the net assimilation (A) and iodine in the basal leaves of two cultivars ("Tigullio" and "Red Rubin") of sweet basil (*Ocimum basilicum* L.) grown hydroponically with different concentrations of potassium iodide (KI; control = 0.1 μM) or potassium iodate (KIO_3) in the nutrient solution. The measurements were taken on the opposite leaves of the 2nd node at the end of the experimental period. Plants were grown hydroponically under greenhouse conditions for 12 days from 9th to 21st May 2016 (*Experiment 4*).

the concentration of total phenols and the antioxidant capacity (DPPH assay) were much higher in "Red Rubin" than in "Tigullio" (**Figures 6C**, **D**): 6.56 ± 0.48 against 2.49 ± 0.04 mg g^{-1} FW of gallic acid equivalents, and 0.74 ± 0.09 and 2.33 ± 0.27 mg FW ml^{-1}, respectively for the inhibitory concentration (IC_{50}).

In addition to the DPPH assay, we performed also the FRAP assay on our samples: the data of the two methods showed a very good correlation for both "Tigullio" and "Red Rubin" (Pearson's coefficients 0.994 and 0.892, respectively).

The cumulated water uptake during *Experiment 4* was also measured (**Figure 7A**). In the control, water uptake was similar in the two cultivars when expressed per plant (**Figure 7A**), while it was significantly higher in "Red Rubin" when expressed on a leaf area basis (**Figure 7B**). For both cultivars, no differences were found among the control and 100 and 200 μM KIO_3. Compared to the control, whole plant water uptake was significantly decreased at 100 and 200 μM KI, while an opposite result was found when water uptake was expressed on a leaf area basis (**Figure 7B**).

DISCUSSION

Effects of Iodine on Plant Growth

In this work, KI and KIO_3 concentrations not higher than 10 μM or 100 μM, respectively, did not influence the total biomass in "Tigullio" or "Red Rubin" cultivars (**Table 2**; **Tables SM1** and **SM2**). Nevertheless, the use of nutrient solution with 50 μM KI or 400 μM KIO_3 resulted in a similar reduction of plant growth (**Table SM2**), thus suggesting that in sweet basil I^- added to the nutrient solution is approximately eight-times more phytotoxic than IO_3^-.

In both cultivars, higher concentrations of KI (100 or 200 μM) in the nutrient solution caused a significant decrease of plant

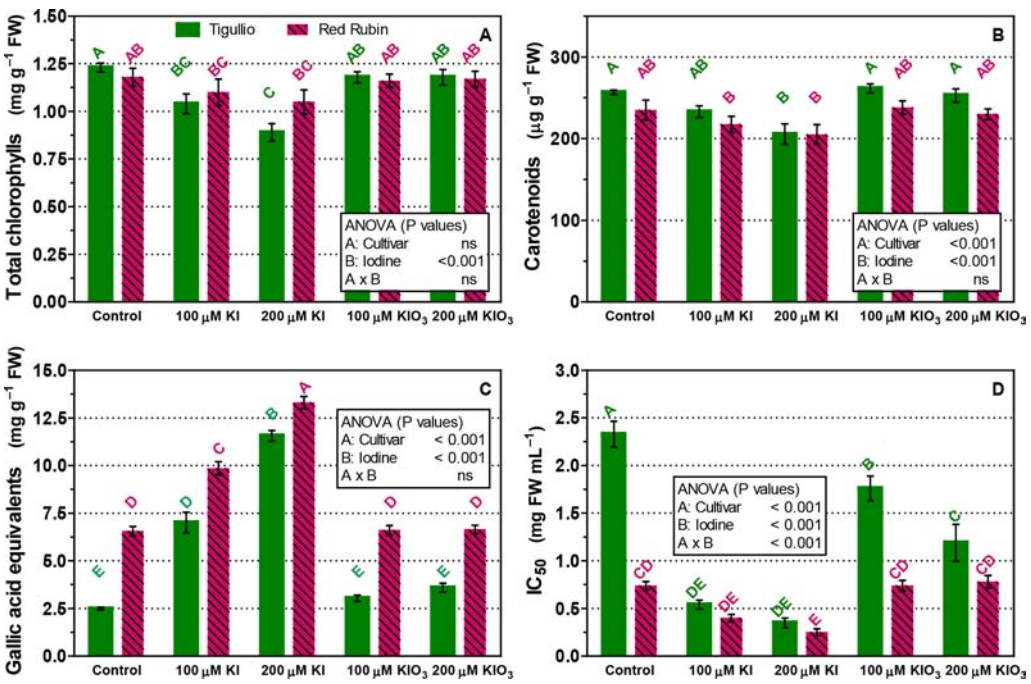

FIGURE 6 | Influence of the concentration of potassium iodide (KI; control = 0.1 μM) or potassium iodate (KIO_3) in the nutrient solution on the concentration of total chlorophylls **(A)**, carotenoids **(B)**, total phenols as gallic acid equivalents **(C)** and antioxidant capacity (IC_{50}) **(D)** in the basal leaves of two cultivars ("Tigullio" and "Red Rubin") of sweet basil (*Ocimum basilicum* L.). The measurements were taken at the opposite leaves of the 2nd node at the end of the experimental period. Each value is the mean of four replicates (± SD); bars with the same letter indicate values that are not significantly different ($P < 0.05$). Plants were grown hydroponically under greenhouse conditions for 12 days from 9th to 21st May 2016 (*Experiment 4*).

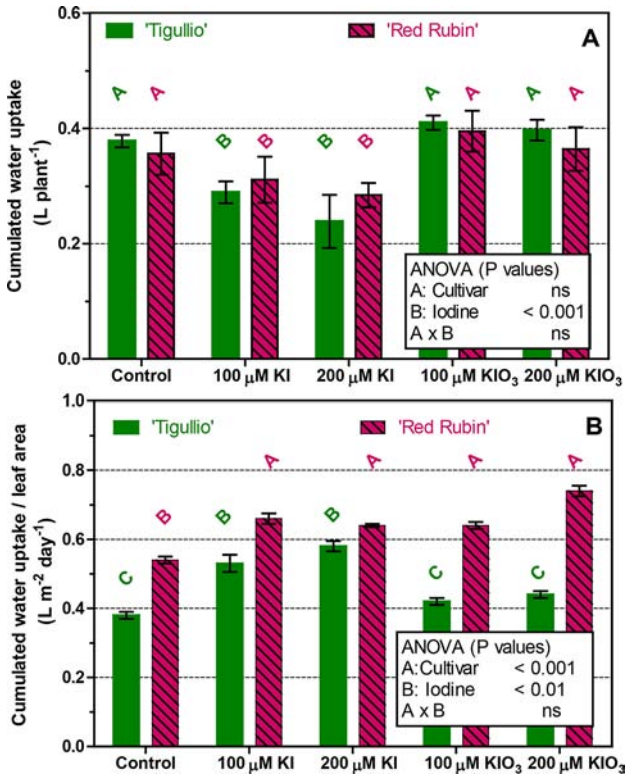

FIGURE 7 | Influence of different concentrations of potassium iodide (KI; control = 0.1 µM) or potassium iodate (KIO_3) in the nutrient solution on the cumulated water uptake (expressed per plant or on leaf area basis) of two cultivars ("Tigullio" and "Red Rubin") of sweet basil (*Ocimum basilicum* L.). Each bar is the mean (± SD) of four replicates; bars with the same letter indicate values that are not significantly different ($P < 0.05$). Plants were grown hydroponically under greenhouse conditions for 12 days from 9th to 21st May 2016 (*Experiment 4*).

height, total dry weight and leaf area compared to the control, and growth inhibition was more severe in "Tigullio" than in "Red Rubin" (**Table 2**; **Table SM2**), suggesting a higher iodine tolerance of the red-leaved cultivar. This conclusion was corroborated by the result of the application of the Maas-Hoffman model to assess the plant response to iodine supply, as a lower slope and a higher critical concentration (the threshold) was computed for "Red Rubin" than for "Tigullio" (**Table 3**).

Several authors found a positive effect of iodine applied to the nutrient solution on plant growth, especially if this element was supplied at low concentration and/or as IO_3^- (Borst Pauwels, 1961 on barley and tomato; Zhu et al., 2003 on spinach; Li et al., 2017 on strawberry). For example, Li et al. (2017) reported an increase of plant biomass in strawberry when iodine was applied to the nutrient solution up to 1.97 µM as I^- and 2.86 µM as IO_3^-. In contrast, detrimental effects were observed when the I^- or IO_3^- concentrations in the nutrient solution were higher than 10–40 µM or 100–200 µM, respectively (Blasco et al., 2008). In our experiments, the addition of KI or KIO_3 to the nutrient solution never produced a significant increase of total biomass with respect to the control, probably due to the much higher concentrations (10 µM or higher) tested in comparison to those reported in the literature.

A decrease in the biomass of iodine treated plants was also reported in lettuce (Blasco et al., 2012; Smoleń et al., 2014a), tomato and potato (Caffagni et al., 2011), as well as in carrot (Smoleń et al., 2014b) and in *Opuntia* (García-Osuna et al., 2014), although in other plant species whose vegetative reserve organs are also harvested, such as onion, iodine seemed to have no effect on plant weight (Dai et al., 2009).

Effects of Iodine on Leaf Photosynthesis and Water Uptake

Toxic iodine concentrations in the nutrient solution significantly reduced the CO_2 assimilation of mature leaves in both basil cultivars, albeit to a lesser extent in "Red Rubin," which had a lower photosynthetic capacity compared to "Tigullio" (**Figure 4A**). In both cultivars, leaf CO_2 assimilation was negatively correlated to leaf iodine content (**Figure 5**); however, the slope of the regression line between these parameters was much lower in "Red Rubin" than in "Tigullio" and this is a further evidence of the greater iodine tolerance of the purple-leaved cultivar. In "Tigullio," the reduction of CO_2 assimilation was attributable to both stomatal and mesophilic limitations as leaf stomatal conductance decreased while the intercellular CO_2 concentrations did not change following iodine stress (**Figure 4C**). On the contrary, only mesophilic limitations were observed in "Red Rubin." Application of 80 µM KI reduced leaf CO_2 assimilation in lettuce and this effect was associated with a reduction of stomatal conductance and total chlorophyll (Blasco et al., 2011).

In "Tigullio" the detrimental effects of 100 and 200 µM KI on leaf CO_2 assimilation were associated to a reduced content of total chlorophylls and carotenoids (**Figures 6A**, **B**), which was also revealed by the occurrence of leaf chlorosis. No significant effects of iodine level were found on the leaf pigment concentrations of "Red Rubin."

The reduction of the content of photosynthetic pigments due to toxic concentration of KI was found in barley (Duborská et al., 2018) and in lettuce (Lawson et al, 2015; Medrano-Macías et al., 2016).

The reduction of stomatal conductance observed in mature leaves of "Tigullio" following KI application was not associated with reduced water uptake as expressed on a leaf area basis, which instead increased in both cultivars (**Figure 7**). The cumulated water uptake of whole plants, which was accounted for 94–96% by transpiration in previous experiments with the same sweet basil cultivars (Pardossi et al., 2015), was decreased by KI application as a result of reduced leaf area.

In all the treatments, "Red Rubin" showed a higher water uptake per leaf area rate than "Tigullio" (**Figure 7B**), probably due to the purple colour of leaves that resulted in a higher average leaf temperature (0.6–1.0°C more than "Tigullio," data not shown), and thus favoured the transpiration process.

Accordingly, the experiments of Voogt et al. (2010; 2014) on the iodine biofortification of lettuce, cucumber, pepper, round and cherry tomato highlighted that the application of iodine did

not alter the water uptake capacity of the crop, and possibly the cumulated water uptake differences could be ascribed to the total leaf area reduction.

Effects of Iodine on Nutrient Uptake

The growth reduction observed in both cultivars exposed to 100 and 200 μM KI was associated with a significant reduction of the leaf concentration of N, P, K (not in "Red Rubin"), and Zn (**Table 4**). Leaf contents of Mg, Mn, and Cu were not affected, while the contents of Fe and Ca were significantly increased. The application of KI also increased the leaf concentration of nitrate.

Similar results were found in lettuce grown in soilless culture by Blasco et al. (2012). These authors found that, compared to the control, the application of 40 μM KI decreased the leaf content of N, P, and K and increased Fe content without significant effects on the leaf concentration of Cu and Mn.

Low iodine fertilizer concentrations (100 μM KI or KIO_3) added in the irrigation water were found to increase P, Mg, Fe, K, Cu, and Mn levels in *Opuntia Ficus-indica* (García-Osuna et al., 2014).

In contrast with our findings, Smoleń et al. (2015a) found a negative correlation in field-grown lettuce between the concentration of iodine and those of Mg, Ca, S, Na, B, Cu, Fe, Mn, Zn, and K (the latter in agreement with our results).

Blasco et al. (2012) suggested that high concentrations of iodine may alter the specific root transporters for NO_3^-, affect P and K uptake or trigger an antagonistic interaction that would explain the reduction of these elements in the leaves of basil plants. Schlorke et al. (2016) suggested that a high iodine content in plant tissues could induce detoxifying mechanisms that increase the activity of oxidizing enzymes and lead to changes in tissue concentrations of Cu, Fe, and Mn. Alternatively, Kato et al. (2013) suggested that IO_3^- may induce reductase activity in the root, which could impact on the bioavailability of mineral nutrients, and that the activation of iodate reductase could induce other responses associated with redox signalling to counteract the effect of IO_3^-.

Indeed, in our work the leaf concentration of all considered nutrients remained within the sufficiency ranges reported for sweet basil (Zheljazkov and Warman, 2003); therefore, the remarkable growth reduction observed in plants treated with KI cannot be explained by iodine-induced mineral deficiencies. Similar conclusions were reported for barley by Duborská et al. (2018) and for lettuce by Blasco et al. (2012) and Smoleń et al. (2014a).

Sweet basil is a vegetable that can accumulate high content of nitrates (Murátiková and Neugebauerová, 2018). The application of KI also increased the leaf concentration of NO_3^- (**Table 4**). An increase in leaf NO_3^- content due to iodine exposure was observed by several authors (Wong and Hung, 2001; Blasco et al., 2012; Smoleń and Sady, 2012; Smoleń et al., 2014a).

Medrano-Macías et al. (2016) suggested that IO_3^- could act as a substrate for widespread enzymes, such as nitrate reductase. Accordingly, Smoleń et al. (2011) reported that nitrate reductase could catalyse the reduction of IO_3^- to I^-, thus interfering with NO_3^- metabolism in plants.

Iodine Accumulation and Tolerance

Plants can absorb iodine by the roots as I^- through ionic channels and chloride transporters that are energized by proton pumps (White and Broadley, 2009). The lower toxicity of IO_3^- observed in our work and in many other species (e.g.: barley, Duborská et al., 2018; cabbage, Weng et al., 2008; lettuce, Blasco et al., 2008; Blasco et al., 2010; Voogt et al., 2010; rice, Mackowiak and Grossl, 1999; spinach, Zhu et al., 2003; Smoleń et al., 2012; tomato, Caffagni et al., 2011; Kiferle et al., 2013) could be related to the necessity for IO_3^- to undergo electrochemical or enzymatic reduction to I^- prior to plant uptake (Zhu et al., 2003; Mackowiak et al., 2005; Kato et al., 2013).

After root absorption, iodine is transported to the shoot mainly through the xylematic flux, while its redistribution through the phloem is low (Mackowiak and Grossl, 1999; Smolen et al., 2014a). Voogt et al. (2014) concluded that i) iodine accumulation in the shoot is dependent on the mass flow of water caused by plant *transpiration* and ii) the differences in iodine distribution across plant species and seasons could be explained by the difference in the transpiration rate.

In our work, iodine uptake and translocation to the leaves of both basil cultivars were dependent on the iodine form and concentration in the nutrient solution (**Figure 3**). The addition of KI to the nutrient solution produced an accumulation of iodine in the leaf tissue approximately eight-times higher than that induced by KIO_3. At the same KI or KIO_3 concentration in the nutrient solution, iodine tissue content in "Red Rubin" was similar or significantly higher than in "Tigullio" (**Table 2** and **Figures 3** and **4D**). This is an evidence that iodine tolerance in "Red Rubin" could not be ascribed to a root iodine-excluding mechanism or to a slower xylem loading of iodine than in "Tigullio," and suggests that the higher iodine tolerance of "Red Rubin" was probably due to a superior ability to tolerate higher concentrations of iodine in leaf tissue.

Zhu et al. (2003) in hydroponically-grown spinach treated with 100 μM KI or KIO_3, calculated the solution-to-leaf-transfer factor (TF_{LEAF}) as the ratio between the iodine concentrations in leaves expressed on a fresh weight basis and in the nutrient solution: it was 19.4 and 2.2 for KI and KIO_3, respectively. In our work (*Experiment 3*) TF_{LEAF} was 14.3 for 200 μM KI and 2.1 for 200 μM KIO_3, in good agreement with the values reported by Zhu et al. (2003) for spinach.

Weng et al. (2008) demonstrated by electron microscopy that in cabbage roots iodine accumulated mainly in the cell wall, while in the leaves iodine was stored in the chloroplast. Some bibliographic evidences confirmed that iodide could easily be oxidized to elemental iodine, which can disrupt the cell membranes of roots and oxidize chlorophylls and carotenoids, thus resulting in leaf chlorosis and reduced CO_2 assimilation (Lawson et al., 2015; Medrano-Macías et al., 2016; Duborská et al., 2018). Blasco et al. (2008; 2010) found that iodine application led to an increase in the concentrations of antioxidant molecules (e.g. ascorbic acid, glutathione, and phenolic compounds) and in the activity of some enzymatic

antioxidants (e.g. superoxide dismutase, catalase, L-galactono dehydrogenase enzymes). In our work, we measured the concentration of total phenols and the antioxidant capacity in mature leaves, which were both higher in "Red Rubin" than in "Tigullio" and increased with leaf iodine content in both genotypes (**Figures 4D** and **6C, D**).

Phenols and anthocyanins are secondary metabolites involved in the plant protection against different types of biotic and abiotic stress, including mineral toxicity (Pardossi et al., 2015; Medrano-Macías et al., 2016). High leaf phenolic concentrations were thought to play an important role in boron tolerance of "Red Rubin" plants (Landi et al., 2014; Pardossi et al., 2015), and phenolic compounds can bind iodine through electrophilic substitution of H in the aromatic ring (Reiller et al., 2006). The anthocyanins contained in the epidermis of "Red Rubin" could act as a photoprotective layer for leaf mesophyll that partly absorbs the incident light energy and may diminish photooxidative damages of the chloroplasts (Landi et al., 2014; Pardossi et al., 2015). Based on these evidences, we can hypothesize that the same mechanisms could be involved also in the iodine tolerance of "Red Rubin." Further work is necessary to verify this assumption.

Iodine Biofiortification

In *Experiment 1*, we found that 10 μM KI did not affect plant growth and increased the leaf iodine content to 295 and 420 mg g^{-1} DW, respectively, in "Tigullio" and "Red Rubin" (**Table 2**). Considering a leaf dry matter content of 7.5% for basil leaves (on average), we calculated that the daily iodine RDA for healthy adults (150 μg day^{-1}) could be satisfied by the assumption of approximately 6.7 g of fresh biofortified leaves of "Tigullio," which corresponds to 7–8 leaves, an amount easy to add to a portion of salad.

Green-leaved sweet basil is also widely used to prepare the worldwide popular sauce "Genovese pesto." Using the above-mentioned data, we calculated the hazardous amount, which is the maximum daily amount of sweet basil below the recommended dietary tolerable upper intake of iodine (1,100 μg day^{-1} for an adult of 70 kg body weight; Smoleń et al., 2019). For "Tigullio" containing 295 mg I kg^{-1} DW, the hazardous amount is 49.7 g of fresh leaves, which is approximately three-fold higher than the amount necessary to prepare a portion of pasta (80 g) dressed with "Genovese pesto" (about 12 g of sauce containing 6 g of fresh basil leaves).

CONCLUSIONS

The detrimental effects of high iodine levels in the nutrient solution on the growth of both basil cultivars "Tigullio" and "Red Rubin" cultivated in hydroponic culture were associated to large iodine accumulation in leaf tissues and a marked reduction of leaf expansion and photosynthesis.

The greater tolerance to iodine toxicity of purple-leaved "Red Rubin" was associated with the ability to withstand higher concentrations of iodine in leaf tissues, rather than to an exclusion mechanism. High leaf concentration of phenolic compounds could play an important role in iodine tolerance of "Red Rubin."

The supply of a nutrient solution containing 10 μM KI could be a simple and effective biofortification protocol for hydroponically-grown sweet basil. Our results also confirmed that this KI concentration is the maximum (in terms of consumer needs) recommended concentration that can be used to enrich sweet basil in iodine. Higher doses may already be harmful to the consumer.

AUTHOR CONTRIBUTIONS

LI: Substantial contributions to the conception or design of the work; drafting the work; interpretation of data and article writing; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. GC and RM: Performed the analyses of mineral ions, chlorophylls, carotenoids and growth analysis; support to article writing; revised the article critically; final approval of the version to be published. CP and CT: Performed the analyses of mineral ions, chlorophylls, carotenoids content in the plant and growth analysis. DS: Performed the analyses of mineral ions and growth analysis; interpretation of data. CK: Performed the analyses of antioxidant capacity, total phenol content, and growth analysis; support to article writing; revised the article critically; final approval of the version to be published. PP: support to article writing; revised the article critically; final approval of the version to be published. AP: conception or design of the work; analysis and interpretation of data; support to article writing; revised the article critically; final approval of the version to be published.

FUNDING

This work was funded by University of Pisa.

ACKNOWLEDGMENTS

We thank Dr. Giorgio Lucchini (Department of Agricultural and Environmental Sciences - Production, Landscape, Agroenergy, University of Milan) for its technical support in iodine plant tissue analyses.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01494/full#supplementary-material

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