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NUTRIENT DELIVERY AND WATER MANAGEMENT FOR PRODUCING LETTUCE (*Lactuca sativa* L.) FOR BIOREGENERATIVE LIFE SUPPORT SYSTEMS

COORDINATER
Prof. Guido D'Urso

CANDIDATE
Christophe El-Nakhel

PROMOTER
Prof. Youssel Rouphael

Co-PROMOTER
Prof. Stefania De Pascale

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1 Chapter 1- Introduction

Plants and bacteria can recycle astronaut waste while growing them food to eat: an approach called bio-regenerative life support.

When humans venture beyond Earth orbit and on to Mars, horticulture will surely follow. Early missions would likely include small-scale plant growing systems to produce fresh vegetables and small fruits to supplement the space travelers diet (Wheeler, 2002).

As mission durations increase, so would the role of horticulture, where plants would provide an increasing proportion of food, oxygen, carbon dioxide removal, and water purification. In all cases, innovative horticultural technologies and approaches will be required. These would include the development of efficient electric lighting and/or solar collectors, innovative design and management concepts for greenhouses and growing modules, capabilities to manage temperature, humidity, and atmospheric composition (e.g., oxygen, carbon dioxide, ethylene, and total pressure), use of techniques to recycle and conserve water and nutrients, and incorporation of novel plant health monitoring concepts. As well, a successful horticultural effort on Mars would require new cultivars that are well suited for the constraints of Martian gardening. Meeting these needs will be challenging but could benefit terrestrial horticultural by inspiring novel technologies that are energy-efficient and miniaturized, as well as through increasing our fundamental understanding of crop physiology (Wheeler, 2002).

1.1 BLSS history

Agricultural systems for space have been discussed since the works of Tsiolkovsky in the early 20th century. Central to the concept, is the use of photosynthetic organisms and light to generate oxygen and food. Research in the area started in 1950s and 60s through the works of Jack Myers and others, who studied algae for O2 production and CO2 removal for the US Air Force and the National Aeronautics and Space Administration (NASA). Studies on algal production and controlled environment agriculture were also carried out by Russian researchers in Krasnoyarsk, Siberia beginning in 1960s including tests with human crews whose air, water, and much of their food were provided by wheat and other crops. NASA initiated its Controlled Ecological Life Support Systems (CELSS) Program ca. 1980 with testing focused on

controlled environment production of wheat, soybean, potato, lettuce, and sweet potato. Findings from these studies were then used to conduct tests in a 20 m^2, atmospherically closed chamber located at Kennedy Space Center. Related tests with humans and crops were conducted at NASA's Johnson Space Center in the 1990s. About this same time, Japanese researchers developed a Controlled Ecological Experiment Facility (CEEF) in Aomori Prefecture to conduct closed system studies with plants, humans, animals, and waste recycling systems. CEEF had 150 m^2 of plant growth area, which provided a near-complete diet along with air and water regeneration for two humans and two goats. The European Space Agency MELiSSA Project began in the late 1980s and pursued ecological approaches for providing gas, water and materials recycling for space life support, and later expanded to include plant testing (Wheeler, 2017). Moreover, the driving element of MELiSSA is the recovering of food, water and oxygen from organic waste carbon dioxide and minerals, using light as source of energy to promote biological photosynthesis. It is an assembly of processes (mechanical grinding, bioreactors, filtration, wet oxidation, etc.) aiming at a total conversion of the organic wastes and CO_2 to oxygen, water and food. It is based on the principle of an "aquatic" lake ecosystem where waste products are processed using the metabolism of plants and algae which in return provide food, air revitalization and water purification. (https://www.esa.int/ESA).

A Canadian research team at the University of Guelph developed a research facility ca. 1994 for space crop research. The Canadian team eventually developed sophisticated canopy-scale hypobaric plant production chambers ca. 2000 for testing crops for space, and have since expanded their testing for a wide range of controlled environment agriculture topics. Most recently, a group at Beihang University in Beijing designed, built and tested a closed life support facility (Lunar Palace1), which included a 69 m^2 agricultural module for air, water, and food production for three humans. As a result of these studies for space agriculture, novel technologies and findings have been produced; this includes the first use of light emitting diodes for growing crops, one of the first demonstrations of vertical agriculture, use of hydroponic approaches for subterranean crops like potato and sweet potato, crop yields that surpassed reported record field yields, the ability to quantify volatile organic compound production (e.g., ethylene) from whole crop stands, innovative approaches for controlling water delivery, approaches for processing and recycling wastes back to crop production systems, and more. The theme of agriculture for space has contributed to, and benefited from

terrestrial, controlled environment agriculture and will continue to do so into the future (Wheeler, 2017).

Humanity's plans to further explore space, strongly suggest the development of bio-regenerative life support systems (BLSS) fully incorporated into space stations, transit vehicles and eventually in habitats on the Moon and Mars. These concepts aim to decrease the re-supply mass by regenerating essential resources for humans through biological processes (Zabel et al., 2015). At present, human life in space flights or in International Space Station (ISS) is guaranteed by a regular resupply of food and water. However, in order to explore deep space with long-term missions and space habitation with increasing crew size, resupply from and return of waste to earth becomes difficult because of the long transport time and high costs associated with mass and volume restrictions for transportation (Clauwaert et al., 2017).

1.2 Plant role in BLSS

Within a BLSS, the cultivation of higher plants takes a crucial role as they can contribute to all major functional aspects (e.g. food production, carbon dioxide reduction, oxygen production, water recycling and waste management). Furthermore, fresh crops are not only beneficial for human physiological health, but also have a positive impact on crew psychological well-being. Adding up these features, higher plants represent a unique asset that makes the investigation of their cultivation in closed systems an essential endeavor. However, cultivation in closed environments is challenging and several key technologies necessary for space-based plant production are not yet space-qualified or remain in early stages of development (Zabel et al., 2015).

The possibility of growing plants in Space is a piece of the mosaic of Space exploration: certainly small, but undeniably essential. In fact, in the "short-term", plants have a practical importance because they play a role in the bioregenerative systems and can provide fresh food. Since the ISS is unlikely ever to support plant systems with more than a few square meters of growing aera, plant cultivation in orbit will be limited to production of some fresh food for diet supplementation, crew recreation, and testing of crop productivity and gas exchange in reduced gravity. Instead, planetary bases have the resources or mission complements to justify full scale BLSS (with current technology it would require 30-40 m^2 of growing area to support one person with 100% water and O_2 and 50% food production). Planet bases take advantage of larger space

and locally available inputs (e.g. regolith as growing substrate for Lunar & Mars bases; CO_2 for plant growth for Mars bases), with dramatic reduction in management and transport costs. A common factor on planet bases in the presence of gravity (1/6 g on Moon and 1/3 g on Mars), which helps Spacefarmers adopt a wide range of gravity-driven drainage strategies, and low atmospheric pressure. The low pressure allows engineers to reduce structural costs and to reduce the risk of gas leakage from Space greenhouses; however, the atmospheric pressure inside the greenhouse must be carefully selected to avoid crop water stress and anoxia whenever adequate amounts of oxygen are not supplied at root level. The use of Planet resources, such as soils or water, and easier waste management/composting of crop residues with processed human excreta would undoubtedly increase chances to produce crops with higher quality standards.

Research studies in the 1980s and early 1990s focused largely on controlled environment production tests of several candidate crops, especially crops rich in carbohydrate and protein, e.g staple crops. The studies typically involved the use of recirculating hydroponic culture systems, electric lighting systems, and careful management of the ambient environment to optimize growth and yields. Similar horticultural and physiological studies have continued through the 1990s. Testing in recent years has also included integration studies, where plants were linked with waste treatment/resource recovery processes, investigations of plant growth and management in atmospherically closed systems, and definition studies for growing plants in Spaceflight environment, such as development of gravity-independent watering systems. The best long-term reliability data come from the biomass production chamber at NASA Kennedy Space Center where multiple crops were grown backtoback in a large, atmospherically closed chamber for over 400 day. But, more testing is needed to assess the long-term reliabilities of these systems and make informed decisions on their life support applications (De Micco et al., 2009).

1.3 Candidate crops for BLSS

Plants (crops) have been used for food by humans for millennia, and of course provide the same atmospheric regeneration functions as algae (Wheeler, 2017). One of the first list of crops suggested for space travelers was drafted at the Biologistics Symposium held at Wright Patterson Air Force Base, USA in 1962 (Wheeler, 2002).

Selection criteria included the ability to grow under relatively low light intensities, compact size, high productivity, and tolerance to osmotic stress from NaCl (from urine recycling). This list included: lettuce, chinese cabbage, cabbage / cauliflower / kale, turnip, Swiss chard, endive, dandelion, radish, New Zealand spinach, tampala, and sweetpotato (Wheeler, 2017). This list focused largely on vegetables and perishable crops that could supplement the diet. Related conferences held about 15 years later generated more comprehensive lists to meet the broader needs of human diets (i.e., a more complete supply of carbohydrate, protein, and fat), as well as taking into consideration potential yields, harvest index (ratio of edible to total biomass), and horticultural requirements, including planting, pollination, harvesting and processing. Most lists contain a mix of "staple" type crops that could provide carbohydrate, protein, and fat, along with a balance of vegetables and small fruits. However, it was recognized that meeting all the dietary needs including micronutrients and vitamins would require large plantings with numerous species, and that using dietary supplements to meet some micronutrient requirements would be more cost effective for near term missions. (Wheeler, 2002). The choice of crops may partly hold the answer to several of the challenges facing seed-to-seed production in microgravity. Crop criteria established for plants grown in space include: the ratio of edible mass to total biomass (harvest index), crop efficiency (per unit area, time, and volume), potential yield (edible mass and O_2 and H_2O production), and the crop's horticultural requirements (planting, harvesting, pollination, processing needs). Salad crops present the highest harvest indices (≈90%) among candidate crops, and low water uptake/transpiration ratio which translates into high humidity input into the space flight environment that can be harnessed, but they cannot be part of a closed system using recycled gray water. Salad crops are highly suitable for chamber cultivation, they are easy to cultivate, they have short growth cycles, they are low ethylene producers and can be picked and eaten fresh, requiring minimal horticultural input from the crew. Moreover, growing salad crops is easily adaptable to the needs of a diverse and renewed diet while adding a palatable and bioactive aspect to it (Kyriacou et al., 2017). Lettuce was nearly omnipresent in crops list suggested or studied for life support systems (Wheeler, 2002), nevertheless, even cultivars of candidate crops undergo a series of selection to choose the appropriate one (Chunxiao & hong, 2008).

1.4 Influence of lettuce genetic material

Epidemiological studies showed a correlation between increased vegetable consumption and reduced risks of chronic diseases, such as cancer, cardiovascular disease and age-related functional decline. These health-beneficial effects are thought to be related to macronutrients, micronutrients and bioactive compounds present in vegetables (Kim et al., 2016).

Lettuce (*Lactuca sativa* L.) is one of the most important leafy vegetables, having the highest rank in both production and economic value among vegetables (Kim et al., 2018) and was classified first between selected crops to be cultivated in both Future Exploration Greenhouse (FEG) at the Antartic Neumayer Station III & International Standard Payload Rack (ISPR) on the International Space Station (ISS) (Dueck et al., 2016). It comes in a variety of colors, sizes and shapes and because of this diversity lettuces can be grouped by their types. A type is a group of cultivars that are morphologically similar, which can be further subdivided into subtypes, which shares more morphological and genetic similarities. A cultivar is a variety selected for desirable traits for cultivation. A variety is a taxonomic rank below species and subspecies, there are six main lettuce types based upon leaf shape, size, texture, head formation, and stem type: (1) crisphead lettuce (var. *capitata* L. nidus jaggeri Helm), (2) butterhead lettuce (var. *capitata* L. nidus tenerrima Helm), (3) romaine or cos lettuce (var. *longifolia* Lam., var. *romana* Hort. in Bailey), (4) leaf or cutting lettuce (var. *acephala* Alef., syn. var. secalina Alef., syn. var. *crispa* L.), (5) stem or stalk (Asparagus) lettuce (var. *angustana* Irish ex Bremer, syn. var. asparagina Bailey, syn. L. angustana Hort. In Vilm.), and (6) Latin lettuce (no scientific name) (Kim et al., 2016). Colour is one of the most important attributes affecting consumer perception of quality. As such, it plays a key role in food preference and acceptability and may even influence taste thresholds and sweetness perception (Lopez et al., 2014)

Lettuce is a rich source of nutritionally important phytoconstituents, including carotenoids, folate, ascorbic acid, and polyphenols. In addition, red-leaf lettuce accumulates a significant amount of anthocyanin pigments, which are known to have potent antioxidant properties and other health benefits (Kim et al., 2018). Since lettuce is generally eaten raw, more nutrients are retained compared to other vegetables that are cooked or processed, such as potatoes (Kim et al., 2016). In recent years, plant breeders have shown great interest in increasing the antioxidant content of lettuce to improve the health promoting properties of these products to meet consumer demand for health

promoting food. The different types of raw material and genotypes of lettuce studied showed differences in phenolic contents (Martínez-Sánchez et al., 2012). Like any other traits, nutrient content of lettuce is determined by genetic difference, environmental influence, and genotype by environment interactions (Mou, 2009). The nutritional value of lettuce varies greatly with types and in addition to differences among horticultural types; there is significant genotypic variation within each type of lettuce.

The nutrient composition of fruits and vegetables is influenced by several pre- and post-harvesting factors, including the genetic makeup of the plant and the stages of harvesting. The composition and quantity of phytochemicals in lettuce also depend on the choice of cultivar, leaf color, and maturity at harvesting (Kim et al., 2018). Nutritional value of lettuce can be enhanced by manipulating cultural practices (e.g., utilization of light emitting diode, improved hydroponic system and adequate fertilization). Therefore, lettuce merits further studies to evaluate nutrient composition under uniform growing environment, development of strategies to enhance nutritional value of lettuce (Kim et al., 2016).

1.5 Nutrient solution management: Macro-elements concentration & composition

In addition to varietal differences, nutritional quality of lettuce may be influenced by environmental factors such as light, temperature, growing season, cultural practices, fertilizer application, and storage conditions. The moisture content of the plant also affects nutrient concentration. Enhancing the nutritional levels of vegetables would improve the nutrient intake without requiring an increase in consumption (Mou, 2009).

Leafy vegetables grown in soilless culture require careful management of fertilizers because of the limited root substrate and high density of seedlings; also, the concentrations of essential plant nutrients in such a medium are frequently insufficient to sustain plant growth. Therefore, optimization of the nutrient solution concentration is required by farmers in order to maximize yield and quality. The total nutrient concentration of the solution used in soilless culture is one of the most important aspects for successful vegetable production. Too high levels of nutrients induce osmotic stress, ion toxicity and nutrient imbalance, while too low levels generally lead to nutrient deficiencies (Fallovo et al., 2009).

Management of nutrient solution composition, particularly the effects of primary (i.e., N, P, K), secondary macronutrients (Ca, Mg, S) as well as plant micronutrients (i.e., Fe, Si) is also considered an important crop management practice in both soil and soilless culture for improving product quality (Rouphael et al., 2012). On the other hand, the excessive N fertilizer application can result in undesirable changes of quality characteristics such as decrease in the concentration of soluble sugars, carotenoids and vitamin C, while increasing the nitrates, TA, and acid:sugar ratio, leading to a downgrading of commercial and nutritional quality. The low phytochemicals content of vegetables grown under excessive N application (i.e., over fertilization) could be expected since plant secondary metabolites (e.g. β-carotene, flavonols, lycopene and phenolics) are stimulated under N starvation conditions although the net photosynthesis is not simultaneously reduced. It is also interesting to note, that a proper N management under soilless culture can lead to an efficient reduction and accumulation of nitrates most notably present in leafy vegetables. Three strategies have been proposed in the scientific literature to cope with the high nitrate accumulation in leafy vegetables: i) replacing nitrate (e.g., calcium nitrate) with chloride (e.g., calcium chloride), ii) nitrate deprivation several days before harvest (DBH, 2–15 days depending on the species), or iii) partial substitution of nitrate with ammonium nitrogen (Rouphael et al., 2018). Moreover, nutritional chemical eustress known as positive stress, can elicit targeted physiological responses aimed at improving the nutritional value of vegetables by triggering the strategic accumulation of desired metabolites in plants as part of the adaptation mechanism to suboptimal conditions (Rouphael & Kyriacou, 2018)

1.6 Importance of biofortification

Trace elements are of main importance, since lot of biological processes depend on it. Working with biological processes and a closed system, cannot be limited to carbon, nitrogen and oxygen, therefore it’s crucial to have trace elements to maintain the system. Somewhere in the loop iron can accumulate on material surfaces or other (Walker & Granjou, 2017), which make an insertion of such elements or others in plants growing cycle of beneficial goals and elements utility such as biofortification of crops. On the other hand, human body deficiencies in micronutrients, also known as *hidden hunger*, have become a serious nutritional issue affecting one-third of human population in developing countries of Africa, Asia and Latin America. Biofortification of vegetable crops with these essential and/or beneficial micronutrients may represent a promising

approach for providing the human diet with selenium (Se), iodine (I), zinc (Zn), iron (Fe) and silicon (Si). Three methods of biofortification in essential and/or beneficial micronutrients have been used for greenhouse vegetables so far, namely the application of the fore-mentioned nutrients with side dressing near the root, with foliar sprays and more efficiently with application in nutrient solution in hydroponic culture (Rouphael et al., 2018).

1.6.1 Iron biofortification

Iron (Fe) is one of the indispensable microelements for life and, although the earth crust is rich in it, iron forms insoluble compounds (White and Broadley, 2009), and its phytoavailable concentration (10^{-17} M) does not reach the optimal range for plant growth (10^{-9}–10^{-4} M) (Sperotto et al., 2012).

Iron is involved in very important processes, in both plants and humans, such as respiration, photosynthesis, and oxygen transport (Sperotto et al., 2012; Abbaspour et al., 2014). In the human body, it exists in two different forms of heme complexes, such as hemoglobin and myoglobin, or in non-heme forms, such as iron–sulfur clusters and other prosthetic groups. Two billion people are anaemic worldwide and according to the World Health Organization (WHO) the main cause is Fe deficient human diet (FAO/WHO, 2001). Fe deficiency is also among the most responsible factors for illnesses worldwide (Gowthami and Ananda, 2017). Fe absorption in the human intestine is inhibited by anti-nutrient molecules such as phytic acid, polyphenols and calcium while it is favoured by molecules such as ascorbic acid and β-carotene that can reduce or chelate Fe, leading to more bioavailable complexes.

Recently, Finklestein et al. (2017), have shown that biofortification with Fe in staple food crops can increase Fe status (serum ferritin concentrations and total body iron) in populations at risk, such as Philippines, India, and Rwanda. According to their work, the beneficial effect has been demonstrated not only in iron deficient youngsters or baseline adults, but also among individuals who are not at risk.

1.6.2 Selenium biofortification

As selenium (Se) is lacking in many human diets, consumption of vegetables containing Se has raised a lot of interest among scientists in the last decade. The Food and Nutrition Board of the Institute of Medicine has suggested a Recommended Dietary Allowance (RDA) of 55 μg day^{-1}, a daily toxic threshold of 400 μg and also estimated

the average dietary intake at which clinical selenosis appears as 1262 μg day^{-1}. It is demonstrated that both leafy vegetables enriched with Se exhibited a longer shelf-life, in association with lower rates of ethylene biosynthesis Furthermore, in senescing lettuce plants, Se has been found to mitigate oxidative stress by preventing the decrease in tocopherol concentration and by stimulating the activity of superoxide dismutase. Differences in accumulation capacity for Se have been reported also in lettuce accessions, confirming the importance of the genetic material in biofortification. Another important factor that could affect Se concentration in vegetables is the chemical form, with sodium selenate and sodium selenite being the most used Se-sources for biofortification. In general, vegetables are more susceptible to selenite than selenite. Plants exposed to selenate can accumulate much more Se in plant tissues than selenite, since selenate is taken up actively by the sulfate trasporters, compared to less efficient phosphate trasporters used in the uptake of selenite. Therefore, when producing vegetables enriched with Se it is crucial to take into consideration several agronomic and environmental factors such as the concentration of Se in the nutrient solution, the exposure time, the genetic material as well as the growing season in order to avoid toxic conditions that might be observed in the diet of some target population groups (vegetarians, infants and elderly) (Rouphael et al., 2018).

1.7 Research objective

Considering the constrains that plants have to face when cultivated in space environments, the aim of the first experiment, was to evaluate the performance, nutritional and functional quality of several lettuce cultivars belonging to the three different types (Romaine, butterhead and leaf lettuce) based upon leaf shape and color, under two light conditions in order to identify the genotypes that are more suitable candidates for incorporation into BLSSs. For this purpose, six lettuce cultivars were grown hydroponically in a Fitotron open-gas-exchange growth chamber under two light regimes (optimal and low light), where growth and physiological parameters, as well as chemical composition (mineral profile, lipophilic and hydrophilic antioxidant compounds) of leaves were assessed. Based on this experiment, we were able to identify a potential candidate cultivar red Salanova for BLSSs, which we adopted in the rest of the experiments with its counterpart green Salanova. Both cultivars were cultivated again, in order to elaborate the evolution of water uptake, morphological and qualitative

data through a complete nineteen days growing cycle. The data can be appreciated by space-faring colonists in order to know in advance water consumption , nutrient accumulation and detecting the adequate maturity stage for harvesting these two butterhead lettuce in order to maintain optimal quality in storage.

Moreover, red and green Salanova where studied in macronutrient deprivation eustress conditions. All growth parameters were standardized and kept uniform while macro-elements concentrations in the nutrient solution were modulated to prompt the accumulation of plants secondary metabolites (e.g. phenolic acids, anthocyanins and carotenoids), with the scope of revealing the eustress threshold level where yield is sustained, while nutritional and functional quality is improved and mineral fertilizers input is reduced. Another experiment was held successively, this time regarding macrocation proportions (K/Ca/Mg) effect on the used lettuce genotypes and the possibility to rise quality level, especially bioactive compounds and achieving a kind of macronutrients biofortification with essential elements such as potassium, calcium and magnesium.

Another aspect was also addressed, which is to cover the needs of certain micronutrients potential deficiencies, or even to depict any increment in plant secondary metabolism under an increase of micronutrient unusual high presence in the nutrient solution. Therefore, two experiments concerning biofortification with Iron and later on with Selenium were carried out with the aim to assess the effect of different applied concentrations on fresh biomass, mineral composition, antioxidant activities, nitrate and ascorbic acid contents as well as on phenolics and carotenoids profile and more importantly to check the extent of the accumulation of these micronutrients in lettuce leaves and setting the boundary between biofortification and toxicity of iron and selenium in Salanova cultivation.

Such findings should procure Bio-regenerative Life-Support Systems with more nutritious lettuce plants as part of a plant-food-based diet able to sustain physical and psychological well-being of space colonists, but more importantly it should provide a set of strategic nutrient solutions for precise purposes, such as saving valuable resources and second choosing the target bioactive molecules needed to be manipulated.

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2 Chapter 2: Performance and bioactive profile of diverse lettuce genotypes grown under optimal and low light conditions: attempt for energy limitation in future Bioregenerative Life Support Systems (BLSSs)

2.1 Abstract

Space farming for fresh food production is a key requirement for the success of long duration space missions and human life sustainment in space colonies. However, several obstacles have to be overleaped including abnormal environmental conditions, and space energy limitations among others. So far, several studies have suggested various crop species highlighting the varied response to environmental constraints in a genotype and cultivar dependent manner. The aim of the present study was to evaluate six lettuce cultivars belonging to different groups and with different leaf colors under low light intensity in order to identify the most promising genotypes for incorporation in controlled ecological life support systems (CELSSs). Baby Romaine plants had a better agronomic performance than the rest of the tested cultivars under low light intensity conditions, indicating a more efficient light harvesting mechanism. Stomatal resistance (rs) increased under low light conditions, especially in the case of Lollo verde and Red oak leaf cultivars, indicating stress conditions, whereas intrinsic water use efficiency (WUEi) was the highest for baby Romaine and red oak leaf cultivars, regardless of light regime. Nitrates content increased under low light intensity especially in the case of Green Salanova and Lollo verde cultivars, while increasing trends were also observed for P and Ca content for baby Romaine and Lollo verde cultivars, respectively. Chicoric acid was the major detected compound, followed by chlorogenic, caffeoyl-tartaric and caffeoyl-meso-tartaric acids. The major phenolic compound (chicoric acid) and total phenolic acids were not affected by light intensity, whereas the rest of the detected phenolic compounds showed a varied response to light intensity. Regarding cultivars response, red oak leaf was mostly affected by low light intensity showing the highest content in chicoric acid and consequently in total phenolic acids content, while under optimal light conditions red Salanova exhibited the highest phenolic profile. In most cases, ascorbic acid content was not affected by light conditions and the tested cultivars

showed a varied response to light intensity. The main detected pigments were β-cryptoxanthin and violaxanthin + neoxanthin, followed by lutein and β-carotene. All the target carotenoids decreased significantly under low light intensity, with red Salanova having a distinct profile of carotenoids. In conclusion, cultivation of mixed lettuce cultivars is the most possible scenario for space farming where some cultivars could provide adequate amounts of fresh biomass while others could contribute to covering daily diet requirements in nutrients and health beneficial compounds.

2.2 Introduction

The rapidly increasing population and depletion of natural resources along with the ongoing climate change have created uncertainty about food security or even the human survival on earth (Turchin and Green, 2018). Space colonization has been proposed as an alternative solution by pioneer aerospace scientists many decades ago and several space programs have focused on life support systems in space through the construction of orbital colonies or the colonization of adjacent to earth plants such as Mars (Walker and Granjou, 2017; Zabel et al., 2016). Although major breakthroughs have been achieved in regard to space engineering and spaceflights during the last decades, the main issue that hinders space life is the use of higher plants in life support systems that could sustain human survival under unfavorable environments (Meinen et al., 2018; Chunxiao and Hong, 2008).

So far, life in space flights and short mission has been supported by dried staple food or nutritional formulas, since fresh food production under space conditions remains a challenge due to several environmental constraints and lack of knowledge of plant physiology under such conditions (Meinen et al., 2018; Kyriacou et al., 2017). Controlled Ecological Life Support Systems (CELSSs) or Bio-regenerative Life Support Systems (BLSSs) have been proposed as efficient means to ensure human survival in space environments in the long-term through sustainable provision of necessary food sources (Guo et al., 2017). The main idea of these systems is based on earth biosphere principles and aims to combine food crops and decomposers for the continuous supply of water and oxygen to space colonists without resupplying from the earth (Zabel et al., 2016). Within this concept, current research programs such as MELiSSA (Micro-Ecological Life Support System Alternative) have focused on creating space habitats that can support human life through the autonomous supply of water, air and food (Walker and Granjou, 2017). However, up to now the most advanced CELSSs can only

achieve regeneration of water and oxygen without being able to support food production under space conditions (Williams et al., 2010). Therefore, there is an urgent need to engineer a space biosphere where fresh food production from higher plants will be able to sustain human life for long term expeditions and space colonies. The major principles of plant cultivation in space environments are the assimilation of CO_2 and the production of O_2, the production of food that can cover daily nutrient requirements of colonizers and the production of clean water through plant transpiration (Monje et al., 2003). Growing fresh food in space from vegetables species has been recognized as key element for the success of CELSSs, considering their importance in human nutrition and their perishable nature (Kyriacou et al., 2017). Apart from a nutritional point of view, cultivation of higher plants in space missions has been associated with beneficial impact and therapeutic effects of human-plant interactions on crew members physiology and psychology or better said mental and overall health status of space colonizers (Guo et al., 2017; Koga and Iwasaki, 2013).

BLSSs are supposed to support crew members needs in regard to food and nutrients requirements, however this has to be achieved under abnormal conditions, such as reduced gravity, gas exchange related issues, extreme temperatures, low light intensity and high irradiation levels (Monje et al., 2003; Kuang et al., 2000). Therefore, earth-based experiments within fully controlled chambers or artificial biospheres such as Biosphere 2 are valuable tools for testing plant and human responses to specific constraints and the obtained results could be forwarded to find application in future space missions (Haeuplik-Meusburger et al., 2014).

So far, various species (i.e., tuber crops, cereals, fruit and leafy vegetables) have been tested as potential candidates for food production in space. The selection criteria for these species were their adaptability under environmental constraints such as low light intensity, small plant size, high nutritional value and harvest index were also considered as factors (Wheeler, 2017; Chunxiao and Hong, 2008). Moreover, space restrictions and energy input requirements (i.e. light) for plant production are of major importance in space food production systems and considered as selection criteria for candidate crops (Meinen et al., 2018).

Research on identification of possible species that could support human life in space environments started since the early 1960s and several experiments have been carried out so far (Chunxiao and Hong, 2008). According to the results of an initial survey for the acceptance of fresh vegetable crop candidates by space station crew members,

lettuce (*Lactuca sativa* L.) was the most preferable crop among the various leafy greens tested (Mauerer et al., 2017). Its leaves constitute a highly nutritious food source, well established in human diet on a daily basis, and the fact that it can be consumed in large quantities could fulfill recommended daily intake for most of the macro- and micro-nutrients (Mou, 2012). Nutritional value and bioactive compounds content of lettuce can be regulated with proper environmental conditions (e.g. light intensity and spectrum, nutrient solution composition, ambient CO_2 concentration etc.).While the great availability of cultivars with very diverse quality features has proven to be the key to successful cultivation of this species in space farms (Park et al., 2012, Kang et al., 2014; Konstantopoulou et al., 2010). Light intensity is associated with several quality parameters of lettuce since it regulates biosynthesis of secondary metabolites and affects visual appearance of leaves (Becker and Kläring, 2016; Zhou et al., 2009; Krumbein et al., 2014). According to Zhou et al.(2009) low light conditions (200-350 μmol m^{-2} s^{-1}) resulted in lower quality of lettuce leaves comparing to high light (1000-1200 μmol m^{-2} s^{-1}) which was attributed to induction of antioxidant mechanisms when plants were subjected to higher than normal light intensities. Moreover, Kitazaki et al. (2018) suggested a metabolomics reprogramming approach for the effect of light intensity on the biosynthetic pathways of flavonoids and phenylpropanoids. In contrast, Urrestarazu et al. (2016) reported that even low light intensities (95 and 117 μmol m^{-2} s^{-1}) can provide sufficient plant growth and high energy efficiency of lettuce, since it is considered a low-light adapted species (Zhen and van Iersel, 2017). The selection of cultivar is equally important since a significant variation in chemical composition and antioxidant compounds content has been reported among lettuce cultivars (López et al., 2013; Kim et al., 2018).

Considering the constrains that plants have to face when cultivated in space environments, the aim of the present study was to evaluate several lettuce cultivars belonging three different types (Romaine, butterhead and leaf lettuce) based upon leaf shape and color under two light conditions, in order to further identify those genotypes that are more suitable candidates for incorporation into CELSSs and space farming. For this purpose, six lettuce cultivars were grown hydroponically in a Fitotron open-gas-exchange growth chamber under two light regimes (optimal and low light), where growth and physiological parameters, as well as chemical composition (mineral profile, lipophilic and hydrophilic antioxidant compounds) of leaves were assessed.

2.3 Materials and methods

2.3.1 Standards and chemicals

Acetonitrile, methanol water and dichloromethane (Merck; Darmstadt, Germany) were used for liquid chromatography diode array detection (LC-DAD) analysis and liquid chromatography tandem mass spectrometry (LC-MS/MS). Ethanol absolute and chloroform were obtained from VWR Chemicals (Radnor, PA); hexane, butylated hydroxytoluene (BHT), formic acid (99% for mass spectrometry) along with analytical standards (chicoric acid, chlorogenic acid, lutein, β-carotene, violaxanthin, neoxanthin, β-cryptoxanthin, and cyanidin) were purchased from Sigma-Aldrich (St. Louis, MO). Ultrapure water was obtained from a Milli-Q Gradient A10 water purification system

2.3.2 Growth chamber environmental control, plant material and closed soilless system management

Two consecutive experiments were conducted in a Fitotron open-gas-exchange growth chamber (28 m^2: 7.0 × 2.1 m × 4.0 m; W × H × D), at the experimental station of the Department of Agricultural Sciences, University of Naples Federico II, Italy.

For light treatments, High Pressure Sodium (HPS) lamps were used with two different light intensities, namely (i) optimal light intensity conditions at 420 μmol m^{-2} s^{-1} Photosynthetic Photon Flux Density (PPFD) (Experiment 1) and (ii) low light intensity conditions at 210 μmol m^{-2} s^{-1} PPFD (Experiment 2). Light intensity treatments (Exps. 1 and 2) were applied at a light/dark regime of 12/12 h, while temperature was regulated at 24/18 °C for light/dark conditions, respectively. Relative humidity was regulated at 65-75% by using a fog system. For CO_2 concentration, ambient conditions (370-410 ppm) were simulated by using one air extractor for air exchange.

Six lettuce cultivars belonging to three main lettuce (*Lactuca sativa* L.) types based upon leaf color and shape (Romaine [(*Lactuca sativa* L. var. *longifolia*], butterhead [*Lactuca sativa* L. var. *capitata*] and leaf lettuce [(*Lactuca sativa* L. var. *crispa*]), were used in both experiments. Common and scientific name, lettuce types and color as well as seed source are reported in supplementary information (Supplementary Table S1).

In both experiments, seedlings were transplanted at the two-true leaf stage in rockwool cubes (7 × 7 × 7 cm) (Delta, Grodan, Roermond, The Netherlands). Plants intra-row and inter-row spacing were 0.15 m and 0.43 m, respectively, accounting for a total plant density of 15.5 plants m^{-2}. Lettuce plants were cultivated in a Nutrient Film

Technique (NFT) growing system. The NFT gullies were 200 cm long, 14.5 cm wide and 8 cm deep, having 1% inclination. Each gully was covered with propylene taps to avoid the evaporation of nutrient solution. The flow rate of nutrient solution was 1.5 L min^{-1} supplied at the top end of each NFT channel and allowed to run slowly down the trough. The excess of nutrient solution was collected in 25 L polypropylene tanks. The composition of the nutrient solution was: 8.0 mM N-NO_3^-, 1.5 mM S, 1.0 mM P, 3.0 mM K, 3.0 mM Ca, 1.0 mM Mg, 1.0 mM NH_4^+ , 15 µM Fe, 9 µM Mn, 0.3 µM Cu, 1.6 µM Zn, 20 µM B, and 0.3 µM Mo, with an electrical conductivity (EC) of 1.4 dS m^{-1} and a pH of 5.9 ± 0.2.

In both growth chamber experiments (optimal and low light intensity experiments), a randomized-complete block design with three replicates was adopted to compare six lettuce cultivars (baby Romaine, green Salanova, Lollo rossa, Lollo verde, red oak leaf or red Salanova). Each experimental unit consisted of one NFT gully with twelve plants each (*n*=216 lettuce plants for each experiment).

2.3.3 Sampling, morphological and physiological parameters

The first and last plant of each experimental unit in the NFT gullies were set as 'guards' and were not included in sampling for morphological, physiological and chemical composition parameters. Three plants per experimental unit were directly frozen in liquid nitrogen and stored at -80 °C for further qualitative analysis; while eight plants per replicate were harvested in order to determine leaf number and measure fresh weight and leaf area, the latter being measured by an electronic area meter (LI-COR 3100C biosciences, Lincoln, Nebraska, USA). Fresh lettuce samples from each experimental unit were dried in a forced-air oven at 70 °C until constant weight (72 h) for dry weight per plant and dry matter content evaluation. Light Use Efficiency (LUE) was also calculated by dividing dry biomass with cumulative daily intercepted PPFD and expressed as g mol^{-1}.

In both experiments, just before harvesting, the net carbon dioxide assimilation rate (A_{CO2}), stomatal resistance (r_s), and transpiration rate (E) were recorded with a portable gas exchange analyzer (LCA-4; ADC BioScientific Ltd., UK) equipped with a broadleaf chamber (cuvette window area of 6.25 cm^2) (Rouphael et al., 2017b). The leaf gas exchange measurements were carried out on fully expanded leaves, using eight replicates for each lettuce cultivars. Intrinsic Water Use Efficiency (WUE_i) was calculated after dividing A_{CO2} by E (Carillo et al., 2019).

2.3.4 Mineral composition analysis

Minerals and nitrates content was recorded in oven-dried lettuce leaf samples according to the method previously described by (Rouphael et al., 2017a). In particular, dried leaf samples were ground with an electrical mill to a 841 μm mesh and 0.25 g of dry tissue were suspended in 50 ml of ultrapure water (Milli-Q, Merck Millipore, Darmstadt, Germany). Then samples were subjected to freeze-thaw with liquid N and incubated at 80 °C for 10 min in a shaking water bath (ShakeTemp SW22, Julabo, Seelbach, Germany). This process was repeated four times. The suspensions were centrifuged at 6000 rpm for 10 min (R-10 M, Remi Elektrotechnik Limited, India), and the supernatants were filtered with a Whatman filter paper (0.20 μm; Whatman International Ltd., Maidstone, U.K.). Ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA) coupled to a conductivity detector was implemented for NO_3-N, P, K, Ca, Mg and Na analysis. The implemented columns were: a) IonPac CG12A (4×250 mm, Dionex, Corporation) guard column and IonPac CS12A (4 × 250 mm, Dionex, Corporation) analytical column for K, Ca, Mg and Na determination, b) IonPac AG11-HC (4 × 50 mm) guard column and IonPac AS11-HC (4 × 250 mm) analytical column for NO_3-N and P analysis. Twenty five μL from filtered extracts were injected into the columns and eluted at a flow rate of 2 mL min^{-1} in isocratic mode for 15 min. The corresponding solvents were NaOH 5 mM for NO_3-N and P and CH_4O_3S 20 mM for K, Ca, Mg and Na. Standard curves for anions and cations in the range 0.05-0.5 mM were used for minerals content in tested samples and results were expressed as g kg^{-1} dry weight (dw), whereas NO_3-N was expressed as mg kg^{-1} on a fresh weight (fw) basis, according to the dry matter content (%) of each sample.

2.3.5 Antioxidant compounds determination

Lipophilic and hydrophilic antioxidant molecules (carotenoids and phenolic compounds) were determined with an HPLC equipment. While ascorbic acid and lipophilic antioxidant activity (LAA) were determined spectrophotometrically. In particular, batch samples of fresh leaves from three plants per experimental unit were frozen in liquid nitrogen immediately after harvest, lyophilized Christ, Alpha 1-4 (Osterode, Germany) and stored at -80 ºC until further analysis.

2.3.6 Extraction and quantification of total acorbic acid and lipophilic antioxidant activity analysis

For total ascorbic acid (TAA) assessment, the method described by Kampfenkel et al. (1995) was implemented. TAA content was determined on the basis of Fe^{3+} reduction to Fe^{2+} by ascorbic acid (AA) and the detection of Fe^{2+} complexes with 2,2-dipyridyl. Samples were pre-incubated in dithiothreitol to reduce dehydroascorbate to ascorbic acid and the latter was determined spectrophotometrically at 525 nm. For quantification of TAA content, calibration curves of standard ascorbate were used and the results were expressed as mg AA 100 g^{-1} fw.

LAA was extracted from freeze-dried leaves (0.2 g) with methanol, and antioxidant activity of this extract was measured with the 2,20-azinobis 3-ethylbenzothiazoline-6-sulfonic acid ABTS method (Pellegrini et al., 1999). The principle of the assay is that the inhibitory response of the radical cation is proportional to the antioxidant concentration, and the reaction is complete at the time point selected of 2.5 min. The absorbance of the solutions was measured at 734 nm. LAA was expressed as mmol of Trolox (6-hydroxy-2,5,7,8-tetramethylchro man-2-carboxylic acid) per 100 g of dw (Fogliano et al., 1999).

2.3.7 Phenolic acids and anthocyanins identification and quantification

Phenolic acids were extracted according to the method described by (Llorach et al., 2008). Freeze-dried samples (400 mg) were extracted in a mixture of methanol/water/formic acid (50/45/5, v/v/v, 12 mL), followed by sonication for 30 min and centrifugation (2500 × *g* for 30 min at 4 °C). The supernatants were collected and centrifuged at 21100 × *g* for 15 min at 4 °C and new supernatants were filtered through 0.22 µm cellulose filters (Phenomenex) before analysis. Hydroxycinnamic derivatives and anthocyanins were separated with a reversed phase C18 column (Prodigy, 250 × 4.6 mm, 5 µm, Phenomenex, Torrance, CA) equipped with a C18 security guard (4.0 × 3.0 mm, Phenomenex). The mobile phases were the following: (A) water formic acid (95:5, v/v) and (B) methanol through the following gradient of solvent B, (t in [min]/[%B]): (0/5), (25/40), (32/40). The flow rate was 1 mL min^{-1} and 20 µL of each extract was injected; LC column was installed onto a binary system (LC-10AD, Shimadzu, Kyoto, Japan), equipped with a DAD (SPD-M10A, Shimadzu, Kyoto, Japan) and a Series 200 auto sampler (Perkin Elmer, Waltham, MA). Standard compounds of chlorogenic acid

and chicoric acid at 330 nm were used for calibration curves of hydroxycinnamic derivatives. Identification of caffeoyl-meso-tartaric acid and caffeoyl-tartaric acid was performed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The chromatographic profiles of reference curves and samples were recorded in multiple reaction monitoring mode (MRM) by using an API 3000 triple quadrupole (ABSciex, Carlsbad, CA). Negative electrospray ionization was used for detection and source parameters were selected as follows: spray voltage -4.2 kV; capillary temperature: 400 °C, dwell time 100 ms; nebulizer gas and cad gas were set to 10 and 12 respectively (arbitrary units). Target compounds $[M-H]^-$ were analyzed using mass transitions given in parentheses: chicoric acid (*m/z* 473→311, 293), chlorogenic acid (*m/z* 353 →191), caffeoyl-tartaric acid (*m/z*311→ 179, 149, retention time 15.8 min), caffeoyl-meso-tartaric acid (*m/z*311→ 179, 149, retention time 17.8 min). Phenolic acids content was expressed as mg 100 g^{-1} dw. Anthocyanins were determined at 520 nm and quantified by using cyanidin as standard compound. The results for anthocyanins were reported as µg of cyanidin equivalent per g of samples on a dw basis.

2.3.8 Target carotenoids extraction and quantification

Carotenoids were assessed according to the method previously described by (Vallverdú-Queralt et al., 2013) after slight modifications. One hundred mg of lettuce freeze dried sample was added to a mixture of ethanol/hexane (4:3, v/v, 2.5 mL) with 1% BHT and the suspension was vortexed at 22 °C for 30 s, sonicated for 5 min in dark conditions, centrifuged (2500 × *g* at 4 °C for 10 min) and filtered (0.45 µm nylon filters; Phenomenex, Torrance, CA). The supernatant was collected in a volumetric flask and the same extraction procedure was repeated for three times. The total amount of extracts of each sample was dried with an air flow of nitrogen and stored at -20 °C until further analysis. Dried extracts were re-dissolved in 1% BHT in chloroform and 20 µL of each sample was injected into a C18 column (Prodigy, 250 × 4.6 mm, 5 µm, Phenomenex, Torrance, CA) equipped with a C18 security guard (4.0 × 3.0 mm, Phenomenex). The used mobile phases were the following: (A) acetonitrile, hexane, methanol, and dichloromethane (4:2:2:2, v/v/v/v) and (B) acetonitrile were used. Flow rate was 0.8 mL min^{-1} through the following gradient of solvent B (t in [min]/[%B]): (0/70), (20/60), (30/30), (40/2). A binary LC10AD system coupled to a DAD (SPD-M10A; Shimadzu, Kyoto, Japan) equipped with a Series 200 autosampler (Perkin Elmer, Waltham, MA) was used for carotenoids quantitation, while for identification and quantification of

peaks commercial standards of violaxanthin, neoxanthin, β-cryptoxanthin, lutein and β-carotene were used for comparison of UV-vis spectra and retention times of eluted compounds at 450 nm. Moreover, to increase the accuracy of quantification intra- and inter-day assays were performed in triplicates and calibration curves were built accordingly. A recovery test was also performed by spiking two random samples with two known amounts of carotenoids (50 and 100 μg mL^{-1}) and taking into account the overestimation due to the presence of the target analytes in the samples. Concentrations of target carotenoids were reported as μg g^{-1} of samples on a dw basis.

2.3.9 Statistical analysis and principal component analysis

All experimental data were subjected to analysis of variance (ANOVA) using the SPSS 20 software package for Windows 2010. Combined analysis of variance was performed using light intensity conditions as a fixed variable (Gomez and Gomez, 1983). Means comparison was performed with the use Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$.

Table S1. Common and scientific name, lettuce type, leaf color and source of the six lettuce (*Lactuca sativa* L.) cultivars considered in this study.

Cultivar name	Scientific name	Lettuce type	Leaf color	Source
Baby Romaine	*Lactuca sativa* L. var. *longifolia*	Romaine	Dark Green	Rijk Zwaan
Green Salanova	*Lactuca sativa* L. var. *capitata*	Butterhead	Light Green	Rijk Zwaan
Lollo rossa	*Lactuca sativa* L. var. *crispa*	Leaf lettuce	Red	Rijk Zwaan
Lollo verde	*Lactuca sativa* L. var. *crispa*	Leaf lettuce	Light green	Nunhems
Red oak leaf	*Lactuca sativa* L. var. *crispa*	Leaf lettuce	Red	Rijk Zwaan
Red Salanova	*Lactuca sativa* L. var. *capitata*	Butterhead	Red	Rijk Zwaan

2.4 Results and discussion

2.4.1 Implications of light intensity and cultivars for morphological and physiological parameters

The functional quality as well as the morpho-physiological parameters of *Lactuca sativa* L. were governed by genetic material and also by Genotype × Environment interaction. In this study, for most of the morphometric parameters measured, significant interactions were observed between the tested factors (Cultivar: C; Light intensity: L) (Table 1). Leaf area, leaf number, and fresh and dry biomass were higher under optimal light conditions with a varied response in terms of cultivar. In particular, red Salanova presented the highest leaf area, while leaf number was the highest for both green and red Salanova cultivars (Table 1). Regarding the fresh yield, the lowest values were reported for green Salanova, Lollo rossa and red oak leaf cultivars under low light conditions (Table 1). Interestingly, the effect of light intensity treatment on lettuce productivity was cultivar-dependent. In fact decreasing light intensity from 420 to 210 µmol m^{-2} s^{-1} PPFD, decreased the fresh yield of baby Romaine, green Salanova, Lollo rossa, Lollo verde, red oak leaf and red Salanova by 36.8%, 64.6%, 65.4%, 54.6%, 58.5% and 60.3%, respectively (Table 1). When shoot dry mass was expressed in g $plant^{-1}$, the recorded values were higher for Lollo verde and red Salanova cultivars under optimal light conditions, whereas when dry matter was expressed in % red oak leaf and baby Romaine exhibited the highest values under optimal and at both optimal and low light intensity conditions, respectively (Table 1).

Significant interactions between the two tested factors were also observed in the studied physiological parameters, with only exception the transpiration rate (E) which was affected by C and L factors without significant interaction between them (Table 2). Net CO_2 assimilation rate (ACO_2) was the highest under optimal light conditions with red Salanova exhibiting the highest overall values. A contrasting trend was observed for stomatal resistance (r_s) which increased under low light intensity conditions, especially in the case of Lollo verde and red oak leaf cultivars (Table 2). On the other hand, when averaged over cultivars, E values were higher by 51.3% under optimal light conditions, while in regards to cultivar effect, the highest E rate was observed for red Salanova plants without being significantly different from baby Romaine and green Salanova cultivars (Table 2). Regarding intrinsic water use efficiency (WUE_i), the highest values

were recorded for baby Romaine and red oak leaf cultivars, regardless of light regime, followed by red Salanova under optimal light. Finally, the highest light use efficiency (LUE) was detected in baby Romaine plants when grown under low light intensity conditions, without however being significantly different from Lollo verde plants grown under optimal light conditions (Figure 1).

Table 1 Growth parameters, fresh yield, dry biomass and leaf dry matter percentage of lettuce grown hydroponically in a Fitotron open-gas-exchange growth chamber in relation to light conditions and cultivar.

Source of variance	Leaf area (cm^2 $plant^{-1}$)	Leaf number (no. $plant^{-1}$)	Fresh biomass (g $plant^{-1}$)	Dry biomass (g $plant^{-1}$)	Dry matter (%)
Cultivar (C)					
Baby Romaine	799 ± 84 d	23.76 ± 1.72 c	61.0 ± 6.2 b	3.41 ± 0.38 a	5.58 ± 0.07 a
Green Salanova	1037 ± 143 b	45.55 ± 5.01 a	60.5 ± 13.0 b	2.83 ± 0.55 c	4.82 ± 0.13 c
Lollo rossa	715 ± 138 e	13.30 ± 0.62 d	61.7 ± 13.5 b	2.91 ± 0.62 c	4.78 ± 0.05 c
Lollo verde	893 ± 133 c	13.81 ± 0.48 d	64.2 ± 11.1 ab	3.33 ± 0.59 a	5.20 ± 0.11 b
Red oak leaf	1035 ± 151 b	37.21 ± 2.88 b	51.9 ± 9.8 c	2.82 ± 0.55 c	5.41 ± 0.09 ab
Red Salanova	1245 ± 214 a	44.28 ± 4.70 a	66.0 ± 12.8 a	3.09 ± 0.56 b	4.76 ± 0.09 c
Light intensity (μmol m^{-2} s^{-1}) (L)					
420 (Optimal)	1271 ± 63 a	35.21 ± 4.23 a	85.4 ± 2.3 a	4.26 ± 0.07 a	5.06 ± 0.11
210 (Low)	637 ± 29 b	24.09 ± 2.31 b	36.4 ± 1.5 b	1.87 ± 0.09 b	5.12 ± 0.07
C × L					
Baby Romaine × Optimal	983 ± 23 d	27.40 ± 0.95 e	74.7 ± 1.2 b	4.24 ± 0.10 b	5.69 ± 0.05 a
Green Salanova × Optimal	1355 ± 19 b	56.67 ± 1.31 a	89.5 ± 0.9 a	4.06 ± 0.06 b	4.58 ± 0.10 f
Lollo rossa × Optimal	1019 ± 48 d	14.53 ± 0.52 g	91.7 ± 3.7 a	4.28 ± 0.14 b	4.68 ± 0.04 ef
Lollo verde × Optimal	1177 ± 81 c	14.80 ± 0.31 g	88.3 ± 5.0 a	4.63 ± 0.19 a	5.29 ± 0.07 bc
Red oak leaf × Optimal	1368 ± 29 b	43.20 ± 2.23 b	73.4 ± 3.5 b	4.03 ± 0.13 b	5.52 ± 0.10 ab
Red Salanova x Optimal	1722 ± 33 a	54.67 ± 1.38 a	94.6 ± 2.3 a	4.33 ± 0.15 ab	4.57 ± 0.05 f
Baby Romaine × Low	614 ± 32 f	20.12 ± 0.81 f	47.2 ± 2.0 c	2.57 ± 0.08 c	5.46 ± 0.08 ab
Green Salanova × Low	718 ± 11 ef	34.42 ± 0.22 c	31.6 ± 0.3 e	1.59 ± 0.04 e	5.06 ± 0.14 cd
Lollo rossa × Low	411 ± 15 g	12.07 ± 0.34 g	31.7 ± 1.3 e	1.54 ± 0.05 e	4.88 ± 0.04 def
Lollo verde × Low	610 ± 30 f	12.82 ± 0.27 g	40.1 ± 2.2 d	2.04 ± 0.06 d	5.10 ± 0.21 cd
Red oak leaf × Low	701 ± 35 ef	31.22 ± 0.84 d	30.4 ± 1.9 e	1.61 ± 0.12 e	5.30 ± 0.14 bc
Red Salanova × Low	768 ± 16 e	33.90 ± 0.79 cd	37.5 ± 0.7 d	1.85 ± 0.03 de	4.94 ± 0.04 de
Significance					
Cultivar (C)	***	***	***	***	***
Light intensity (L)	***	***	***	***	NS
C × L	***	***	***	***	**

NS, **, *** Non-significant or significant at $P \leq 0.01$, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (P = 0.05). All data are expressed as mean ± standard error, $n = 3$.

Table 2. Physiological parameters (net CO_2 assimilation rate [A_{CO2}]; stomatal resistance [r_s]; transpiration rate [E]; intrinsic Water Use Efficiency [WUE_i]) of lettuce grown hydroponically in a Fitotron open-gas-exchange growth chamber in relation to light conditions and cultivar.

Source of variance	A_{CO2} ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	r_s ($m^2\ s^1\ mol^{-1}$)	E ($mol\ H_2O\ m^{-2}\ s^{-1}$)	WUEi ($\mu mol\ CO_2\ mol^{-1}\ H_2O$)
Cultivar (C)				
Baby Romaine	10.49 ± 0.45 a	5.00 ± 0.41 cd	2.60 ± 0.13 ab	4.08 ± 0.11 b
Green Salanova	7.85 ± 0.49 c	5.15 ± 0.51 cd	2.67 ± 0.13 ab	2.93 ± 0.12 d
Lollo rossa	5.99 ± 0.56 d	5.92 ± 0.62 b	2.51 ± 0.15 b	2.36 ± 0.16 e
Lollo verde	4.06 ± 0.43 e	5.72 ± 0.93 bc	2.26 ± 0.16 c	1.75 ± 0.13 f
Red oak leaf	9.55 ± 0.59 b	8.03 ± 0.57 a	2.21 ± 0.16 c	4.40 ± 0.15 a
Red Salanova	10.07 ± 0.80 a	4.94 ± 0.65 d	2.76 ± 0.16 a	3.58 ± 0.11 c
Light intensity ($\mu mol\ m^{-2}\ s^{-1}$) (L)				
420 (Optimal)	9.99 ± 0.37 a	3.70 ± 0.21 b	2.98 ± 0.06 a	3.39 ± 0.13 a
210 (Low)	5.76 ± 0.32 b	8.15 ± 0.26 a	1.97 ± 0.05 b	2.96 ± 0.17 b
C × L				
Baby Romaine × Optimal	12.06 ± 0.30 b	3.64 ± 0.23 de	3.03 ± 0.12	4.03 ± 0.17 ab
Green Salanova × Optimal	9.57 ± 0.32 c	3.36 ± 0.29 def	3.05 ± 0.15	3.19 ± 0.17 c
Lollo rossa × Optimal	8.01 ± 0.24 e	4.08 ± 0.30 d	2.93 ± 0.14	2.79 ± 0.16 cd
Lollo verde × Optimal	5.59 ± 0.15 g	2.33 ± 0.15 f	2.79 ± 0.13	2.03 ± 0.11 e
Red oak leaf × Optimal	11.68 ± 0.27 b	6.24 ± 0.44 c	2.73 ± 0.15	4.38 ± 0.24 ab
Red Salanova x Optimal	13.01 ± 0.31 a	2.53 ± 0.14 ef	3.35 ± 0.05	3.90 ± 0.12 b
Baby Romaine × Low	8.72 ± 0.08 d	6.53 ± 0.35 c	2.12 ± 0.08	4.14 ± 0.14 ab
Green Salanova × Low	5.91 ± 0.12 g	7.16 ± 0.25 bc	2.25 ± 0.05	2.64 ± 0.10 d
Lollo rossa × Low	3.71 ± 0.23 h	7.99 ± 0.76 b	2.05 ± 0.14	1.89 ± 0.19 ef
Lollo verde × Low	2.33 ± 0.27 i	9.53 ± 0.51 a	1.66 ± 0.07	1.43 ± 0.19 f
Red oak leaf × Low	7.16 ± 0.21 f	10.05 ± 0.50 a	1.63 ± 0.07	4.42 ± 0.18 a
Red Salanova × Low	6.76 ± 0.21 f	7.65 ± 0.22 b	2.09 ± 0.01	3.23 ± 0.11 c
Significance				
Cultivar (C)	***	***	***	***
Light intensity (L)	***	***	***	***
C × L	***	***	NS	**

NS, **, *** Non-significant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (P = 0.05). All data are expressed as mean ± standard error, $n = 3$.

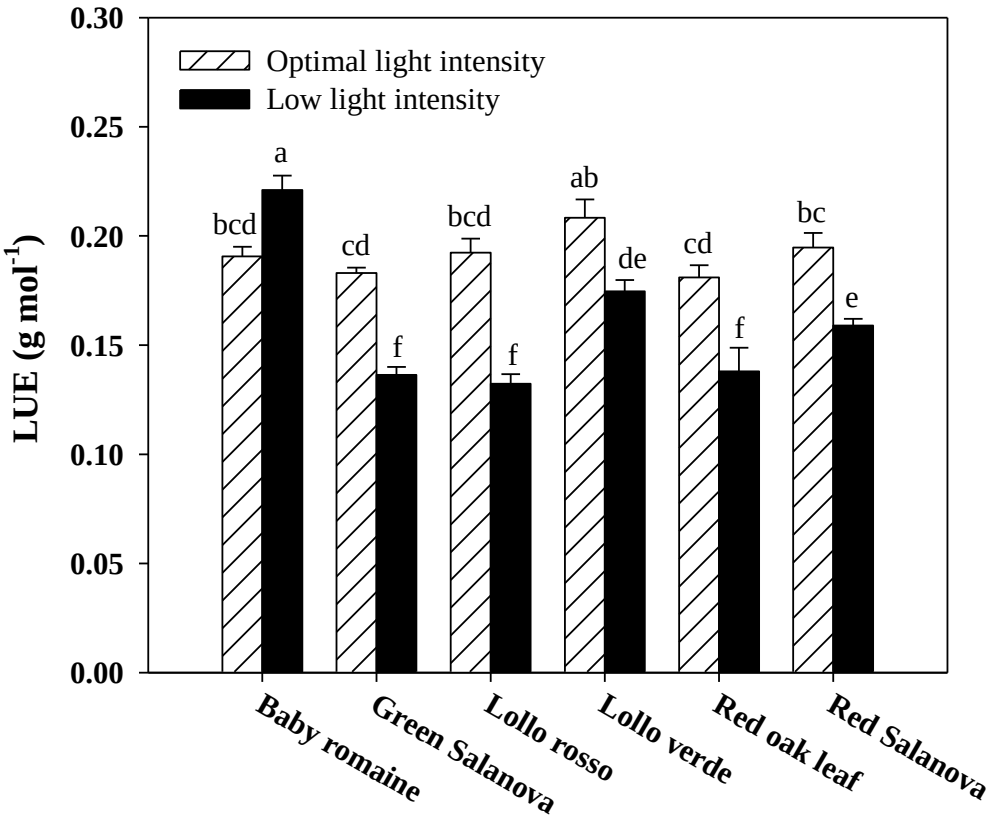

Figure 1. Light Use Efficiency (LUE) of lettuce grown hydroponically in a Fitotron open-gas-exchange growth chamber in relation to light conditions and cultivar. All data are expressed as mean ± standard error, n = 3. Different letters above each bar indicate significant differences according to Duncan's multiple-range test (P = 0.05).

2.4.2 Implications of light intensity and cultivars for nitrate and mineral Profile

Nitrate content and mineral composition as a function of cultivar and light intensity are presented in Table 3. Low light intensity resulted in an increase of nitrates content, especially in the case of green Salanova and Lollo verde cultivars. Among the minerals analyzed, K was by far the most abundant, irrespective of cultivar and light intensity treatment, ranging from 58.2 to 70.9 g kg^{-1} dw, followed by Ca (4.8-13.5 g kg^{-1} dw), P (4.8-7.6 g kg^{-1} dw), Mg (2.0-2.9 g kg^{-1} dw) and finally Na (0.3-0.8 g kg^{-1} dw) (Table 3).

Neither cultivar nor light intensity regime had a significant effect on K and Na concentration in lettuce leaves (avg. 64.4 and 0.4 g kg^{-1} dw). Regarding the minerals content, a cultivar-dependent response to light conditions was observed with phosphorus and calcium content being the highest for baby Romaine and Lollo verde plants grown under low light intensity conditions, respectively (Table 3). In contrast, magnesium content increased under optimal light conditions without significant differences being observed for most of the tested cultivars between the applied light

regimes except for the case of green and red Salanova plants were significant differences were detected (Table 3).

Table 3. Nitrate, phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sodium (Na) concentrations of lettuce grown hydroponically in a Fitotron open-gas-exchange growth chamber in relation to light conditions and cultivar.

Source of variance	NO_3 (mg kg^{-1} fw)	P (g kg^{-1} dw)	K (g kg^{-1} dw)	Ca (g kg^{-1} dw)	Mg (g kg^{-1} dw)	Na (g kg^{-1} dw)
Cultivar (C)						
Baby Romaine	2494 ± 54 b	6.28 ± 0.62 a	64.26 ± 1.30	6.73 ± 0.88 cd	2.56 ± 0.09 bc	0.33 ± 0.04
Green Salanova	2699 ± 136 ab	5.86 ± 0.22 b	68.00 ± 1.36	9.71 ± 0.77 b	2.31 ± 0.19 d	0.37 ± 0.03
Lollo rossa	2081 ± 160 c	5.56 ± 0.26 bc	65.03 ± 3.22	6.14 ± 0.49 d	2.62 ± 0.09 bc	0.36 ± 0.06
Lollo verde	2806 ± 177 a	5.16 ± 0.19 cd	62.04 ± 1.18	10.94 ± 1.16 a	2.91 ± 0.08 a	0.55 ± 0.14
Red oak leaf	2084 ± 34 c	5.08 ± 0.17 d	60.44 ± 1.10	7.44 ± 0.69 c	2.70 ± 0.06 b	0.31 ± 0.01
Red Salanova	2108 ± 114 c	5.61 ± 0.17 bc	66.43 ± 2.65	6.01 ± 0.43 d	2.45 ± 0.11 cd	0.30 ± 0.02
Light intensity (μmol m^{-2} s^{-1}) (L)						
420 (Optimal)	2275 ± 56 b	5.16 ± 0.10 b	65.15 ± 1.08	6.27 ± 0.37 b	2.66 ± 0.05 a	0.41 ± 0.05
210 (Low)	2482 ± 124 a	6.02 ± 0.21 a	63.58 ± 1.35	9.38 ± 0.58 a	2.56 ± 0.08 b	0.33 ± 0.01
C × L						
Baby Romaine × Optimal	2419 ± 90 cde	4.90 ± 0.03 de	66.56 ± 1.76	4.78 ± 0.14 g	2.38 ± 0.07 cd	0.32 ± 0.08
Green Salanova × Optimal	2423 ± 38 cde	5.42 ± 0.13 cd	65.08 ± 0.60	8.05 ± 0.32 cd	2.65 ± 0.08 abcd	0.33 ± 0.02
Lollo rossa × Optimal	1974 ± 69 e	5.68 ± 0.11 bc	66.19 ± 1.65	5.14 ± 0.16 gh	2.67 ± 0.04 abc	0.42 ± 0.13
Lollo verde × Optimal	2461 ± 61 cd	4.82 ± 0.16 de	60.55 ± 2.05	8.42 ± 0.53 c	2.96 ± 0.09 a	0.76 ± 0.23
Red oak leaf × Optimal	2152 ± 4.0 cde	4.71 ± 0.05 e	62.71 ± 0.82	5.96 ± 0.40 fg	2.70 ± 0.11 abc	0.30 ± 0.02
Red Salanova x Optimal	2223 ± 221 cde	5.42 ± 0.32 cd	69.81 ± 4.77	5.30 ± 0.59 gh	2.61 ± 0.11 abcd	0.31 ± 0.03
Baby Romaine × Low	2570 ± 29 bc	7.65 ± 0.18 a	61.95 ± 0.17	8.68 ± 0.20 c	2.74 ± 0.07 abc	0.33 ± 0.06
Green Salanova × Low	2975 ± 121 ab	6.31 ± 0.17 b	70.92 ± 0.65	11.38 ± 0.34 b	1.98 ± 0.02 e	0.40 ± 0.04
Lollo rossa × Low	2189 ± 335 cde	5.44 ± 0.55 cd	63.87 ± 6.91	7.13 ± 0.42 de	2.57 ± 0.20 bcd	0.30 ± 0.02
Lollo verde × Low	3151 ± 185 a	5.50 ± 0.20 cd	63.52 ± 0.77	13.47 ± 0.17 a	2.86 ± 0.13 ab	0.35 ± 0.02
Red oak leaf × Low	2015 ± 31 de	5.45 ± 0.08 cd	58.18 ± 0.51	8.93 ± 0.11 c	2.71 ± 0.05 abc	0.32 ± 0.02
Red Salanova × Low	1993 ± 60 e	5.80 ± 0.10 bc	63.04 ± 0.90	6.72 ± 0.31 ef	2.30 ± 0.15 de	0.30 ± 0.01
Significance						
Cultivar (C)	***	***	NS	***	***	NS
Light intensity (L)	**	***	NS	***	*	NS
C × L	**	***	NS	***	**	NS

NS,*,**, *** Non-significant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (P = 0.05). All data are expressed as mean ± standard error, $n = 3$.

2.4.3 Implications of light intensity and cultivars for hydrophilic and lipophilic antioxidant molecules and antioxidant capacity

Phenolic compounds concentrations as well as the total phenolic acids are presented in Table 4. Four phenolic acids were detected with their total concentration differing between tested lettuce cultivars, light intensity regimes and their combinations. Chicoric acid was the major detected compound, followed by chlorogenic acid, whereas caffeoyl-tartaric and caffeoyl-meso-tartaric acids were quantified in lower amounts (Table 4). Moreover, significant differences on individual and total phenolic acid content were observed mostly concerning the cultivar effect, whereas light conditions had an impact only on the less abundant compounds and not on chicoric acid and total phenolic acids content. Red oak leaf was the cultivar that was mostly affected by low light intensity showing the highest content for most of the individual phenolic acids including chicoric and chlorogenic acids and consequently for total phenolic acids content (Table 4). Interestingly, red Salanova which was the second best cultivar in regard to chicoric and total phenolic acids content was beneficially affected by optimal light conditions (highest concentrations of caffeoyl tartaric, chlorogenic, chicoric and caffeoyl-meso-tartaric acids) and thus indicating a cultivar-dependent response of lettuce to light regime (Table 4). Expectedly, anthocyanins were detected only in red pigmented lettuce cultivars (Lollo rossa, red oak leaf and red Salanova), where read oak leaf grown under low light intensity conditions exhibited the highest values (Table 4). Similarly to phenolic compounds, total ascorbic acid a water-soluble antioxidant in lettuce was significantly affected by the two tested factors (data not shown). In most cases, TAA content was not affected by light conditions, while the tested cultivars showed a varied response (Figure 2). The only exception was cv. Lollo verde where the reduction of light intensity from 420 to 210 μmol m^{-2} s^{-1} PPFD had a detrimental effect on ascorbic acid content resulting in a four-fold reduction comparing to optimal conditions.

LAA was significantly affected by the two tested factors (cultivars and light intensity) but not by their interaction. As for the light intensity treatment mean effect averaged over cultivars, LAA was beneficially affected by optimal light conditions (+22.1%) compared to low light regime (Figure 3). Regardless of the light intensity treatment, red Salanova and red oak leaf cultivars had the highest LAA content, followed by baby Romaine (Figure 3)

In the current study, all the carotenoids detected are presented in Table 5. The main detected pigments were β-cryptoxanthin and violaxanthin + neoxanthin, followed by

lutein and β-carotene (Table 5). significant (C) × (L) interaction was noted with respect to the concentrations of carotenoids compounds. Optimal light conditions had a beneficial effect on the biosynthesis of most of the detected soluble pigments which was higher in baby Romaine (for violaxanthin + neoxanthin, lutein, β-cryptoxanthin and β-carotene) and read Salanova (for violaxanthin + neoxanthin and lutein). Interestingly, a significant and sharp spike in leaf violaxanthin + neoxanthin, lutein, β-cryptoxanthin and β-carotene concentrations were observed in red Salanova grown under low light intensity compared to the remaining five cultivars (Table 5).

Table 4. Phenolic acids composition, total phenolic acids and anthocyanins of lettuce grown hydroponically in a Fitotron open-gas-exchange growth chamber in relation to light conditions and cultivar.

Source of variance	Caffeoyl tartaric acid (mg 100 g^{-1} dw)	Chlorogenic acid (mg 100 g^{-1} dw)	Chicoric acid (mg 100 g^{-1} dw)	Caffeoyl-meso-tartaric acid (mg 100 g^{-1} dw)	∑ phenolic acids (mg 100 g^{-1} dw)	Anthocyanins (μg cyanidin eq. g^{-1} dw)
Cultivar (C)						
Baby Romaine	6.04 ± 0.52 bc	4.09 ± 0.79 c	26.7 ± 3.95 b	1.22 ± 0.40 d	38.0 ± 5.51 c	n.d.
Green Salanova	3.97 ± 0.67 c	2.91 ± 0.06 c	16.1 ± 2.94 b	0.43 ± 0.07 d	23.4 ± 3.58 c	n.d.
Lollo rossa	9.43 ± 1.75 a	14.58 ± 1.86 c	86.0 ± 5.98 a	6.12 ± 1.81 c	116.2 ± 8.23 b	6.22 ± 1.45 b
Lollo verde	7.40 ± 1.28 ab	3.00 ± 0.68 c	31.4 ± 3.14 b	0.58 ± 0.09 d	42.3 ± 5.01 c	n.d.
Red oak leaf	8.70 ± 2.69 a	66.19 ± 20.94 a	106.9 ± 30.36 a	11.45 ± 3.45 b	193.3 ± 57.19 a	16.35 ± 2.23 a
Red Salanova	4.79 ± 0.46 c	48.03 ± 6.03 b	96.8 ± 21.58 a	21.73 ± 6.40 a	171.3 ± 33.86 a	6.25 ± 1.67 b
Light intensity (μmol m^{-2} s^{-1}) (L)						
420 (Optimal)	4.58 ± 0.41 b	18.42 ± 5.12 b	56.1 ± 11.60	8.61 ± 3.09 a	87.7 ± 19.54	n.a
210 (Low)	8.86 ± 1.01 a	27.84 ± 9.55 a	65.2 ± 12.83	5.23 ± 1.62 b	107.1 ± 24.17	n.a
C × L						
Baby Romaine × Optimal	5.30 ± 0.69 de	2.92 ± 0.49 e	20.1 ± 1.92 cd	0.67 ± 0.25 e	29.0 ± 2.75 fg	n.d.
Green Salanova × Optimal	2.58 ± 0.28 e	3.01 ± 0.08 e	9.8 ± 0.56 d	0.30 ± 0.05 e	15.6 ± 0.82 g	n.d.
Lollo rossa × Optimal	6.29 ± 1.06 cde	18.13 ± 1.96 cde	89.7 ± 7.77 b	10.10 ± 0.71 c	124.2 ± 9.80 c	3.58 ± 0.78 c
Lollo verde × Optimal	5.24 ± 0.64 de	1.62 ± 0.37 e	26.6 ± 1.66 cd	0.44 ± 0.14 e	33.9 ± 2.15 fg	n.d.
Red oak leaf × Optimal	3.05 ± 0.41 de	24.53 ± 5.77 cd	45.9 ± 8.01 c	5.33 ± 1.59 cde	78.8 ± 15.58 cdef	11.46 ± 0.58 b
Red Salanova x Optimal	4.99 ± 1.00 de	60.34 ± 4.85 b	144.5 ± 7.08 a	34.83 ± 5.75 a	244.6 ± 18.26 b	9.87 ± 0.95 b
Baby Romaine × Low	6.78 ± 0.57 cd	5.26 ± 1.24 de	33.2 ± 5.64 cd	1.76 ± 0.67 e	47.0 ± 7.97 efg	n.d.
Green Salanova × Low	5.35 ± 0.49 de	2.81 ± 0.01 e	22.4 ± 1.74 cd	0.56 ± 0.06 e	31.1 ± 1.91 fg	n.d.
Lollo rossa × Low	12.57 ± 2.07 ab	11.03 ± 0.96 de	82.4 ± 10.23 b	2.13 ± 0.16 de	108.1 ± 13.31 cd	8.87 ± 1.70 b
Lollo verde × Low	9.56 ± 1.78 bc	4.39 ± 0.52 de	36.1 ± 4.92 cd	0.72 ± 0.05 e	50.7 ± 7.08 defg	n.d.
Red oak leaf × Low	14.35 ± 2.05 a	107.85 ± 20.56 a	168.0 ± 28.59 a	17.56 ± 4.43 b	307.8 ± 54.83 a	21.24 ± 0.80 a
Red Salanova × Low	4.58 ± 0.19 de	35.72 ± 2.63 c	49.1 ± 2.38 c	8.62 ± 0.07 cd	98.0 ± 5.10 cde	2.64 ± 0.21 c
Significance						
Cultivar (C)	***	***	***	***	***	**
Light intensity (L)	***	*	NS	*	NS	-
C × L	***	***	***	***	***	***

NS,*,**, *** Nonsignificant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. n.d. not detected. n.a. not applicable. Different letters within each column indicate significant differences according to Duncan's multiple-range test (P = 0.05). All data are expressed as mean ± standard error, $n = 3$

Table 5. Composition of carotenoids profile of lettuce grown hydroponically in a Fitotron open-gas-exchange growth chamber in relation to light conditions and cultivar.

Source of variance	Violaxanthin + neoxanthin (μg violaxanthin eq. g^{-1} dw)	Lutein (μg g^{-1} dw)	β-cryptoxanthin (μg g^{-1} dw)	β-carotene (μg g^{-1} dw)
Cultivar (C)				
Baby Romaine	843.7 ± 119 b	608.9 ± 61.6 b	1031.7 ± 95.5 ab	372.5 ± 79.2 b
Green Salanova	597.2 ± 53.3 c	242.8 ± 23.1 c	444.5 ± 19.5 c	163.1 ± 27.7 c
Lollo rossa	490.4 ± 31.1 d	237.8 ± 14.3 c	408.9 ± 26.4 c	191.4 ± 34.4 c
Lollo verde	431.4 ± 15.1 d	180.9 ± 7.10 d	257.3 ± 18.2 d	129.6 ± 16.6 d
Red oak leaf	831.9 ± 52.1 b	611.0 ± 38.6 b	947.1 ± 42.3 b	359.0 ± 28.9 b
Red Salanova	996.0 ± 19.1 a	739.2 ± 13.9 a	1046.2 ± 29.2 a	404.1 ± 24.4 a
Light intensity (μmol m^{-2} s^{-1}) (L)				
420 (Optimal)	788.2 ± 58.2 a	485.7 ± 59.2 a	741.4 ± 89.9 a	346.0 ± 33.6 a
210 (Low)	608.6 ± 51.9 b	387.8 ± 50.8 b	637.2 ± 73.2 b	193.8 ± 24.6 b
C × L				
Baby Romaine × Optimal	1102.6 ± 55.3 a	746.0 ± 9.26 a	1228.9 ± 82.1 a	547.6 ± 27.5 a
Green Salanova × Optimal	707.3 ± 32.9 e	287.9 ± 18.0 e	479.0 ± 22.5 e	223.1 ± 14.1 f
Lollo rossa × Optimal	559.2 ± 7.10 fg	268.2 ± 9.90 ef	466.5 ± 13.1 ef	267.3 ± 10.4 e
Lollo verde × Optimal	458.5 ± 12.0 gh	184.9 ± 11.6 g	233.8 ± 23.5 g	165.7 ± 8.98 g
Red oak leaf × Optimal	886.9 ± 61.6 cd	673.0 ± 43.5 b	986.6 ± 43.5 bc	417.5 ± 26.1 c
Red Salanova x Optimal	1014.8 ± 31.5 ab	754.5 ± 26.8 a	1053.4 ± 63.6 b	455.1 ± 16.6 b
Baby Romaine × Low	584.7 ± 27.0 f	471.8 ± 9.76 d	834.5 ± 4.88 d	197.4 ± 4.54 fg
Green Salanova × Low	487.1 ± 31.5 fgh	197.7 ± 18.0 g	410.0 ± 14.8 ef	103.1 ± 6.38 h
Lollo rossa × Low	421.6 ± 9.08 h	207.3 ± 0.92 fg	351.3 ± 3.99 fg	115.4 ± 7.20 h
Lollo verde × Low	404.3 ± 16.2 h	176.9 ± 10.0 g	280.8 ± 23.4 g	93.5 ± 2.62 h
Red oak leaf × Low	776.8 ± 82.3 de	549.0 ± 41.7 c	907.5 ± 74.0 cd	300.5 ± 9.12 e
Red Salanova × Low	977.1 ± 21.9 bc	723.8 ± 3.75 ab	1039.0 ± 13.6 b	353.0 ± 9.91 d
Significance				
Cultivar (C)	***	***	***	***
Light intensity (L)	***	***	***	***
C × L	***	***	***	***

*** Significant at $P \leq 0.001$, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test ($P = 0.05$). All data are expressed as mean ± standard error, $n = 3$.

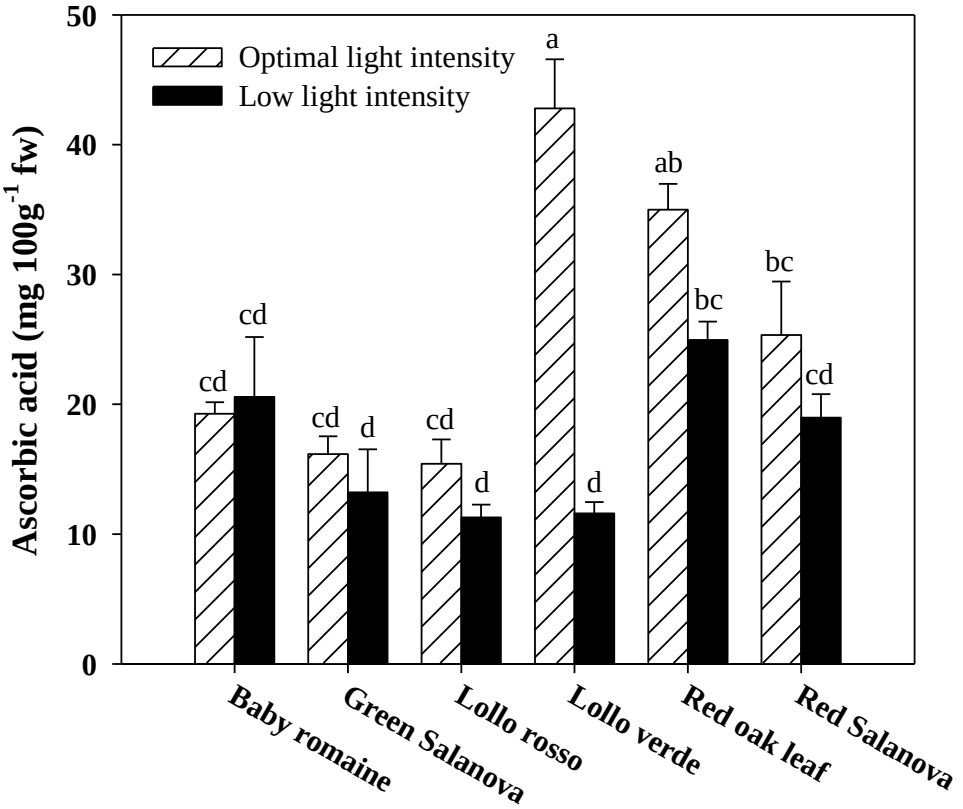

Figure 2. Total ascorbic acid of lettuce grown hydroponically in a Fitotron open-gas-exchange growth chamber in relation to light conditions and cultivar. All data are expressed as mean ± standard error, n = 3. Different letters above each bar indicate significant differences according to Duncan's multiple-range test (P = 0.05).

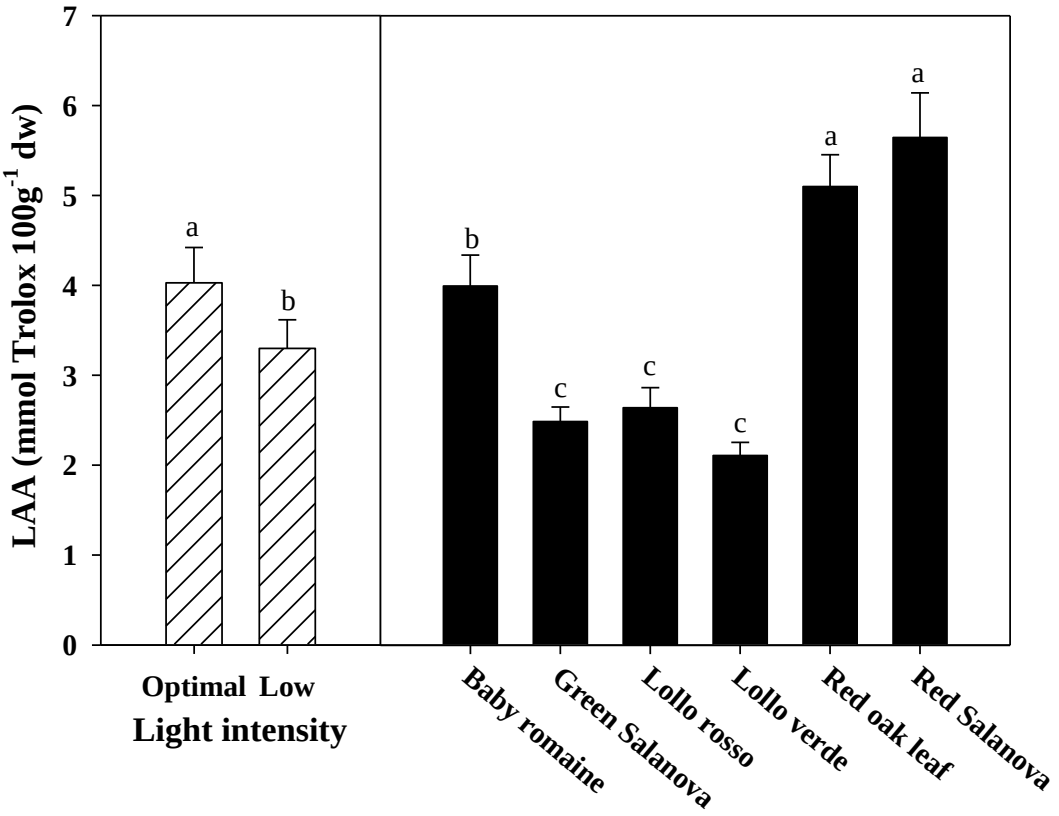

Figure 3. Mean effects of light conditions and cultivar on lipophili antioxidant activity (LAA) of lettuce grown hydroponically in a Fitotron open-gas-exchange growth chamber in relation to light conditions and cultivar. All data are expressed as mean ± standard error, n = 3. Different letters above each bar indicate significant differences according to Duncan's multiple-range test (P = 0.05).

Space colonization can be only achieved through the integration of controlled ecological life support systems (CELSSs) or bio-regenerative life support systems (BLSSs) in space biospheres that could support human life for long time periods without replenishment of supplies from the Earth. However, space farming is a very challenging task since higher plants have to be grown under abnormal conditions and key obstacles have to be overcome, mostly regarding environmental constrains (e.g. microgravity, low pressure, excessive radiation and so forth) and space and energy limitations, as well as in terms of covering crew member nutritional needs through the production of functional and biofortified food products (Guo et al., 2017; Kyriacou et al., 2017). Several crops have been suggested for space farming based on specific criteria and lettuce has been identified as a candidate leafy vegetable crop in several research studies (Meinen et al., 2018; Massa et al., 2017). The present study evaluated the response of six lettuce cultivars under optimal and low light intensity in order to

identify the most promising genotypes in regard to yield components, physiology parameters, mineral profile and bioactive compounds content.

Low light intensity had a detrimental effect on fresh biomass yield, regardless of cultivar, although baby Romaine plants performed better (reduction of 36% and 39% on fresh and dry weight basis, respectively) than the rest of the tested cultivars under such conditions in terms of fresh and dry biomass yield (reduction between 55 to 65% on both fresh and dry basis) and dry matter content, demonstrating that baby Romaine cv. show physiological acclimation in response to their limited light environment (Table 1). This response could be explained by the higher net assimilation rate (A_{CO2}), intrinsic water use efficiency (WUE_i), and lower stomatal resistance (r_s) values recorded for baby Romaine cultivar under low light intensity conditions, indicating a more efficient light harvesting mechanism (higher LUE) of the genotype under the specific environmental constraint (Table 2 and Figure 1). These results are in consistence with the study of (Fu et al., 2012) who did not detect great differences in the yield of romaine lettuce under similar light conditions, also suggesting that LUE was the highest at 200 μmol m^{-2} s^{-1} which was also the case in our study for baby Romaine plants. Other researchers highlighted the effect of photoperiod and light quality along with light intensity on lettuce growth and development which altogether define light conditions (Kang et al., 2014; Muneer et al., 2014). Therefore, the light effect on biomass yield has multiple aspects to be considered, especially in space farming where every light parameter (photoperiod, quality and intensity) has to be optimized for higher biomass production and better LUE.

Light intensity affected nitrate nitrogen and minerals content in a genotype dependent manner (Table 3). As expected, low light intensity resulted in an increase of nitrates content, especially for green Salanova and Lollo verde cultivars, since nitrate reductase activity which transforms nitrates to nitrites is associated with light intensity among other factors (Colla et al., 2018; Petropoulos et al., 2011). Another putative mechanism behind the accumulation of nitrate under low light intensity could be attributed to the fact that key enzymes such as glutamate synthase and glutamine synthetase are inhibited, whereas apragine synthetase involved in stabilizing nitrate for transport and storage is stimulated (Colla et al., 2018). However, even in the case of these two cultivars (green Salanova and Lollo verde) the detected nitrates content was within the limits set by EU commission for safe lettuce consumption (EU regulation: No 1258/2011).

Phosphorus, and calcium content increased under low light intensity, whereas potassium and sodium remained unaffected and magnesium content decreased (Table 4). Under low photosynthetically active radiation conditions vegetables tended to concentrate key macronutrients, especially P and Ca in their storage organs as a result of lower crop productivity. In the current study, high fresh yield triggered by favorable environmental conditions (optimal light intensity), may have accelerate lettuce biomass accumulation, thus decrease the macro-minerals (P and Ca) concentration due to the dilution effect (Stagnari et al., 2014; Pérez-López et al., 2015). Minerals intake through diet is essential for human health and vegetable cultivation in space farming should aim in providing products with enhanced mineral content. For this purpose, cations antagonism has to be attentively considered when adjusting the nutrient solution composition in order to combine the best agronomic performance with increased minerals and low nitrates content.

The major phenolic compound (chicoric acid) and total phenolic acids were not affected by light intensity, whereas the rest of the detected phenolic compounds showed a varied response to this factor (Table 4). This is an important finding of the study, since phenolic compounds are associated with beneficial to health properties and increased intake from low amounts of food is a desirable feature for selecting species suitable for space farming. Similar results have been reported in the literature, where a reduction of light intensity had a significant effect on phenolic compounds content of red-leaf lettuce depending on the structure of the compounds (Becker et al., 2013). Phenolic compounds are a key element for bioactive and functional properties of lettuce, although a relatively high content is associated with bitterness and a reduction in consumer acceptance (Bunning et al., 2010). Stress conditions such as non-optimal light intensity may induce the biosynthesis of phenolic compounds as a defense mechanism to stressors and although mild stress is beneficial to quality without affecting severely plant growth and yield, severe or prolonged stress may have a detrimental effect (Pérez-López et al., 2018). Genotype response also has to be considered, since the results showed a varied response of the tested cultivars under different light regimes (Table 4). Similarly, literature reports also suggest a great variation in phenolic compounds content among the various lettuce genotypes (Bunning et al., 2010; Llorach et al., 2008). Our finding suggested that all red-leaf lettuce cultivars tested have a distinct profile in phenolic acids, in particular under optimal light conditions red Salanova can be used as a nutrient-dense food. On the other hand, red oak leaf had the highest phenolic

compounds content overall when grown under low light intensity which could be attributed to severe stress as also indicated by the low fresh biomass yield and the high stomatal resistance (Table 1 and 2, respectively). Therefore, although initially a high phenolic compounds content might seem attractive, space farming constrains also have to be considered and the golden ratio between quality and fresh biomass yield must be achieved.

The interest in fat-soluble pigments such as carotenoids is not recent, owing to their beneficial effects on human well-being in particular human vision during future space missions (Kyriacou et al., 2017). Soluble pigments content, especially β-carotene which is a precursor of vitamin A and zeaxanthin and lutein which contribute to vision protection of crew members from excessive radiation in space conditions were higher when optimal light intensity was applied regardless of cultivar (Table 5). Similar results have been reported by Lefsrud et al (2008) and Li et al. (2009) who showed that key carotenoids (β-carotene and lutein) in spinach and kale leaves were significantly higher under optimal (300 μmol m^{-2} s^{-1}) than under low irradiation conditions (100 μmol m^{-2} s^{-1}). Furthermore, red Salanova contained consistently high amounts of pigments regardless of light intensity which is an extremely important finding from a nutritional point of view due to the antioxidant properties of these compounds (Cohu et al., 2014)(Kitazaki et al., 2018). Similar trends were observed for ascorbic acid content and lipophilic antioxidants which also decreased under low light intensity conditions (Figures 2 and 3). For almost all the tested genotypes no significant differences were observed between low and optimal light intensity, whereas only Lollo verde showed almost a fourfold decrease in ascorbic acid content under low light intensity (Figure 2). Despite the fact that lettuce is not considered a rich source of ascorbic acid, the great variation among the existing cultivars allows its complementary role to ascorbic acid intake along with other food sources(Mampholo et al., 2016; Bunning et al., 2010), especially when considering that several species have to be incorporated in CELSSs to sustain human life and fulfill nutrient requirements of crew members and space colonizers(Chunxiao and Hong, 2008; Qin et al., 2008). So far, the literature has confirmed the effect of light quality on pigments content (Kołton et al., 2014; Ouzounis et al., 2015), as well the increase of anthocyanins under excessive light intensity as a protection mechanism for leaf chlorophylls (Feild et al., 2001). However, Fu et al. (2017) suggested that very low light intensity (60-140 μmol m^{-2} s^{-1}) may also increase carotenoids content, a finding which has to be further investigated since energy saving

through implementation of low light intensity is of major importance for space farming. Another approach was the one proposed by Cohu et al. (2014) who tested the effect of low light intensity supplemented with short intervals of high light intensity pulses and found that these short pulses may trigger carotenoids biosynthesis and zeaxanthin in particular in *Arabidopsis thaliana* plants. Such a practice could be very useful in space conditions where energy consumption is a key element. Moreover, Mampholo et al. (2016) reported a great variation in β-carotene among the lettuce cultivars, which according to Mou (2012) could be partly attributed to head structure and the function of β-carotene as a complementary to chlorophylls light harvesting compound. Therefore, the effect of light conditions in space environments has to be further investigated through the evaluation of various genotypes in order to find cultivars that are acclimatized to such conditions and produce biofortified with soluble pigments fresh produce.

2.5 Conclusion

Space farming for fresh food production is the next breakthrough to be achieved for a successful outcome of long duration space missions and space colonization. However, cultivation of higher plants under space conditions entails consideration of several parameters with contrasting effects on plant growth and physiology and produce quality as well. For example, space limitations and low light intensity in space shuttles or space stations requires high light use efficiency without compromising fresh biomass yield and quality of the final product. The results of our study, supported the existing research reports who suggest lettuce as a candidate crop for space farming. The great variation among the existing cultivars allows for the selection of genotypes with highly efficient light harvesting mechanisms in order to provide sufficient fresh biomass yield, while at the same time quality may increase through the increase of antioxidant compounds such as soluble pigments, ascorbic acid and phenolic compounds and the decrease of antinutrients such as nitrates. The most promising of the tested cultivars under low light intensity conditions was baby Romaine which showed the best agronomic performance in terms of fresh biomass yield and physiology parameters. Moreover, the same cultivar contained an increased content of P and Ca under low light intensity, without nitrates content being affected by light regime. Regarding the bioactive properties, red colored cultivars such as Red oak leaf and Red Salanova had the highest content in phenolics and soluble pigments, respectively while both of them showed the highest lipophilic

antioxidant activity, regardless of light regime. Therefore, it can be concluded that cultivation of mixed lettuce cultivars is the most possible scenario for space farming where some cultivars could provide adequate amounts of fresh biomass while others could contribute to covering daily diet requirements in health beneficial compounds.

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3 Chapter 3: Water requirements, physiological and qualitative aspects of red and green butterhead lettuce grown in closed soilless system: better understanding of Salanova as a candidate cultivar for life support systems

3.1 Abstract

Plant production in space journeys is crucial to be self-sufficient and provide a proper dietary intake to sustain physical and psychological well-being of space colonists, as well as contributing to atmosphere revitalizing, water purification and waste product recycling. Choosing the appropriate cultivar is as important as the species selection, since such choice can influence the obtained fresh biomass, water use efficiency (WUE), growing cycle duration, qualitative features and more importantly quality conservation after harvest. Two differently pigmented butterhead *Lactuca sativa* L. (red and green Salanova) were assessed in term of morphometric, mineral, bioactive compounds and physiological parameters. The experiment was carried out in a controlled-environment growth chamber using a closed soilless system (Nutrient Film technique). Red Salanova registered a biomass of 130 g at harvesting (19 DAT), 22.1 % bigger than green Salanova, with a water uptake of 1.42 L during the full growing cycle and a WUE of 91.9 g L^{-1}, 13.8 % higher than the green cultivar. At harvesting, green Salanova had accumulated more P, K, Ca, Mg and 37.2 % more nitrate than red Salanova that in its turn had higher relative water content, leaf total and osmotic potential and higher SPAD index. Red Salanova as well exhibited at harvest around two-fold greater lipophilic antioxidant activity and total phenols, and around six-fold greater ascorbic acid. These latter characteristics improved the antioxidant capacities of red Salanova allowing it to use more efficiently light, and have better yield and overall performance than the green one. Moreover, the high phenolics and ascorbic acid contents of red Salanova represent natural sources of antioxidants to enrich human diet, and more convenient agronomic requirements, and make it an appropriate cultivar candidate for bio-regenerative life support systems.

3.2 Introduction

When humans rove far from Earth orbit, horticulture will doggedly follow (Wheeler, 2002). Deep space voyages cannot lean on conveyance from Earth, this umbilical reliance and replenishment will not be an option anymore (Walker & Granjou, 2017). Therefore, in order to extend space journeys, humans during their missions should be able to provide proper dietary intake (Kyriacou et al., 2017), by being self-sufficient and producing fresh food that is crucial for retaining physical (Dueck et al., 2016) and psychological well-being (Dueck et al., 2016, Zabel et al., 2016). A plant-food-based diet is premium to nourish body and soul (Lampe, 1999), making sustainable plant production in space a primary objective of research activities (Wolff et al., 2014). Therefore, in order to support numerous crew members for long-duration space missions Bio-regenerative Life-Support Systems (BLSS) have been designed to eventually eliminate the need to rely on resupply from Earth (Dueck et al., 2016). A life support system is pivotal for regenerating all survival essentials (Wolff et al., 2014). In it, higher plants play an essential nutritional role, as atmosphere revitalizer through CO_2 absorption and O_2 emission, water purifier through transpiration (Wolff et al., 2014; Zabel et al., 2016; Kyriacou et al., 2017; Walker & Granjou, 2017; Wheeler, 2017) and organic wastes recycling via mineral nutrition (Wolff et al., 2014).

Higher plants growth chamber in BLSS denote the compartment IVb. It stands for a paramount mantle in MELiSSA's (Micro-Ecological Life Support System Alternative) loop. This latter aims the fulfilment of a viable ecological 'niche' for humans in the outer space, still functional notwithstanding the utter disconnection from the Earth, focusing on the interaction of the organism within its environment as unit of life (Walker & Granjou, 2017). An appropriate selection of the species (crop) for this compartment can supply food as portion of the produced biomass (Wheeler, 2017) and provide a myriad of nutrients including biologically active compounds with antioxidant, antibacterial and antiviral effects able to stimulate the immune system (Lampe, 1999). The main general shared criteria through which the candidate cultures for the space are selected are: broad nutritional coverage, harvest index, crop efficiency and potential yield (Chunxiao & Hong, 2008; Wolff et al., 2014; Kyriacou et al., 2017; Wheeler, 2017). Namely, salad crops have a very high harvest index, low water uptake/transpiration ratio, brief growing cycle and little crew attention to grow it (Kyriacou et al., 2017). Lettuce was nearly omnipresent in crops list suggested or studied for life support systems (Wheeler, 2002), and topped chart scores of space/time

efficiency, harvest index, light/energy use efficiency and handling time, as well as scoring the highest among selected crops to be cultivated in the Future Exploration Greenhouse (FEG) at Neumayer Station III and in the International Standard Payload Rack (ISPR) on the International Space Station (ISS) (Dueck et al., 2016). Nevertheless, even cultivars of candidate crops undergo a series of selection to choose the appropriate ones (Chunxiao & Hong, 2008). Moreover, lettuce nutrient composition and bioactive compounds vary among type and pigmentation as well (Kim et al., 2016) which can influence the selection. On the other hand, water and nutrient management are demanding features for plant cultivation in life support systems. Therefore, recirculating hydroponic systems are favored (Wolff et al., 2014) to remove water and nutrient stress, improve production, obtain higher water use and dispense less nutrients (Wheeler, 2017), leading to an effective resource management (Putra & Yuliando, 2015). Such inputs emphasize on the importance of a continuous ground experiment in order to monitor lettuce water absorption during a growing cycle in a closed loop hydroponic system. To our knowledge no previous work has focused on measuring Salanova lettuce water uptake, physiological and qualitative aspects on three days interval basis and covering the full growing cycle.

Based on this approach, the purpose of this this paper was to elaborate the evolution of two differently pigmented butterhead lettuce water uptake, morphological, physiological and qualitative data through a complete nineteen days growing cycle. The experiment was carried out in a Fitotron growth chamber in a closed soilless system of nutrient film technique (NFT). The gained data can be appreciated by space-faring colonists in order to know in advance the water consumption of butterhead lettuce cv. Salanova, nutrient accumulation and detecting the adequate maturity stage for harvesting in order to maintain optimal quality in storage, and more importantly these findings are appreciated by terrestrial controlled environment agriculture.

3.3 Materials and methods

3.3.1 Plant material, growth chamber conditions, experimental design and harvesting schedule

Two butterhead lettuce cultivars (*Lactuca sativa* L. var. capitata) green Salanova® and red Salanova® (Rijk Zwaan, Der Lier, The Netherlands) were cultivated for nineteen days in a controlled closed soilless system. The experiment was carried out in a

28 m^2 open-gas-exchange growth chamber (7.0 m × 2.1 m × 4 m; W × H × D), at the Department of Agricultural Sciences of the University of Naples Federico II, Italy. Lettuce plants were cultivated in a Nutrient Film Technique (NFT) growing system, consisting of propylene gullies covered with white polyethylene film to avoid evaporation of the nutrient solution (NS) and to reflect the incident light. The gullies where 200 cm long, 14.5 cm wide and 8 cm deep each, having a sloping degree of 1%. The NS was delivered by submerged pumps at a constant flow of 1.5 $L.min^{-1}$ and then was collected in 25 L polypropylene tanks by gravity dependent flow. The NS consisted of a modified Hoagland & Arnon formulation: 9.0 mM $N\text{-}NO_3^-$, 2.0 mM S, 1.0 mM P, 4.0 mM K, 4.0 mM Ca, 1.0 mM Mg, 1.0 mM NH_4^+, 15 µM Fe, 9 µM Mn, 0.3 µM Cu, 1.6 µM Zn, 20 µM B, and 0.3 µM Mo. The electrical conductivity (EC) of the nutrient solution was 1.5 dS m^{-1}, respectively, while the pH of the nutrient solutions was monitored daily and maintained at 6.0 ± 0.2. Two weeks after sowing, lettuce seedlings were transplanted in rockwool cubes (7 × 7 × 7cm) (Delta, Grodan, Roermond, The Netherlands) placed into the gullies with an intra-row spacing of 15 cm and an inter-row spacing of 43 cm, making a density of 15.5 plants per square meter.

Light was supplied by high pressure sodium lamps, with an intensity of 420 µmol m^{-2} s^{-1} according to a light/dark regime of 12/12h with corresponding temperature and relative humidity (RH) of 24/18 °C and 60/80 %, respectively, the latter being maintained by a fog system. The experiment was carried out at ambient CO_2 concentration (370-410 ppm) and air circulation and dehumidification were guaranteed by two HVAC systems.

Treatments of the two Salanova cultivars were arranged in a randomized complete-block design with three replicates, making a total of 216 plants divided in 36 experimental units made of six plants each. Treatments where six harvests separated by three days interval, starting at four days after transplanting (DAT) and ending at 19 DAT. All measurements and analysis where executed at each harvest.

3.3.2 Sampling, growth analysis, and SPAD index measurement

Plants were sampled six times during the crop cycle at 4, 7, 10, 13, 16 and 19 DAT, noting that at 1 DAT part of the seedlings was harvested. At each date, harvested plants

were in a part frozen in liquid nitrogen and stored at -80 °C for qualitative analysis, and in a part used for biometric measurements such as leaf number, fresh weight and leaf area, the latter being measured by an Area Meter (LI-COR 3100C biosciences, Lincoln, Nebraska, USA). The Soil Plant Analysis Development (SPAD) index was measured on young healthy leaves by means of a portable chlorophyll meter SPAD-502 (Konica-Minolta, Japan) of three representative plants per experimental unit. Measurements were averaged to a single SPAD value per each replicate.

3.3.3 Water uptake, water use efficiency and relative growth rate

Water level of all the tanks was measured on a three-day-basis interval, in order to detect water uptake evolution of the plants through the 19 days growing cycle. Then this volume was divided by three (number of days) and then by number of plants per gully in order to express daily water uptake in mL $plant^{-1}$ day^{-1}, while cumulative water uptake was expressed in Liter per plant. Water use efficiency (WUE) was calculated by dividing fresh yield of the plant by the volume of consumed water, and expressed in grams of fresh yield per liter. While relative growth rate (RGR) was calculated based on the following formula: $RGR = (\log_e W_2 - \log_e W_1) / (t_2 - t_1)$, where W is leaf dry matter and t the sampling date.

3.3.4 Water potential and relative water content

Leaf total water potential (Ψ_{tot}) was measured on 4 cm leaf discs punched from young fully expanded leaves using a Dewpoint potentiaMeter (WP4C, Decagon Devices, Pullman, WA). Leaf osmotic potential (Ψ_π) was measured after freezing and thawing leaf discs. While turgor pressure or pressure potential (Ψ_p) was estimated as the difference between Ψ_{tot} and Ψ_π, assuming that the matric potential is equal to zero. Leaf relative water content (RWC) was measured based on Colla et al. (2008) with slight modifications. Briefly, each repetition consisted of 10 discs of 8 mm each, which were excised from the interveinal areas and weighed to determine fresh weight (FW), then floated in distilled water for 12 h to retrieve turgidity and re-weighted to determine turgid weight (TW). Finally samples were dried at 80 °C for 48 h to determine dry weight (DW). RWC was calculated based on the following formula: RWC % = ((FW-DW)/ (TW-DW)) × 100.

3.3.5 Dry matter, total nitrogen and mineral content analysis

Leaf samples were oven dried at 70 ˚C for three days, until reaching a constant weight, and then weighed on an analytical balance (Denver Instruments, Denver, Colorado, USA) for dry weight determination, and finally dry matter (DM) percentage was calculated as DM = 100 × Dry weight/Fresh weight. Red and green Salanova leaf samples were ground separately in a Wiley Mill to pass through a 841 microns screen, then 250 mg of the ground tissues were analyzed by ion chromatography for mineral content: N, P, K, Ca, Mg and S as described in details by Rouphael et al. (2017), mineral content was expressed in mg per g of dry weight. As for nitrate, it was expressed in mg per kg of fw according to the dry matter of each sample. While total nitrogen was determined on one g of dried samples by Kjeldahl method (Bremner, 1965).

3.3.6 Analysis of lipophilic antioxidant activity

Lipophilic antioxidant activity (LAA) was determined by using a radical cation assay, extracting 200 mg of lyophilized material by methanol. Based on Pelligrini et al. (1999), 2,2'-azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method was used to measure LAA. The principle of the assay is that the inhibitory response of the radical cation is proportional to the antioxidant concentration and the reaction is complete at the time point selected of 2.5 min. A UV–Vis spectrophotometer was used to measure the absorbance reduction of the solutions at 734 nm wavelength to determine LAA. Results were expressed as mmol Trolox (6-hydroxy-2,5,7,8-tetramethylchroman- 2-carboxylic acid) per 100 g dw (Pellegrini et al., 1999).

3.3.7 Analysis of total ascorbic acid and total phenols

Total ascorbic acid (TAA) which is the sum of ascorbic acid (AA) and dehydroascorbic (DHA) was assessed by spectrophotometric detection on fresh plant tissues. The assay is based on the reduction of Fe^{3+} to Fe^{2+} by AA and the spectrophotometric detection of Fe^{2+} complexes with 2,2-dipyridyl. DHA is reduced to ASA by pre-incubation of the sample with dithiothreitol. (Kampfenkel et al., 1995). Quantification was performed by UV–Vis spectrophotometry (Hach DR 2000; Hach Co.,

Loveland, Colorado, USA) at 525 nm and the results were expressed as mg AA per 100 g fw.

Total phenolic content was determined in 60% methanol/water (w/v) extracts, according to the Folin-Ciocalteu procedure (Singleton et al., 1999) using gallic acid as standard. Then 100 μL of the supernatant was combined with 400 μL of 7.5% sodium carbonate/water (w/v), samples were shaken for 15 min and then incubated for 30 min at room temperature. Absorption was measured at 765 nm using a UV-Vis spectrophotometer, and the results were expressed as mg gallic ac. eq. 100 g^{-1} dw.

3.3.8 Statistical analysis

The obtained data were subjected to analysis of variance (ANOVA) using the software package SPSS 13 for Windows 2001. Means comparison was performed with the use of Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$. Moreover, comparison between the two cultivars was done using t-test. The Pearson correlation analysis were performed by SigmaPlot 12 software (Systat Software Inc.) according to Ferchichi et al. (2018). The principal component analysis (PCA) was assessed using Minitab 16.8 statistical software (Carillo et al., 2019a). The score plot and loading matrix were determined based on the first and second principal components (PCs). A heatmap was generated using the https://biit.cs.ut.ee/clustvis/ online program package with Euclidean distance as the similarity measurement and hierarchical clustering with complete linkage. Morpho-physiological and quality parameters, as well as mineral composition data were visualized using a false color scale, with red indicating an increase and blue a decrease of values (Carillo et al., 2019b).

3.4 Results

3.4.1 Growth response, water uptake and water use efficiency

Leaf area, fresh and dry biomass of both cultivars red and green Salanova showed an exponential increase during the 19 days growing cycle (Figure 1). A significant higher increase of red Salanova leaf area in comparison to green Salanova was obvious since 13 DAT and increased even more until 19 DAT, marking almost a 35.6 % higher value. It is explained by the significant difference in the daily leaf area increase that is dominated by the red cultivar since 13 DAT (Table 1). Fresh biomass exhibited a

significant difference between cultivars from 16 till 19 DAT for red Salanova, with a yield of 130.2 g plant^{-1}, 22.1 % bigger than green Salanova that registered 106.6 g plant^{-1} at harvesting (Figure 1B). Both cultivars had no significant difference in terms of fresh biomass daily increase till 13 DAT, while during the subsequent growth period red Salanova showed significant higher daily increase (Table 1). As for dry biomass, it was significantly higher for the green cultivar almost in all the growing cycle, except around 16 DAT where red Salanova exhibited a significant higher increment (Fig. 1C), which is evidenced in the daily changes presented in details in Table 1 with an increase of 0.6 g plant^{-1} day^{-1} for red Salanova in comparison to 0.41 g plant^{-1} day^{-1} for green Salanova. Dry biomass at harvest was 5.4 and 5.7 g plant^{-1} for red and green Salanova, respectively.

Considering Figure 1D, both cultivars showed an increase in RGR until 7 DAT, where red Salanova registered 0.41 mg mg^{-1} day^{-1}, 24.2 % greater than the green cultivar. After that, RGR of both cultivars decreased gradually over the growing cycle, with red being significantly higher from 13 to 16 DAT, but significantly decreasing less than green cultivar towards 19 DAT and reaching 0.09 mg mg^{-1} day^{-1} at harvest, instead the green cultivar showed a slight increase of RGR at the end of the growing cycle and reached 0.13 mg mg^{-1} day.

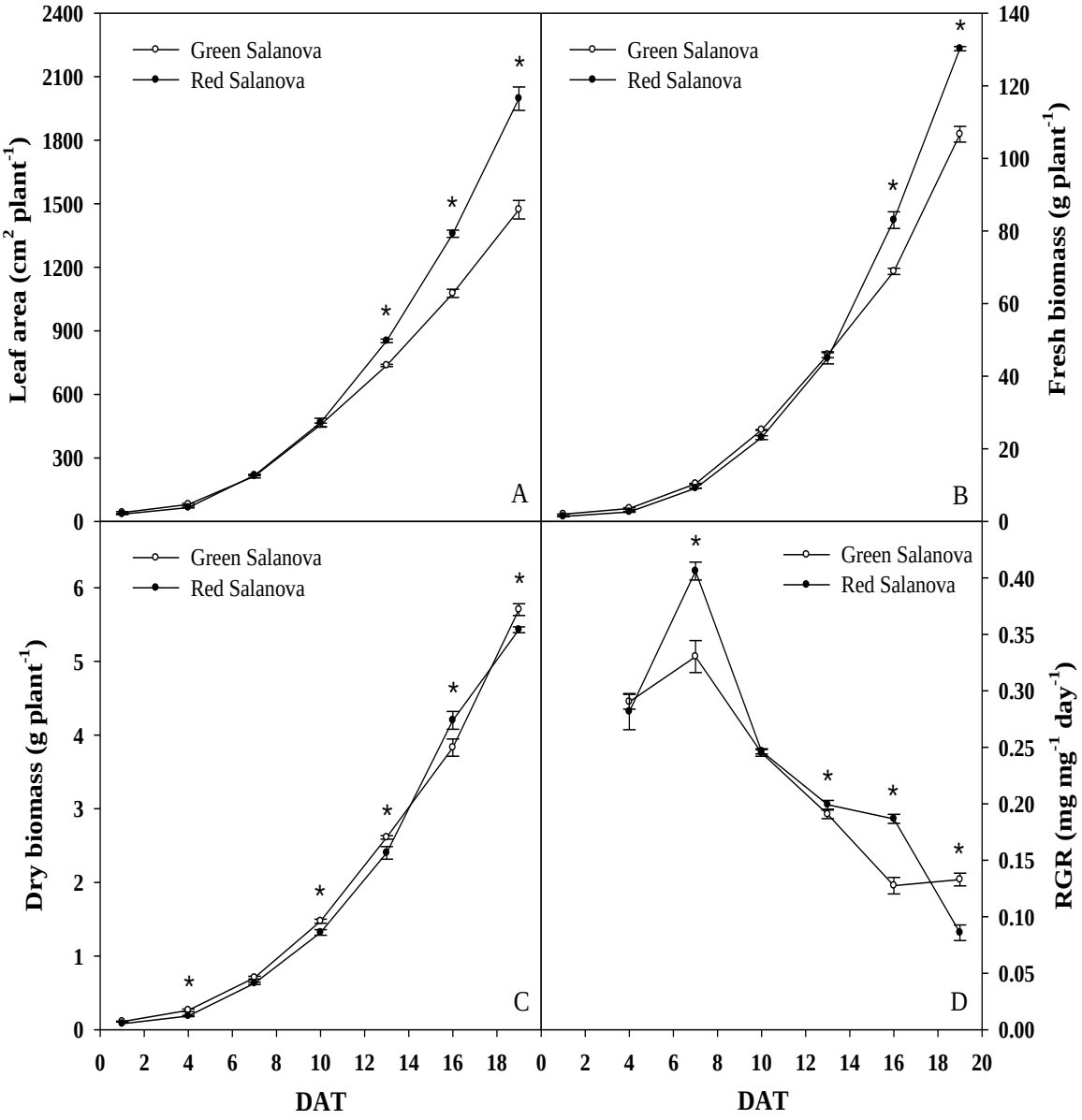

Figure 1. Evolution of leaf area (A), fresh biomass (B), dry biomass (C) and RGR (D) of red and green Salanova during the growing cycle. The values are means of three replicates. Astericks indicate a significant difference between cultivars.

Table 1. Analysis of variance and mean comparisons for daily increase on a three days basis interval of leaf area, fresh and dry biomass, water uptake, total N and macro-elements (P, S, K, Ca and Mg) concentrations per plant of red and green Salanova, and mean daily increase.

Daily variables	Cultivar	4 DAT	7 DAT	10 DAT	13 DAT	16 DAT	19 DAT	*Significance*	Mean
Leaf Area	Green Salanova	12.81 f	44.66 e	80.80 d	93.36 c	113.49 b	131.72 a	***	79.47
(cm^2 $plant^{-1}$ day^{-1})	Red Salanova	10.38 f	50.86 e	82.82 d	128.79 c	168.53 b	212.70 a	***	109.01
	t-test	*0.008*	*0.002*	*0.772*	*0.002*	*0.001*	*0.006*		*0.001*
Fresh biomass	Green Salanova	0.53 e	2.26 d	4.94 c	6.91 b	7.64 b	12.59 a	***	5.81
(g $plant^{-1}$ day^{-1})	Red Salanova	0.42 f	2.18 e	4.64 d	7.29 c	12.68 b	15.74 a	***	7.16
	t-test	*0.232*	*0.217*	*0.141*	*0.454*	*0.000*	*0.020*		*0.000*
Dry biomass	Green Salanova	0.05 e	0.15 d	0.26 c	0.38 b	0.41 b	0.62 a	***	0.31
(g $plant^{-1}$ day^{-1})	Red Salanova	0.04 f	0.15 e	0.23 d	0.36 c	0.60 b	0.41 a	***	0.30
	t-test	*0.035*	*1.000*	*0.036*	*0.338*	*0.005*	*0.002*		*0.045*
Water uptake	Green Salanova	29.47 d	35.43 cd	54.71 c	89.50 b	104.00 b	128.45 a	***	73.59
(ml $plant^{-1}$ day^{-1})	Red Salanova	26.97 f	35.47 e	57.13 d	94.76 c	123.98 b	134.04 a	***	78.72
	t-test	*0.055*	*0.976*	*0.477*	*0.068*	*0.000*	*0.743*		*0.145*
Total N	Green Salanova	2.32 e	6.73 d	8.79 c	18.39 a	13.97 b	19.31 a	***	11.58
(mg $plant^{-1}$ day^{-1})	Red Salanova	1.41 e	5.79 d	12.35 c	17.23 b	25.31 a	18.54 b	***	13.44
	t-test	*0.013*	*0.026*	*0.002*	*0.407*	*0.002*	*0.322*		*0.020*
P	Green Salanova	0.15 e	0.75 d	1.15 c	1.81 b	1.89 b	2.91 a	***	1.44
(mg $plant^{-1}$ day^{-1})	Red Salanova	0.05 e	0.53 d	1.08 c	0.37 d	2.56 a	1.38 b	***	1.00
	t-test	*0.004*	*0.009*	*0.333*	*0.002*	*0.017*	*0.000*		*0.002*
S	Green Salanova	0.02 d	0.12 c	0.02 d	0.32 b	0.17 c	0.41 a	***	0.18
(mg $plant^{-1}$ day^{-1})	Red Salanova	0.02 c	0.09 c	0.27 b	0.19 b	0.48 a	0.20 b	***	0.21
	t-test	*0.741*	*0.082*	*0.001*	*0.039*	*0.001*	*0.041*		*0.139*
K	Green Salanova	3.73 d	7.82 c	15.24 b	16.52 b	15.31 b	47.24 a	***	17.65
(mg $plant^{-1}$ day^{-1})	Red Salanova	1.89 d	7.22 c	9.07 c	6.42 c	30.05 a	24.44 b	***	13.18
	t-test	*0.019*	*0.019*	*0.000*	*0.001*	*0.002*	*0.000*		*0.005*
Ca	Green Salanova	0.59 e	1.43 d	2.72 b	3.17 b	2.02 c	6.57 a	***	2.75
(mg $plant^{-1}$ day^{-1})	Red Salanova	0.26 c	1.37 bc	1.73 b	1.19 bc	4.67 a	0.94 bc	***	1.69
	t-test	*0.020*	*0.389*	*0.005*	*0.000*	*0.020*	*0.000*		*0.003*
Mg	Green Salanova	0.18 d	0.51 cd	0.74 c	1.08 b	0.51 cd	1.47 a	***	0.75
(mg $plant^{-1}$ day^{-1})	Red Salanova	0.10 d	0.44 c	0.76 b	0.39 c	1.18 a	0.24 cd	***	0.52
	t-test	*0.004*	*0.210*	*0.832*	*0.002*	*0.022*	*0.007*		*0.035*

NS,*,**, *** Non-significant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Different letters in rows (intragroup comparisons were performed only regarding DAT) indicate significant differences according to Duncan's multiple-range test (P = 0.05). Cultivars are compared according to Student's t-test.

Cumulative water uptake as well, demonstrated an exponential increase along the growing cycle as illustrated in Figure 2A, with a significant higher uptake noted for red Salanova starting at 16 until 19 DAT. This increase was clarified in Table 1 where a significant 19.2 % higher increase of daily water uptake was registered at 16 DAT for the red cultivar, while no significant difference was mentioned for the rest of the growing cycle. Total water uptake at the end of the growing cycle registered 1.42 and 1.32 L plant^{-1} for red and green Salanova, respectively. As follows from Figure 2B, WUE displayed a quick increase the first 10 days after transplanting, then slowed down for green Salanova and formed a type of plateau between 10 and 16 DAT, while red Salanova WUE had a continuous increase all along the growing cycle, with significant higher values starting at 14 DAT in comparison to green Salanova that had significant higher WUE from transplant till 14 DAT. At the end of the growing cycle, red Salanova had a WUE of 92 g fresh biomass L^{-1}, 13.8% higher than green Salanova WUE that registered 80.8 g fresh biomass L^{-1}.

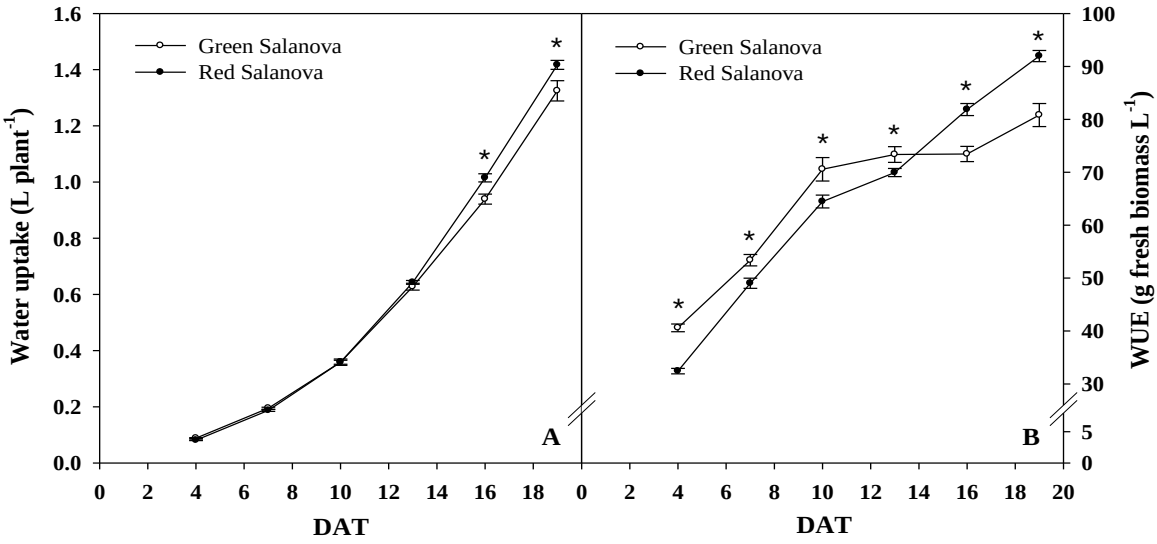

Figure 2. Evolution of water uptake (A) and WUE (B) of red and green Salanova during the growing cycle. Data are means of three replicates. Astericks indicate a significant difference between cultivars. Vertical bars indicate ±S.E. of the means, their absence indicates that the size was less than the symbol.

Mean daily increase of leaf area, fresh biomass and dry biomass was significantly higher for red Salanova, while there was no significant difference in water uptake

between the two cultivars, which can explain why the red cultivar had higher WUE at harvesting (Table 1).

Water uptake (L plant^{-1}) was linearly correlated with leaf area (cm^2 plant^{-1}) ($r = 0.99$; $p<0.001$), while fresh biomass (g plant^{-1}) was positively correlated with WUE ($r=0.88$; $p<0.001$) as shown in Figures 3A and 3B for both cultivars with $r^2=0.967$.

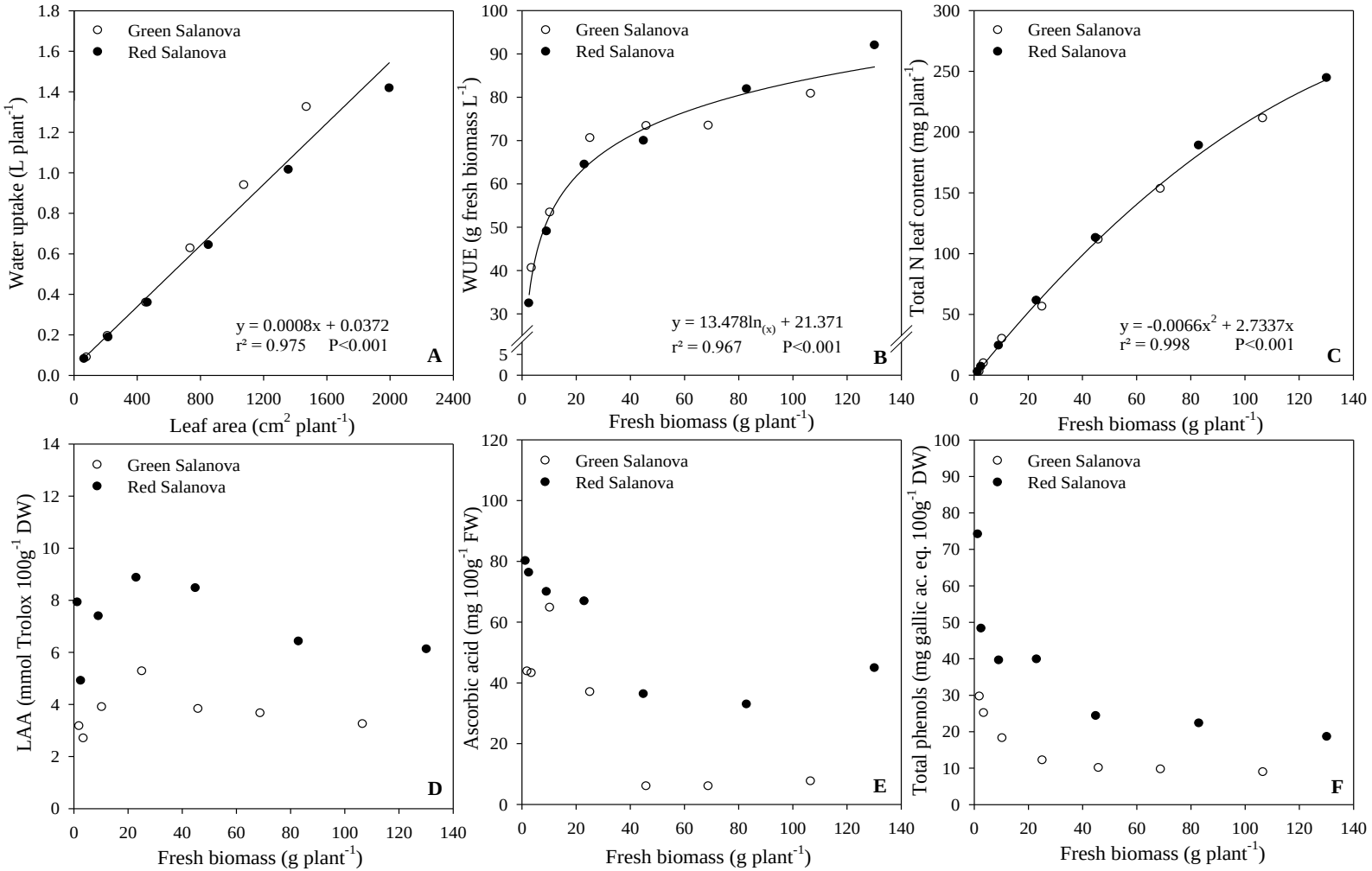

Figure 3. Relationship between water uptake and leaf area (A), WUE and fresh biomass (B), total N leaf content and fresh biomass (C), LAA and fresh biomass (D), TAA and fresh biomass (E), total phenols and fresh biomass (F) for red and green Salanova during the growing cycle.

3.4.2 Leaf macro-mineral composition

Figure 4 depicts leaf mineral concentration varying throughout the growing cycle in function of dry weight. Nitrogen and Sulfur where significantly higher in red Salanova since 8 DAT, with total N concentration being almost steady around 45 mg g^{-1} dw, while its concentration in green salanova had a decrease trend since 13 DAT and reached 37 mg g^{-1} dw at harvesting. Moreover, total N leaf content (mg $plant^{-1}$) showed a quadratic correlation with fresh biomass (g $plant^{-1}$) for both cultivar with $r^2 = 0.998$ (Figure 3C).

As for sulfur leaf concentration, it showed a peak of 0.89 mg g^{-1} dw at 10 DAT in red Salanova and a sudden decrease in green Salanova reaching 0.34 mg g^{-1} dw, which is explained by S daily increase at the same date that registered only 0.02 mg $plant^{-1}$ day^{-1} compared with 0.27 mg $plant^{-1}$ day^{-1} for red Salanova (Table 1). Then sulfur concentration stabilized around 0.72 mg g^{-1} dw and 0.55 mg g^{-1} dw for red and green Salanova, respectively, from 13 DAT.

Instead, phosphorus, potassium, calcium and magnesium were significantly more concentrated in the green cultivar during the growing cycle. P was nearly steady in green Salanova since 7 DAT ranging around 4.55 mg g^{-1} dw, while in red Salanova it varied between 2.73 and 4.12 mg g^{-1} dw and stabilized since 16 DAT at 3.39 mg g^{-1} dw (Figure 4B). As for K, it varied slightly in green salanova and was 56.8 mg g^{-1} dw at harvesting, while in red Salanova it was around at 44.9 mg g^{-1} dw (Figure 4D). Ca leaf concentration had an overall decrease trend in both cultivars to reach around 8.86 and 5.77 mg g^{-1} dw in green and red Salanova, respectively, at harvesting (Figure 4E). Mg had as well a similar decrease trend and registered 2.4 and 1.8 mg g^{-1} dw, respectively, in green and red Salanova (Figure 4F). The red cultivar had a sudden decrease at 13 DAT in P, K, Ca and Mg concentration, which is explained by a clear decrease in these macro-elements daily accumulation at the same date and coinciding with an increment of daily increase of leaf area and fresh biomass. Macro-elements exhibited the highest daily increase at 16 DAT in red Salanova, whilst in green Salanova at 19 DAT. P, K, Ca and Mg had significant higher daily accumulation in green Salanova (44%, 34%, 62% and 44%, respectively), but its daily dry biomass increase was only 3% higher than red Salanova, these facts lead us to assume that red Salanova had better nutrient use efficiency (data not shown).

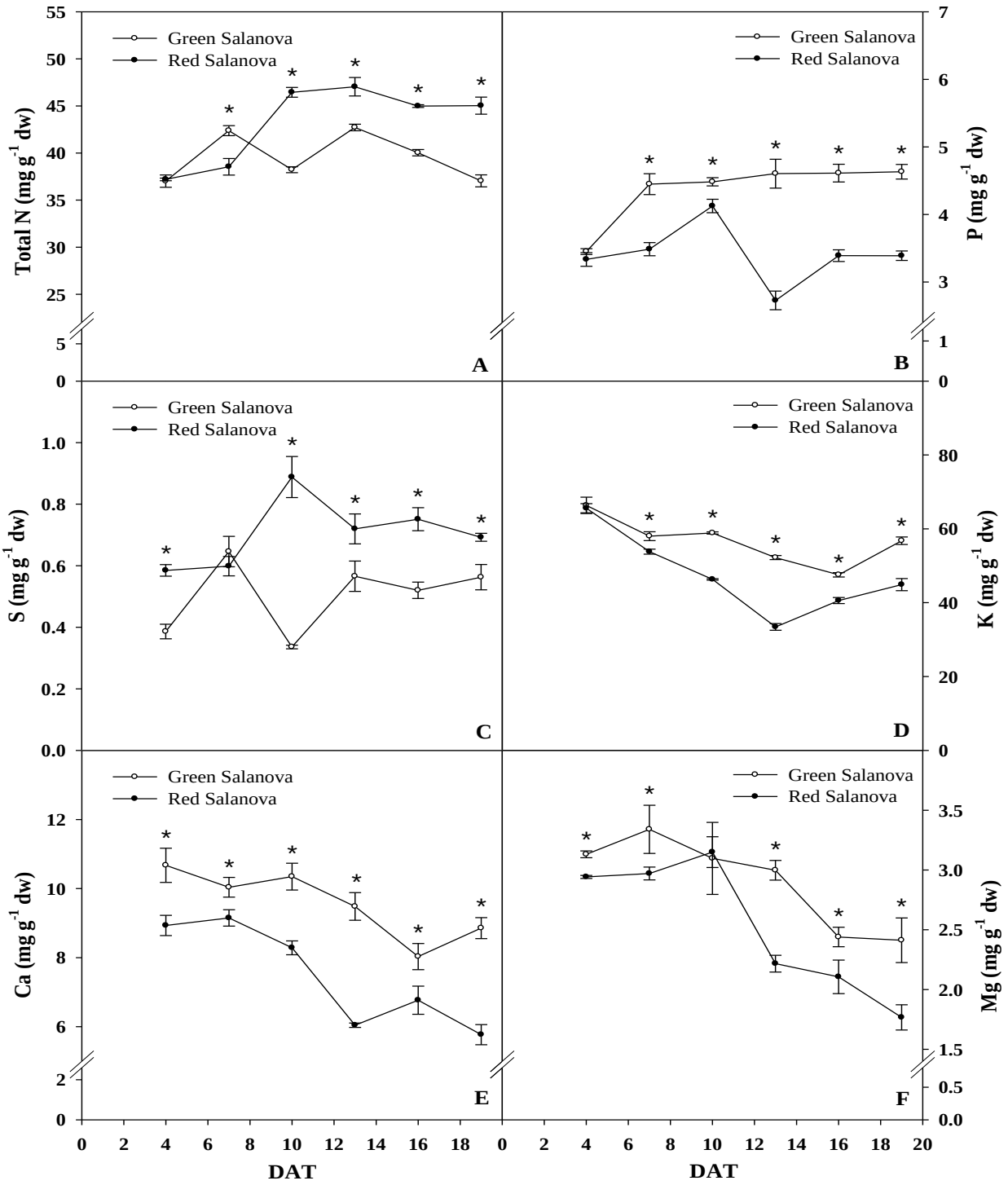

Figure 4. Evolution of total N (A), phosphorus (B), sulfur (C), potassium (D), calcium (E) and magnesium (F) concentrations of red and green Salanova during the growing cycle. Data are means of three replicates. Stars indicate a significant difference between cultivars. Vertical bars indicate ±S.E. of the means, their absence indicates that the size was less than the symbol.

3.4.3 Relative water content, leaf water potential and SPAD index

RWC expresses the absolute amount of water that plants require to reach artificial full saturation, hence there is a relationship between RWC and water potential that changes according to plant material age (Gonzalez & Gonzalez-milar, 2001). The tissue water content was expressed in amount of water per unit weight of water at full hydration because this method is more accurate than others. Considering Table 2, RWC percentage was significantly higher in green Salanova at the beginning of the growing cycle, while at 13 DAT there was no significant difference between the two cultivars, then at 16 DAT RWC was 12.5% higher in red Salanova which is in correspondence with daily water uptake that was 19.2% higher in red Salanova and significantly not different at DAT 13 (Table 1). RWC percentage had a gradual increase trend along the growing cycle in red Salanova, while in the green cultivar it increased till reaching 88.8% at 10 DAT, and then decreased and almost stabilized at around 83.7% at harvest, whereas red Salanova at harvest registered 97.1%.

Quality of perishable products like lettuce can be characterized by leaf water potential, an absolute value ranging between zero and the wilting point (Galindo et al., 2004). Ψ_{tot} in Table 2 demonstrated the same tendency as RWC % for both cultivars, increasing gradually in red Salanova to reach -0.52 MPa at harvest, whilst green Salanova registered the highest Ψ_{tot} -0.59 MPa at 10 DAT and -0.78 MPa at harvest. As well, leaf osmotic potential had the same drift during the growth, reaching -0.24 MPa at 10 DAT in green Salanova and -0.5 MPa at harvest, whilst red Salanova registered the highest Ψ_{π} -0.21 MPa at harvest.

On the contrary, leaf turgor pressure was the highest at the beginning of the growing cycle and declined gradually until harvest in red Salanova (Table 2). SPAD index reported in Table 2 was significantly higher in the red cultivar during all the growing cycle, and both cultivars showed a significant gradual increase of SPAD until harvest. At 19 DAT, SPAD was 33.4 % and 19.0 % higher than 4 DAT in green and red Salanova, respectively.

Table 2. Analysis of variance and mean comparisons for RWC, leaf total potential, leaf osmotic potential, leaf turgor pressure and SPAD index on a three days basis interval of red and green Salanova.

Physiological parameters	Cultivar	4 DAT	7 DAT	10 DAT	13 DAT	16 DAT	19 DAT	*Significance*
RWC	Green Salanova	68.41 d	78.45 c	88.85 a	84.97 ab	83.81 b	83.71 b	***
(%)	Red Salanova	61.77 f	69.94 e	75.25 d	82.40 c	94.25 b	97.13 a	***
	t-test	*0.000*	*0.002*	*0.000*	*0.277*	*0.000*	*0.000*	
Leaf total potential	Green Salanova	-1.15 d	-0.78 c	-0.59 a	-0.66 b	-0.78 c	-0.78 c	***
(MPa)	Red Salanova	-1.30 e	-0.85 cd	-0.98 d	-0.72 bc	-0.61 ab	-0.52 a	***
	t-test	*0.002*	*0.246*	*0.003*	*0.441*	*0.001*	*0.000*	
Leaf osmotic potential	Green Salanova	-0.65 c	-0.55 bc	-0.24 a	-0.46 b	-0.47 b	-0.50 b	***
(MPa)	Red Salanova	-0.66 d	-0.25 ab	-0.37 c	-0.26 ab	-0.30 bc	-0.21 a	***
	t-test	*0.635*	*0.000*	*0.019*	*0.015*	*0.005*	*0.000*	
Leaf turgor pressure	Green Salanova	0.50 a	0.23 cd	0.35 b	0.20 d	0.31 bc	0.28 bcd	***
(MPa)	Red Salanova	0.64 a	0.60 a	0.61 a	0.46 b	0.31 c	0.32 c	***
	t-test	*0.008*	*0.000*	*0.002*	*0.001*	*0.964*	*0.264*	
SPAD index	Green Salanova	23.81 c	24.75 c	25.71 bc	25.78 bc	28.54 b	31.76 a	***
	Red Salanova	39.42 d	39.39 d	41.83 c	43.86 b	44.98 b	46.90 a	***
	t-test	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*	

NS,*,**, *** Non-significant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Different letters in rows (intragroup comparisons were performed only regarding DAT) indicate significant differences according to Duncan's multiple-range test ($P = 0.05$). Cultivars are compared according to Student's t-test.

3.4.4 Qualitative parameters

Some of the lowest manifesting nitrate concentration genotypes are butterhead varieties (Burns et al., 2010). In Table 3, nitrate content tends to accumulate more in butterhead green cultivar compared to the red one, notwithstanding that both concentrations are under the maximum levels of nitrate defined for leafy salad crop by the European Communities Commisssion, 2001. In both cultivars, nitrate is at its lowest levels at the beginning of the growing cycle and increased gradually. Around mid-cycle (from 10 to 13 DAT), red Salanova showed a nitrate decrease, while green Salanova had the same shift but between 13 and 16 DAT. Nitrate concentration then increased again to reach 1175 and 1871 mg kg^{-1} fw at harvesting in red and green Salanova, respectively.

Dry matter percentage had no significant difference between the two cultivars till 10 DAT where afterwards green Salanova earned the greater percentage in comparison to red Salanova. Both cultivars had a decreasing trend along the growing cycle, particularly at harvest, red Salanova dry matter percentage was 4.2% compared to 5.4% in green Salanova (Table 3).

Lipophilic antioxidant activity (LAA) was significantly higher in the red cultivar during all the growing cycle, with a little increase in both cultivars in mid-cycle and stabilizing at harvesting at 3.3 and 6.1 mmol Trolox 100 g^{-1} dw in green and red Salanova, respectively. Total phenols and ascorbic acid were higher in red Salanova during all the growing cycle, with a decrease trend noted for total phenols in both cultivars along the growth (Table 3). Total phenols at harvesting were 18.6 and 8.9 mg gallic ac. eq. 100 g^{-1} dw in red and green Salanova, respectively. Total ascorbic acid in red Salanova registered a gradual decrease till 16 DAT and afterwards a significant increase to reach 44.9 mg 100 g^{-1} fw at harvest. Whereas, in green Salanova it increased at 7 DAT to decrease later on but with a drastic trend starting 13 DAT, registering 7.6 mg AA 100 g^{-1} fw almost 6 fold less concentrated than red Salanova. Moreover qualitative aspects (LAA, TAA and total phenols) of red and green Salanova exhibited no correlation with fresh biomass as shown in Figure 3 (D, E and F, respectively).

Table 3. Analysis of variance and mean comparisons for nitrate, dry matter, LAA, total phenols and ascorbic acid on a three days basis interval in red and green Salanova.

Qualitative parameters	Cultivar	4 DAT	7 DAT	10 DAT	13 DAT	16 DAT	19 DAT	*Significance*
Nitrate	Green Salanova	1323 b	1727 a	1927 a	1478 b	1319 b	1871 a	***
(mg kg^{-1} fw)	Red Salanova	786 c	1079 b	772 c	536 d	1109 ab	1175 a	***
	t-test	*0.000*	*0.008*	*0.000*	*0.000*	*0.007*	*0.000*	
Dry matter	Green Salanova	7.35 a	6.81 b	5.85 c	5.68 cd	5.56 cd	5.35 d	***
(%)	Red Salanova	7.12 a	6.87 b	5.72 c	5.34 d	5.06 e	4.17 f	***
	t-test	*0.339*	*0.742*	*0.221*	*0.014*	*0.015*	*0.000*	
LAA	Green Salanova	2.70 c	3.90 b	5.27 a	3.83 b	3.66 b	3.25 bc	***
(mmol Trolox 100 g^{-1} dw)	Red Salanova	4.91 c	7.39 ab	8.87 a	8.47 a	6.42 bc	6.12 bc	**
	t-test	*0.005*	*0.024*	*0.002*	*0.003*	*0.007*	*0.017*	
Total phenols	Green Salanova	25.10 a	18.22 b	12.15 c	10.06 d	9.66 d	8.90 d	***
(mg gallic ac. eq. 100 g^{-1} dw)	Red Salanova	48.27 a	39.55 b	39.84 b	24.33 c	22.29 c	18.57 c	***
	t-test	*0.005*	*0.001*	*0.000*	*0.000*	*0.000*	*0.000*	
Total ascorbic acid	Green Salanova	43.20 b	64.76 a	36.95 b	5.99 c	5.98 c	7.61 c	***
(mg AA 100 g^{-1} fw)	Red Salanova	76.26 a	69.95 ab	66.83 b	36.28 d	32.91 d	44.86 c	***
	t-test	*0.001*	*0.263*	*0.005*	*0.000*	*0.000*	*0.000*	

NS,*,**, *** Non-significant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Different letters in rows (intragroup comparisons were performed only regarding DAT) indicate significant differences according to Duncan's multiple-range test ($P = 0.05$). Cultivars are compared according to Student's t-test

3.4.5 Heat map analysis

The aggregated data heat-map analysis (Figure 5) identified two main clusters corresponding to red Salanova from 4 to 10 DAT and to green Salanova at 4 and 7 DAT on the left, and all the other treatments on the right. This indicated that the growth stage was the main clustering factor responsible for the different effects. Indeed, two separated sub-clusters could be defined under both the first and the second clusters, which illustrated the variety × stage interaction. In particular, red Salanova at 10 DAT is separated from all the other treatments on the left, due to the higher total nitrogen (N), LAA and S and lower nitrate and K, while green and red Salanova clustered separately on the right side because of the lower macronutrients (nitrate, Ca, Mg, K and P), and higher SPAD index, leaf osmotic potential, N, LAA and S. Additionally, red Salanova at 19 DAT showed the highest SPAD index, leaf osmotic potential, leaf total potential, WUE, RWC, and the lowest RGR, DM, Ca and Mg. This latter treatment manifested in common with green Salanova at 19 DAT the highest dry biomass, water uptake, leaf area (LA) and fresh biomass. On the other hand, the green treatments from 10 to 19 DAT showed the lowest phenols, TAA, LAA, S, SPAD index, and leaf turgor pressure of the sub-cluster (Figure 5).

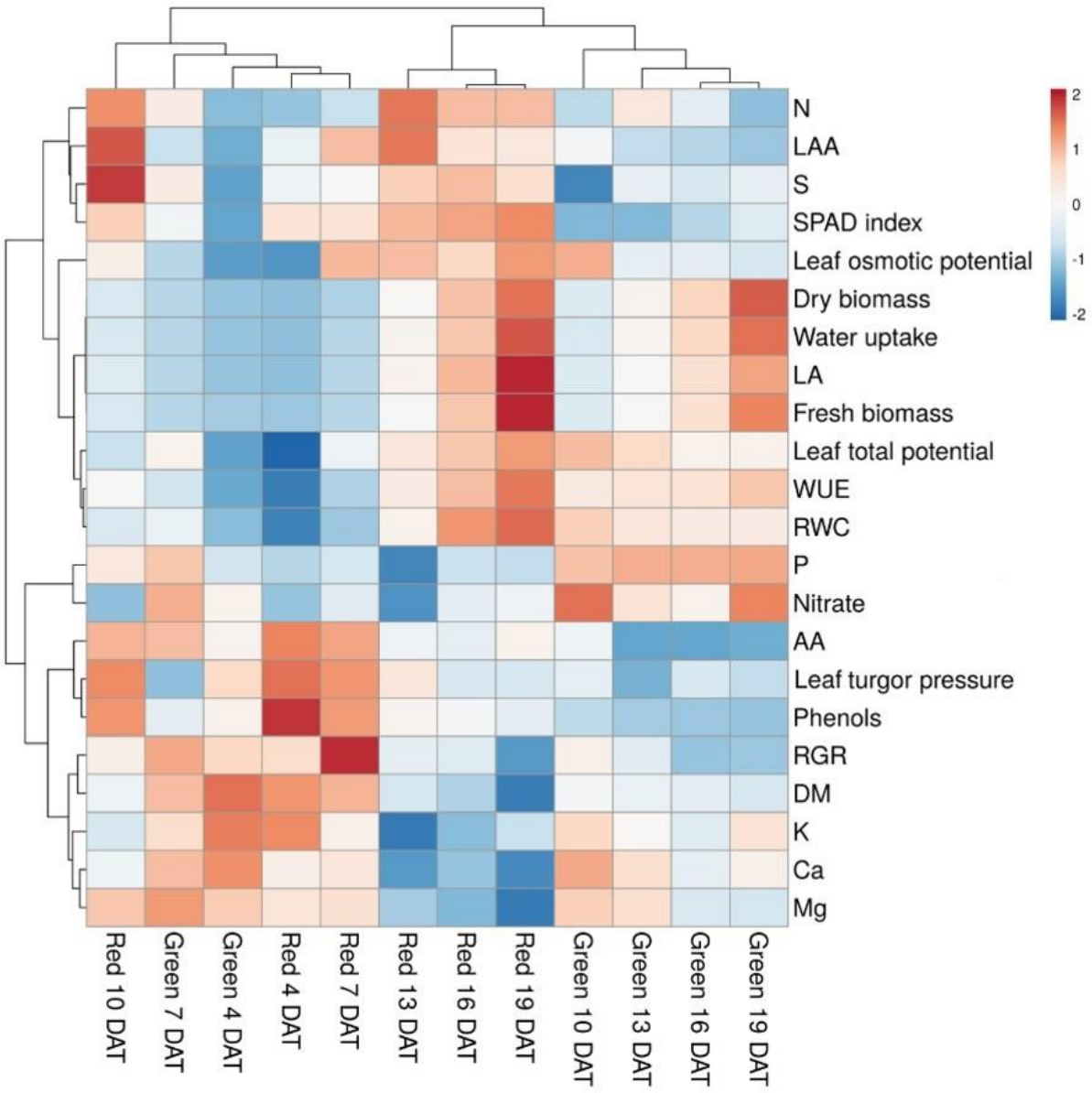

Figure 5. Cluster heat map analysis summarizing the responses of red and green Salanova to growth in a controlled-environment chamber using a closed soilless system (NFT). It was generated using the https://biit.cs.ut.ee/clustvis/ online program package with Euclidean distance as the similarity measure and hierarchical clustering with complete linkage.

3.4.6 Principal component analysis

The score plot and loading matrix based on the first and second principal components PC1 and PC2 are reported in Figure 6. The first two PCs were associated with eigen values higher than 1 which explained 80% of the cumulative variance. With PC1 and PC2 accounting for 51.8% and 28.2%, respectively. PC1 was strongly and positively correlated with morphometric traits (leaf area, fresh biomass and dry biomass) and water uptake ability (WUE, RWC, water uptake, leaf total and osmotic potential); and negatively correlated with RGR, DM, Mg, Ca, K, leaf turgor pressure and total phenols. In addition, PC2 was positively associated with nitrate, P, Ca and K, and negatively correlated with SPAD index, LAA and phenols.

Furthermore, based on the loading matrix, our PCA indicated that variation in biomass was mostly aligned with water uptake and WUE, while variation in nitrate content was not correlated to fresh biomass (Figure 6). In fact, the correlation analysis showed that fresh biomass was strongly positively correlated to leaf area and water uptake ($r=0.99$; $P<0.001$), to WUE ($r=0.88$; $P<0.001$) and RWC ($r=0.79$; $P<0.01$); while there was a strong negative correlation between fresh biomass and Mg ($r=0.87$; $P<0.001$). Nitrate content was positively correlated to the P content ($r=0.77$; $P<0.01$) and negatively to LAA ($r=0.70$; $p<0.05$) and SPAD index ($r=0.66$; $P<0.05$), while no correlation was found with fresh biomass ($r=15$; $p>0.05$). SPAD index and LAA were positively correlated ($r=0.75$; $p<0.01$).

Moreover, the score plot deriving from the PCA clearly highlighted that plant growth stage contributed to the separation of component 1 (PC1), while plant cultivar contributed to separation of PC2, with the green cultivar on the positive side and the red cultivar on the negative side of PC2. For instance, red Salanova plants at 19 DAT were characterized by improved fresh biomass, LA, water uptake, WUE, N, SPAD index, LAA and phenols, while green Salanova plants at 19 DAT were characterized by high nitrate and P content (Figure 6).

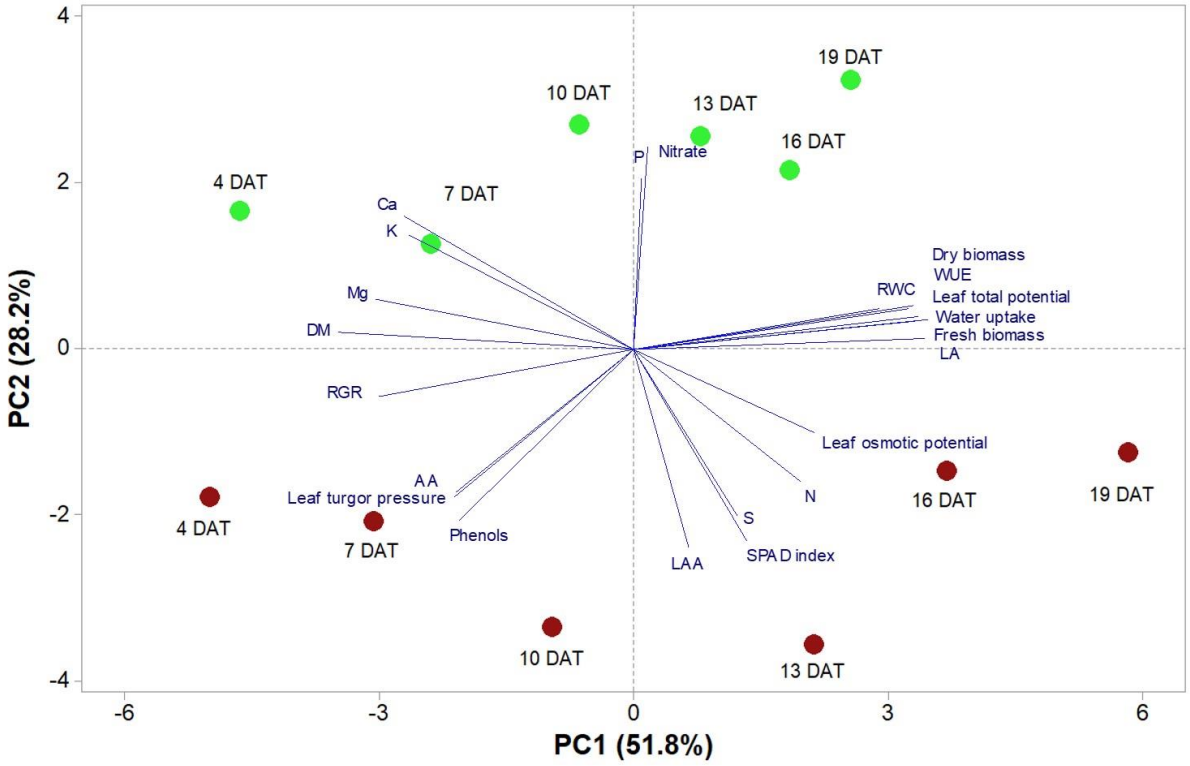

Figure 6. Principal component loading plot and scores of principal component analysis (PCA) of growth parameters (RGR, fresh biomass, dry biomass, dry matter (DM), leaf area (LA) and leaf number (LN)), qualitative parameters (Nitrate, LAA, TAA, total phenols), water requirement (WUE, water uptake, RWC, leaf total and osmotic potential and leaf turgor pressure), total N (N), mineral concentrations and SPAD index, in green and red Salanova grown in a controlled-environment growth chamber using a closed soilless system (NFT).

3.5 Discussion

Long-term human space exploration missions require a vital self-support system for two main reasons, (i) room and consequently resources are limited, (ii) continuous supply of fresh vegetable-based food can provide a proper daily intake of nutrients and phytochemicals supporting human health and well-being.

It is well known that plants are able to grow and reproduce in microgravity (Wolff et al., 2014). Moreover, a closed soilless cultivation can allow achieving *in situ* up to 10 times higher plant yields than in open field (Lages Barbosa et al., 2015), without compromising the quality of products and limiting the problem of water and nutrient loss (Rouphael and Colla, 2005). This system can also contribute to refresh air and provide clean water (Wolff et al., 2014), therefore being able to feed and sustain a crew of astronauts for months.

Among plant species selected to be used in life support systems, lettuce (*Lactuca sativa* L.) together with spinach, make the most promising species to be grown in NFT soilless system because of its quicker growth and higher nutrient use efficiency (Sharma et al., 2019). In fact, 8-10 harvests per year can be obtained using a maximum daily light integral (DLI) of 17 mol m^{-2} d^{-1}, with the possibility to boost the rate of production by further increasing DLI (Brechner & Both, 2013).

The present experiment dealing with two differently pigmented butterhead *Lactuca sativa* Salanova can provide fundamental knowledge on the most suitable lettuce cultivar to be grown in NFT, which showed the highest yield and fastest growth, which can maximize growth cycles per time, and implement BLSS under a DLI of 18.144 mol m^{-2} d^{-1}.

Red and green Salanova showed an exponential growth in the first week, higher in the red one, in line with a previous study on hydroponically grown lettuce from Albornoz & Lieth (2016). In agreement with the data of the same paper, RGR of both cultivars showed a decrease after the first week, initially less steep in red Salanova, even if at harvest green Salanova showed a higher RGR than red one. Such variation in RGR between the two cultivars might imply that the two cultivars had a different maturity stage at harvest.

Red cultivar was also characterized by higher water uptake and WUE, which linearly correlated with the increase of leaf area, since the increase in cell volume, basic to growth, requires water uptake (Hsiao et al., 1976). In particular, leaf turgor pressure (Ψ_p) has a crucial role in cell growth, since it is the physical force needed to maintain enlargement (Hsiao et al., 1976; Galindo et al., 2004), this latter being favored by turgor pressure reduction as a result of wall relaxation (Cosgrove, 1993). In this view, Ψ_p was significantly higher in the red cultivar than in the green one from 7 to 13 DAT, showing a higher capacity of the red cultivar to grow and expand its leaves in that growth stage.

Red Salanova showed also a RWC higher than 94% since 16 DAT, while the green one stood at 83-84% from 13 DAT onward. Since a RWC between 90 and 100% is coupled to stomata closure and cutback of growth and cellular expansion (Gonzalez & Gonzalez-milar, 2001), it might be that red Salanova had reached its maturation at 16 DAT unlike green Salanova. A good understanding of the evolution of water status indices is crucial during vegetable growth for choosing the appropriate harvesting date close as much as possible to the optimal maturity stage. In fact, advanced mature lettuce maintains better RWC in storage, more likely due to cellular osmolytes production that

preserve turgor pressure and osmoregulate the cytosolic compartments, thus enhancing and maintaining leaf hydration level (Barg et al., 2009). In fact, when RWC falls, also bound water content decreases (Wilson & Rose, 1966), bound water being another important water status index. In addition, small molecules (<500 Da) that are directly or indirectly involved in osmotic balance, can also contribute to scavenge superoxide anion radicals, singlet oxygen, and hydrogen peroxide stabilizing and protecting membranes and macromolecules, and therefore improving lettuce postharvest quality and shelf life (Carillo, 2018).

However, while potential-driven water uptake and turgor-driven cell expansion are critical regulation tasks mainly played by potassium (Mengel and Arneke, 1982), an increment of plant growth rate implies also a higher demand for nutrients used for new biomass synthesis. Indeed, protein synthesis, storage and energy distribution and nucleic acid synthesis and its growth regulation role require importantly nitrogen, phosphorus and sulfur (Albornoz & Lieth, 2017). RGR, in fact, correlates with plants N requirement, both decreasing during growth, since non-photosynthetic materials that increase faster with plants growth, hold less N than photosynthetically-active surfaces (Broadley et al., 2003).

Initially, both cultivars had a gradual daily increase of macro-elements since transplant, rationalized by the amplification in biomass production (Albornoz & Lieth, 2016). However, the red cultivar had a sudden decrease at 13 DAT in P, K, Ca, Mg and nitrate concentration, which more than being explained by a decrease in these macro-elements daily accumulation due a genetic weakness, where roots could have been unable to provide the necessary elements to support the urging quick growth, was probably a consequence of a dilution due to leaf area and fresh yield increase in the same period. The sudden nutrient dilution and the consequent shift in nutrients accumulation can imply that red Salanova reached its peak of growth faster than its green counterpart did, with the possibility to extend the latter's growing cycle in order to obtain more fresh biomass. Moreover, a decline in plant nutrient demand is foreseen with plant age especially for N, P and K (Albornoz & Lieth, 2017).

Plant growth was accompanied by a nitrate concentration attenuation that peaked at 13 and 16 DAT, for red and green Salanova, respectively, while when commercial maturity got closer nitrate concentration rise again (Broadley et al., 2003). As in fact, shoot nitrate concentration decreases during middle stages because of the development of new leaves that have less nitrate and more organic solutes. Such leaves are

characterized by a reduced transpiration rate that limits nitrate delivery through xylem, but receive more sugars through phloem to help maintaining turgor (Burns et al., 2010). Whereas, the increase of NO_3-N in plants late growth can be due to leaves self-shading that reduces light incidence and therefore energy for nitrate reduction (Broadley et al., 2003). However, nitrate was not the limiting factor blocking the growth of green cultivar since its nitrate concentration was much higher than that of red one. On the contrary, this can be another index showing that red Salanova reached maturity earlier than green Salanova or that the green variety was not able to use efficiently the available nitrogen.

Nitrogen use efficiency (NUE) and growth, if not by nitrate/nitrogen itself, can be influenced by light (Burns et al., 2010). When light, and in particular the DLI, exceeds the light saturation point of lettuce, it can cause photoinhibition and decrease NUE compromising lettuce growth and development, and significantly affecting fresh yield (Weiguo et al., 2012; Sublett et al., 2018).

The optimal DLI for lettuce plants ranges between 10 and 17 mol m^2 d^{-1} and we have used 18.1 DLI to boost lettuce growth. However, there are lettuce varieties which do not tolerate DLI >17. In fact, the data clearly evidence that green Salanova performed less well during cultivation in the chosen conditions compared to red one especially in parameters related to light.

Green Salanova was, in fact, characterized by a lower total phenols and ascorbic acid content during all the growing cycle, with a decrease trend noted for total phenols along the growth, in contrast with Chudichudet et al. (2011) who found that total phenols increased with plant age. Phenols can serve as sunscreens and, together with ascorbate, as scavengers of reactive oxygen species (ROS) for protecting young expanding leaves more prone to light damage (Carillo et al., 2019). LAA was also significantly lower in the green cultivar during all the growing cycle, with a little increase in both cultivars in mid-cycle, while stabilizing at harvesting. Therefore, green Salanova, less able to cope with ROS produced under high DLI, could undergo electron transport chain (ETC) over-reduction and generation of $^1O_2$ at level of photosystem II (Asada, 2006). Moreover, $^1O_2$ can be responsible for the initiation of a genetic program, mediated by the proteins EXECUTOR 1 and 2 pathways, which limits growth in plants, and eventually causes programmed cell death (Lee et al., 2007). The inhibition of growth and the lower turgor potential could account also for the reduced expansion of

leaf area, which is useful for absorbing less light and cope with the excess of light (Nobel et al., 1977).

However, an ubiquitous photosynthetic protection response that plant can enact under high light to reduce high light damages, is the synthesis of the Early Light Induced Protein (ELIP) which is thought to act as a photoprotectant, inhibiting chlorophylls synthesis and therefore reducing photon capture proteins of antenna complexes, and therefore photosynthetic activity (Tzvetkova-Chevolleau et al., 2007). Accordingly, chlorophylls content and photosynthesis decreased in green Salanova, as proved by lower SPAD index compared to red cultivar, while nitrate, not used for chlorophyll and photosynthetic apparatus synthesis, accumulated in green Salanova.

These data encourage us to choose red over green Salanova to play the role of candidate cultivar for BLSSs, since at the high light chosen for speeding up growth in an environment where fast repetitive growing cycles are essential, it grows and perform better. The red cultivar ability to use efficiently the higher DLI for reaching maturity stage faster, makes it more likely to conserve its sensory qualities after storage compared to the green one, since maturity stage at harvest contributes in maintaining quality attributes (Barg et al., 2009). In particular, its high content of phenolics and ascorbic acid represents not only a notable fount of dietary antioxidants (Chutichudet et al., 2011), but, has also the potential to delay shelf-life. In fact, ascorbic acid has been always used for its antioxidant and stabilizing abilities in food industries (Varvara et al., 2016), but, above all, it has a strong potential for preventing phenolic compound degradation in fresh-cut lettuce (Chudichudet et al., 2011).

3.6 Conclusion

Agriculture for space topic has handed out lot of benefits and technologies for controlled environment agriculture and *vice versa*. Such as volume constraints that steered for the selection of shorter crops with high harvest indices which is well valued in plant factories on Earth (Wheeler, 2017). Our findings highlighted that red salanova cultivar reached maturity faster than green salanova at the chosen DLI, implying a shorter harvest cycle to attain target weight and maturity stage requirement as well for maintaining better quality attributes after harvest in case storage of the commodity is an option in human life support systems. A short time to grow this cultivar leave space for other new growing cycle in brief periods, leading to a less consumption of water and

minerals for reaching target produce. Our results indicate that fresh biomass, WUE, LAA, total phenols and ascorbic acid where higher in red salanova, as well as having 37.2% less nitrate than green Salanova. These qualitative findings along the horticulture requirements present red Salanova as a new candidate cultivar for BLSS, yet further experiments should be held in order to determine the contribution of this cultivar in air regeneration and water recycling.

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4 Chapter 4: Macronutrient deprivation eustress elicits differential secondary metabolites in red and green-pigmented butterhead lettuce grown in closed soilless system

4.1 Abstract

Through precise control of the nutrient solution (NS), closed soilless systems effectuate targeted manipulation of plant secondary metabolites that constitute health-promoting components of human daily diet. An NFT system was presently employed to assess the effect of NS macronutrient-based concentration (full, half- and quarter-strength corresponding to EC 1.5, 0.75 and 0.5 dS m-1) on the bioactive profile of red and green-pigmented Salanova® butterhead lettuce. Half-strength NS reduced fresh yield of both cultivars by 14%, while quarter-strength NS reduced the yield of green and red Salanova by 24% and 38%, respectively. However, moderate nutrient stress (half-strength NS) boosted red Salanova concentrations of ascorbate, chlorogenic, chicoric, caffeoyl-meso-tartaric and total phenolic acids, and anthocyanins by 266%, 199%, 124%, 251%, 162% and 380%, respectively compared to control full-strength NS. Nutritional eustress and appropriate cultivar selection constitute effective means to increase phytochemical content and optimize year-round production of lettuce in closed soilless systems.

4.2 Introduction

Plant foods are a source of vitamins and minerals, but also a notable source of health-promoting phytochemicals, called plant secondary metabolites (PSM) (Della Penna, 1999), which are organic (Fang et al., 2011) small biomolecules (Owalabi et al., 2018) deriving from primary metabolites which partake in diverse physiological activities and hold a crucial role in plant defense and plant-environment interaction (Kennedy and Wightman, 2011; Verma and Shukla, 2015). PSM are often signature components of certain plant species or genera and play a major role in plant stress and defense responses or in central and secondary metabolism (Della Penna, 1999; Kennedy and Wightman, 2011). This pool of versatile phytochemicals is divided into three major groups depending on their biosynthetic pathway: terpenoids, alkaloids and

phenylpropanoids/allied phenolic compounds, plus other minor groups such as amines, cyanogenetic glycosides, glucosinolates and psoralens (Fang et al., 2011). With plant-rich diets, the latter affect positively human health and decrease mortality by preventing a range of common diseases like macular degeneration, cancer and cardiovascular diseases (Della Penna, 1999; Khanam et al., 2012). PSM are also considered biosynthetic end-products able to accumulate in plants at high levels, unlike primary metabolites, and are of major benefit to humans as medicinal active ingredients (Verma and Shukla, 2015). In fact, many antibiotics, anti-tumor agents and antivirals are derivates of PSM, as are many food additives and some herbicides and phyto-toxins used in agriculture (Akula and Ravishankar, 2011; Verma and Shukla, 2015).

With the rise of consumer interest in functional foods, the food industry is on the quest for naturally occurring functional ingredients through plant breeding/genetic modification (Owalabi et al., 2018; Rouphael et al., 2018) and by elicitation methods (Baenas et al., 2014; Owalabi et al., 2018) such as plant nutrient deprivation (Rouphael and Kyriacou, 2018; Kennedy and Wightman, 2011). Lots of factors are implicated in controlling PSM, including: genetic, ontogenic, morphogenetic and environmental factors divided into biotic and abiotic (Verma and Shukla, 2015). Abiotic factors are ramified into diverse types of plant stress including flooding, drought, temperature (heat, chilling and frost), radiation, nutrient deprivation as well as seasonal variation (Akula and Ravishankar, 2011;Verma and Shukla, 2015).

On the other hand, controlled environments (i.e, greenhouse and indoor growing modules) can facilitate the year-round production of fresh vegetables with consistent functional quality by providing highly stable and replicable growth conditions (Rouphael et al., 2018; Rouphael and Kyriacou, 2018). In particular, soilless culture (i.e., nutrient film technique [NFT], floating raft system), which is the most contemporary plant cultivation system, makes efficient use of the nutrient solution to maximize crop yield and to produce high-value fresh vegetables (Asaduzzaman et al., 2015; Kyriacou and Rouphael; 2018, Rouphael et al., 2018; Rouphael and Kyriacou, 2018). Appropriate management of the nutrient solution composition is an important pre-harvest factor that can modulate the organoleptic and functional aspects of horticultural products (Kyriacou and Rouphael, 2018). Nutritional chemical *eustress* (e.g, mild to moderate salinity and nutrient stress), also known as positive stress, can elicit targeted physiological responses aimed at improving the nutritional value of vegetables by triggering the strategic accumulation of desired metabolites in plants as

part of the adaptation mechanism to suboptimal conditions (Rouphael and Kyriacou, 2018).

Although lettuce is a favored, widely consumed vegetable, it is not praised enough for its nutritional value, most likely due to its high (~95%) water content (Kim et al., 2016). Lettuce is a source of health promoting phyto-constituents such as carotenoids, folate, vitamin C and polyphenols (Kim et al., 2018), and its nutrient composition can in fact equal that of other vegetables regarded as nutritious, depending on the lettuce genotype (Kim et al., 2016) and predominantly on leaf pigmentation (Kim et al., 2018). Organoleptic and nutritional value along with production aspects such as space/time yield efficiency, harvest index, nutrient use efficiency and light use efficiency, nominate lettuce a candidate crop for future innovative cultivation in bio-regenerative life support systems (Dueck et al., 2016) and widespread vertical farming (Sarkar and Majumder, 2015) often referred to as *plant factory*.

Previous studies (Chen et al., 1997; Alberici et al., 2008; Fallovo et al., 2009; Genuncio et al., 2012) have demonstrated the effect of reduced nutrient solution levels on yield and select quality traits of greenhouse leafy vegetables, including lettuce. Certain studies in controlled environments focused on nitrogen levels only, such as Quadir et al. (2017) and Becker et al. (2015), or on nitrogen and phosphorus levels, such as Galieni et al. (2015). Nevertheless, nothing is known about the interaction between macronutrient deprivation, applied as reduced macronutrient levels in the nutrient solution, and cultivar, in respect to lettuce PSM profiles.

Based on this framework, the objective of this work was to study macronutrient deprivation *eustress* on two lettuce genotypes of distinct pigmentation, red and green butterhead lettuce. These were cultivated in growth chambers using a closed soilless system of nutrient film technique (NFT) in which all growth parameters were standardized and kept uniform while macro-elemental concentrations in the nutrient solution were modulated to prompt the accumulation of PSM (e.g. phenolic acids, anthocynanins and carotenoids) that were subsequently profiled and quantified. The acquired data can be valorized by growers to set the eustress threshold level where yield is sustained while nutritional and functional quality is improved and mineral fertilizers input is reduced.

4.3 Materials and methods

4.3.1 Plant Material, Growing Conditions, Experimental Design and Nutrient Solution Management

A nineteen-day experiment was conducted in a 28 m^2 open-gas-exchange growth chamber (7.0 m×2.1 m×4.0 m; W×H ×D) at the Department of Agricultural Sciences, University of Naples Federico II, south Italy. Two butterhead lettuce cultivars (*Lactuca sativa* L. var. *capitata*) green Salanova® and red Salanova® (Rijk Zwaan, Der Lier, The Netherlands) were cultivated in a nutrient film technique system (NFT), consisting of propylene gullies covered with propylene taps to avoid evaporation of the nutrient solution. Each gully was 200 cm long, 14.5 cm wide and 8.0 cm deep and was equipped with one faucet to provide the nutrient solution at a constant flow of 1.5 L min^{-1}, maintained by submerged pumps. The nutrient solution was collected in 25 L polypropylene tanks by gravity-dependent flow, due to the gullies' 1% inclination. Two weeks after sowing, lettuce seedlings were transplanted in 7×7×7 cm Rockwool cubes (Delta, Grodan, Roermond, The Netherlands) and placed into the gullies at an intra-row spacing of 0.15 m with the gullies set 0.43 cm apart, making a density of 15.5 plants m^{-2}. Artificial light was provided by High Pressure Sodium (HPS) lamps, with an intensity of 420 µmol m^{-2} s^{-1} according to a light/dark regime of 12/12h. Air temperature was set at 24/18° C (light/dark) with corresponding relative humidity of 60-80% maintained by a fog system. The experiment was carried out at ambient carbon dioxide concentration (370-410 ppm) and air change and dehumidification were facilitated by two HVAC systems.

The experiment was designed as a factorial combination of cultivar (red and green Salanova) and nutrient solution concentration (full-strength, half-strength and quarter-strength). The six combinatorial treatments were arranged in a randomized complete-block design with three replicates, making a total of 18 experimental units with twelve plants each (n=216 green and red Salanova plants).

The full strength nutrient solution consisted of the following macro and micronutrients: 9.0 mM N-NO_3^-, 2.0 mM S, 1.0 mM P, 4.0 mM K, 4.0 mM Ca, 1.0 mM Mg, 1.0 mM NH_4^+, 15 µM Fe, 9 µM Mn, 0.3 µM Cu, 1.6 µM Zn, 20 µM B, and 0.3 µM Mo. The half and quarter nutrient solutions had 50% and 25% of the macronutrients while micronutrient concentrations were kept the same as in the full strength nutrient solution. The three nutrient solution concentration treatments were prepared using de-

mineralized water. The electrical conductivity (EC) of the full, half and quarter strength nutrient solutions in the polypropylene tanks were 1.5, 0.75 and 0.5 dS m^{-1}, respectively, while the pH of the nutrient solutions was monitored daily and maintained at 6.0±0.2.

4.3.2 Sampling, growth analysis, and light use efficiency

The first and last plant of each experimental unit in the NFT gullies were set as 'guards' and were not sampled. Two plants per replicate were directly frozen in liquid nitrogen and stored at -80 °C for further qualitative analysis; while eight plants per replicate were harvested in order to determine leaf number and measure fresh weight and leaf area, the latter being measured by an Area Meter (LI-COR 3100C biosciences, Lincoln, Nebraska, USA). Light Use Efficiency (LUE) was calculated as shoot dry biomass divided by cumulative daily intercepted Photosynthetic Photon Flux Density (PPFD) and expressed as g mol^{-1}.

4.3.3 Leaf dry matter and mineral content analysis

Leaf plant tissues were dried at 70 °C for 72 h until they reached a constant weight, and the corresponding shoot dry biomass and leaf dry matter content (%) were determined. The dried leaf tissues were ground separately at 0.5 mm in a Wiley Mill.

Total nitrogen concentration in red and green lettuce tissue was determined by the established Kjeldahl method (Bremner, 1965). Mineral profile analysis, including macronutrients and sodium, was performed according to Kyriacou et al. (2019) with slight modifications. Briefly, 250 mg of the dried tissues were suspended in 50 ml of ultrapure water (Milli-Q, Merck Millipore, Darmstadt, Germany) and shaken in a water bath (ShakeTemp SW22, Julabo, Seelbach, Germany) at 80 °C for 10 min. The suspension was centrifuged at 6000 rpm for 10 min (R-10 M, Remi Elektrotechnik Limited, India), then filtered through a 0.45 μm nylon syringe filter (Phenomenex, Torrance, CA, USA) and analysed by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA) coupled to a conductivity detector. An IonPac CG12A (4×250 mm, Dionex, Corporation) guard column and IonPac CS12A (4×250 mm, Dionex, Corporation) analytical column were used for the K, Ca and Mg analysis, while for N-NO_3 and P determination, an IonPac AG11-HC guard (4×50 mm) column and IonPac AS11-HC analytical column (4×250 mm) were adopted.

4.3.4 Analysis of hydrophilic antioxidant activity

In order to assess comprehensively the hydrophilic antioxidant activity (HAA) of green and red Salanova butterhead lettuce, 200 mg of lyophilized samples were extracted with distilled water and their antioxidant activity was determined by the N,N-dimethyl-p-phenylenediamine (DMPD) method (Fogliano et al., 1999). The principle of the assay is that in the presence of a suitable oxidant solution, DMPD can form a stable, chromophore radical cation ($DMPD.^{+}$). Antioxidant compounds (AO) which are able to transfer a hydrogen atom to $DMPD.^{+}$ quench the color and produce a discoloration of the solution proportional to their amount, hence a linear inhibition of color formation can be observed in the presence of antioxidant compounds extracted from vegetable samples. The HAA were determined by UV–Vis spectrophotometry: the absorbance of the solution was measured at 505 against external standard linear calibration and expressed as mmol AA equivalent per 100 g of dry weight (dw) (Fogliano et al., 1999).

4.3.5 Analysis of total ascorbic acid

Total ascorbic acid (TAA), as the sum of ascorbic (AA) and dehydroascorbic (DHA) acids, was assessed by spectrophotometric analysis of fresh plant tissues. The assay is based on the reduction of Fe^{3+} to Fe^{2+} by AA and the spectrophotometric detection of Fe^{2+} complexes with 2,2-dipyridyl (Kampfenkel et al., 1995). DHA is reduced to AA by pre-incubation of the sample with dithiothreitol. As in the case of HAA, total ascorbic acid was also measured by UV–Vis spectrophotometry. The absorbance of the solution was measured at 525 nm, and data were expressed as mg AA per 100 g fresh weight (fw) of butterhead lettuce.

4.3.6 Phenolic acids and anthocyanins extraction, identification and quantification

Phenolic acids were extracted according to Llorah et al. (2008). Twelve ml of methanol/water/formic acid (50/45/5, v/v/v, 12 ml) were added to 400 mg of freeze-dried samples, sonicated for 30 min before centrifuged for 30 min at 2500 g and 4°C. The supernatants were collected centrifuged once more at 21100 g for 15 min at 4°C. Samples were filtered through 0.22 µm cellulose filters (Phenomenex) before LC

analysis. Separation of hydroxycinnamic derivatives and anthocyanins was performed on an LC binary system (LC-10AD, Shimadzu, Kyoto, Japan), equipped with a Diode Array Detector (SPD-M10A, Shimadzu, Kyoto, Japan) and a Series 200 autosampler (Perkin Elmer, Waltham, MA). Separation was achieved on a reversed phase C18 column (Prodigy, 250×4.6 mm, 5 µm, Phenomenex, Torrance, CA), equipped with a C18 security guard (4.0×3.0 mm, Phenomenex), by using as mobile phases (A) water formic acid (95:5, v/v) and (B) methanol in the following gradient of solvent B, (t in [min]/[%B]): (0/5), (25/40), (32/40). The flow rate was isocratic at 1 ml min^{-1} and injection volume was 20 µl. Calibration curves of hydroxycinnamic derivatives were performed by using chlorogenic acid and chicoric acid at 330 nm. Identification of caffeoyl-meso-tartaric acid and caffeoyl-tartaric acid was performed by LC-MS/MS experiments. The chromatographic profiles of reference curves and samples were recorded in multiple reaction monitoring mode (MRM) by using an API 3000 triple quadrupole (ABSciex, Carlsbad, CA). Negative electrospray ionization was used for detection, and source parameters were selected as follows: spray voltage -4.2 kV, capillary temperature 400 °C, dwell time 100 ms, nebulizer gas and cad gas were set to 10 and 12 respectively (arbitrary units). Target compounds $[M-H]^-$ were analyzed using mass the transitions in parentheses: chicoric acid (*m/z* 473→311, 293), chlorogenic acid (*m/z* 353 →191), caffeoyl tartaric acid (*m/z*311→ 179, 149, retention time 15.8 min), caffeoyl meso tartaric acid (*m/z*311→ 179, 149, retention time 17.8 min). The concentration of phenolic acids was reported as mg per 100 g of dry weight (dw) of butterhead lettuce. Within the same LC-DAD chromatographic runs, anthocyanins were monitored at 520 nm, and the concentration was calculated by using cyanidin as reference standard. The results were reported as µg of cyanidin equivalent per g of dw.

4.3.7 Carotenoids extraction, identification and quantification

The carotenoids profile was produced as previously reported (Allverdú-Queralt et al., 2013) with slight modifications. To 0.1 g of freeze-dried samples were added 2.5 ml of ethanol/hexane (4:3, v/v) with 1% BHT. The suspension was vortexed for 30 s at 22°C, then sonicated for 5 min in the dark. The supernatants from three rounds of centrifugation at (2500 g, 4°C, 10 min) were collected and filtered through 0.45 µm nylon filters (Phenomenex, Torrance, CA), dried under a gentle flow of nitrogen and stored at -20°C until analysis. Before analysis, the dried extracts were dissolved in 1%

BHT in chloroform. Separation of carotenoids was performed on a binary LC10AD system equipped with a SPD-M10A DAD (Shimadzu, Kyoto, Japan), a 200 Series autosampler (Perkin Elmer, Waltham, MA) and a C18 column (Prodigy, 250×4.6 mm, 5 µm, Phenomenex, Torrance, CA) connected to a C18 security guard (4.0×3.0 mm, Phenomenex). The injection volume was 20 µl and mobile phases used were: (A) acetonitrile, hexane, methanol, and dichloromethane (4:2:2:2, v/v/v/v) and (B) acetonitrile. An isocratic flow rate was set at 0.8 mL min^{-1} and the following step gradient of solvent B was built: (t in [min]/[%B]): (0/70), (20/60), (30/30), (40/2). Identification of the peaks was achieved by comparison of UV-vis spectra and retention times of eluted compounds with pure standards at 450 nm. Quantitation was performed by using violaxanthin, neoxanthin, β-cryptoxanthin, lutein and β-carotene as reference standards. Three separate sets of calibration curves were built, each set was injected three times in the same day (intra-day assay) and three times in three different days (inter-day assay). The accuracy was reported as the discrepancies between the calibration curves performed intra-day and inter-day and the results were expressed as relative standard deviation RSD (%). A recovery test was performed by spiking two samples with known amounts of carotenoids (50 and 100 µg mL^{-1}) and accounting for the overestimation due to the target analytes already present in the samples. Concentrations were reported as µg per g dw except for violaxanthin + neoxanthin, which were reported in µg violaxanthin equivalents per g dw.

4.3.8 Statistics and principal component analysis

All data were subjected to analysis of variance (ANOVA) using the SPSS 20 software package (www.ibm.com/software/analytics/spss). Duncan's multiple range test (DMRT) was performed for mean comparisons on each of the significant ($p < 0.05$) variables measured. Principal component analysis (PCA) was conducted on growth analysis parameters, mineral profile and secondary metabolites and to individuate nutritional and functional quality parameters that were most effective in discriminating between butterhead lettuce genotypes (green and red-pigmented) and nutrient solution concentrations (full, half and quarter strength) by using Minitab 16.2.1 statistical software. Calculations and analyses were also carried using the appropriate functions within SPSS 20 software package in order to determine the score plot and loading matrix based on the first and second principal components (PC1 and PC2).

4.4 Results and discussion

4.4.1 Growth, fresh yield, leaf dry matter and light use efficiency

Nutritional and functional quality as well as yield of *Lactuca sativa.* L are governed by genetic differences and also by Genotype × Management practice interaction (Mou, 2009; Rouphael et al., 2018). In this study, green and red Salanova cultivars were assessed from a yield and functional quality point of view as per their response to three levels of nutrient solution electrical conductivity (EC) mediated by major macronutrients.

For most of the morphometric parameters measured no significant interaction between cultivar (C) and nutrient solution concentration (S) was recorded, except for the case of marketable fresh yield (Table 1). In fact, our morphological data (leaf area and number, shoot dry biomass, leaf dry matter content) clearly indicated that the effect of nutrient solution concentration exceeded that of the genetic material (red or green-pigmented cultivar) (Table 1). The highest marketable fresh yield was recorded in red Salanova irrigated with full strength nutrient solution (NS), followed by the same genotype receiving half strength solution. The half-strength NS had similar effects on the fresh biomass of both cultivars, which decreased by around 14% in comparison to the control, while the quarter-strength NS was more detrimental on the red cultivar which decreased in fresh biomass by 37.9% as opposed 24.0% in the green cultivar (Table 1).

Regardless of the NS concentration, the cultivar red Salanova exhibited higher leaf area, shoot dry biomass and light use efficiency (LUE) than green Salanova by 23.5%, 24.8% and 20.0%, respectively (Table 1). As for the NS concentration mean effect on both cultivars, all growth parameters showed a significant decrease when the NS concentration was reduced, with the lowest values recorded at quarter-strength, whilst an opposite tendency was noted for leaf dry matter percentage that was 17.4% higher at quarter-strength NS than that at full and half-strength NS (Table 1). The above findings, are in line with Fallovo et al. (2009), who found a significant decrease in yield, leaf area index and total dry biomass of lettuce (*Lactuca sativa* L. var. *acephala*) grown in floating raft system when NS electrical conductivity (EC) decreased from 2.0 to 0.3 dS m^{-1}; as well as with Genuncio et al. (2012), who found a significant decrease in lettuce

292 fresh weight when the NS EC decreased from 1.8 to 0.92 dS m^{-1}. Contrarily, neither
293 Vernieri et al. (2006) observed a decrease in rocket leaf area when NS was decreased 4-
294 fold nor Chen et al. (1997) in lettuce shoot fresh weight when NPK levels were reduced
295 100-fold. The reduction of marketable fresh yield in plants receiving half- and
296 especially quarter-strength NS could be associated both to an osmotic effect and to
297 nutrient deficiency, in particular N and Mg (Fallovo et al., 2009; Table 2), leading to
298 smaller leaves, lower stature and consequently to a significant reduction in marketable
299 yield. In fact, Kposell and co-workers (2007) reported that reduced N in the NS is the
300 most critical nutrient factor affecting growth and altering plant composition compared
301 to other macro or microelements.

Table 1. Growth parameters, fresh yield and light use efficiency (LUE) of hydroponically grown green and red butterhead lettuce Salanova in relation to nutrient solution concentration mediated by major macronutrients.

Source of variance	Leaf area (cm^2 $plant^{-1}$)	Leaf number (no. $plant^{-1}$)	Fresh biomass (g $plant^{-1}$)	Dry biomass (g $plant^{-1}$)	Leaf dry matter (%)	LUE (g mol^{-1})
Cultivar (C)						
Green Salanova	1109± 50.4 b	57.95± 1.94	66.84± 3.20 b	3.27± 0.15 b	4.91± 0.11	0.15± 0.007 b
Red Salanova	1370± 58.0 a	57.66± 1.77	86.58± 5.98 a	4.08± 0.19 a	4.82± 0.25	0.18± 0.009 a
Nutrient solution concentration (S)						
Full strength (1.4 dS m^{-1})	1388± 69.7 a	61.63± 1.71 a	90.70± 6.67 a	4.03± 0.27 a	4.50± 0.26 b	0.18± 0.012 a
Half strength (0.75 dS m^{-1})	1247± 87.0 b	58.66± 2.2 ab	77.83± 6.00 b	3.67± 0.32 ab	4.70± 0.11 b	0.17± 0.014 ab
Quarter strength (0.5 dS m^{-1})	1082± 50.6 c	53.12± 1.14 b	61.59± 1.81 c	3.33± 0.16 b	5.40± 0.11 a	0.15± 0.007 b
C × S						
Green Salanova × full strength	1252± 23.5	62.30± 1.89	76.51± 1.45 c	3.72± 0.08	4.87± 0.08	0.17± 0.003
Green Salanova × half strength	1091± 99.9	58.11± 4.08	65.91± 5.77 d	3.06± 0.35	4.64± 0.23	0.14± 0.016
Green Salanova × quarter strength	985± 43.6	53.44± 2.27	58.11± 1.49 d	3.03± 0.05	5.22± 0.09	0.14± 0.002
Red Salanova × full strength	1525± 71.2	60.96± 3.25	104.90± 4.37 a	4.34± 0.50	4.13± 0.44	0.19± 0.023
Red Salanova × half strength	1403± 58.8	59.22± 2.70	89.75± 2.11 b	4.28± 0.06	4.77± 0.05	0.19± 0.002
Red Salanova × quarter strength	1180± 36.4	52.80± 1.14	65.08± 1.42 d	3.63± 0.18	5.57± 0.15	0.16± 0.008
Significance						
Cultivar (C)	***	NS	***	**	NS	**
Nutrient solution concentration (S)	***	*	***	*	**	*
C × S	NS	NS	**	NS	NS	NS

NS, **, *** Non-significant or significant at $p \leq 0.01$, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (p= 0.05). All data are expressed as mean ± standard deviation, $n = 3$.

4.4.2 Mineral Profile and Nitrate Content

Leaf macronutrient concentrations as a function of cultivar and NS concentration are reported in Table 2. Among the minerals analyzed, K was by far the most abundant, irrespective of cultivar and NS concentration, ranging from 51.2 to 61.2 g kg^{-1} dw, followed by N (38.4-48.7 g kg^{-1} dw), Ca (9.0-13.4 g kg^{-1} dw), P (4.1-5.8 g kg^{-1} dw), Mg (2.4-3.3 g kg^{-1} dw), and finally Na (0.5-0.9 g kg^{-1} dw) (Table 2). Significant interaction between the two experimental factors was recorded with respect to total N and K, whereas Ca, Mg and Na were only influenced by NS concentration and P content was significantly affected by both main factors with no significant C × S interaction (Table 2). Specifically, total N was not affected by NS deprivation in green Salanova, whereas it decreased linearly with the NS concentration reduction in red Salanova. As for K, it significantly decreased at quarter-strength NS in red Salanova, while in green Salanova it was significantly higher only with half-strength NS (Table 2). Disregarding NS concentration, the highest value of P in leaf tissue was recorded in green Salanova (Table 2). Notwithstanding the cultivar effect, butterhead lettuce plants grown with quarter-strength NS incurred significantly decreased P content, while an opposite tendency was recorded for calcium, magnesium and sodium (Table 2).

Table 2. Total nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sodium (Na) concnetrations of hydroponically green and red butterhead lettuce Salanova in relation to nutrient solution concentration mediated by major macronutrients.

Source of variance	Total N (g $100g^{-1}$ dw)	P (g kg^{-1} dw)	K (g kg^{-1} dw)	Ca (g kg^{-1} dw)	Mg (g kg^{-1} dw)	Na (g kg^{-1} dw)
Cultivar (C)						
Green Salanova	44.0 ± 0.71	5.20 ± 0.24 a	52.87 ± 2.36	12.26 ± 0.60	2.98 ± 0.09	0.68 ± 0.06
Red Salanova	44.2 ± 1.60	4.79 ± 0.21 b	53.41 ± 3.22	11.81 ± 0.71	2.85 ± 0.14	0.77 ± 0.07
Nutrient solution concentration (S)						
Full strength (1.50 dS m^{-1})	47.1 ± 0.82 a	5.47 ± 0.23 a	55.42 ± 2.94 b	9.65 ± 0.42 b	2.63 ± 0.10 b	0.59 ± 0.04 b
Half strength (0.75 dS m^{-1})	44.6 ± 0.91 b	5.27 ± 0.12 a	59.92 ± 0.82 a	13.08 ± 0.44 a	2.83 ± 0.08 b	0.69 ± 0.06 b
Quarter strength (0.50 dS m^{-1})	40.4 ± 1.00 c	4.24 ± 0.16 b	44.09 ± 1.53 c	13.38 ± 0.18 a	3.29 ± 0.07 a	0.90 ± 0.08 a
C × S						
Green Salanova × full strength	45.5 ± 0.62 b	5.83 ± 0.07	51.24 ± 0.66 bc	10.25 ± 0.60	2.82 ± 0.02	0.58 ± 0.06
Green Salanova × half strength	43.9 ± 1.71 b	5.38 ± 0.21	61.23 ± 1.00 a	13.08 ± 0.94	2.86 ± 0.16	0.60 ± 0.02
Green Salanova × quarter strength	42.5 ± 0.40 b	4.39 ± 0.32	46.14 ± 2.53 cd	13.45 ± 0.25	3.27 ± 0.08	0.86 ± 0.15
Red Salanova × full strength	48.7 ± 0.32 a	5.10 ± 0.36	59.59 ± 5.02 a	9.06 ± 0.39	2.43 ± 0.11	0.59 ± 0.06
Red Salanova × half strength	45.3 ± 0.80 b	5.16 ± 0.12	58.60 ± 0.79 ab	13.08 ± 0.32	2.80 ± 0.06	0.78 ± 0.11
Red Salanova × quarter strength	38.4 ± 1.10 c	4.10 ± 0.10	42.04 ± 1.02 d	13.30 ± 0.31	3.31 ± 0.13	0.94 ± 0.10
Significance						
Cultivar (C)	NS	**	NS	NS	NS	NS
Nutrient solution concentration (S)	***	***	***	***	***	*
C × S	**	NS	*	NS	NS	NS

NS, **, *** Non-significant or significant at $p \leq 0.01$, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (p= 0.05). All data are expressed as mean ± standard deviation, $n = 3$.

Nitrate content recorded among the six treatments was within the maximum nitrate limit for the commercialization of lettuce according to Commission regulation (EU) No 1258/2011; however, NO_3 content was only affected by NS concentration (Table 3). In fact, when averaged over cultivar, NO_3 content decreased by 34.6% at quarter-strength NS (1190 mg kg^{-1} fw) in comparison to the full and half-strength NS (avg. 1821 mg kg^{-1} fw) (Table 3). Shinohara and Suzuki (1981) also observed a significant decrease of lettuce leaf nitrate and total N as a result of reducing the NS concentration from 50% to 25%. Nitrate leaf content also is in concord with the results of Vernieri et al. (2006) and Fallovo et al. (2009). Furthermore, the results on leaf macronutrients obtained by Fallovo et al. (2009) at EC = 0.3 dS m^{-1} (i.e., 2 mequiv L^{-1}) were compatible with our results that showed phosphorus and potassium concentration decreased significantly at quarter-strength NS (EC = 0.5 dS m^{-1}). Similar results were obtained by Rouphael et al. (2012) on cardoon (*Cynara cardunculus* L. var. *altilis* DC.) and artichoke [*Cynara cardunculus* L. subsp. *scolymus* (L.) Hegi] leaves, where potassium concentration decreased at the lowest two NS concentrations (2 and 20 mequiv L^{-1}). However, disparate trends to ours with respect to calcium and magnesium were recorded in the works of both Rouphael et al. (2012) and Fallovo et al. (2009). An explanation for these differences might be sought in the species and/or cultivar and environmental conditions the plants were grown in, as well as in the different growing systems employed (floating raft culture vs. Nutrient Film Technique).

Interestingly, potassium concentration in leaves has shown a positive and significant correlation ($p<0.01$) with carotenoids content, in particular lutein, β-cryptoxanthin and β-carotene (Pearson's coefficient of 0.98, 0.99 and 0.97, respectively). Under half-strength NS K concentration peaked, while under quarter-strength NS K was significantly lower than the control full-strength NS treatment. This tendency could be explained considering the importance of potassium in carotenoid biosynthesis, by influencing key enzymes such as phosphofructokinase and pyruvate kinase (Rouphael et al., 2018). Another minor positive correlation was also noted between Mg leaf content and chicoric acid, which is a derivate of caffeic acid that in turn is influenced by Mg (Supplementary Figure 1) (Rouphael et al., 2018). As for sodium, its leaf concentration increased with the reduction of the NS concentration, and was negatively correlated with potassium leaf content. This can be explained by the substitution of K^+ shortage by Na^+ in non-specific functions such as the maintenance of vacuolar osmotic potential (Wakeel et al., 2011). A spike in leaf Ca^{2+} concentration observed under both moderate and severe NS stress levels, in

comparison to the control treatment, is justified by the role of calcium signaling in plant response to nutrient stress (Akula and Ravishankar, 2011). This higher concentration is detected by Ca^{2+}-sensors or calcium-binding proteins that can trigger calcium-dependent protein kinases responsible of regulating many stress-responsive genes (Tuteja and Mahajan, 2007). Such metabolic reshuffling can influence the efflux and influx of all macro and micro-elements and obviously the final leaf mineral status/concentration.

Table 3. Nitrate, hydrophilic antioxidant activity and total ascorbic acid content of hydroponically grown green and red butterhead lettuce Salanova in relation to nutrient solution concentration mediated by major macronutrients.

Source of variance	Nitrate (mg kg^{-1} fw)	HAA (mmol AA. eq. 100 g^{-1} dw)	Total ascorbic acid (mg AA 100 g^{-1} fw)
Cultivar (C)			
Green Salanova	1720 ± 108	2.08 ± 0.09	15.55 ± 0.47 b
Red Salanova	1501 ± 167	2.10 ± 0.04	34.41 ± 7.15 a
Nutrient solution concentration (S)			
Full strength (1.50 dS m^{-1})	1717 ± 156 a	1.90 ± 0.08 c	16.90 ± 0.72 c
Half strength (0.75 dS m^{-1})	1925 ± 77 a	2.28 ± 0.04 a	38.52 ± 10.82 a
Quarter strength (0.50 dS m^{-1})	1190 ± 119 b	2.10 ± 0.05 b	19.52 ± 1.82 b
C × S			
Green Salanova × full strength	1669 ± 88	1.78 ± 0.12	16.71 ± 1.08 c
Green Salanova × half strength	2075 ± 50	2.34 ± 0.05	14.39 ± 0.04 c
Green Salanova × quarter strength	1416 ± 133	2.13 ± 0.04	15.56 ± 0.33 c
Red Salanova × full strength	1764 ± 334	2.02 ± 0.04	17.09 ± 1.19 c
Red Salanova × half strength	1775 ± 67	2.23 ± 0.04	62.64 ± 1.85 a
Red Salanova × quarter strength	964 ± 44	2.06 ± 0.08	23.49 ± 0.82 b
Significance			
Cultivar (C)	NS	NS	***
Nutrient solution concentration (S)	***	***	***
C × S	NS	NS	***

NS, **, *** Non-significant or significant at $p \leq 0.01$, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (p= 0.05). All data are expressed as mean ± standard deviation, $n = 3$.

4.4.3 Hydrophilic antioxidant activity, ascorbic acid and phenolic compounds

Phenolic compounds have always captivated the attention of researchers since they are linked to sensory quality, healthcare and cardiovascular function and constitute components of the quotidian diet (Kennedy, 2019). Moreover, lettuce is a rich source of polyphenols and ascorbic acid (Kim et al., 2018). The phenolic profiles of red and green lettuce are very similar, except that the latter contains lower concentrations of these compounds compared to red lettuce, and does not produce anthocyanins (Becker et al., 2015) that are characteristic of red-leaf lettuce and contribute to its antioxidant properties. Anthocyanin accumulation in the leaves, turns their color from green to green/red or simply red, and the accumulation correlates with colorimetric redness (Kim et al., 2018). In addition, ascorbic acid is drawn into several biochemical mechanisms and is known for its antioxidant capacity in reducing oxidative free radicals (Lopez et al., 2014; Kim et al., 2016).

In our work, ascorbic acid, which is the major form of vitamin C in plants (Kim et al., 2016), demonstrated significant C × S interaction since green Salanova incurred no significant changes in response to NS treatments whereas in red Salanova half- and quarter-strength NS increased TAA content by 266.5% and 37.4% in comparison to the full strength control, respectively (Table 3). As for HAA, which ranged from 1.8 to 2.3 mmol AA eq. 100 g^{-1} dw, no significant cultivar or C × S interaction effects were observed (Table 3); however, reducing NS concentration to half- and quarter-strength significantly increased HAA content, with the highest increase (+20%) observed at the 50% level (Table 3). Our results of ascorbic acid are not in agreement with Shinohara and Suzuki (1981) who observed an increase in ascorbic acid accumulation when shifting the NS from half to quarter strength, while our highest accumulation was recorded at half strength.

Concentrations of phenolic compounds are reported in Table 4. Four phenolic acids were detected with their total concentration differing berween tested butterhead lettuce cultivars, NS concentrations and their combinations (Table 4). The most abundant compound was chicoric acid, followed by chlorogenic acid, while caffeoyl-meso-tartaric and caffeoyl-tartaric acid were detected in lower amounts. As shown in Table 4, all phenolic acids showed an interaction between the two tested factors, except for caffeoyl tartaric acid. The latter being the only phenolic acid richer in green Salanova, whereas all other phenolic acids accumulated preferentially in red Salanova regardless of the NS concentration. As a result, the sum of phenolic acids in red Salanova (547.8 mg 100 g^{-1}

dw) was higher by 263.3% than the corresponding sum in green Salanova (150.8 8 mg $100g^{-1}$ dw; Table 4). Furthermore, moderate and severe nutrient stress had no effect on chlorogenic acid and caffeoyl meso tartaric acid in green Salanova, but a major impact on red Salanova, where they increased respectively by 192.9% and 79.3% at 50% NS concentration and by 250.8% and 213.5% at 25% NS concentration (Table 4). Chicoric acid increased by 60.4% in green Salanova plants treated with quarter-strength NS compared to the full and half-strength treatments, whereas in red Salanova it increased on average by 109.8%% with half- and quarter-strength NS compared to the control (Table 4). Based on the above findings, it is apparent that the sum of phenolic acids spiked by 161.6% at 50% NS and by 100.2% at 25% NS in red Salanova, though it was statistically invariable in green Salanova (Table 4). Expectedly, anthocyanins were detected only in red Salanova, where their concentration increased by 380.7% and 336.5 % at moderate and severe nutrient stress conditions, respectively (Table 4). These findings are in agreement with Rouphael et al. (2012), who established that phenolic acids and flavonoids concentration varies as a function of nutrient stress and genotype. Gershenzon (1984) also reported that anthocyanins in *Lactuca sativa* L. where positively influenced by N deficiency, while Akula and Ravishanka (2011) attributed anthocyanins accumulation to nutrient deficiency in general.

The current results on phenolics corroborate those of Rouphael et al. (2012), that marked increase in phenolic compounds (specifically chlorogenic acid) and flavonoids is elicited by low solution concentrations (4 and 20 mequiv. L^{-1}). Our data are also in agreement with Alberici et al. (2008), who found a significant increase in the total phenols of lettuce cultivated in a floating raft system under reduced nutrient solution, depending on cultivation season. Analogous increase in the concentration of chicoric acid and chlorogenic acid in lettuce were noted by Qadir et al. (2017) and Becker et al. (2015) under reduced N concentration. Such results imply that N deficiency is a major mediator of phenolic acids accumulation, which is in line with Chishaki and Horiguchi (1997) who observed the most marked effect on phenolic acid levels in rice plants in response to N deficiency, followed by P deficiency and lastly by K deficiency which had a minor effect.

Moreover, anthocyanins and the sum of phenolic acids exhibited significant (p<0.01) positive correlation with HAA (Pearson's coefficient of 0.98, 0.99 and 0.97, respectively), since all three variables increased significantly at half and quarter-strength, in comparison to the control treatment, with the highest levels attained in red Salanova irrigated with half-strength NS. These correlations can be traced to the activation by stress treatments (heat

shock, chilling and high light) of genes such as phenylalanine ammonia-lyase (PAL) and L-galactose dehydrogenase involved respectively in the biosynthesis of phenolics and ascorbic acid, which contribute to lettuce HAA (Oh et al., 2009). The activation of these genes might also be triggered by other kinds of stress, such as nutrient deprivation in the current case, more precisely nitrogen deficiency, which is in line with the findings of Becker et al. (2015), who declared that the expression of phenylalanine genes is more extensive under nitrogen depletion.

Gershenzon (1984) explained that high phenolic concentration is an outcome of *de novo* synthesis, rather than a deceleration of primary metabolism, whereby nutrient deprivation can promote the synthesis of phenolic compounds by raising the activity of PAL or by increasing its precursors such as phenylalanine. Moreover, when protein synthesis slows down in such stress conditions, unused carbohydrates are rerouted to phenolic synthesis (Akula and Ravishankar, 2011). The accumulation of these phenolics in plants, is an adaptation to stress since they serve as internal growth inhibitors and slow down plant metabolism within the scope of survival (Gershenzon, 1984).

Table 4. phenolic acids composition, total phenolic acids and anthocyanins of hydroponically grown green and red butterhead lettuce Salanova in relation to nutrient solution concentration mediated by major macronutrients.

Source of variance	Caffeoyl tartaric acid (mg 100 g^{-1} dw)	Chlorogenic acid (mg 100 g^{-1} dw)	Chicoric acid (mg 100 g^{-1} dw)	Caffeoyl meso tartaric acid (mg 100 g^{-1} dw)	∑ phenolic acids (mg 100 g^{-1} dw)	Anthocyanins (μg cyanidin eq. g^{-1} dw)
Cultivar (C)						
Green Salanova	21.70 ± 1.62 a	12.02 ± 1.05 b	111.52 ± 9.84 b	5.59 ± 0.62 b	150.84 ± 12.68 b	n.d.
Red Salanova	12.15 ± 1.19 b	217.87 ± 33.38 a	246.48 ± 28.45 a	71.34 ± 11.10 a	547.84 ± 71.63 a	34.96 ± 6.21
Nutrient solution concentration (S)						
Full strength (1.50 dS m^{-1})	12.48 ± 2.05 c	61.72 ± 23.90 c	115.67 ± 15.43 b	16.14 ± 5.60 b	206.01 ± 41.08 b	n.a.
Half strength (0.75 dS m^{-1})	16.94 ± 2.25 b	173.12 ± 73.55 a	208.35 ± 51.18 a	51.40 ± 21.08 a	449.81 ± 143.7 a	n.a.
Quarter strength (0.50 dS m^{-1})	21.37 ± 2.48 a	109.99 ± 42.47 b	212.98 ± 29.06 a	47.86 ± 17.86 a	392.20 ± 86.66 a	n.a.
C × S						
Green Salanova × full strength	17.01 ± 0.17	9.20 ± 0.24 d	89.01 ± 1.97 c	4.28 ± 0.21 d	119.50 ± 2.04 d	n.d.
Green Salanova × half strength	21.37 ± 2.36	11.61 ± 0.91 d	96.76 ± 4.16 c	4.56 ± 0.34 d	134.31 ± 6.86 d	n.d.
Green Salanova × quarter strength	26.72 ± 1.44	15.25 ± 1.77 d	148.80 ± 9.19 b	7.94 ± 0.52 d	198.71 ± 10.23 d	n.d.
Red Salanova × full strength	7.94 ± 0.62	114.24 ± 9.93 c	142.33 ± 21.82 b	28.00 ± 3.99 c	292.51 ± 30.87 c	10.31 ± 1.49 c
Red Salanova × half strength	12.50 ± 0.20	334.63 ± 31.03 a	319.94 ± 25.08 a	98.24 ± 5.17 a	765.32 ± 60.38 a	49.56 ± 0.47 a
Red Salanova × quarter strength	16.02 ± 0.18	204.73 ± 6.37 b	277.16 ± 4.26 a	87.78 ± 0.53 b	585.69 ± 3.14 b	45.00 ± 0.62 b
Significance						
Cultivar (C)	***	***	***	***	***	-
Nutrient solution concentration (S)	***	***	***	***	***	-
C × S	NS	***	***	***	***	***

NS, **, *** Non-significant or significant at $p \leq 0.01$, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (p= 0.05) All data are expressed as mean ± standard deviation, $n = 3$.

The interest in fat-soluble pigments such as carotenoids is not recent, owing to their beneficial effects on human well-being (Kim et al., 2016; Baenas et al., 2014). Carotenoids are C_{40} isoprenoid polyene plant compounds divided into oxygenated xyanthophylls and hydrocarbon carotenes (Kospell et al., 2007), which are pigments of the light harvesting complex of lettuce photosystem II, also engaged in non-photochemical quenching (heat dissipation) and are also potent Reactive Oxygen Species (ROS) scavengers (Kospell et al., 2007; Becker et al., 2015). The dietary intake of carotenoids is related with reduction of degenerative diseases, more specifically chronic eye impairments, including cataract and macular degeneration (Kopsell et al., 2007) while their bioactive value is also studied for the prevention of cancers and cardiovascular diseases (Baenas et al., 2014).

In the current study, all the carotenoids detected are presented in Table 5. Violaxanthin + neoxanthin, lutein, β-cryptoxanthin and β-carotene were higher in red Salanova than in green Salanova by 64.1%, 122.0%, 127.4% and 80.0%, respectively, regardless of the NS concentration. No significant (C) × (S) interaction was noted with respect to the concentrations of carotenoids compounds. Averaged over both cultivars, moderate nutrient stress (half-strength NS) increased the accumulation of violaxanthin + neoxanthin compared to full and quarter-strength treatments. Furthermore, the application of 50% of the macronutrient concentration in the NS increased β-cryptoxanthin by 10.4% and 33.7% and β-carotene by 10.7% and 19.4%, respectively, compared to full and quarter-strength NS treatments (Table 5).

The Fallovo et al. (2009) findings, which showed no effect of nutrient solution concentration on carotenoids, are not in harmony with the results of our study. Similarly, Vernieri et al. (2006) found that rocket total carotenoids were unchanged at NS 25% and decreased significantly at NS 10% in comparison to the control treatment, unlike Alberici et al. (2008) who found a significant increase in lettuce carotenoid content with the reduction of the NS concentration in one of the seasons studied. Our results cast new light onto the findings of certain authors, reviewed by Rouphael et al. (2018), who claimed that antioxidants responded more strongly to light and temperature than to nutrient solution electrical conductivity. Furthermore, Rouphael and co-workers as well as Becker et al. (2015) also mentioned that it is also expected to have a stimulation of PSMs such as β-carotene due to N starvation conditions. Although such N deficiency would be expected to decrease carotenoids concentration in lettuce leaves because they are part of a photosystem down-sized by lack of chlorophyll, their

concentration increased because of ROS formation in mineral-deficient plant roots up to a certain limit (Becker et al., 2015).

Table 5. Composition of soluble pigments of hydroponically grown green and red butterhead lettuce Salanova in relation to nutrient solution concentration mediated by major macronutrients.

Source of variance	Violaxanthin + neoxanthin (μg violaxanthin eq. g^{-1} dw)	Lutein (μg g^{-1} dw)	β-Cryptoxanthin (μg g^{-1} dw)	β-carotene (μg g^{-1} dw)
Cultivar (C)				
Green Salanova	680.32 ± 29.81 b	303.43 ± 15.18 b	444.65 ± 26.07 b	217.10 ± 12.79 b
Red Salanova	1116.39 ± 47.59 a	673.54 ± 25.09 a	1011.37 ± 41.03 a	390.65 ± 12.77 a
Nutrient solution concentration (S)				
Full strength (1.50 dS m^{-1})	813.66 ± 80.79 b	505.87 ± 79.98 a	747.14 ± 121.04 b	307.79 ± 39.87 b
Half strength (0.75 dS m^{-1})	1007.52 ± 102.98 a	538.83 ± 90.69 a	822.15 ± 140.45 a	340.66 ± 37.99 a
Quarter strength (0.50 dS m^{-1})	873.89 ± 122.57 b	420.75 ± 80.65 b	614.74 ± 122.71 c	263.18 ± 40.79 c
C × S				
Green Salanova × full strength	643.09 ± 11.68	327.33 ± 5.26	477.96 ± 5.99	219.16 ± 6.57
Green Salanova × half strength	784.66 ± 32.28	337.62 ± 0.75	511.24 ± 10.32	257.90 ± 1.82
Green Salanova × quarter strength	613.20 ± 33.11	245.33 ± 13.37	344.76 ± 15.92	174.24 ± 12.76
Red Salanova × full strength	984.23 ± 58.36	684.41 ± 8.90	1016.32 ± 27.58	396.43 ± 7.06
Red Salanova × half strength	1230.37 ± 48.13	740.04 ± 25.32	1133.07 ± 43.10	423.42 ± 19.09
Red Salanova × quarter strength	1134.57 ± 77.84	596.17 ± 39.64	884.72 ± 46.34	352.11 ± 15.66
Significance				
Cultivar (C)	***	***	***	***
Nutrient solution concentration (S)	**	***	***	***
C × S	NS	NS	NS	NS

NS, **, *** Non-significant or significant at $p \leq 0.01$, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (p= 0.05) All data are expressed as mean ± standard deviation, $n = 3$.

4.5 Principal component analysis

To obtain an in-depth overview of crop productivity, growth parameters, mineral profile as well as nutritional and functional quality traits of the studied green- and red-pigmented butterhead lettuce in response to nutrient solution concentration mediated by major macronutrients (full strength, FS; half strength, HS; quarter strength, QS) a principal component analysis (PCA) was conducted for all the agronomical and physico-chemical composition parameters measured and discussed above. The first three principal components (PCs) were associated with eigen values > 1 and explained 94.5% of the cumulative variance, with PC1 accounting for 47.5%, PC2 for 37.4% and PC3 for 9.6% (Supplementary Table S1). PC1 was positively correlated to all target carotenoids, yield on fresh and dry basis, LUE, chlorogenic acid, caffeoyl-meso-tartaric, anthocyanins and total phenolics content. PC1 was also negatively correlated to caffeoyl-tartaric acid content. Moreover, PC2 was positively correlated to chicoric acid, leaf dry matter, Na, Mg and Ca content; and negatively correlated to nitrate, and major macro-minerals (N, P and K) (Supplementary Table S1). Furthermore, the loading matrix indicates the correlations among the examined quanti-qualitative traits. In our study, variation in chlorogenic acid and β-carotene contents were most closely aligned with total ascorbic acid and lutein content, respectively, whereas variation in chicoric acid content was not correlated to nitrate content (Figure 1).

The effectiveness of PCA for plotting species and/or cultivar characterization as well as its interpretive usefulness on multiple nutritional and functional quality traits in relation to several preharvest factors has been reported previously in a series of scientific studies.[42-45] This was also the case in our controlled experiment, since the score plot of the PCA integrated information on the bioactive profile of the tested butterhead cultivars exposed to different nutrient solution concentration: green-pigmented lettuce distinguished for caffeoyl-tartaric acid, leaf dry matter percentage, Ca, Mg and Na content; whereas red-pigmented Salanova was superior in yield, yield components, target lipophilic and hydrophilic antioxidant molecules as well as in total phenolic acids (Figure 1). Particularly, the red-pigmented lettuce treated with half-strength NS was positioned on the positive side of PC1 in the upper right quadrant of the PCA score plot as it delivered leaves of premium quality with high concentration of hydrophilic and lipophilic antioxidants (Figure 1). Moreover, red-pigmented Salanova lettuce irrigated with full strength NS was characterized by higher yield on fresh and dry weight basis, growth parameters as well as

N and K content. Green Salanova lettuce grown under quarter-strength NS was positioned in the upper left quadrant, characterized overall by higher caffeoyl-tartaric acid and bivalent cations (Ca and Mg) (Figure 1). Finally, the lower left quadrant depicted treatments (green lettuce treated with full and half strength NS) characterized by the lowest nutritional and functional quality (Figure 1). The PCA carried out in this experiment facilitated a broad view of yield and quality traits reinforced by ion chromatography and HPLC outputs and enabled the identification of phenotypic variation patterns with respect to significant nutritional and functional quality characteristics underscored by variation in the genetic material and nutrient solution concentration.

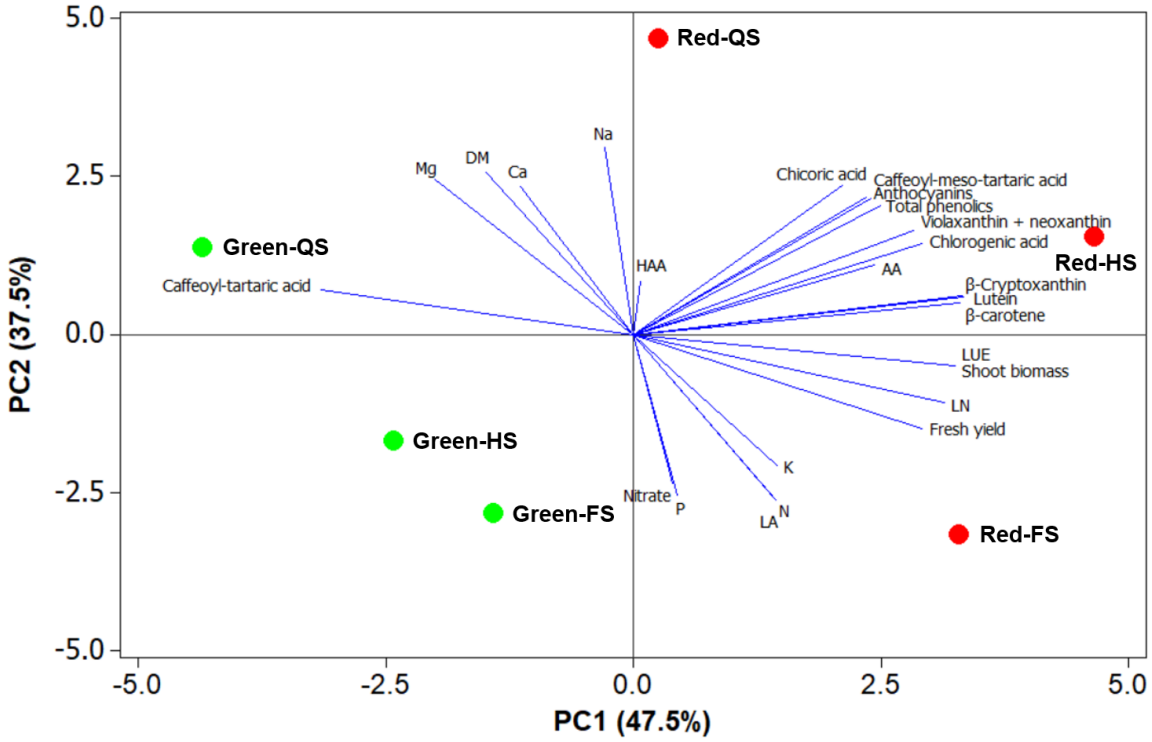

Figure 1. Loading plot and scores of principal component analysis (PCA) for growth parameters, fresh yield, shoot dry biomass mineral concentrations (nitrate, P, K, Ca, Mg and Na), lipophilic and hydrophilic antioxidant molecules (target phenolic acids and total phenolics, anthocyanins, ascorbic acid and carotenoids) in green and red butterhead lettuce Salanova grown under different nutrient solution concentration mediated major macronutrients (Full strength [FS], half strength [HS], quarter strength [QS].

Supplementary Table S1. Eigen values, relative and cumulative proportion of total variance, and correlation coefficients for growth parameters, mineral profile, nutritional and functional traits of butterhead lettuce with respect to the three principal components.

Principal components	PC1	PC2	PC3
Eigen value	11.8	9.3	2.4
Percentage of variance	47.5	37.4	9.6
Cumulative variance	47.5	84.9	94.5
Eigen vectors [a]			
β-Cryptoxanthin	**0.968**	0.198	0.005
Lutein	**0.964**	0.200	-0.030
β-carotene	**0.956**	0.164	0.066
Shoot biomass	**0.944**	0-.159	-0.264
LUE	**0.943**	-0.157	-0.267
Caffeoyl-tartaric acid	**-0.916**	0.230	0.259
LN	**0.913**	-0.348	-0.204
Violaxanthin +	**0.847**	0.472	0.120
Fresh yield	**0.847**	-0.482	-0.127
Chlorogenic acid	**0.820**	0.539	0.132
Total phenolics	**0.722**	0.670	0.111
AA	**0.705**	0.364	0.361
Na	-0.085	**0.970**	-0.104
N	0.416	**-0.854**	0.070
LA	0.410	**-0.842**	-0.034
DM	-0.431	**0.837**	-0.221
P	0.128	**-0.825**	0.165
Mg	-0.582	**0.805**	-0.069
Chicoric acid	0.612	**0.773**	0.092
Ca	-0.332	**0.768**	0.537
Nitrate	0.114	**-0.765**	0.628
Anthocyanins	**0.682**	**0.711**	0.034
Caffeoyl-meso-tartaric	**0.697**	**0.705**	0.032
K	0.422	**-0.674**	0.599
HAA	0.022	0.274	**0.905**

4.6 Conclusion

Epidemiologic data emphasize the association of high vegetable-rich diet with low risk of chronic diseases, due to their high and varied contribution in bioactive compounds to the human diet. Our work supports the idea of Rouphael and Kyriacou (2018) that closed soilless cultivation systems provide a consistent and optimized year round production, an efficient and precise path of nutritional *eustress* that facilitates the strategic accumulation of bioactive compounds complementing or even substituting expensive breeding programs. We demonstrated that a 50% decrease of macronutrient input in the NS caused a marginal decrease in yield of both Salanova cultivars (~14%), but on the other side outstandingly improved qualitative characteristics, pronouncedly in red Salanova. The latter showed a 266% increase in ascorbic acid, 162% in total phenolic acids, 381% in anthocyanins, 25% in violaxanthin + neoxanthin, 8% in lutein, 11% in β-cryptoxanthin and 7% in β-carotene, compared to the control treatment. These results showcase how such a strategy can increase nutrient use efficiency and modulate secondary metabolism to improve the functional quality of red butterhead lettuce. Cultivation in a half-strength nutrient solution can boost the nutraceutical characteristics of red Salanova and make it a nutrient-dense food.

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5 Chapter 5: The bioactive profile of lettuce produced in a closed soilless system as configured by combinatorial effects of genotype and macrocation supply composition

5.1 Abstract

In the present study, the effect of cultivar and macrocation proportions (K/Ca/Mg) on bioactive compounds content of hydroponically cultivated lettuce was evaluated. Two lettuce cultivars (red and green-pigmented Salanova®) were grown in a fully controlled growth chamber (Fitotron®). Fresh weight and color attributes were higher in green Salanova and S_K treated plants, while elevated macrocation proportions (S_K, S_{Ca}, and S_{Mg}) affected corresponding minerals and P and Na content. Ascorbic acid was higher for S_{Ca} and S_{Mg} treatments, while red Salanova and S_{Ca} and S_{Mg} treated plants contained lower amounts of nitrates. Chicoric and chlorogenic acid were the main phenolic compounds in S_{Mg}, and S_{Ca} and S_{Mg} red Salanova plants, respectively. Moreover, red Salanova plants treated with elevated Mg (S_{Mg}) contained higher amounts of pigments. In conclusion, nutrient solution management could be used as an effective cultural practice to increase bioactive properties and quality of hydroponically grown lettuce.

5.2 Introduction

Within the coming decades, global crop production will confront severe pressure from increasing population, ensuing climate change and depletion of natural resources; therefore, corrective measures are imperative to ensure food security (Shahbaz et al., 2012) However, apart from the urgent issue of food security there is also a rising demand for crop products of high nutritional value and enhanced bioactive content (Rouphael and Kyriacou, 2018). This is especially the case for vegetable products which, due to their high content in vitamins, polyphenols and antioxidant compounds, play a vital role in the human diet and are increasingly pivotal in components consumers' awareness of functional food quality and bioactive value (Micha et al., 2015). Accordingly, over the past two decades, food nutritionists and scientists have re-evaluated the wider significance quality aspects associated to bioactive phytochemical content and related health-promoting properties of fresh products that enter the horticultural supply chain (Khanam et al., 2012). Recently, Kyriacou and Rouphael (2017) identified the significance of intrinsic (dictated by

genotypic, agroenvironmental and postharvest factors) and extrinsic (influenced by socioeconomic and marketing factors) characteristics that configure the quality of fresh vegetables and suggested ways of controlling these factors towards optimizing quality. Controlled environments and, particularly, closed soilless hydroponic systems are considered the most effective production system in regards to quality control, especially for fresh leafy vegetables (Rouphael and Kyriacou, 2018; Rouphael et al., 2018). For example, Fallovo et al. (2009) reported that the macronutrient composition of nutrient solutions has a significant impact on chemical the composition of *Lactuca sativa* L. var. *acephala*. Moreover, according to Fanasca et al. (2006) the adjustment of nutrient solution cationic proportions (K/Ca/Mg) has a significant impact on tomato fruit quality with a cation-specific effect on quality parameters (e.g. increased Ca improved yield and reduced blossom end rot; increased Mg and K improved the levels of antioxidant compounds). In a recent study, Barickman et al. (2016) investigated the effect of increased K content in nutrient solution on lettuce mineral composition and demonstrated the importance of macrocation proportions on yield and quality. In contrast, Luna et al. (2013) pointed out that genotype and growing conditions have a stronger effect than nutrient solution composition on the post-harvest quality of lettuce, although the nutrient solutions tested were within the optimum range for plant nutrition. Moreover, considering the important role of vegetables in the human diet as sources of mineral elements, an appropriate regulation of nutrient solution composition in soilless hydroponic systems could affect the biofortification of mineral content in vegetable tissues (Karley and White, 2009). Przybysz et al. (2016) evaluated the potential of lettuce biofortification with Mg and suggested that cultivar selection is crucial to achieve high Mg content in leaf tissues without undesirable side-effects on quality. Another factor to be considered when adjusting nutrient solution composition is the interaction between cations due to antagonistic or synergistic effects that may cause nutrient imbalance in plant tissues which can severely affect quality (Zhang et al., 2017).

On a global scale, lettuce (*Lactuca sativa* L.) constitutes the most important leafy vegetable with approximately 27 million metric tons produced annually (FAOSTAT, 2019). It is usually consumed in various types of salads (mixed and/or fresh-cut salads, and baby leaf products) and is considered one of the most popular vegetables in the world (Kim et al., 2016). Dependent on the leaf type and growing conditions, its edible leaves present high nutritional value (Baslam et al., 2013). Fallovo et al. (2009) and Mou (2012) reported

significant differences in macro- and micronutrients content among lettuce genotypes of variable head/leaf types and colors, while Qin et al. (2018) and Mou (2005) detected significant variation in polyphenolic composition and carotenoids content among several lettuce cultivars. Similarly, Seo et al. (2009) suggested a strong correlation of the nutrient solution S, Mg and P contents with the sesquiterpene lactones content, responsible for lettuce bitter taste, and consequently for consumers acceptance and overall product marketability. Moreover, Llorach et al. (2008) underlined the importance of lettuce varietal type/ genotype for the phenolic composition and antioxidant capacity, suggesting that the higher antioxidant capacity of the red-pigmented types than the green-pigmented ones was due to their higher content of bioactive compounds.

Although a lot of information in the scientific literature is currently available concerning the management of nutrient solution concentration (NaCl and/or macronutrients concentration) for achieving biofortification with essential elements, there is scarcity of information concerning the effects of macronutrient proportions and especially their interaction with cultivar effect for leafy vegetables such as lettuce. For this purpose, an experiment was carried out under controlled environment conditions in order to investigate the effect of genotype (red-pigmented and green pigmented Salanova® cultivars) and macrocation proportions (K/Ca/Mg) on the bioactive compounds content of hydroponically cultivated butterhead lettuce.

5.3 Material and methods

5.3.1 Standards and chemicals

Acetonitrile, methanol water and dichloromethane for liquid chromatography diode array detection (LC-DAD) analysis and liquid chromatography tandem mass spectrometry (LC-MS/MS) were obtained from Merck (Darmstadt, Germany). Ethanol absolute and chloroform were obtained from VWR Chemicals (Radnor, PA); hexane, butylated hydroxytoluene (BHT), formic acid (99% for mass spectrometry) along with analytical standards (chicoric acid, chlorogenic acid, lutein, β-carotene, violaxanthin, neoxanthin, β-cryptoxanthin, and cyanidin) were purchased from Sigma-Aldrich (St. Louis, MO). Ultrapure water was obtained from a Milli-Q Gradient A10 water purification system.

5.3.2 Growth chamber conditions, plant material, experimental design and nutrient solution management

The experiment was carried out in a 28 m^2 open-gas-exchange growth chamber (7.0 × 2.1 m × 4.0 m, W × H × D), located at the experimental farm of the Department of Agricultural Sciences, University of Naples Federico II, Italy. Light was provided by High Pressure Sodium lamps, with an intensity of 420 µmol/m^2/s according to a light/dark regime of 12/12 h. Temperature was set at 24/18 °C (light/dark) and relative humidity was kept at 60-80% respectively, by using a fog system. The experiment was carried out at ambient CO_2 concentration (370-410 ppm), and air exchange was performed by means of one air extractor.

Two cultivars of butterhead lettuce (*Lactuca sativa* L. var. *capitata*), green Salanova and red Salanova (Rijk Zwaan, Der Lier, The Netherlands) were grown in a Nutrient Film Technique (NFT) growing system. Nutrient solution was pumped and delivered at a flow rate of 1.5 L/min at the top end of each NFT channel and allowed to run slowly down the trough, while the excess of nutrient solution was collected in 25 L polypropylene tanks. The NFT gullies were 200 cm long, 14.5 cm wide and 8 cm deep, with 1% inclination. Lettuce seedlings were transplanted in rockwool cubes (7 × 7 × 7 cm) (Delta, Grodan, Roermond, The Netherlands). Plants intra-row and inter-row spacing was 15 cm and 43 cm, respectively accounting for a total plant density of 15.5 plants/m^2. Each gully was covered with propylene taps to avoid the evaporation of nutrient solution.

Six treatments derived from a factorial combination of two lettuce cultivars (green and red Salanova) and three nutrient solutions of different cationic proportions (S_K =0.68 K/0.16 Ca/0.16 Mg; S_{Ca}= 0.16 K/0.68 Ca/0.16 Mg; S_{Mg} = 0.16 K/0.16 Ca/0.68). The treatments were arranged in a randomized complete-block design with three replications per treatment, amounting to a total of 18 experimental units. Each experimental unit consisted of 12 plants, yielding a total number of 216 green and red Salanova plants. The first two and last two plants of each NFT channel (i.e. experimental unit) were considered as guards, and the morphological, colorimetric and chemical composition measurements were conducted on each of the eight remaining plants per experimental unit.

In all nutrient solution (S) treatments, the macro-anion proportions were as following: 0.80 NO_3/0.10 SO_4/0.10 H_2PO_4, and the ratio between anions (NO_3^- + SO_4^{2-} + $H_2PO_4^-$) and cations (K^+ + Ca^{2+} + Mg^{2+}) was equal to one. The total macronutrients concentration was 24 meq/L. In all treatments, the concentrations of macroanions (proportion × total

concentration; expressed in mM) were: NO_3^- (9.6), SO_4^{2-} (0.6), $H_2PO_4^-$ (1.2); the concentrations of macrocations expressed in mM were: K^+ (8.11), Ca^{2+} (0.97), Mg^{2+} (0. 97) for S_K treatment; K^+ (1.94), Ca^{2+} (4.06), Mg^{2+} (0.97) for S_{Ca} treatment; K^+ (1.94), Ca^{2+} (0.97), Mg^{2+} (4.06) for S_{Mg} treatment. The micronutrients concentrations (expressed in μM) in all nutrient solution treatments were as following: 15 Fe, 9 Mn, 0.3 Cu, 1.6 Zn, 20 B and 0.3 Mo. The electrical conductivity (EC) and pH were adjusted at 1.5 ± 0.1 dS/m and 6.0, respectively.

5.3.3 Growth parameters, marketable yield, leaf colour measurements and collection of lettuce samples

Nineteen days after transplantation (DAT), the number of leaves per plant was recorded on eight red and eight green lettuce plants per experimental unit. The total leaf area per plant was measured using an electronic area meter (Li-Cor3000, Li-Cor, Lincoln, NE, USA). The fresh yield of red and green-pigmented lettuce was recorded immediately after harvest. Samples of fresh leaf tissues were dried at 70 °C for 3 d until they reached a constant weight, which was determined on an analytical balance (Denver Instruments, Denver, Colorado, USA) for dry biomass assessment. Light use efficiency (LUE) was expressed as the shoot dry biomass divided by cumulative daily intercepted photosynthetically active radiation. Just before harvest, leaf color was measured on the upper part of eight leaves per replication, using an 8 mm-aperture Minolta CR-300 Chroma Meter (Minolta Camera Co. Ltd, Osaka, Japan). The Chroma meter was calibrated with a Minolta standard white plate before sampling red and green butterhead lettuce leaves. The Commission internationale de l'éclairage (CIE) color space parameters recorded in the present study were lightness (L*; ranging from 0 = black to 100 = white) and chroma ($C^* = (a^{*2}+b^{*2})^{1/2}$).

Oven-dried leaf tissues were used for mineral composition profile determination. For the identification and quantification of lipophilic (carotenoids) and hydrophilic (ascorbic acid and phenolic compounds) antioxidant compounds by HPLC, fresh leaf samples from four plants per experimental plot were instantly frozen in liquid nitrogen and stored at -80 ºC before lyophilized in a Christ, Alpha 1-4 (Osterode, Germany) freeze drier.

5.3.4 Mineral composition analysis

The desiccated green and red butterhead leaf tissues were ground in a Wiley Mill to pass through an 841 µm screen and used for macro-mineral profile analysis and sodium content as described in details by Rouphael et al. (2017). Briefly, 0.25 g of dry tissue were suspended in 50 ml of ultrapure water (Milli-Q, Merck Millipore, Darmstadt, Germany) and subjected to four freeze-thaw cycles in liquid N followed by incubation in a shaking water bath (ShakeTemp SW22, Julabo, Seelbach, Germany) at 80 °C for 10 min. The suspension was centrifuged at 6000 rpm for 10 min (R-10 M, Remi Elektrotechnik Limited, India), then filtered through a 0.20 µm filter paper (Whatman International Ltd., Maidstone, U.K.). Nitrate, phosphorus, potassium, calcium, magnesium and sodium were separated and quantified by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA) coupled to a conductivity detector. An IonPac CG12A (4×250 mm, Dionex, Corporation) guard column and IonPac CS12A (4 × 250 mm, Dionex, Corporation) analytical column were used for the K, Ca, Mg and Na analysis, while for NO_3-N and P determination, an IonPac AG11-HC guard (4 × 50 mm) column and IonPac AS11-HC analytical column (4 × 250 mm) were adopted. Injection volume was 25 µL and a flow rate of 2 mL/min was set in isocratic mode for a total run time of 15 min. The solvents used were NaOH 5 mM for NO_3-N and P and CH_4O_3S 20 mM for K, Ca, Mg and Na. Ion concentrations were determined against standard curves of anions or cations in the range 0.05-0.5 mM and expressed as g/kg dry weight (dw). NO_3-N concentration was expressed in mg/kg fresh weight (fw) on the basis of each sample's original dry weight content (dw), while P, K, S, Ca, Mg and Na concentrations were expressed in g/kg dw.

5.3.5 Extraction and quantification of ascorbic acid

Total ascorbic acid (TAA) was assessed by spectrophotometric assay based on the reduction of Fe^{3+} to Fe^{2+} by ascorbic acid (AA) and the spectrophotometric detection of Fe^{2+} complexes with 2,2-dipyridyl based on the protocol of Kampfenkel et al. (1995). Dehydroascorbate was first reduced to ascorbic acid by pre-incubation of the sample in dithiothreitol. Quantification was performed at 525 nm against an external ascorbate standard calibration curve in the range of 5-100 µmol/mL and the results were expressed as mg AA 100 g fw.

5.3.6 Phenolic acids and anthocyanins identification and quantification

Phenolic acids as hydroxycinnamic derivatives were extracted according to the procedure previously reported by Llorach et al. (2008). A mixture of methanol/water/formic acid (50/45/5, v/v/v, 12 mL) was added to 400 mg of freeze-dried samples. The suspensions were sonicated for 30 min and then subjected to centrifugation (2500 × g for 30 min at 4 °C). The supernatants were collected for a second centrifugation at 21100 × g for 15 min at 4 °C; before LC analysis, each sample was filtered through 0.22 µm cellulose filters (Phenomenex). Separation of hydroxycinnamic derivatives and anthocyanins was achieved through a reversed phase C18 column (Prodigy, 250 × 4.6 mm, 5 µm, Phenomenex, Torrance, CA) equipped with a C18 guard column (4.0 × 3.0 mm, Phenomenex) by using (A) water formic acid (95:5, v/v), and (B) methanol as solvents for building the following gradient of solvent B, (t in [min]/[%B]): (0/5), (25/40), (32/40). The flow rate was 1 mL/min and 20 µL of each extract was injected; LC column was installed onto a binary system (LC-10AD, Shimadzu, Kyoto, Japan), equipped with a DAD (SPD-M10A, Shimadzu, Kyoto, Japan) and a Series 200 auto-sampler (Perkin Elmer, Waltham, MA). Calibration curves of hydroxycinnamic derivatives were produced by using standard compounds of chlorogenic acid and chicoric acid at 330 nm. Identification of caffeoyl-meso-tartaric acid and caffeoyl-tartaric acid was performed by LC-MS/MS. The chromatographic profiles of reference curves and samples were recorded in multiple reaction monitoring mode (MRM) by using an API 3000 triple quadrupole (ABSciex, Carlsbad, CA). Negative electrospray ionization was used for detection and source parameters were selected as follows: spray voltage -4.2 kV; capillary temperature: 400 °C, dwell time 100 ms; nebulizer gas and cad gas were set to 10 and 12 respectively (arbitrary units). Target compounds $[M-H]^-$ were analyzed using mass transitions given in parentheses: chicoric acid (*m/z* 473→311, 293), chlorogenic acid (*m/z* 353 →191), caffeoyl tartaric acid (*m/z*311→ 179, 149, retention time 15.8 min), caffeoyl-meso-tartaric acid (*m/z*311→ 179, 149, retention time 17.8 min). The concentration of phenolic acids was expressed as mg 100/g dw. Within the same LC-DAD chromatographic runs, anthocyanins were monitored at 520 nm and their concentration was calculated by using cyanidin as reference standard. The results were reported as µg of cyanidin equivalent per g of sample dw.

5.3.7 Carotenoids extraction and quantification

Carotenoids were monitored as previously reported by Vallverdú-Queralt et al. (2013) with slight modifications. A mixture of ethanol/hexane (4:3, v/v, 2.5 mL) with 1% BHT were added to 0.1 g of freeze dried samples. The suspension was vortexed at 22 °C for 30 s and sonicated for 5 min in the dark. After centrifugation (2500 × g, 4 °C, 10 min) and filtration through 0.45 µm nylon filters (Phenomenex, Torrance, CA), the supernatants were collected in a volumetric flask; the procedure was repeated three times. The extracts were dried under a gentle flow of nitrogen and stored at -20 °C until analysis. The dried extracts were dissolved in 1% BHT in chloroform and 20 µL of each sample was injected onto a C18 column (Prodigy, 250 × 4.6 mm, 5 µm, Phenomenex, Torrance, CA) equipped with a C18 security guard (4.0 × 3.0 mm, Phenomenex). The following mobile phases: (A) acetonitrile, hexane, methanol, and dichloromethane (4:2:2:2, v/v/v/v) and (B) acetonitrile were used. Carotenoids were eluted at 0.8 mL/min through the following gradient of solvent B (t in [min]/[%B]): (0/70), (20/60), (30/30), (40/2). Quantitation of carotenoids was achieved through a binary LC10AD system connected to a DAD, SPD-M10A (Shimadzu, Kyoto, Japan) equipped with a Series 200 autosampler (Perkin Elmer, Waltham, MA). Quantification was performed by using violaxanthin, neoxanthin, β-cryptoxanthin, lutein and β-carotene as reference standards. The identification of peaks was achieved by comparison of UV-vis spectra and retention times of eluted compounds with pure standards at 450 nm. Three separate sets of calibration curves were built, each set was injected three times in the same day (intraday assay) and three times in three different days (inter-day assay). The accuracy was reported as the discrepancies between the calibration curves performed intra-day and inter-day and the results are expressed as relative standard deviation RSD (%). A recovery test was performed by spiking two samples with two known amounts of carotenoids (50 and 100 µg/mL concentration) and taking into account the overestimation due to the target analytes already present in the samples. Concentrations were reported as µg/g of sample dw.

5.3.8 Statistical analysis

Experimental data were subjected to analysis of variance (two-way ANOVA) using the software package SPSS 13 for Windows 2001. To separate treatment means within each measured parameter, the Duncan's Multiple Range Test was performed at $P \leq 0.05$.

Principal component analysis (PCA) was performed on leaf morphometric traits, mineral composition, ascorbic acid, phenolics and carotenoids profile and to individuate quality traits that were most effective in discriminating between genetic materials (green and red-pigmented butterhead lettuce) and cationic proportions (K/Ca/Mg) in the nutrient solution by using Minitab 16.2.1 statistical software. Calculations and analyses were also carried using the appropriate functions within SPSS in order to derive the score plot and loading matrix based on the first and second principal components (PCs).

5.4 Results and discussion

5.4.1 Fresh yield, biomass production and colorimetric attributes

The results regarding growth and yield parameters of red and green Salanova butterhead lettuce are presented in Table 1. For most of the measured parameters no significant interaction between cultivar (C) and nutrient solution composition (S) was observed, except for the case of leaf number per plant and dry biomass. In particular, red Salanova plants had higher leaf area, whereas green Salanova plants showed higher fresh and dry biomass weight per plant, as well as the highest values for light use efficiency (LUE), lightness (L*) and Chroma (Table 1). Regarding the effect of nutrient solution composition (S), increased K proportions (S_K) resulted in higher leaf area, fresh biomass weight per plant and Chroma values, while increased proportions of K (S_K) and Mg (S_{Mg}) had a beneficial effect on lightness (L*) values (Table 1). Dry biomass and LUE were not affected by nutrient solution. Especially for leaf number and dry matter content (%), a significant interaction between the tested factors was observed wherein the highest leaf number and dry matter content was recorded on S_K-treated and S_{Mg}-treated green Salanova plants, respectively (Table 1). The results of this study are in agreement with those reported by Barickman et al. (2016) who observed a positive quadratic response between K levels and fresh weight of red Romaine lettuce plants. However, they also mentioned that positive effects were recorded up to a certain K content and excessive amounts of K resulted in hampered plant growth probably due to elevated electrical conductivity (EC) values (Barickman et al., 2016). Similarly, according to Fallovo et al. (2009) nutrient solution concentration and growing season may affect growth parameters such as yield, dry biomass, leaf area index, and shoot/root ratio. In contrast, Fallovo et al. (2009) did not find a significant effect of elevated K levels on growth parameters of hydroponically grown

lettuce plants. These inconsistencies in the scientific literature could be the result of differences in the composition of nutrient solutions regarding cation and anion proportions as well as in their final concentrations. Both of these parameters are crucial for plant growth since they may result in competition for cation uptake (Barickman et al., 2016) and elevated EC values (Fanasca et al., 2006). Besides, the observed differences in color attributes and LUE could be associated with genotypic differences, although elevated K levels may also have a beneficial effect. It is worth noting that no significant effects on LUE were recorded in respect to nutrient solution composition. It could be expected that elevated Mg would increase photosynthetic rate and, consequently, LUE compared to the other treatments. However, as already reported high Mg rates may impair photosynthesis due to inhibition of K transportation and interference with Mg homeostasis within the chloroplast (Shaul, 2002). This could be the case in our study where elevated Mg levels (S_{Mg}) were accompanied with low K content in plant tissues (see results in Table 2).

Table 1. Growth parameters, fresh yield, shoot dry biomass and leaf colorimetric traits of hydroponically grown butterhead lettuce in relation to nutrient solution composition and cultivar.

Source of variance	Leaf area (cm^2 $plant^{-1}$)	Leaf number (no. $plant^{-1}$)	Fresh biomass (g $plant^{-1}$)	Dry biomass (g $plant^{-1}$)	Dry matter (%)	LUE (g mol^{-1})	L*	Chroma
Cultivar (C)								
Green Salanova	1165 ± 19 b	59 ± 1.28 a	80.57 ± 2.25 a	3.73 ± 0.04 a	4.61 ± 0.07 a	0.17 ± 0.002 a	55.85 ± 0.50 a	38.29 ± 0.46 a
Red Salanova	1213 ± 30 a	55 ± 0.93 b	77.01 ± 2.30 b	3.50 ± 0.07 b	4.52 ± 0.07 b	0.16 ± 0.003 b	45.93 ± 0.32 b	29.30 ± 0.47 b
Nutrient solution (S)								
S_K	1267 ± 29 a	60 ± 2.10 a	86.57 ± 1.74 a	3.74 ± 0.07	4.32 ± 0.04 c	0.17 ± 0.003	51.73 ± 2.36 a	35.07 ± 2.02 a
S_{Ca}	1177 ± 13 b	57 ± 0.66 b	77.16 ± 1.54 b	3.58 ± 0.05	4.64 ± 0.04 b	0.16 ± 0.002	49.94 ± 2.05 b	32.77 ± 2.05 b
S_{Mg}	1123 ± 15 b	54 ± 0.73 c	72.65 ± 1.11 c	3.53 ± 0.09	4.74 ± 0.06 a	0.16 ± 0.004	51.02 ± 2.35 ab	33.55 ± 2.07 b
C × S								
Green Salanova × S_K	1229 ± 5	64 ± 0.47 a	87.94 ± 1.69	3.85 ± 0.03	4.38 ± 0.06 c	0.17 ± 0.001	56.84 ± 1.06	39.40 ± 0.92
Green Salanova × S_{Ca}	1167 ± 11	57 ± 0.26 b	80.09 ± 1.72	3.67 ± 0.05	4.59 ± 0.03 b	0.17 ± 0.003	54.52 ± 0.15	37.33 ± 0.53
Green Salanova × S_{Mg}	1099 ± 3	56 ± 0.30 bc	73.69 ± 2.05	3.66 ± 0.02	4.87 ± 0.02 a	0.16 ± 0.001	56.21 ± 0.67	38.14 ± 0.55
Red Salanova × S_K	1305 ± 51	56 ± 2.10 bc	85.21 ± 3.22	3.63 ± 0.12	4.26 ± 0.04 c	0.16 ± 0.005	46.62 ± 0.71	30.74 ± 0.94
Red Salanova × S_{ca}	1187 ± 24	56 ± 1.37 b	74.23 ± 0.55	3.49 ± 0.01	4.70 ± 0.05 b	0.16 ± 0.001	45.35 ± 0.28	28.21 ± 0.12
Red Salanova × S_{Mg}	1146 ± 24	53 ± 0.43 c	71.60 ± 0.92	3.39 ± 0.16	4.61 ± 0.06 b	0.15 ± 0.007	45.82 ± 0.46	28.96 ± 0.31
Significance								
Cultivar (C)	*	***	*	**	*	**	***	***
Nutrient solution (S)	***	***	***	NS	***	NS	*	**
C × S	NS	*	NS	NS	**	NS	NS	NS

S_K, S_{Ca}, S_{Mg}, nutrient solution with high proportion of K, Ca, and Mg, respectively.

NS,*,**, *** Non-significant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test ($P = 0.05$). All data are expressed as mean ± standard deviation, $n = 3$.

5.4.2 Nitrate content and mineral profile

The nitrate content recorded among the six treatments was within the maximum nitrate limit for the commercialization of lettuce according to Commission regulation (EU) No 1258/2011; however, NO_3 content was affected by both cultivar (C) and nutrient solution (S) composition, without significant C × S interaction (Table 2). Green Salanova plants contained significantly higher (16.4%) amounts of NO_3 than red Salanova plants, while increased levels of K (S_K) resulted in almost double NO_3 content compared to the other cation treatments (Table 2). Similar results have been reported by Yoshida et al. (2014) who also suggested a positive correlation between K levels in nutrient solution and NO_3 content in lettuce leaves. The same trend has been observed in cucumber leaves where increased K rates correlated with increased NO_3 content (Ruiz and Romero, 2002). It is well confirmed that K plays a major role in NO_3 uptake through potassium malate decarboxylation which takes place in roots and provides the adequate bicarbonate ions to be exchanged with nitrate during uptake (Ivashikina and Feyziev, 1998). Therefore, the adjustment of macrocation proportions in nutrient solution provides a cost-effective means to reduce nitrate content and consequently improve the quality and safety of fresh lettuce.

It is well known that the intake of macro-minerals through dietary sources on a daily basis is vital to human health in order to avoid nutritional disorders and metabolic impairments; moreover, macro-minerals are considered crucial components of human homeostasis and metabolism (Levander, 1990). In fact, Levander (1990) based on two surveys carried out in Finland and the USA, demonstrated that the contribution of vegetables to dietary intake of phosphorus, potassium, calcium, magnesium and sodium is 7-11%, 31-35%, 5-7%, 18-24% and 11%, respectively. Taking into consideration the high annual per capita consumption of lettuce in several EU countries and also around the world, the contribution of this important leafy vegetable to human dietary intake of minerals is of high importance. Among the minerals analysed, K was by far the most abundant, irrespective of cultivar and cationic proportions in the nutrient solution, ranging from 40.1 to 80.9 g/kg dw, followed by Ca (3.8-15.7 g/kg dw), P (3.9-5.4 g/kg dw), Mg (1.7-7.7 g/kg dw), and finally Na (0.6-1.7 g/kg dw) (Table 2).

A significant interaction between the tested factors was observed in the case of K, Ca and Na, whereas P and Mg were affected only by nutrient solution composition (Table 2). As it could be anticipated, increased cation content in nutrient solution resulted in

increased content of the corresponding minerals in leaf tissues, e.g. S_K for K, S_{Ca} for Ca, and S_{Mg} for Mg content (Table 2). Na content was the highest in red Salanova plants treated with elevated Ca levels (S_{Ca}). Finally, P content was the highest in plants treated with elevated K levels (S_K). The results of this study demonstrated the antagonism between cations such as K and Mg, Mg and Ca, and Ca and K, whereas the effect of genotype was less profound. According to Hermans et al. (2013), the enrichment of plants with Mg results in depletion of Ca although a varied response was observed between head and romaine lettuce types. Moreover, Barickman et al. (2016) reported that excessive K rates result in depolarization of the cytosol and in reduction of the driving force for the uptake of other cations such as Ca and Mg through plasma membrane transporters (Rietra et al. (2017). Similarly to our study, increased levels of K or Mg content in the nutrient solution resulted in a decrease of Ca content in hydroponically grown tomato plants, whereas P content increased only when high levels of K were applied (Gunes et al., 1998).

Table 2. Nitrate, phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sodium (Na) concnetrations of hydroponically grown butterhead lettuce in relation to nutrient solution composition and cultivar.

Source of variance	NO_3 (mg kg^{-1} fw)	P (g kg^{-1} dw)	K (g kg^{-1} dw)	Ca (g kg^{-1} dw)	Mg (g kg^{-1} dw)	Na (g kg^{-1} dw)
Cultivar (C)						
Green Salanova	1604 ± 137 a	4.36 ± 0.28	56.93 ± 6.06	9.11 ± 1.70 a	4.16 ± 0.88	0.81 ± 0.06 b
Red Salanova	1378 ± 185 b	4.46 ± 0.23	58.73 ± 8.03	6.32 ± 1.12 b	4.27 ± 0.90	1.18 ± 0.14 a
Nutrient solution (S)						
S_K	2122 ± 61 a	5.37 ± 0.12 a	85.73 ± 2.41 a	5.77 ± 0.65 b	1.93 ± 0.09 c	0.89 ± 0.05 b
S_{Ca}	1184 ± 70 b	4.11 ± 0.04 b	41.51 ± 0.68 c	13.24 ± 1.14 a	3.05 ± 0.14 b	1.27 ± 0.21 a
S_{Mg}	1167 ± 86 b	3.75 ± 0.11 c	46.24 ± 1.14 b	4.15 ± 0.22 c	7.67 ± 0.27 a	0.83 ± 0.11 b
C × S						
Green Salanova × S_K	2136 ± 83	5.40 ± 0.10	80.93 ± 1.22 b	7.17 ± 0.33 c	2.12 ± 0.04	0.96 ± 0.03 bc
Green Salanova × S_{Ca}	1338 ± 12	4.12 ± 0.07	42.94 ± 0.29 cd	15.70 ± 0.47 a	2.75 ± 0.06	0.86 ± 0.12 bc
Green Salanova × S_{Mg}	1338 ± 85	3.56 ± 0.13	46.91 ± 1.78 c	4.47 ± 0.38 d	7.60 ± 0.56	0.62 ± 0.06 c
Red Salanova × S_K	2108 ± 106	5.33 ± 0.25	90.53 ± 2.14 a	4.37 ± 0.08 d	1.74 ± 0.03	0.83 ± 0.07 bc
Red Salanova × S_{ca}	1029 ± 25	4.10 ± 0.05	40.09 ± 0.42 d	10.77 ± 0.45 b	3.34 ± 0.07	1.68 ± 0.19 a
Red Salanova × S_{Mg}	997 ± 31	3.95 ± 0.03	45.58 ± 1.71 c	3.83 ± 0.06 d	7.74 ± 0.24	1.05 ± 0.10 b
Significance						
Cultivar (C)	***	NS	NS	***	NS	***
Nutrient solution (S)	***	***	***	***	***	**
C × S	NS	NS	**	***	NS	**

S_K, S_{Ca}, S_{Mg}, nutrient solution with high proportion of K, Ca, and Mg, respectively.
NS, **, *** Non-significant or significant at $P \leq 0.01$, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test ($P = 0.05$). All data are expressed as mean ± standard deviation, $n = 3$.

5.4.3 Hydrophilic antioxidants: total ascorbic acid and phenolics profile

TAA content was influenced only by nutrient solution composition. In particular, elevated levels of Ca (S_{Ca}) and Mg (S_{Mg}) resulted in higher concentrations of TAA than elevated K levels (S_K) (Table 3). According to Fuad Mondal et al. (2017), increasing K rates resulted in increased ascorbic acid content in hydroponically grown strawberry fruit, while Hermans et al. (2013) reported similar results for muskmelon fruit. In contrast, Fanasca et al. (2006) did not observe a significant effect of cultivar, nutrient solution composition (i.e., cationic proportions K/Ca/Mg) or their combinatorial effect on ascorbic acid content of tomato fruit. Moreover, ascorbic acid content was negatively correlated with nitrates content (Mozafar, 1993), which was also the case in our study for plants treated with elevated K levels (S_K) where high nitrates content was associated with low contents of ascorbic acid. However, in contrast to our study Llorach et al. (2008) and Baslam et al. (2013) reported significant differences in ascorbic acid content between different types of lettuce. This difference could be attributed to the fact that both cultivars in our study belong to the same lettuce type and differ only in leaf color rather than representing different varietal types.

Concentrations of phenolic compounds are reported in Table 3. Four phenolic acids were found with a total concentration differing between tested cultivars, macrocation treatments and their combinations (Table 3). The most abundant compound was chicoric acid, followed by chlorogenic acid, while caffeoyl-meso-tartaric and caffeoyl-tartaric acid were detected in lower amounts. Chicoric acid is a typical phenolic acid detected in the *Asteraceae* family while chlorogenic acid is widely distributed in the plant kingdom (Petropoulos et al., 2017). With the exception of caffeoyl-tartaric acid, red Salanova plants contained higher amounts of phenolic compounds than green Salanova plants, especially in response to elevated Ca (S_{Ca}) and elevated Mg (S_{Mg}) treatments (Table 3). Luna et al. (2013) and Baslam et al. (2013) have also reported differences in phenolic compounds composition between green and red-pigmented lettuce cultivars. Moreover, the total phenolic acids content in our study was highest in Salanova plants treated with elevated Ca (S_{Ca}) and Mg (S_{Mg}) levels. Similarly, Khanam et al. (2012) detected only phenolic acids in lettuce leaves, whereas Llorach et al. (2008) identified caffeic acid derivatives, flavones and flavonols. These differences could be attributed to differences in the extraction method (Azmir et al., 2013), as well as to differences in the genotypes tested (Luna et al., 2013).

Considering the significant effects of cultivar and nutrient solution composition on phenolic compounds content, elevated Mg and/or Ca contents in nutrient solution can be used as a cost effective tool to increase bioactive content of hydroponically grown lettuce, especially for red-pigmented cultivars.

Table 3. total ascorbic acid content and phenolic acids composition of hydroponically grown butterhead lettuce in relation to nutrient solution composition and cultivar.

Source of variance	Total ascorbic acid (mg AA 100 g^{-1} fw)	Caffeoyl-tartaric acid (mg 100 g^{-1} dw)	Chlorogenic acid (mg 100 g^{-1} dw)	Chicoric acid (mg 100 g^{-1} dw)	Caffeoyl-meso-tartaric acid (mg 100 g^{-1} dw)	∑ phenolic acids (mg 100 g^{-1} dw)
Cultivar (C)						
Green Salanova	9.45 ± 0.87	7.58 ± 1.08 a	6.34 ± 0.81 b	42.30 ± 6.23 b	2.48 ± 0.35 b	58.69 ± 8.39 b
Red Salanova	10.61 ± 0.96	5.19 ± 0.42 b	75.48 ± 6.22 a	94.01 ± 6.13 a	42.54 ± 2.40 a	217.2 ± 14.7 a
Nutrient solution (S)						
S_K	7.50 ± 0.67 b	4.70 ± 0.46 b	27.36 ± 10.5 b	47.71 ± 10.7 c	17.38 ± 7.22 b	97.15 ± 28.1 c
S_{Ca}	10.18 ± 0.65 a	5.39 ± 0.29 b	47.49 ± 18.7 a	68.78 ± 14.3 b	25.63 ± 10.35 a	147.3 ± 43.1 b
S_{Mg}	12.41 ± 0.97 a	9.05 ± 1.27 a	47.87 ± 17.3 a	87.97 ± 10.1 a	24.51 ± 9.36 a	169.4 ± 35.5 a
C × S						
Green Salanova × S_K	7.00 ± 1.27	5.33 ± 0.79 bc	3.86 ± 0.26 d	24.06 ± 1.31 e	1.28 ± 0.19 d	34.53 ± 2.44 e
Green Salanova × S_{Ca}	9.78 ± 1.07	5.67 ± 0.35 bc	5.82 ± 0.11 cd	36.88 ± 1.52 d	2.52 ± 0.25 d	50.89 ± 1.71 d
Green Salanova × S_{Mg}	11.57 ± 1.04	11.73 ± 0.54 a	9.34 ± 0.20 c	65.95 ± 1.31 c	3.62 ± 0.06 d	90.64 ± 1.65 c
Red Salanova × S_K	8.00 ± 0.65	4.06 ± 0.06 c	50.87 ± 1.30 b	71.35 ± 2.72 c	33.48 ± 1.23 c	159.8 ± 3.99 b
Red Salanova × S_{ca}	10.58 ± 0.90	5.11 ± 0.46 bc	89.17 ± 1.58 a	100.7 ± 3.06 b	48.74 ± 1.11 a	243.7 ± 1.08 a
Red Salanova × S_{Mg}	13.25 ± 1.70	6.38 ± 0.76 b	86.40 ± 1.97 a	110.0 ± 5.29 a	45.40 ± 1.44 b	248.2 ± 9.42 a
Significance						
Cultivar (C)	NS	***	***	***	***	***
Nutrient solution (S)	**	***	***	***	***	***
C × S	NS	**	***	**	***	***

S_K, S_{Ca}, S_{Mg}, nutrient solution with high proportion of K, Ca, and Mg, respectively.
NS, **, *** Non-significant or significant at $P \leq 0.01$, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test ($P = 0.05$). All data are expressed as mean ± standard deviation, $n = 3$.

5.4.4 Carotenoids profile and anthocyanins

It is well established that several efforts through conventional breeding and genetic transformation have been made to improve lipophilic antioxidants such as carotenoids, while the improvement through nutrient solution management could also provide comparable results in a cost-effective manner (Kyriacou and Rouphael, 2018). In the current study, the carotenoids composition and anthocyanins content are presented in Table 4. Data showed that red Salanova plants contained higher amounts of all the detected compounds compared to green Salanova plants, particularly plants treated with elevated Mg (S_{Mg}) levels. However, especially for β-carotene content, no significant differences were observed between red Salanova plants treated with elevated K (S_K) and Mg (S_{Mg}) levels. The higher carotenoids and anthocyanins content of red-pigmented plants was expected since pigmentation of plant tissues is due to the presence of such compounds (Llorach et al., 2008). However, despite the genotype effect, the importance of the present results lies in the content increase of pigments such as xanthophylls, when Mg-enriched nutrient solution is applied, yielding final products of evidently better visual quality. Magnesium (Mg) fertilization has been associated with anthocyanin accumulation and increased pigmentation, especially in ornamental plants (Nissim Levi et al., 2007). The enhanced coloration in response to Mg application could be attributed to increased stability of anthocyanin molecules over and above their *de novo* synthesis, since complexation of Mg with vacuolar pigments enhances the pigments' stability and improves the coloration of plant tissues (Nissim Levi et al., 2007). Considering the bioactive properties of anthocyanins, nutrient solution management could be used as a cost-effective cultural practice to increase their content in red-pigmented lettuce and further improve the bioactive properties and visual quality of the final product.

Table 4. Composition of soluble pigments of hydroponically grown butterhead lettuce in relation to nutrient solution composition and cultivar.

Source of variance	Violaxanthin + neoxanthin (μg violaxanthin eq. g^{-1} dw)	Lutein (μg g^{-1} dw)	β-Cryptoxanthin (μg g^{-1} dw)	β-carotene (μg g^{-1} dw)	Anthocyanins (μg cyanidin eq. g^{-1} dw)
Cultivar (C)					
Green Salanova	440.6 ± 9.14 b	180.8 ± 5.71 b	325.5 ± 14.6 b	110.2 ± 4.36 b	n.d.
Red Salanova	869.4 ± 30.2 a	565.0 ± 20.7 a	942.8 ± 34.6 a	319.5 ± 15.3 a	12.94 ± 1.61
Nutrient solution (S)					
S_K	653.1 ± 106 b	373.1 ± 87.9 ab	618.5 ± 147 b	213.8 ± 49.6	n.a.
S_{Ca}	616.3 ± 67.3 c	348.7 ± 67.3 b	600.0 ± 104 b	200.5 ± 34.1	n.a.
S_{Mg}	695.6 ± 117 a	397.0 ± 104 a	684.1 ± 165 a	230.2 ± 58.9	n.a
C × S					
Green Salanova × S_K	417.2 ± 0.93 d	176.7 ± 0.89 d	289.4 ± 5.39 e	103.1 ± 1.01 c	n.d.
Green Salanova × S_{Ca}	468.3 ± 18.1 d	199.0 ± 10.8 d	372.2 ± 25.3 d	126.2 ± 5.76 c	n.d.
Green Salanova × S_{Mg}	436.2 ± 3.01 d	166.7 ± 0.43 d	315.0 ± 9.89 de	101.2 ± 0.44 c	n.d.
Red Salanova × S_K	888.9 ± 4.61 b	569.5 ± 6.98 b	947.6 ± 16.3 b	324.5 ± 6.83 a	7.55 ± 0.62 c
Red Salanova × S_{ca}	764.4 ± 19.2 c	498.4 ± 12.2 c	827.8 ± 36.7 c	274.8 ± 16.3 b	13.07 ± 1.29 b
Red Salanova × S_{Mg}	955.0 ± 34.3 a	627.2 ± 28.3 a	1053 ± 7.12 a	359.2 ± 26.4 a	18.21 ± 0.90 a
Significance					
Cultivar (C)	***	***	***	***	-
Nutrient solution (S)	***	*	**	NS	-
C × S	***	***	***	**	***

S_K, S_{Ca}, S_{Mg}, nutrient solution with high proportion of K, Ca, and Mg, respectively.

NS,*,**, *** Non-significant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. n.d. not detected, n.a. not applicable. Different letters within each column indicate significant differences according to Duncan's multiple-range test ($P = 0.05$). All data are expressed as mean ± standard deviation, $n = 3$.

5.4.5 Principal component analysis

To obtain a comprehensive overview of the nutritional and functional quality profiles of the studied green- and red-pigmented butterhead lettuce in response to the cationic proportions (K/Ca/Mg) in the nutrient solution, a principal component analysis (PCA) was conducted for all the agronomical and chemical composition traits measured and discussed above. The first three principal components (PCs) were associated with Eigen values higher than 1 and explained 95.9% of the cumulative variance, with PC1 accounting for 57.4%, PC2 for 29.7% and PC3 for 8.8% (Table 5). PC1 was positively correlated to chicoric, chlorogenic, caffeoyl-meso-tartaric and total phenolic acids, anthocyanins, ascorbic acid, and carotenoids (β-cryptoxanthin, Lutein, β-carotene, violaxanthin and neoxanthin) content. PC1 was also negatively correlated to agronomical traits (fresh yield, shoot biomass and leaf number), as well as to nitrates content. Moreover, PC2 was positively correlated to nitrates content, leaf area, P and K contents; and negatively correlated to ascorbic acid, dry matter content, caffeoyl-tartaric acid and Mg (Table 5). Furthermore, the loading matrix indicates the correlations among the examined quanti-qualitative traits, where in two vectors with an angle <90° are positively correlated, whereas an angle >90° designates variables negatively correlated. In our experiment, variation in chlorogenic and anthocyanins contents were most closely aligned with total phenolic acids content, whereas variation in ascorbic acid content was not correlated to nitrate content (Figure 1).

The effectiveness of PCA for plotting species and/or cultivar characterization as well as its interpretive usefulness on multiple nutritional and functional quality traits has been reported previously in a series of research papers (Cardarelli et al., 2017 and references cited therein). This was the case in our study, since the score plot of the PCA highlighted crucial information on the nutritional and functional quality of the tested butterhead cultivars exposed to different macronutrient proportions, with green-pigmented lettuce distinguished for yield, plant growth parameters, colorimetric traits, nitrate, P and K contents; whereas the red-pigmented cultivar was superior in target lipophylic and hydrophilic antioxidant molecules as well as in total phenolic acids (Figure 1). Particularly, the red-pigmented lettuce treated with a high proportion of Ca and Mg was positioned on the positive side of PC1 in the upper and lower right quadrants of the PCA score plot and delivered leaves of premium quality with high

concentration of hydrophilic and lipophilic antioxidants (Figure 1). Green lettuce grown under high proportion of K was positioned in the upper left quadrant, characterized overall by higher plant growth parameters (leaf number, fresh yield and shoot dry biomass) and LUE (Figure 1). Finally, the lower left quadrant depicted treatments (green lettuce treated with high proportions of Ca and Mg) characterized by high levels of key colorimetric traits (L* and C*) as well as the highest caffeoyl-tartaric acid content (Figure 1). The PCA carried out in this study facilitated a broad view of yield and quality traits reinforced by ion chromatography and HPLC outputs and enabled the identification of phenotypic variation patterns with respect to significant nutritional and functional quality characteristics underscored by variation in the genetic material and nutrient solution composition.

Table 5. Eigen values, relative and cumulative proportion of total variance, and correlation coefficients for each morphometric, nutritional and functional traits of butterhead lettuce with respect to the three principal components.

Principal components	PC1	PC2	PC3
Eigen value	14.3	7.4	2.1
Percentage of variance	57.4	29.7	8.8
Cumulative variance	57.4	87.1	95.9
Eigen vectors [a]			
Shoot biomass	**-0.983**	0.142	0.027
Chicoric acid	**0.980**	-0.067	0.094
LUE	**-0.979**	0.159	0.019
Total phenolics	**0.977**	0.132	-0.024
Anthocyanins	**0.951**	0.203	-0.007
Chlorogenic acid	**0.949**	0.239	-0.115
C*	**-0.930**	-0.336	0.101
Caffeoyl-meso-tartaric	**0.929**	0.329	-0.087
L*	**-0.898**	-0.417	0.079
β-Cryptoxanthin	**0.882**	0.442	0.130
Lutein	**0.870**	0.473	0.124
β-carotene	**0.867**	0.465	0.140
Violaxanthin +	**0.865**	0.453	0.179
LN	**-0.822**	0.262	-0.259
Fresh yield	**-0.719**	**0.694**	0.010
TAA	**0.713**	**-0.669**	0.128
Nitrate	**-0.674**	**0.660**	0.319
LA	-0.143	**0.976**	0.014
DM	0.244	**-0.933**	-0.116
P	-0.410	**0.897**	0.082
Caffeoyl-tartaric acid	-0.124	**-0.857**	0.429
K	-0.418	**0.794**	0.432
Mg	0.444	**-0.739**	0.460
Ca	-0.254	-0.135	**-0.885**
Na	0.533	0.212	**-0.701**

[a]Boldface factor loadings are considered highly weighed

[b] LUE, light use efficiency; LN, leaf number, TAA, total ascorbic acid; LA, leaf area; DM, dry matter.

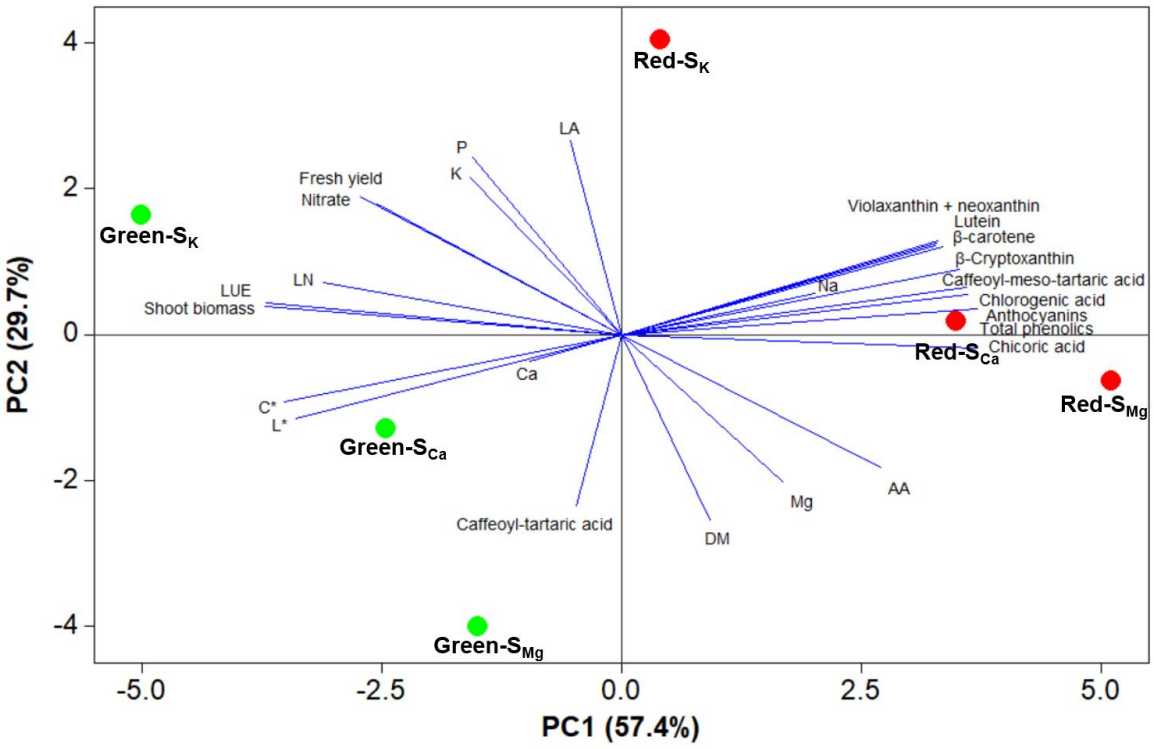

Figure 1. Principal component loading plot and scores of principal component analysis (PCA) of colorimetric data (L*, C*), growth parameters, fresh yield, shoot dry biomass mineral concentrations (NO_3, P, K, Ca, Mg and Na), lipophilic and hydrophilic antioxidant molecules (target phenolic acids and total phenolics, anthocyanins, total ascorbic acid and carotenoids) in green and red butterhead lettuce Salanova grown under different cationic proportions (K/Ca/Mg).

5.5 Conclusion

Nutrient solution management in hydroponic systems is crucial not only for the improvement of yield and growth parameters but also for modifying chemical composition attributes. The results of the present study demonstrated that by altering macrocation proportions in the nutrient solution (K/Ca/Mg proportions) it is possible to increase the concentrations of the respective macrominerals in lettuce, thus facilitating the production of biofortified fresh products of enhanced nutritive value. Moreover, the present results showed the importance of nutrient solution composition on bioactive properties, growth parameters and yield attributes of two lettuce cultivars. Elevated K

levels had a beneficial effect on fresh weight and color attributes, especially in green Salanova plants, while elevated macrocation proportions affected corresponding minerals and P and Na content. Considering that ascorbic acid and nitrates contents are important quality features for lettuce, the present results showed that by increasing Ca and Mg proportions it is possible to improve the quality of the final product. Macrocation proportions and genotype also affected hydrophilic and lipophilic antioxidants. Chicoric and chlorogenic acid were the most abundant phenolic compounds while their content increased significantly at elevated Ca and Mg levels. Finally, pigmentation of red lettuce increased under elevated Mg (S_{Mg}) treatment accompanied by higher content of anthocyanins, xanthophylls and β-carotene. Overall, management of nutrient solution macrocation proportions could be an effective cultural practice to increase bioactive properties and quality of hydroponically grown lettuce, while cultivar selection remains a key factor for obtaining premium quality products.

5.6 References

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6 Chapter 6: Iron Biofortification of Red and Green Pigmented Lettuce in Closed Soilless Cultivation Impacts Crop Performance and Modulates Mineral and Bioactive Composition

6.1 Abstract

Consumer demand for vegetables of fortified mineral and bioactive content is on the rise, driven by the growing interest of society in fresh products of premium nutritional and functional quality. Biofortification of leafy vegetables with essential micronutrients such as iron (Fe) is an efficient means to address the human micronutrient deficiency known as hidden hunger. Morphometric analysis, lipophilic and hydrophilic antioxidant capacities of green and red butterhead lettuce cultivars in response to Fe concentration in the nutrient solution (0.015 control, 0.5, 1.0 or 2.0 mM Fe) were assessed. The experiment was carried out in a controlled-environment growth chamber using a closed soilless system (Nutrient Film Technique). The percentage of yield reduction in comparison to the control treatment was 5.7%, 13.5% and 25.3% at 0.5, 1.0 and 2.0 mM Fe, respectively. Irrespective of the cultivars, the addition of 1.0 mM and especially 2 mM Fe in the nutrient solution induced an increase in the Fe concentration of lettuce leaves by 20.5% and 53.7%, respectively. No significant effects of Fe application on phenolic acids and carotenoid profiles were observed in green Salanova. However, increasing Fe concentration in the nutrient solution to 0.5 mM triggered a spike in chlorogenic acid and total phenolics in red Salanova lettuce by 110.1% and 29.1% compared to the control treatment, respectively; moreover, increased accumulation of caffeoyl meso tartaric phenolic acid by 31.4% at 1.0 mM Fe and of carotenoids violaxanthin, neoxanthin and β-carotene by 37.0% at 2.0 mM Fe were also observed in red Salanova compared to the control (0.015 mM Fe) treatment. Red Salanova exhibited higher yield, P and K contents, ascorbic acid, phenolic acids and carotenoid compounds than green Salanova. Nutrient solution management in soilless culture and Fe biofortification in particular could serve as effective cultural practices for producing Fe-enriched lettuce of premium quality, notwithstanding cultivar selection being a critical underlying factor for obtaining high quality products.

6.2 Introduction

Food obligations and unbalanced diets lead to malnutrition that causes up to 3 million children deaths each year (Murgia et al., 2012). This phenomenon, known as *hidden hunger* is affecting both industrial and developing countries. In fact, a diet poor in meat or fish, but also the cultivation in poor soils or where nutrients are not phytoavailable, negatively affect human health, causing deficiencies in vitamins and in essential and/or beneficial micronutrients (Murgia et a., 2012; White and Broadley, 2009; Przybysz et al., 2016). Iron (Fe) is one of the indispensable microelements for life and, although the earth crust is rich in it, iron forms insoluble compounds (White and Broadley, 2009), and its phytoavailable concentration (10^{-17} M) does not reach the optimal range for plant growth (10^{-9}–10^{-4} M) (Sperotto et al., 2012).

Iron is involved in very important processes, in both plants and humans, such as respiration, photosynthesis, and oxygen transport (Sperotto et al., 2012; Abbaspour et al., 2014). In the human body, it exists in two different forms of heme complexes, such as hemoglobin and myoglobin, or in non-heme forms, such as iron–sulfur clusters and other prosthetic groups. Two billion people are anaemic worldwide and according to the World Health Organization (WHO) the main cause is Fe deficient human diet (FAO/WHO, 2001)[6]. Fe deficiency is also among the most responsible factors for illnesses worldwide (Gowthami and Ananda, 2017). Fe absorption in the human intestine is inhibited by anti-nutrient molecules such as phytic acid, polyphenols and calcium while it is favoured by molecules such as ascorbic acid and β-carotene that can reduce or chelate Fe, leading to more bioavailable complexes.

Biofortification is a way to address hidden hunger by increasing the nutritional content of plants edible parts. Recently, Finklestein et al. (2017), have shown that biofortification with Fe in staple food crops can increase Fe status (serum ferritin concentrations and total body iron) in populations at risk, such as Philippines, India, and Rwanda. According to their work, the beneficial effect has been demonstrated not only in iron deficient youngsters or baseline adults, but also among individuals who are not at risk.

Biofortification can be achieved through mineral fertilisation, breeding or biotechnological approaches (Murgia et al., 2012; Abbaspour et al., 2014), however

each of these solutions meets limitations. For instance, the excessive use of fertilizers contributes to soil pollution or turns minerals into insoluble forms. Fertilization also requires a continuous supply of the element and consequently raises production cost. Moreover, high mineral concentrations can turn into stress conditions for plants. Excessive amounts of Fe can lead to phytotoxicity and growth inhibition, as demonstrated in many cultures such as rice (Pereira et al., 2013; de Souza Pinto et al., 2016; Frei et al., 2016), potato (Adamski et al., 2011), wheat (Agarwal et al., 2006; Li et al., 2012), tea (Hemalatha and Venkatesan, 2011). On the other hand, the spread of biofortified transgenic crops in many countries must undergo procrastinated procedures before its legal distribution to the public [1]. Hydroponic cultivation systems eliminate or reduce problems of nutrient phyto-availability. They have long been seen as an answer to the urgent need to produce food for an increasing global population (Rouphael et al., 2018; Rouphael and Kyriacou, 2018), since they allow the management of plant nutritional status during growth through the effective control of water and nutrient supply. In fact, manipulating the nutrient solution in terms of concentration or composition demonstrably improved the yield or quality of zucchini squash (Rouphael and Colla, 2005), cucumber (Rouphael et al., 2016), lettuce (Fallovo et al., 2009; Rouphael et al 2016a), artichoke (Colla et al., 2013), cardoon (Borgognone et al., 2016), and tomato (Fanasca et al., 2006).

Lettuce (*Lactuca sativa* L.) is one of the most cultivated and consumed leafy vegetables in the world, appreciated for its organoleptic properties and its content of health-promoting molecules, such as minerals, vitamins, terpenoids, as well as carotenoids, phenolic acids and flavonoids (Llorach et al., 2008; Kim et al., 2016; Kyriacou et al., 2019). Among the different pre-harvest factors (i.e., agricultural practices, developmental stages, climatic control) the genetic factor is considered the major determinant of variation in nutraceutical properties (Nicolle et al., 2004a; Mou, 2005; Lebeda et al., 2007c; Colonna et al., 2016; Rouphael et al., 2012a; Rouphael et al., 2017). The response of lettuce to iron biofortification was investigated only in terms of yield and iron status, under soil cultivation (Tyksinski and Komosa, 2008; Kozik et al., 2011). Essentially nothing is known about Fe biofortification under closed soilless cultivation (i.e., Nutrient Film Technique) where the constant exposure of the root system to Fe fortified nutrient solution could maximize Fe uptake, translocation and accumulation in edible parts. In addition, the efficiency of biofortification may depend

upon several interacting parameters, in particular cultivar and application rate (Rouphael et al., 2018; Rouphael and Kyriacou, 2018). To our knowledge, no information is available on how biofortification with an essential micronutrient such as Fe could differentially modulate the nutritional and functional quality of lettuce, accounting for potential interaction with tested cultivars.

In view of this background, our aim was to assess the effect of different iron application rates within the nutrient solution on growth parameters, fresh yield, mineral composition, antioxidant activities, nitrate and ascorbic acid contents as well as on phenolics and carotenoids profiles of green and red pigmented butterhead lettuce grown in NFT system under controlled environment. The obtained information will assist the scientific community as well as growers of leafy vegetables in identifying optimum cultivar-application rate combinations for achieving high nutritional and functional value, and in understanding the boundary between biofortification and iron toxicity in lettuce.

6.3 Materials and Methods

6.3.1 Growth chamber conditions, lettuce cultivars and experimental design

The experiment was carried out in a 28 m^2 controlled-environment growth chamber (7.0 × 2.1 m × 4.0 m; W × H × D), situated at the experimental station of the Department of Agricultural Sciences, University of Naples Federico II, Italy. Artificial light was provided by High Pressure Sodium lamps, with an intensity of 420 µmol m^{-2} s^{-1} according to a light/dark regime of 12/12h. Temperature was set at 24/18°C (light/dark) and relative humidity was 60-80% respectively, the latter being maintained by a fog system. The experiment was carried out at ambient carbon dioxide concentration (370-410 ppm), and air exchange was performed by means of an air extractor.

Two cultivars of lettuce (*Lactuca sativa* L. var. *capitata*) green Salanova® and red Salanova® (Rijk Zwaan, Der Lier, The Netherlands) were grown in a closed soilless system based on the Nutrient Film Technique (NFT). The nutrient solution being collected in polypropylene reservoir tanks of 25 L and recirculated with a constant flow of 1.5 L min^{-1} by submerged pumps. The gullies were 200 cm long, 14.5 cm wide and 8 cm deep, with a 1% slope. Lettuce seedlings were transplanted in rockwool cubes (7 × 7 × 7

cm) (Delta, Grodan, Roermond, The Netherlands). Lettuce seedlings were spaced 15 cm apart between rockwool cubes and 30 cm apart between gullies, giving a plant density of 22 plants per square meter. Each gully was covered with propylene taps to avoid evaporation of the nutrient solution.

The growth chamber experiment was designed as a factorial combination of two butterhead lettuce cultivars (red and green pigmented) and four concentrations of Fe in the nutrient solution (0.015 mM control treatment and three concentrations of 0.5, 1.0 and 2.0 mM Fe). The basic nutrient solution nutrient was a modified Hoagland and Arnon formulation. The composition of the basic nutrient solution was: 8.0 mM N-NO_3^-, 1.5 mM S, 1.0 mM P, 3.0 mM K, 3.0 mM Ca, 1.0 mM Mg, 1.0 mM NH_4^+ , 15 µM Fe, 9 µM Mn, 0.3 µM Cu, 1.6 µM Zn, 20 µM B, and 0.3 µM Mo, with an electrical conductivity (EC) of 1.4 dS m^{-1} and a pH of 5.8 ± 0.2. The biofortified Fe nutrient solution had the same basic nutrient composition plus an additional 0.5, 1.0 and 2.0 mM Fe. Fe biofortification was initiated three days after transplanting (DAT). Fe was added as Iron chelate EDDHA 6% ortho-ortho (Revive Total, Italpollina S.p.a., Rivoli Veronese, Italy).

Eight treatments derived from the factorial combinations of two butterhead lettuce cultivars (red and green Salanova) and four Fe concentrations in the nutrient solution (0.015-control, 0.5, 1.0 or 2.0 mM). Treatments were arranged in a randomized complete-block design amounting to a total of 24 experimental units with twelve plants each (288 green and red Salanova plants in total).

6.3.2 Growth analysis, biomass determination and light use efficiency

Nineteen DAT, all green and red Salanova lettuce plants were harvested. The number of leaves per plant was determined and the total area was measured by a LI-COR 3100C area meter (Biosciences, Lincoln, Nebraska, USA). Leaf tissues were dried at 70 °C for 72 h until they reached a constant weight and weighed again to determine the corresponding shoot dry biomass. The leaf dry matter percentage was also calculated. Finally, Light Use Efficiency (LUE) was expressed as the shoot dry biomass divided by cumulative daily intercepted Photosynthetically Active Radiation (PAR).

6.3.3 Collection of samples for mineral and nutritional quality analyses

Part of the dried leaf tissue of green and red Salanova plants was used for macro-mineral and FE analyses. For the identification and quantification of total ascorbic acid, lipophilic antioxidant activity, phenolic acids and carotenoid compounds by spectophotometry and HPLC-DAD, fresh samples of three plants per experimental unit were instantly frozen in liquid nitrogen and stored at -80 °C before lyophilizing them in a Christ, Alpha 1-4 (Osterode, Germany) freeze drier.

6.3.4 Mineral analysis by ion chromatography and ICP-OES

For mineral analysis, 250 mg of dried green and red butterhead lettuce leaves were ground at 0.5 mm in a Wiley Mill, and then suspended in 50 ml of ultrapure water (Milli-Q, Merck Millipore, Darmstadt, Germany) and shaken in water bath (ShakeTemp SW22, Julabo, Seelbach, Germany) at 80 °C for 10 min. The solution was centrifuged at 6000 rpm for 10 min (R-10 M, Remi Elektrotechnik Limited, India), then filtered through a 0.45 μm nylon syringe filter (Phenomenex, Torrance, CA, USA) and analysed by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA) coupled to a conductivity detector. An IonPac CG12A (4 × 250 mm, Dionex, Corporation) guard column and IonPac CS12A (4 × 250 mm, Dionex, Corporation) analytical column were used for the K, Ca and Mg analysis, while for N-NO3 and P determination, an IonPac AG11-HC guard (4×50 mm) column and IonPac AS11-HC analytical column (4 × 250 mm) were adopted, as detailed in Rouphael et al. (2017b). N-NO_3 was expressed as mg kg^{-1} fresh weight (fw) on the basis of each sample's original dry weight content (dw), while P, K, Ca and Mg were expressed as g kg^{-1} dw.

In addition to macro-minerals analysis, the Fe content was also measured in green and red Salanova leaf tissue. Each sample was subjected to a first phase of acid digestion performed using a commercial high-pressure laboratory microwave oven (Mars plus CEM, Italy) operating at an energy output of 1800 W. Approximately 300 mg of each dry sample was inserted directly into a microwave-closed vessel. Two milliliters of 30% (m/m) H_2O_2, 0.5 ml of 37% HCl and 7.5 ml of HNO_3 69% solution were added to each vessel. The heating program was performed in one step: temperature was ramped linearly from 25 to 180 °C in 37 min, then held at 180 °C for 15 min. After the digestion

procedure and subsequent cooling, samples were transferred into a Teflon beaker and total volume was made up to 25 mL with Milli-Q water. The digest solution was then filtered (DISMIC 25HP PTFE syringe filter, pore size 0.45 µm, Toyo Roshi Kaisha, Ltd., Japan) and stored in a screw cap plastic tube (Nalgene, New York). Blanks were prepared in each lot of samples. The reagents of superpure grade, used for the microwave-assisted digestions, were: hydrochloric acid (36% HCl), nitric acid (69% HNO_3) and hydrogen peroxide (30% H_2O_2) (Merck, Darmstadt, Germany). High-purity water (18 MΩ cm^{-1}) from a Milli-Q water purification system (Millipore, Bedford, USA) was used for the dilution of the standards, for preparing samples throughout the chemical process, and for final rinsing of the acid-cleaned vessels, glasses, and plastic utensils. The accuracy of the measurements was assessed using standard reference materials trace metals: tomato leaves (SRM 1573a).

Fe quantification was performed using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) with an axially viewed configuration (8000 DV, PerkinElmer, Shelton, CT, USA) equipped with an ultrasonic nebulizer. To assess Fe concentration, calibration standards were prepared, treated equally to samples before dilution. For detection we have chosen the frequency with the lowest interferences, high analytical signal and background ratio, line at 259.9 nm.

6.3.5 Total ascorbic acid analysis

The total ascorbic acid defined as dehydroascorbic (DHA) and ascorbic (ASA) acid was determined by UV–Vis spectrophotometry (Hach DR 2000; Hach Co., Loveland, Colorado, USA) as described by Kampfenkel et al. (1995). Briefly, 400 mg of sample fresh plant tissues were extracted with TCA 6%. Two hundred µl of the extract was solubilized with 2,2-dipyridyl (14454, Sigma-Aldrich, St.Louis, MO, USA). The assay is based on the reduction of Fe^{3+} to Fe^{2+} by total ascorbic acid and the spectrophotometric detection of Fe^{2+} complexes with 2,2-dipyridyl. DHA is reduced to ASA by pre-incubation of the sample with dithiothreitol (DTT). The absorbance of the solution was measured at 525 nm, and data were expressed as mg ascorbic acid per 100 g fw.

6.3.6 Lipophilic antioxidant activity analysis

The lipophilic antioxidant activity (LAA) was extracted with methanol and the antioxidant capacity of this extract was measured with the 2,20-azinobis 3-ethylbenzothiazoline-6-sulfonic acid ABTS method (Pellegrini et al., 1999). Similarly to the total ascorbic acid, LAA was determined by UV–Vis spectrophotometry. The absorbance of the solutions was measured at 734 nm. LAA fraction was expressed as mmol Trolox (6-hydroxy-2,5,7,8-tetramethylchro man-2-carboxylic acid) per 100g dw.

6.3.7 Phenolic acids and anthocyanins identification and quantification

Four hundred mg of lyophilized samples was solubilized in a solution of methanol/water/formic acid (50/45/5, v/v/v, 12 ml) as described by Llorach et al. (2008) to determine phenolic acids as hydroxycinnamic derivatives. The suspensions were sonicated for 30 min and then subjected to centrifugation (2500g for 30 min at 4°C). After a second centrifugation of supernatants at 21100g for 15 min at 4°C, samples were filtered through 0.22 µm cellulose filters (Phenomenex). A reversed phase C18 column (Prodigy, 250 × 4.6 mm, 5 µm, Phenomenex, Torrance, CA) equipped with a C18 security guard (4.0 x 3.0 mm, Phenomenex) was utilized for the separation of hydroxycinnamic derivatives and anthocyanins. Twenty µl of each extract was injected and the following mobile phases was used: (A) water formic acid (95:5, v/v) and (B) methanol through the following gradient of solvent B, (t in [min]/[%B]): (0/5), (25/40), (32/40). The flow rate was 1 mL min^{-1}. LC column was installed onto a binary system (LC-10AD, Shimadzu, Kyoto, Japan), equipped with a DAD (SPD-M10A, Shimadzu, Kyoto, Japan) and a Series 200 autosampler (Perkin Elmer, Waltham, MA). Chlorogenic and chicoric acids at 330 nm were used for the calibration curves of hydroxycinnamic derivatives. Identification of caffeoyl-meso-tartaric acid and caffeoyl-tartaric acid was performed by LC-MS/MS experiments.

The chromatographic profiles of reference curves and samples were recorded in multiple reaction monitoring mode (MRM) by using an API 3000 triple quadrupole (ABSciex, Carlsbad, CA). Negative electrospray ionization was used for detection and source parameters were selected as follows: spray voltage -4.2 kV; capillary temperature: 400 °C, dwell time 100 ms, nebulizer gas and cad gas were set to 10 and 12 respectively (arbitrary units). Target compounds [M-H]- were analyzed using mass transitions given in

parentheses: chicoric acid (*m/z* 473→311, 293), chlorogenic acid (*m/z* 353 →191), caffeoyl tartaric acid (*m/z* 311→ 179, 149, retention time 15.8 min), caffeoyl-meso-tartaric acid (*m/z* 311→ 179, 149, retention time 17.8 min). The concentration of phenolic acids was reported as mg 100 g^{-1} of dw.

Anthocyanins were also measured within the same LC-DAD chromatographic runs, at 520 nm and the concentration calculated by using cyanidin as reference standard to calculate the concentration. The results were reported as µg of cyanidin equivalent per g of samples.

6.3.8 Carotenoids identification and quantificat*ion*

One gram of lyophilized samples was used to determine carotenoids content following the method of Vallverdú-Queralt et al. (2013) with slight modifications. Samples were solubilized in ethanol/hexane (4:3, v/v, 2.5 ml) with 1% BHT, vortexed at 22°C for 30 s and sonicated for 5 min in the dark. Then, the solution was centrifuged (2500 g, 4°C, 10 min) and filtred through 0.45 µm nylon syringe filters (Phenomenex, Torrance, CA, USA). The extracts were dried in N and the dried extracts were dissolved in 1% BHT in chloroform. Twenty µl of each sample was injected onto a C18 column (Prodigy, 250 × 4.6 mm, 5 µm, Phenomenex, Torrance, C A, USA) with a C18 security guard (4.0 × 3.0 mm, Phenomenex). Two mobile phases were used: (A) acetonitrile, hexane, methanol, and dichloromethane (4:2:2:2, v/v/v/v) and (B) acetonitrile. Carotenoids were eluted at 0.8 mL min-1 through the following gradient of solvent B (t in [min]/[%B]): (0/70), (20/60), (30/30), (40/2). Carotenoids were quantified by a binary LC-10AD system connected to a DAD (SPD-M10A, Shimadzu, Kyoto, Japan) equipped with a Series 200 auto-sampler (Perkin Elmer, Waltham, MA, USA). Violaxanthin, neoxanthin, β-cryptoxanthin, lutein and β-carotene were used as reference standards. Identification of the peaks was achieved by comparison of UV-vis spectra and retention times of eluted compounds with pure standards at 450 nm. Three separate sets of calibration curves were built, each set was injected three times in the same day (intraday assay) and three times in three different days (interday assay). The accuracy was reported as the discrepancies between the calibration curves performed intraday and interday and the results were expressed as relative standard deviation RSD (%). A recovery test was performed spiking two samples with two known amounts of carotenoids (50 and 100 µg

mL^{-1} final concentration) and taking into account the overestimation due to the target analytes already present in the samples. The concentration of the target carotenoids was expressed as µg g-1 dw.

6.4 Statistics

All morphometric, nutritional and functional quality data were subjected to analysis of variance (two-way ANOVA) using IBM SPSS 20 software package (www.ibm.com/software/analytics/spss). Duncan's multiple range test was performed for mean comparisons on each of the significant ($p < 0.05$) variables measured.

6.5 Results and Discussion

6.5.1 Growth response, fresh yield, dry matter and light use efficiency

Inter and intra-specific genetic variability is among the most important preharvest factors which influence lettuce's phenotypic and biochemical traits. Lettuce presents, within the same species, a variety of colors, sizes, textures and shapes (Lebeda et al., 2004a,b; Lebeda et al., 2014). In our study, two cultivars of green and red Salanova were evaluated from a productive and nutritional point of view in response to different iron concentrations in the nutrient solution.

For leaf number per plant, marketable fresh yield and leaf dry matter percentage no significant interaction between cultivar (C) and iron nutrient solution concentration (I) was observed, whereas leaf area, dry biomass and light use efficiency (LUE) were significantly affected by the interaction of these two factors (Table 1). Irrespective of the iron concentration in the nutrient solution, the red Salanova had higher marketable yield and percentage dry matter than those recorded in green Salanova plants by 9.3% and 7.0%, respectively (Table 1). Analogous genotypic variation in marketable fresh yield and leaf dry matter content has been previously demonstrated over seven iceberg cultivars ('Equinos', 'Ice Castle', 'Metalia', 'Num 189', 'Silvinas', 'Ombrinas' and 'Vanguardia'; Rouphael et al., 2017).

When averaged over both Salanova cultivars, the percentage of yield reduction in comparison to the control treatment (0.015 mM Fe) was 5.7%, 13.5% and 25.3% at 0.5, 1.0 and 2.0 mM Fe concentration in the nutrient solution, respectively, whereas an opposite trend was observed for the leaf dry matter percentage (Table 1). Furthermore,

the highest total leaf area was recorded in red Salanova treated with both 0.015 and 0.5 mM Fe. Although the highest shoot dry biomass was recorded in red Salanova treated with 0.015 mM Fe, increasing the Fe concentration in the nutrient solution from 0.015 to 2.0 decreased the dry biomass with more detrimental effect recorded in red-pigmented butterhead lettuce. Specifically, the percentage of dry biomass reduction in comparison to control plants (0.015 mM Fe) ranged from 4.3% to 9.7% in green Salanova and from 10.4% to 18.1% in red Salanova at 1.0 and 2.0 mM Fe concentration in the nutrient solution, respectively (Table 1). Similarly to the effects on shoot dry biomass, the LUE in red Salanova under control Fe treatment (0.015 mM) exhibited the highest values (Table 1)

Table 1. Analysis of variance and mean comparisons for leaf area, leaf number, fresh yield, shoot dry biomass, leaf dry matter percentage and Light Use Efficiency (LUE) for green and red Salanova butterhead lettuce grown under increasing iron concentration in the nutrient solution.

Source of variance	Leaf area (cm^2 $plant^{-1}$)	Leaf number (no. $plant^{-1}$)	Fresh yield (g $plant^{-1}$)	Dry biomass (g $plant^{-1}$)	Dry matter (%)	LUE (g mol^{-1})
Cultivar (C)						
Green Salanova	1070± 18.6 b	45.94 ± 0.90 b	60.73 ± 2.08 b	3.21 ± 0.06 b	5.32 ± 0.11 b	0.14 ± 0.002 b
Red Salanova	1211± 21.2 a	54.46 ± 0.67 a	66.37 ± 2.26 a	3.76 ± 0.09 a	5.69 ± 0.09 a	0.17 ± 0.004 a
Iron (mM Fe) (I)						
0.015	1196± 50.8 a	50.10 ± 2.10	71.50 ± 1.92 a	3.72 ± 0.20 a	5.19 ± 0.16 c	0.167 ± 0.009 a
0.5	1185± 25.5 a	49.68 ± 1.74	67.40 ± 1.09 b	3.59 ± 0.09 ab	5.32 ± 0.08 c	0.161 ± 0.004 ab
1.0	1091± 26.2 b	48.66 ± 2.65	61.85 ± 2.25 c	3.43 ± 0.13 b	5.55 ± 0.07 b	0.155 ± 0.006 b
2.0	1090± 39.3 b	52.36 ± 2.03	53.44 ± 1.19 d	3.19 ± 0.10 c	5.96 ± 0.11 a	0.143 ± 0.005 c
C × I						
Green Salanova × 0.015 mM Fe	1089± 27.9 de	45.57 ± 0.50	67.67 ± 0.78	3.29 ± 0.04 cd	4.86 ± 0.11	0.148 ± 0.002 cd
Green Salanova × 0.5 mM Fe	1135± 19.3 cd	46.80 ± 1.99	66.13 ± 0.52	3.41 ± 0.06 c	5.16 ± 0.05	0.153 ± 0.002 c
Green Salanova × 1.0 mM Fe	1052± 41.4 ef	42.96 ± 1.35	57.51 ± 2.46	3.15 ± 0.08 de	5.49 ± 0.12	0.142 ± 0.004 de
Green Salanova × 2.0 mM Fe	1004± 10.6 f	48.45 ± 1.92	51.61 ± 1.74	2.97 ± 0.05 e	5.77 ± 0.09	0.134 ± 0.002 e
Red Salanova × 0.015 mM Fe	1302± 28.9 a	54.62 ± 1.10	75.34 ± 1.76	4.15 ± 0.13 a	5.51 ± 0.11	0.187 ± 0.006 a
Red Salanova × 0.5 mM Fe	1236± 17.9 ab	52.57 ± 1.69	68.66 ± 2.02	3.76 ± 0.07 b	5.48 ± 0.06	0.169 ± 0.003 b
Red Salanova × 1.0 mM Fe	1130± 14.5 cd	54.35 ± 0.86	66.20 ± 0.67	3.72 ± 0.04 b	5.62 ± 0.04	0.167 ± 0.002 b
Red Salanova × 2.0 mM Fe	1177± 13.0 bc	56.28 ± 1.31	55.26 ± 0.83	3.40 ± 0.06 c	6.16 ± 0.11	0.153 ± 0.003 c
Significance						
Cultivar (C)	***	***	***	***	***	***
Iron (I)	***	NS	***	***	***	***
C × I	*	NS	NS	*	NS	*

ns,*, *** Nonsignificant or significant at $P \leq 0.05$, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (P = 0.05). All data are expressed as mean ± standard error, n = 3.

In the current study, diamino-di- (ortho-hydroxy phenyl acetic) acid (o, o-EDDHA) was used as iron chelate, because the final amount of dissolved Fe released in solution, is greater than other forms of Fe chelates and it can be considered as a good supplement in nutrient solutions for soilless cultivation (Urrestarazu et al., 2008; Roosta et al., 2015a; Roosta et al., 2015b). Roosta et al. (2015a) have reported increases in the number of leaves and total leaf area of *Capsicum annuum* L, after the application of 10 μM of EDDHA to the nutrient solution. Similar results have been also observed by the same authors for four lettuce varieties grown with 20 μM Fe in an NFT hydroponic system.

The results on growth parameters, yield and shoot dry biomass of this study are in agreement with those reported by Filho et al.(2015), who cultivated *Cichorium intybus* in an NFT using increased iron concentrations in the nutrient solution (0.9, 2.7, 8.3, and 25 mg L^{-1}), where plant height, leaf number per plant, as well as plant fresh and dry weight were reduced as iron concentration increased. The same authors were able to identify the optimal Fe range (2.7 to 8.3 mg L^{-1}), while the 25 mg L^{-1} application rate had the most harmful effects on plant growth and productivity. While in fact iron is essential for plant growth, it is also involved in the Fenton reaction that leads to the formation of Reactive Oxygen Species (ROS), which in turn, can lead to cell destruction, because they react with polyunsaturated fatty acids, proteins and nucleic acids (De Dorlot et al., 2005; Briat et al., 2015; Przybysz et al., 2016) . In the De Dorlodot et al. (De Dorlot et al., 2005) study, three concentrations of Fe^{2+} (0, 125 and 250 mg L^{-1}) were used for greenhouse cultivation of rice plants in hydroponic system. The intermediate dose of 125 mg L^{-1} produced the maximum fresh and dry weights, whereas at the higher dose of 250 mg L^{-1} a significant reduction in both fresh and dry plant weights was incurred, as well as in water content. According to the authors' observations, plants can implement resistance mechanisms to ferrous iron toxicity, since inside the roots they can oxidize and precipitate ferrous iron into ferric iron before it enters the vascular tissues and reaches the leaves (De Dorlot et al., 2005). Other intrinsic detoxification mechanisms against iron are enzymatic oxidations of external ferrous iron. In many cases, ferrous iron is retained and blocked in the root tissues. If iron excess reaches the leaves, it is stored in the apoplast in the form of hydroxides, in the vacuoles where the storage is favored by acidity and chelating organic acids, or in the plastids containing the iron-storage ferritin (De Dorlot et al., 2005). On the other

hand, Fe^{2+} iron stress can be reduced by superoxide dismutase, which reduces O_2^- producing H_2O_2. Presumably, if these mechanisms are impacted, plants are subject to iron toxicity phenomena, leading to stunted growth and yield (Przybysz et al., 2016).

6.5.2 Nitrate content, mineral composition and iron biofortification

The nitrate nitrogen (NO_3-N) content recorded among the eight treatments was within the maximum nitrate content allowable for the commercialization of fresh lettuce (4,000-5,000 mg $NO_3^-kg^{-1}$ fw; depending on harvest period and/or growing conditions) according to Commission regulation (EU) No 1258/2011 (EU, Commission Regulation, 2011). The NO_3-N content varied considerably across the eight treatments (C × I interaction) with the highest nitrate concentration found in green Salanova treated with 2 mM Fe (Table 2). In the current experiment, increasing the Fe concentration in the nutrient solution from 0.015 to 2.0 mM increased the nitrate content in red Salanova (by 8.8%) but especially in green Salanova (by 27.3%) plants (Table 2). Similar results have been reported by Liu et al. (2011) who also suggested a positive correlation between Fe supplementation and nitrate content in hydroponically grown lettuce leaves.

It has been demonstrated that a number of dietary macro-minerals such as P, K, Ca and Mg are crucial components of the human diet due their multifaceted nutraceutical properties such as, lowering blood pressure and hypertension (K), promoting bone health and reducing osteoporosis (P, Ca and Mg) (Kim et al., 2016). Among the four macro-minerals analyzed, K was by far the most abundant irrespective of cultivar and iron concentration in the nutrient solution, ranging from 59.7 to 72.0 g kg^{-1} dw, followed by Ca (4.5-7.2 g kg^{-1} dw), P (4.7-5.3 g kg^{-1} dw), and finally Mg (1.6-2.4 g kg^{-1} dw) (Table 2). Our results on the mineral profile of green and red-pigmented butterhead lettuce were proximate to those reported by the National Nutrient Database for Standard References (USDA, 2015) and by several authors (Kawashima and Soares, 2003; Baslam et al., 2013; Kim et al., 2016) on green and red leaf lettuce including the butterhead type: K (48-72 mg g^{-1} dw), P (4-6 mg g^{-1} dw), Ca (1.4-2.8 mg g^{-1} dw) and Ca (4-10 mg g^{-1} dw).

In our study, a significant interaction between the tested factors was observed in the case of Ca and Mg, whereas P and K were affected only by cultivar and iron concentration in the nutrient solution with no C × I interaction (Table 2). The P and K

concentrations in red Salanova were higher ($p< 0.001$) by 7.7% and 10.1%, respectively than those observed in green Salanova, whereas an opposite trend was recorded for Ca. Moreover, Ca and Mg concentrations were the highest in red Salanova treated with 0.015 mM Fe, whereas the lowest values were also observed in the red-pigmented cultivar treated with 2.0 mM Fe (Table 2). In fact, Fe can cause alteration of mineral composition status due to the competition between Mg and Fe ions for occupying the chlorophyll ring. Moreover, as shown by De Dorlodot et al. (2005), the contents of rice plants grown under soilless conditions in P, Ca, and Mg were reduced by iron application rates of 125 and especially 250 mg L^{-1}, as compared to the control. As pointed out by the same authors, this alteration of plants mineral status can be linked to the direct competition between iron and other cations due to a lack of specific carriers for these ions, as in the case of K reduction. Furthermore, K, Ca and Mg reductions had been observed in radish, broccoli, alfalfa, and mung bean after the nutrient solution was enriched with Fe (3).

The importance of iron for human health is linked to the synthesis of hemoglobin and oxygen transport. Plants contain iron only in trace amounts, hence particular attention is given to this mineral from a human diet perspective, especially from vegans who place vegetables are at the core of their diet. Moreover, plants partly contain Fe in non-heme (non-chelated) forms which are less bioavailable than the heme Fe found in animal-based foods (Kim et al., 2016). However, iron biofortification can be achieved in leafy vegetables including lettuce (Rouphael and Kyiacou, 2018), though its effectiveness can vary between species/cultivars (Przybysz et al., 2016; Li et al., 2018). In our study, the red leaf lettuce cultivar accumulated 45.5% more Fe than the green one (Figure 1), which is in agreement with previous results on red and green-pigmented lettuce (Kim et al., 2016; Mou, 2005; Baslam et al., 2013). Inversely to macro-minerals, the Fe content recorded in the current experiment differed from the values reported in butterhead lettuce by Kawashima and Soares (2003) (100 µg g^{-1} dw) and Baslam and co-workers (2013) (75.8-112 mg kg^{-1} dw). Such differences in Fe content reported in the scientific literature could be associated to different farming practices, environmental conditions as well as to the cultivars tested (Colonna et al., 2016). Regardless of cultivar, the addition of 1.0 mM and especially 2.0 mM Fe in the nutrient solution lettuce leaves incurred a significant increase of iron content by 20.5% and 53.7% (Figure 1), demonstrating that the production of Fe-enriched lettuce using closed

soilless cultivation is feasible. However, elucidating the physiological and especially the molecular mechanisms facilitating Fe uptake in interaction with genotype pose the future challenge confronting the horticultural industry before achieving the production of leafy vegetables of superior functional quality.

Table 2. Analysis of variance and mean comparisons leaf mineral composition in green and red Salanova butterhead lettuce grown under increasing iron concentraton in the nutrient solution.

Source of variance	NO_3 (mg kg^{-1} fw)	P (g kg^{-1} dw)	K (g kg^{-1} dw)	Ca (g kg^{-1} dw)	Mg (g kg^{-1} dw)
Cultivar (C)					
Green Salanova	2277 ± 65 a	4.80 ± 0.08 b	62.67 ± 0.69 b	6.47 ± 0.21 a	2.01 ± 0.05
Red Salanova	2105 ± 27 b	5.17 ± 0.05 a	69.01 ± 0.92 a	5.73 ± 0.30 b	2.06 ± 0.09
Iron (mM Fe) (I)					
0.015	1991 ± 17 c	4.84 ± 0.12 b	67.32 ± 1.70 a	7.08 ± 0.17 a	2.30 ± 0.07 a
0.5	2228 ± 47 b	5.10 ± 0.12 a	68.57 ± 1.65 a	6.50 ± 0.26 b	2.14 ± 0.02 b
1.0	2192 ± 56 b	4.85 ± 0.11 b	65.42 ± 1.49 b	5.69 ± 0.24 c	1.97 ± 0.06 c
2.0	2351 ± 92 a	5.15 ± 0.08 a	62.05 ± 1.08 c	5.12 ± 0.31 d	1.74 ± 0.05 d
C × I					
Green Salanova × 0.015 mM Fe	1999 ± 16 de	4.58 ± 0.03	63.71 ± 1.09	7.01 ± 0.36 a	2.17 ± 0.10 b
Green Salanova × 0.5 mM Fe	2267 ± 94 b	4.88 ± 0.10	65.12 ± 0.99	7.05 ± 0.17 a	2.09 ± 0.01 bc
Green Salanova × 1.0 mM Fe	2295 ± 63 b	4.67 ± 0.18	62.18 ± 0.31	6.03 ± 0.36 b	1.93 ± 0.03 cd
Green Salanova × 2.0 mM Fe	2545 ± 64 a	5.09 ± 0.15	59.67 ± 0.19	5.79 ± 0.15 b	1.84 ± 0.03 d
Red Salanova × 0.015 mM Fe	1983 ± 33 e	5.10 ± 0.09	70.93 ± 0.47	7.16 ± 0.10 a	2.43 ± 0.03 a
Red Salanova × 0.5 mM Fe	2190 ± 24 bc	5.33 ± 0.09	72.02 ± 0.87	5.95 ± 0.04 b	2.19 ± 0.01 b
Red Salanova × 1.0 mM Fe	2089 ± 29 cde	5.03 ± 0.04	68.66 ± 0.70	5.35 ± 0.22 b	2.00 ± 0.13 bcd
Red Salanova × 2.0 mM Fe	2157 ± 30 bcd	5.21 ± 0.07	64.43 ± 0.37	4.45 ± 0.11 c	1.63 ± 0.04 e
Significance					
Cultivar (C)	***	***	***	***	NS
Iron (I)	***	**	***	***	***
C × I	**	NS	NS	*	*

ns,*,**, *** Nonsignificant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (P = 0.05). All data are expressed as mean □ standard error, n = 3.

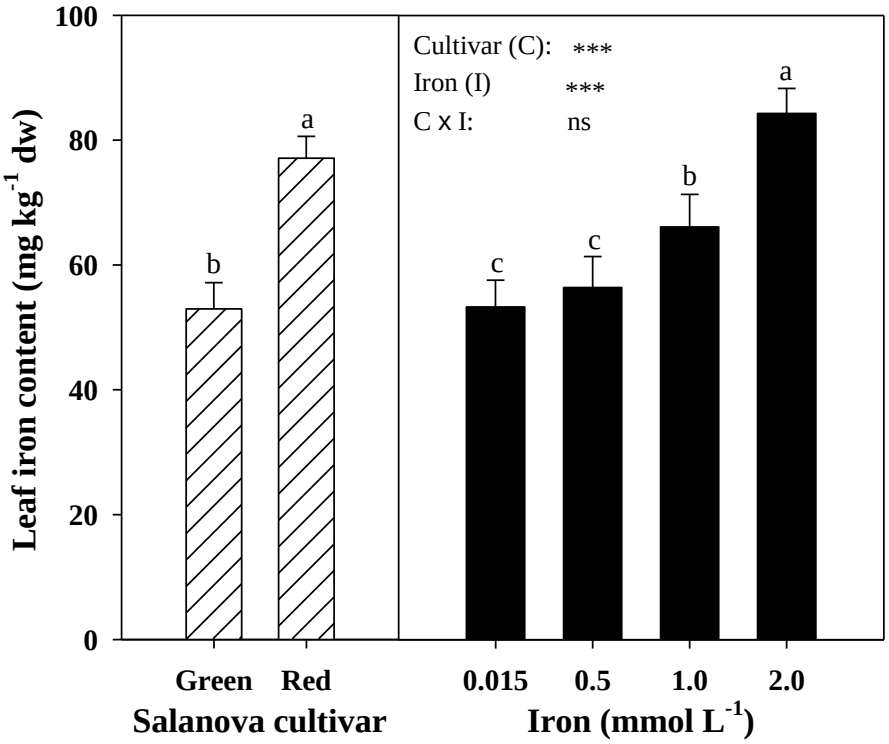

Figure 1. Mean effects of cultivar and iron concentration in the nutrient solution on iron accumulation in lettuce leaves. Different letters indicate significant differences according to Duncan's test ($P < 0.05$). The values are means of three replicates. Vertical bars indicate ± SE of means.

6.5.3 Hydrophilic antioxidants: total ascorbic acid and phenolics profile

Ascorbic acid and phenolic compounds such as phenolic acids and flavonoids represent the main water-soluble antioxidant molecules in lettuce (Kim et al., 2016). The most important role played by ascorbic acid is its radical scavenging power derived from its oxidation to the dehydroascorbate form (Smirnoff, 2018). Ascorbate plays an important role in Fe uptake, thanks to its ability to reduce Fe^{3+}. It has been verified that the presence of ascorbate in the apoplast reduces extracellular Fe^{3+}, facilitating Fe-uptake (Smirnoff, 2018). In the current study, the total ascorbic acid including ascorbic and dehydroascorbic acid varied considerably across treatments, with the highest values (19.4 mg AA 100 g^{-1} fw) found in red Salanova treated with 0.5 mM Fe and to a lesser extent under 1.0 and 2.0 mM Fe (avg. 13.4 mg AA 100 g^{-1} fw (Figure 2). On the other hand, the application of Fe at 0.5 and 1.0 mM in the nutrient solution did not improve the ascorbic acid in green-pigmented lettuce but at 2.0 mM Fe the concentration of this important antioxidant molecule increased by 62.7% (Figure 2). Our findings are in accordance with other previous works, where the concentration and activity of enzymatic and non-enzymatic antioxidant systems increases with the iron content of plants (Przybysz et al., 2016). For instance, ascorbic acid rose by 150% and 80% in alfalfa and broccoli, respectively when the iron concentration in the nutrient solution increased (Przybysz et al., 2016).

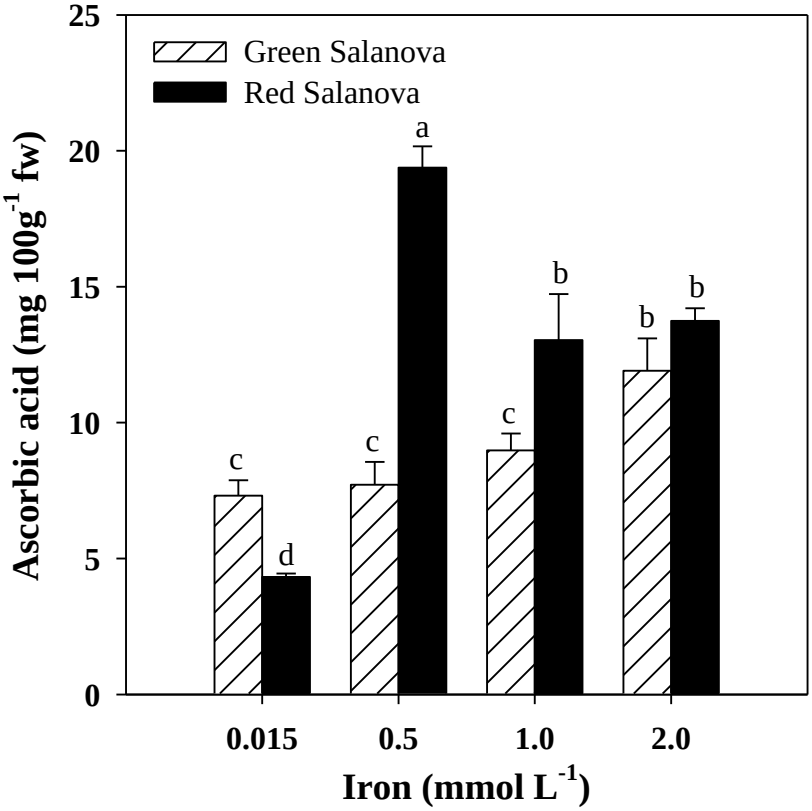

Figure 2. Effects of cultivar and iron concentration in the nutrient solution on total ascorbic acid in lettuce leaves. Different letters indicate significant differences according to Duncan's test ($P < 0.05$). The values are means of three replicates. Vertical bars indicate ± SE of means.

Phenolic compounds including mainly phenolic acids and flavonoids refer to an important group of secondary metabolites having great antioxidant activity and beneficial effects against chronic diseases, such as inflammation, diabetes and some types of cancer (Kim et al., 2016). The HPLC-DAD analysis of the lettuce extracts provides a quanti-qualitative evaluation of the phenolic compounds profile (Table 3). Across treatments, the most abundant compound was chicoric acid, followed by chlorogenic acid, while caffeoyl-meso-tartaric and caffeoyl-tartaric acid were detected in lower concentrations. Moreover, significant differences in the cultivars' response to Fe solution enrichment were found for the target and total phenolic acids, as reflected by the C × I interaction (Table 3). Except for caffeoyl tartaric acid, the greatest accumulation of chlorogenic, chicoric, caffeoyl meso tartaric acids as well as total phenolic acids were observed in red Salanova leaves in comparison to green-pigmented lettuce, which is in agreement with the previous findings of Llorach et al. (2008), Colonna et al. (2016) and Kim et al. (2016).

Concerning the Fe nutrient solution management, no significant effects on target and total phenolic acids were observed in green Salanova with the increasing Fe application rate. Contrarily to the green-pigmented butterhead lettuce, increasing the iron concentration in the nutrient solution to 0.5 mM induced a significant increase in the chlorogenic acid and total phenolics contents of red-pigmented lettuce by 110.1% and 29.1%, respectively compared to the control treatment (0.015 mM); and a higher accumulation (+31.4%) of caffeoyl meso tartaric acid was also observed in red Salanova at 1.0 mM in comparison to the control treatment (Table 3). Since phenolic compounds are considered to be iron bioavailibility inhibitors, their increase could be linked to iron-excess stress (Przybysz et al., 2016). The same type of correlation between the increased iron (i.e., Fe-EDDHA) and phenolic concentrations has been demonstrated on several horticultural species such as broccoli, mung beans, grape berries and radish (Przybysz et al., 2016; Shi et al., 2018) .

Anthocyanins, which constitute a subgroup of flavonoids responsible for red-purple pigmentation in different *Lactuca sativa* L. types (Kim et al., 2016), were expectedly detected only in red-pigmented Salanova (Table 3), as previously demostrated by Llorach et al. (2008). The application of 2.0 mM Fe in the nutrient solution elicited significant increase in the anthocyanin contents of plants compared to those treated with 0.015 and 1.0 mM Fe, whereas treatment with 0.5 mM exhibited intermediate values (Table 3). Our results are in accordance with those of Mohammadi et al. (2018) wherein anthocyanin concentration in peppermint increased by 11.5 % with 0.5g L^{-1} Fe_2O_3 treatment in comparison to the control treatment (0 g L^{-1} Fe_2O_3). Considering the significant influence of the genetic material (red vs. green pigmented cultivar) and iron application rate on phenolic acids and flavonoids (i.e, anthocyanins), mild to high (0.5-2.0 mM) Fe application in nutrient solution can be used as a cost effective tool to increase the phytochemicals content of hydroponically grown lettuce, especially for red-pigmented cultivars, although such practice is likely to precipitate anti-nutritive effects that may counteract Fe bioavailability in human subjects.

Table 3. Analysis of variance and mean comparisons for target phenolic acids, total phenolic acids and anthocyanins in green and red Salanova butterhead lettuce grown under increasing iron concentration in the nutrient solution.

Source of variance	Caffeoyl tartaric acid (mg 100 g^{-1} dw)	Chlorogenic acid (mg 100 g^{-1} dw)	Chicoric acid (mg 100 g^{-1} dw)	Caffeoyl meso tartaric acid (mg 100 g^{-1} dw)	∑ phenolic acids (mg 100 g^{-1} dw)	Anthocyanins (μg cyanidin eq. g^{-1} dw)
Cultivar (C)						
Green Salanova	15.92 ± 1.92 a	7.30 ± 0.68 b	72.62 ± 6.47 b	2.26 ± 0.40 b	98.10 ± 8.71 b	n.d.
Red Salanova	6.62 ± 0.92 b	58.74 ± 8.48 a	114.89 ± 15.2 a	28.92 ± 2.26 a	209.17 ± 21.61 a	14.51 ± 1.81
Iron (mM Fe) (I)						
0.015	10.81 ± 3.57 b	26.77 ± 7.67 c	117.18 ± 12.7 a	16.29 ± 5.33 a	171.05 ± 22.3 a	n.a.
0.5	8.81 ± 0.71 b	48.90 ± 19.3 a	106.71 ± 21.9 a	17.09 ± 6.72 a	181.51 ± 46.5 a	n.a.
1.0	8.79 ± 1.85 b	13.04 ± 3.73 d	45.47 ± 4.83 b	19.16 ± 8.20 a	86.46 ± 8.75 b	n.a.
2.0	16.68 ± 3.49 a	43.37 ± 15.4 b	105.66 ± 15.7 a	9.81 ± 3.86 b	175.51 ± 30.0 a	n.a.
C × I						
Green Salanova × 0.015 mM Fe	18.28 ± 2.83	9.66 ± 0.55 e	89.92 ± 7.07 bc	4.39 ± 0.31 d	122.25 ± 8.74 c	n.d.
Green Salanova × 0.5 mM Fe	10.00 ± 0.15	5.59 ± 0.31 e	61.41 ± 14.5 cd	2.11 ± 0.36 d	79.11 ± 15.1 c	n.d.
Green Salanova × 1.0 mM Fe	12.65 ± 1.39	4.70 ± 0.43 e	52.85 ± 5.46 cd	1.27 ± 0.07 d	71.46 ± 7.29 c	n.d.
Green Salanova × 2.0 mM Fe	22.74 ± 4.68	9.25 ± 0.15 e	86.32 ± 11.2 bc	1.25 ± 0.14 d	119.56 ± 15.9 c	n.d.
Red Salanova × 0.015 mM Fe	3.33 ± 0.12	43.88 ± 1.15 c	144.44 ± 4.90 a	28.19 ± 0.56 b	219.85 ± 5.54 b	8.76 ± 0.58 c
Red Salanova × 0.5 mM Fe	7.61 ± 1.04	92.21 ± 2.00 a	152.01 ± 12.4 a	32.07 ± 1.10 b	283.90 ± 10.1 a	18.03 ± 1.69 b
Red Salanova × 1.0 mM Fe	4.93 ± 0.49	21.37 ± 0.17 d	38.10 ± 5.68 d	37.05 ± 3.96 a	101.46 ± 10.2 c	9.15 ± 0.56 c
Red Salanova × 2.0 mM Fe	10.62 ± 1.48	77.48 ± 5.64 b	125.00 ± 27.3 ab	18.37 ± 1.07 c	231.47 ± 33.7 b	22.11 ± 1.59 a
Significance						
Cultivar (C)	***	***	***	***	***	-
Iron (I)	**	***	***	***	***	-
C × I	NS	***	**	***	***	***

NS, **, *** Nonsignificant or significant at $P \leq 0.01$, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (P = 0.05). n.d. not detected, n.a. not applicable. All data are expressed as mean □ standard error, n = 3.

6.5.4 Lipophilic antioxidant activity and target carotenoids profile

LAA was significantly affected by C × I interaction. In the current experiment, the LAA ranged from 2.6 to 7.1 mmol Trolox 100 g^{-1} dw, with the highest values recorded in green lettuce at 2.0 mM and red lettuce treated with 1.0 and 2.0 mM Fe (Figure 3). The LAA derives mainly from lipophillic pigments which constitute an important quality trait in fresh vegetables, since these antioxidant molecules prevent the formation of free radicals in both plants and humans (Khanam et al., 2012). A putative mechanism involved in the accumulation of LAA under moderate to high Fe concentrations (1.0 and 2.0 mM) could be associated to the fact that excess of iron can be absorbed by ferritin, preventing the formation of Reactive Oxygen Species (ROS) and increasing plants antioxidant activity (Li et al., 2018).

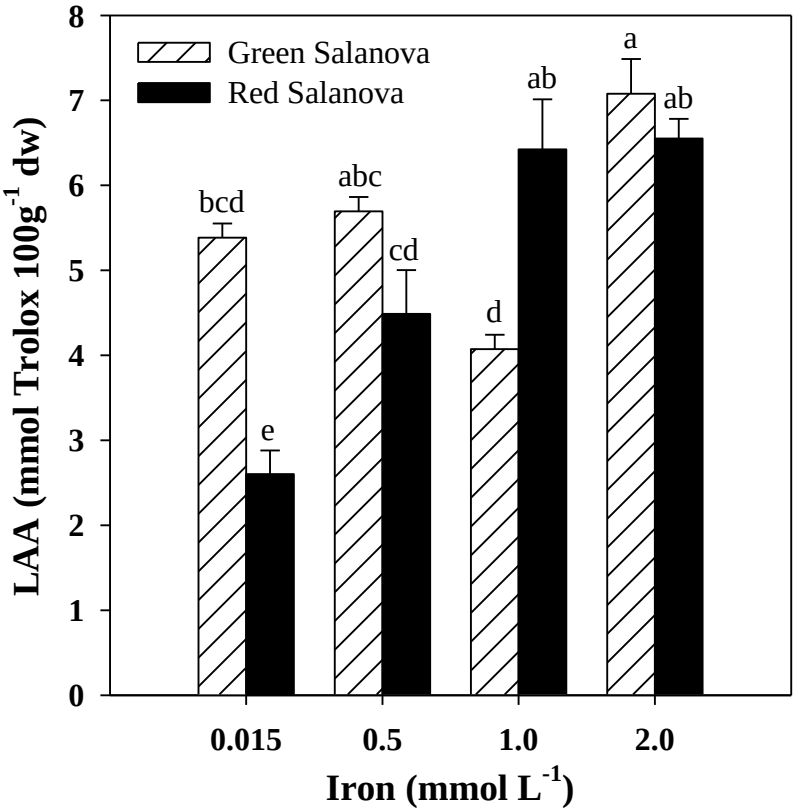

Figure 3. Effects of cultivar and iron concentration in the nutrient solution lipophilic antioxidant activity (LAA) in lettuce leaves. Different letters indicate significant differences according to Duncan's test ($P < 0.05$). The values are means of three replicates. Vertical bars indicate ± SE of means.

Carotenoids, being lipid-soluble pigments, contribute to the yellow-orange color of fruits and vegetables and constitute important antioxidant components of lettuce (Kim et al., 2016). In the current study, the carotenoid composition as a function of cultivars and Fe concentration in the nutrient solution are displayed in Table 4, wherein red Salanova plants contained higher amounts of all the detected compounds compared to green Salanova, verifying that the content in lipophilic pigments of butterhead lettuce is readily reflected in leaf pigmentation.

Irrespective of the Fe treatments, the most abundant carotenoid compounds were violaxanthin + neoxanthin, and β-cryptoxanthin, followed by lutein, while β-carotene was detected in lower levels (Table 4). Moreover, when averaged over both lettuce cultivars the highest lutein and β-cryptoxanthin concentrations were recorded at 2.0 mM and at both 1.0 and 2.0 mM Fe, respectively(Table 4). Similarly to the Fe effects observed on target phenolic acids, limited and non significant variation on violaxanthin + neoxanthin and β-carotene concentrations in response to increasing Fe concentration in the nutrient solution was observed in green Salanova. On the other hand, our results also demonstrated that the addition of 2.0 mM Fe to the nutrient solution elicited significant increase (39.2% and 30.8%) of violaxanthin + neoxanthin and β-carotene compared to the control treatment in red Salanova, whereas treatments with 0.5 and 1.0 mM exhibited intermediate values (Table 4). An explanation to the premium quality of red Salanova in terms of carotenoids could be associated with the activation of molecular and physiological mechanisms necessary for adaptation to suboptimal conditions (excess of Fe), such as the biosynthesis and accumulation of secondary metabolites, for instance, carotenoids (Rouphael and Kyriacou, 2018). These antioxidant compounds are known to contribute to ROS scavenging and cellular water homeostasis (Orsini et al., 2016) and, more interestingly, these secondary metabolites are also important owing to their health-promoting effects

Table 4. Analysis of variance and mean comparisons for target phenolic acids, total phenolic acids and anthocyanins in green and red Salanova butterhead lettuce grown under increasing iron concentration in the nutrient solution.

Source of variance	Violaxanthin + neoxanthin (μg violaxanthin eq. g^{-1} dw)	Lutein (μg g^{-1} dw)	β-Cryptoxanthin (μg g^{-1} dw)	β-Carotene (μg g^{-1} dw)
Cultivar (C)				
Green Salanova	612.38 ± 15.1 b	256.32 ± 11.3 b	426.83 ± 17.7 b	230.76 ± 7.80 b
Red Salanova	1273.74 ± 49.0 a	604.69 ± 18.8 a	1072.76 ± 25.1 a	416.17 ± 13.7 a
Iron (mM Fe) (I)				
0.015	827.73 ± 109 c	394.17 ± 76.9 b	720.56 ± 135 b	290.70 ± 36.7 c
0.5	901.60 ± 156 b	421.91 ± 88.5 b	701.54 ± 146 b	298.67 ± 43.1 c
1.0	962.97 ± 147 b	426.15 ± 65.6 b	775.42 ± 143 a	334.95 ± 34.4 b
2.0	1079.95 ± 184 a	479.79 ± 87.0 a	801.66 ± 160 a	369.53 ± 52.9 a
C × I				
Green Salanova × 0.015 mM Fe	587.73 ± 10.1 de	228.53 ± 16.9	425.23 ± 24.3	208.80 ± 1.80 e
Green Salanova × 0.5 mM Fe	552.42 ± 5.10 e	226.00 ± 15.0	380.36 ± 53.2	202.97 ± 0.60 e
Green Salanova × 1.0 mM Fe	635.33 ± 24.0 de	283.48 ± 21.2	458.20 ± 39.4	259.43 ± 8.10 d
Green Salanova × 2.0 mM Fe	674.03 ± 7.40 d	287.28 ± 13.2	443.52 ± 14.8	251.82 ± 3.50 d
Red Salanova × 0.015 mM Fe	1067.72 ± 41.9 c	559.82 ± 43.0	1015.89 ± 63.4	372.60 ± 5.80 c
Red Salanova × 0.5 mM Fe	1250.77 ± 21.1 b	617.82 ± 24.5	1022.72 ± 42.4	394.36 ± 11.6 b
Red Salanova × 1.0 mM Fe	1290.60 ± 24.0 b	568.83 ± 26.3	1092.64 ± 32.6	410.46 ± 12.6 b
Red Salanova × 2.0 mM Fe	1485.87 ± 76.2 a	672.31 ± 25.0	1159.80 ± 9.10	487.24 ± 10.1 a
Significance				
Cultivar (C)	***	***	***	***
Iron (I)	***	*	**	***
C × I	**	NS	NS	***

NS,*,**, *** Nonsignificant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (P = 0.05). All data are expressed as mean □ standard error, n = 3.

6.6 Conclusion

Nowadays, consumers, scientists, nutritionists and vegetable growers are looking for functional foods with beneficial effects on human health and longevity. Our findings highlighted that biofortification of butterhead lettuce with an essential micronutrient such as Fe could be facilitated by closed soilless cultivation due to the constant exposure of root apparatus to the fortified nutrient solution. Our results indicate that fresh yield, shoot dry biomass, mineral composition (P and K) as well as hydrophilic (ascorbic acid, chlorogenic, chicoric, caffeoyl meso tartaric acids, total phenolics and anthocyanins) and lipophilic (violaxanthin + neoxanthin, lutein, β-cryptoxanthin and β-carotene) antioxidant molecules were strongly affected by genotype, with higher nutritional and functional quality traits recorded in the red-pigmented cultivar. Our findings also demonstrated the possibility to produce Fe-enriched lettuce (by 21-54%) by adding 1.0 mM and especially 2.0 mM Fe, with an acceptable however reduction in fresh marketable yield (13.5% and 25.2% at 1.0 and 2.0 mM Fe, respectively). The addition of Fe in the nutrient solution differentially modulated the functional quality in green and red pigmented lettuce. The application of 0.5 and 1.0 mM Fe in the nutrient solution improved target phenolic acids as well as total phenolics, and the application of 2.0 mM Fe enhanced the carotenoids profile of red Salanova, whereas no significant improvements in functional quality traits were observed in green Salanova. Overall, Fe biofortification facilitated by closed soilless cultivation could be used as an effective and sustainable tool for producing functional food, especially in urban farms triggered by the rising food demand and the malnutrition owing to unbalanced diets.

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7 Chapter 7: Selenium biofortification enhances nutritional quality of two differently pigmented baby lettuces grown in closed soilless cultivation

7.1 Abstract

Selenium (Se) is an essential trace element for human nutrition, as it constitutes the key component of selenoproteins with fundamental biological functions, including the prevention of cardiovascular disorders and cancer, and thus currently used in biofortification programs. In this study, morphometric analysis, mineral composition, antioxidant activitiy, phenolic acids and carotenoids content of two differently pigmented lettuce (*Lactuca sativa* L.) cultivars in response to six Se concentrations (0, 8, 16, 24, 32, 40 μM as sodium selenate) in the nutrient solution were investigated. The experiment was conducted in an open-gas-exchange growth chamber using a closed soilless system. In the green cultivar, all selenium treatments caused on average a slight reduction (9%) in fresh yield, while in the red one this decrease was observed only at 32 and 40 μM Se (11% and 21% respectively). Leaf selenium content increased significantly with Se application in both cultivars, in particular, the red leaf lettuce accumulated 57% more Se than the green one. Regardless the cultivar, the addition of 16 μM Se in the nutrient solution improved the content of all detected phenolic acids, and at the same dose in red Salanova®, a substantial increase in anthocyanins content (184%) was also recorded. In green Salanova®, selenium applications have slightly reduced the overall carotenoids content, while in the red cultivar the dose 32 μM Se triggered a spike in violaxanthin + neoxanthin, lutein and β-cryptoxanthin by 38.6%, 27.4% and 23.1% compared to the control, respectively. Our results demonstrate the feasibility of using lettuce crops in selenium biofortification programs and that the managing of nutrient solutions in closed soilless cultivation system can be used as an effective and sustainable tool to produce Se-enriched foods with high nutraceutical value.

7.2 Introduction

Selenium is not considered as an essential mineral nutrient for higher plants (Terry et al., 2000; Sors et al., 2005; Pilon-Smits and Quinn, 2010), nevertheless several

studies demonstrate the "beneficial" effect of Se at low concentrations in improving photo-oxidative stress tolerance, delaying senescence and stimulating plant yield (Pennanen et al., 2002; Hartikainen, 2005; Lyons et al., 2009). The anti-oxidative function of Se is related to an enhanced glutathione peroxidase (GSH-Px) activity and a decreased lipid peroxidation in Se treated plants, like soybean (Djanaguiraman et al., 2005), ryegrass (Hartikainen el al., 2000) and lettuce (Xue et al., 2001; Hartikainen, 2005). At proper levels, Se is able to reduce oxidative stress caused by internal factors such as oxygen radicals produced by photosynthesis or respiration (Hartikainen, 2005) and promote plant growth in different crops like ryegrass (Hartikainen el al., 2000), potato (Turakainen et al., 2004), soybean (Djanaguiraman et al., 2005) and lettuce (Xue et al., 2001; Rios et al., 2010; Ramos et al., 2010). Malorgio et al. (2009) have found that Se addition to the nutritive solution decreases the ethylene production with consequent improvement of the quality and shelf-life of lettuce and chicory grown in a floating system.

While selenium is considered "beneficial" for plants, this element is deemed essential for animal and human nutrition, as it constitutes the key component of selenoenzymes and selenoproteins with fundamental biological functions (Rayman, 2002). Low dietary intake of Se has been considered to be the key factor in development of serious human illnesses, such as cardio-vascular diseases, viral infections and cancer (Salonen et al., 1982; Willet et al., 1991; Rayman, 2000; Combs, 2001; Finley, 2005). Selenium deficiency has been estimated to affect up to 1 billion people worldwide (Jones et al., 2017). The most serious consequences have been reported in China, the UK, Eastern Europe, Africa and Australia (Chen et al., 2002; Lyons et al., 2004), where large areas of crop soil have low bioavailability of this element and consequently a low Se supply in food. Combs (2001) reports that selenium deficiency is highly correlated with Se content in agricultural crops. The result is the low transfer of this element to the food chain in which plants are at the first level.

The Recommended Dietary Allowance (RDA) of selenium for adult men and women is 55 μg day^{-1} (Johnson et al., 2003), however, Burk et al. (2006) have found that Se supplementation of 200 μg day^{-1}, reduces the risks of cancer of the prostate, lung and colon. Plants can provide a significant source of this element in human diet by using selenium biofortification. This latter is the process to increase the bioavailable content of essential elements in edible plant parts through agricultural intervention or

genetic selection (White and Broadky, 2005). In this sense, recent works have demonstrated that Se fertilization increase the content of this element in rice (Chen et al., 2002), wheat (Lyons et al., 2004), radish (Pedrero et al., 2006), spinach (Ferrarese et al., 2012), potato (Turakainen et al., 2004), bean (Hermosillo-Cereceres et al., 2011), soybean (Yang et al., 2003), pea (Jerše et al., 2018), lamb's lettuce (Hawrylak-Nowak et al., 2018) and lettuce (Esringu et al., 2015; Businelli et al., 2015 Smolen et al., 2016; Silva et al., 2017, 2018). Se fertilization is a relatively low-cost approach to the prophylaxis of consumer's nutrient deficiency. Several countries, such as Finland, Malawi, Australia and New Zealand, have supported this strategy through biofortification programs, where it was demonstrated that Se fertilization boosted the content of selenium in human tissue and body fluids of the population (Arthur, 2003; Eurola el al., 2004; Chilimba et al., 2012).

Higher plant roots uptake Se mainly as selenate and selenite. Selenate is transported across the plasma membrane of root cells, using the same assimilation pathways of sulphate, through the enzyme sulphate permease (Terry et al., 2000; Hawkesford and Zhao, 2007), while selenite is assimilated by phosphate transporters (Li et al., 2008). The selectivity of these transporters is species-dependent and affected by soil sulphate concentration, salinity, pH and redox potential in the soil (Combs, 2001; White et al., 2004). Nevertheless, selenate is more soluble, less phytotoxic and easily transported and accumulated in crops than selenite, as confirmed in several studies (Smokolji et al., 2005; Lyons et al., 2005; Hawrylak-Nowak, 2013). Depending on species, plant transform inorganic selenium into organic selenium forms, such as selenocystine, selenomethionine, selenomethyl selenocysteine, selenocystathione, γ-glutamyl selenomethylselenocystine, selenomethylselenomethionine and selenohomocysteine (Guo and Wu, 1998; Reeves and Baker, 2000; Montes-Bayón et al.; 2002).

At high concentrations, Se has phytotoxic effects by inhibiting growth and modifying the nutritional characteristics of plants (Hartikainen el al., 2000). Regarding the nutraceutical value, in several studies it has been found that Se application affect the secondary metabolism in plants by increasing tocopherol, flavonoids, phenolic compounds, ascorbic acid and vitamin A (Hartikainen el al., 2000; Xu et al., 2003; Rios et al., 2008; Businelli et al., 2015).

Selenium phytotoxicity is attributable to non-specific incorporation of selenocysteine and selenomethionine which replace their sulphur analogues compounds

in plant proteins (Ellis and Salt, 2003). However, plants can exhibit a quite tolerance to selenium by activating detoxification mechanisms, such as the selenomethylselenocysteine production that is a nonproteinogenic seleno aminoacids (Montes-Bayón et al., 2002; Pedrero et al., 2007) or through the synthesis of compounds like dimethyl diselenide, diethyl selenide and dimethyl selenide which are volatilized (Dauchy et al., 1994).

Vegetables are widely used in biofortification studies. Lettuce (*Lactuca sativa* L.) in particular, is the most produced and consumed leafy vegetables in the world (Baslam et al., 2013; Hawrylak-Nowak, 2013). It has attained a central role in human nutrition as it combines palatable organoleptic properties with a rich content of nutraceutical compounds and a low content of dietary fats, which makes lettuce an attractive low-calorie food (Kim et al., 2016). The nutritional value of lettuce have been attributed to phenolic acids, carotenoids, flavonoids and vitamins B_9, C and E, as well as minerals content (Kim et al., 2016). Moreover, since lettuce is generally eaten raw, more nutrients are retained compared to cooked foods and, in the specific case of Se biofortified vegetables, it has been shown that food processing, such as boiling, baking or grilling, diminishes the selenium concentration due to volatility and solubility (Dumont et al., 2006; Sager, 2006). Therefore, being also one of the most easily cultivated vegetables both in soil and in hydroponic systems, lettuce can certainly be considered a valid candidate for Se biofortification programs.

There are several biofortification techniques, such as dosing Se to the soil or substrate, foliar spray with Se solution and hydroponic cultivation with Se enriched nutrient solution (Smrkolj et al., 2007; Puccinelli et al., 2017; Wiesner-Reinhold et al., 2017). The selection should consider different aspects including possible harmful effects on the environment, as the run-off of used Se fertilizers that can consequently cause accumulation of this element in groundwater. On the other hand, hydroponic cultivation, especially in closed-loop systems, have several advantages: (i) environmental spread of selenium is minimized, (ii) Se uptake is higher than other methods, as the constant exposure of the roots to fortified nutrient solution and the absence of micronutrient-soil interactions maximizing accumulation of this element in the edible parts, (iii) product quality is standardized through precise management of the concentration and composition of nutrient solution, (iv) it requires only very small amounts of selenium, and no modification of conventional closed soilless cultivation

technique is required thus ensuring no additional cost (Puccinelli et al., 2017; Wiesner-Reinhold et al., 2017; Rouphael and Kyriacou, 2018).

Taking into account these considerations, the effects of sodium selenate application was evaluated in this present work at different doses on two lettuce cultivars with different pigmentation grown in closed soilless cultivation system. The aim of this study was to identify the appropriate Se concentration of the nutrient solution in order to maximize the accumulation of selenium and enhance the nutraceutical characteristics without causing a loss of yield in lettuce.

7.3 Materials and methods

7.3.1 Growth chamber conditions, lettuce cultivars and experimental design

Two butterhead lettuce (*Lactuca sativa* L. var. capitata) cultivars with different leaf pigmentation, green Salanova® and red Salanova® (Rijk Zwaan, Der Lier, The Netherlands), were cultivated in a 28 m^2 open-gas-exchange growth chamber (7.0 x 2.1m x 4.0 m, W x H x D) situated at the experimental station of the University of Naples Federico II, located in Bellizzi, Salerno province, Italy.

The lighting of the growth chamber was provided by High Pressure Sodium lamps (Master SON-T PIA Plus 400W, Philips, Eindhoven, The Netherlands) with a photosynthetic photon flux density (PPFD) of 420 ± 20 µmol m^{-2} s^{-1}, measured at leaf height using a spectral radiometer (MSC15, Gigahertz-Optik, Turkenfeld, Germany). Day/night temperatures of 24/18 °C were established with a 12 h photoperiod and a relative air humidity of 60−80% respectively. The experiment was carried out at ambient CO_2 concentration (390 ± 20 ppm), while air exchange and dehumidification were guaranteed by two HVAC systems. Plants were grown in nutrient film technique (NFT) consisted of eighteen rigid polyvinyl chloride (PVC) gullies (14.5 cm wide, 8 cm deep and 200 cm long), with a 1% slope. The gullies were at 60 cm above floor level and each of them was fed by a separate 25 L plastic reservoir tank containing the nutrient solution (NS). Continuous recirculation (1.5 L min^{-1}) of the NS was provided by a submerged pump (NJ3000, Newa, Loreggia, PD, Italy) into each reservoir tank. Plant material was supplied by a certified nursery company (Società Agricola Punzi

S.r.l., Eboli, SA, Italy). The 20-days-old lettuce seedlings were transplanted in rockwool cubes (7 x 7 x 7cm, Delta, Grodan, Roermond, The Netherlands) and transferred into the gullies with an intra-row spacing and inter-row spacing of 15 and 43 cm respectively, corresponding to a density of 15.5 plants m^{-2}. Each gully was covered with PVC lids in order to avoid the nutrient solution evaporation. The NS was a modified Hoagland and Arnon formulation, prepared with osmotic water and containing: 8.0 mM NO_3-N, 1.0 mM S, 0.70 mM P, 2.5 mM K, 3.0 mM Ca, 0.7 mM Mg, 1.0 mM NH_4-N, 15 µM Fe, 9 µM Mn, 0.3 µM Cu, 1.6 µM Zn, 20 µM B, and 0.3 µM Mo. EC and pH were controlled daily and kept in a range of 1.4-1.5 dS m^{-1} and 5.8-6.2 respectively.

The experimental design was a randomized complete-block factorial design 6 x 2, in which the main factor was selenium concentration in the nutrient solution (0, 8, 16, 24, 32, 40 µM as sodium selenate from Sigma-Aldrich, St. Louis, MO, USA) and the other factor was the cultivar (green and red Salanova®), with tree replicates, totaling of 36 experimental units with six plants each (216 plants in total).

7.3.2 Growth analysis and biomass determination

Nine plants per treatment were harvested at 19 DAT. Number of leaves and fresh weight of the aerial plant parts was determined, then leaf area was measured by an area meter (LI-COR 3100C, Biosciences, Lincoln, Nebraska, USA).

Leaf dry weight was determined after desiccation in a forced-air oven at 70 °C to constant weight (around 72 h) and then weighed with an analytical balance (Denver Instruments, Denver, Colorado, USA). Leaf dry matter was determined according to the official method 934.01 of the Association of Official Analytical Chemists (AOAC, 2005).

7.3.3 Collection of samples for mineral and nutritional quality analyses

Part of the dried leaf tissue of green and red Salanova® plants was used for macro-mineral and selenium analyses. For the identification and quantification of hydrophilic antioxidant activity, phenolic acids and carotenoid compounds by spectophotometry and HPLC-DAD, fresh samples of three plants per experimental unit were instantly frozen in liquid nitrogen and stored at -80 °C before lyophilizing them in a Christ, Alpha 1-4 (Osterode, Germany) freeze drier.

7.3.4 Mineral analysis by ion chromatography and ICP-OES

Cations and anions were determined by liquid ion exchange chromatography (ICS 3000 Dionex Sunnyvale, CA, USA) with conductimetric detection, as described previously by Rouphael et al. (2017). Briefly, 250 mg of dried sample ground at 0.5 mm in a Wiley Mill (IKA, MF 10.1, Staufen, Germany) were suspended in 50 ml of ultrapure water (Milli-Q, Merck Millipore, Darmstadt, Germany) and stirred in shaking water bath (ShakeTemp SW22, Julabo, Seelbach, Germany) at 80°C for 10 minutes. The mixture was centrifuged at 6000 rpm for 10 min (R-10M, Remi Elektrotechnik Limited, India), then filtered through a 0.45 μm syringe filter (Phenomenex, Torrance, CA, USA). Chromatographic separation of Na, K, Mg, Ca was achieved in isocratic mode (20mM methanesulphonic acid) on an IonPac CS12A analytical column (4×250 mm, Dionex Sunnyvale, CA, USA) equipped with an IonPac CG12A precolumn (4×250 mm, Dionex Sunnyvale, CA, USA) and a self-regenerating suppressor CERS500 (4 mm, Dionex Sunnyvale, CA, USA). Nitrates, phosphorus and sulphur were detected in gradient mode (1mM-50mM KOH) on an IonPac ATC-HC anion trap (9×75 mm, Dionex Sunnyvale, CA, USA), and an AS11-HC analytical column (4×250 mm, Dionex Sunnyvale, CA, USA) equipped with an AG11-HC precolumn (4×50 mm, Dionex Sunnyvale, CA, USA) and a self-regenerating suppressor AERS500 (4 mm, Dionex Sunnyvale, CA, USA). Ions were expressed as g kg^{-1} dw and NO3-N as mg kg^{-1} fw.

In addition to macro-minerals analysis, Se content was also measured in green and red Salanova® leaf tissue. Each sample was subjected to a first phase of acid digestion performed using a commercial high-pressure laboratory microwave oven (Mars plus CEM, Italy) operating at an energy output of 1800 W. Approximately 300 mg of each dry sample was inserted directly into a microwave-closed vessel. Two milliliters of 30% (m/m) H_2O_2, 0.5 ml of 37% HCl and 7.5 ml of HNO_3 69% solution were added to each vessel. The heating program was performed in one step: temperature was increased linearly from 25 to 180 °C in 37 min, at the end the temperature was held at 180 °C for 15 min. After the digestion procedure and subsequent cooling, samples were transferred into a Teflon beaker and total volume was made up to 25 mL with Milli-Q water. The digest solution was then filtered (DISMIC 25HP PTFE syringe filter (pore size = 0.45 mm, Toyo Roshi Kaisha, Ltd., Japan) and stored in a screw cap plastic tube (Nalgene, New York). Blanks were prepared in each lot of samples. All experiments were performed in triplicate. The reagents of superpure grade, used for the microwave-

assisted digestions, were: hydrochloric acid (36% HCl), nitric acid (69% HNO_3) and hydrogen peroxide (30% H_2O_2) (Merck, Darmstadt, Germany). High-purity water (18 MΩ cm^{-1}) from a Milli-Q water purification system (Millipore, Bedford, USA) was used for the dilution of the standards, for preparing samples throughout the chemical process, and for final rinsing of the acid-cleaned vessels, glasses, and plastic utensils. The accuracy of the measurements was assessed using standard reference materials trace metals: tomato leaves (SRM 1573a). Selenium quantification was performed using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) with an axially viewed configuration (8000 DV, PerkinElmer, Shelton, CT, USA) equipped with an Hydride Generation system for Se quantification. 25 mL of digested material was pre-reduced by concentrated HCl (5 ml, superpure grade) followed by heating at 90°C for 20 minutes. After pre-reduction, the solution was diluted to 50 mL in plypropylene vial with deionized water (18 MΩ cm^{-1}). In order to assess the Se concentration were prepared calibration standards, treated in same way before dilution. For detection we have chosen the frequency with the lowest interferences, and high analytical signal and background ratio, the Se (I) line at 196.06 nm.

7.3.5 Analysis of hydrophilic antioxidant activity

The hydrophilic antioxidant activity (HAA) was determined following the N,N-dimethyl-p-phenylenediamine (DMPD) method (Fogliano et al., 1999). Freeze-dried leaves (0.200 g) were extracted with distilled water, then 2 mL of oxidant solution DMPD was added to 20 µl of the extracted sample. DMPD in acidic condition forms a stable and coloured radical cation (DMPD.+). The presence of antioxidant compounds in the samples can transfer an hydrogen atom to the DMPD.+ producing a decolaration of DMPD solution in the same proportion of their amount, and the linear inhibition of colour was measured at 505 nm. The HAA concentration was calculated using an external standard calibration curve, expressed as mmol ascorbic acid equivalent per 100 g dw

7.3.6 Phenolic acids and anthocyanins identification and quantitation

Four hundred mg of lyophilized samples was solubilized in a solution of methanol/water/formic acid (50/45/5, v/v/v, 12 ml) as described by Llorach et al. (2008) to determine phenolic acids as hydroxycinnamic derivatives. The suspensions were sonicated for 30 min and then subjected to centrifugation (2500g for 30 min at 4°C).

After a second centrifugation of supernatants at 21100g for 15 min at 4°C, sample were filtered by 0.22 µm cellulose filters (Phenomenex). A reversed phase C18 column (Prodigy, 250 × 4.6 mm, 5 µm, Phenomenex, Torrance, CA) equipped with a C18 security guard (4.0 x 3.0 mm, Phenomenex) was utilized for the separation of hydroxycinnamic derivatives and anthocyanins. Twenty µl of each extract was injected and the following mobile phases was used: (A) water formic acid (95:5, v/v) and (B) methanol through the following gradient of solvent B, (t in [min]/[%B]): (0/5), (25/40), (32/40). The flow rate was 1 mL min^{-1}. LC column was installed onto a binary system (LC-10AD, Shimadzu, Kyoto, Japan), equipped with a DAD (SPD-M10A, Shimadzu, Kyoto, Japan) and a Series 200 autosampler (Perkin Elmer, Waltham, MA). Chlorogenic and chicoric acids at 330 nm were used for the calibration curves of hydroxycinnamic derivatives. Identification of caffeoyl-meso-tartaric acid and caffeoyl-tartaric acid was performed by LC-MS/MS experiments.

The chromatographic profiles of reference curves and samples were recorded in multiple reaction monitoring mode (MRM) by using an API 3000 triple quadrupole (ABSciex, Carlsbad, CA). Negative electrospray ionization was used for detection and source parameters were selected as follows: spray voltage -4.2 kV; capillary temperature: 400 °C, dwell time 100 ms, nebulizer gas and cad gas were set to 10 and 12 respectively (arbitrary units). Target compounds [M-H]- were analyzed using mass transitions given in parentheses: chicoric acid (*m/z* 473→311, 293), chlorogenic acid (*m/z* 353 →191), caffeoyl tartaric acid (*m/z* 311→ 179, 149, retention time 15.8 min), caffeoyl-meso-tartaric acid (*m/z* 311→ 179, 149, retention time 17.8 min). The concentration of phenolic acids was reported as mg 100 g^{-1} of dw.

Anthocyanins were also measured within the same LC-DAD chromatographic runs, at 520 nm and the concentration calculated by using cyanidin as reference standard to calculate the concentration. The results were reported as µg of cyanidin equivalent per g of dw.

7.3.7 Carotenoids identification and quantification

One gram of lyophilized samples was used to determine carotenoids content following the method of Vallverdú-Queralt et al. (2013) with slight modifications. Samples were solubilized in ethanol/hexane (4:3, v/v, 2.5 ml) with 1% BHT, vortexed at 22°C for 30 s and sonicated for 5 min in the dark. Then, the solution was centrifugated

(2500 g, 4°C, 10 min) and then filtred by 0.45 µm nylon syringe filters (Phenomenex, Torrance, CA, USA). The extracts were dried in N and the dried extracts were dissolved in 1% BHT in chloroform. Twenty µl of each sample was injected onto a C18 column (Prodigy, 250 × 4.6 mm, 5 µm, Phenomenex, Torrance, C A, USA) with a C18 security guard (4.0 × 3.0 mm, Phenomenex). Two mobile phases were used: (A) acetonitrile, hexane, methanol, and dichloromethane (4:2:2:2, v/v/v/v) and (B) acetonitrile. Carotenoids were eluted at 0.8 mL min-1 through the following gradient of solvent B (t in [min]/[%B]): (0/70), (20/60), (30/30), (40/2). Carotenoids were quantified by a binary LC-10AD system connected to a DAD (SPD-M10A, Shimadzu, Kyoto, Japan) equipped with a Series 200 auto-sampler (Perkin Elmer, Waltham, MA, USA). Violaxanthin, neoxanthin, β-cryptoxanthin, lutein and β-carotene were used as reference standards. Identification of the peaks was achieved by comparison of UV-vis spectra and retention times of eluted compounds with pure standards at 450 nm. Three separate sets of calibration curves were built; each set was injected three times in the same day (intraday assay) and three times in three different days (interday assay). The accuracy was reported as the discrepancies between the calibration curves performed intraday and interday and the results were expressed as relative standard deviation RSD (%). A recovery test was performed spiking two samples with two known amounts of carotenoids (50 and 100 µg mL^{-1} final concentration) and taking into account the overestimation due to the target analytes already present in the samples. The concentration of the target carotenoids were expressed as µg g^{-1} of dw.

7.3.8 Statistics

All morphometric, nutritional and functional quality data were subjected to analysis of variance (two-way ANOVA) using IBM SPSS 20 software package (www.ibm.com/software/analytics/spss). Duncan's multiple range test was performed for mean comparisons on each of the significant ($p < 0.05$) variables measured.

7.4 Results and discussion

7.4.1 Growth response, fresh yield and dry matter

Genetic material is the main pre-harvest factor that strongly affects the biometric characteristics as well as the biosynthesis, the composition and accumulation of bioactive compounds (Rouphael et al. 2012, 2017). In our study, total leaf area, number of leaves per plant, dry biomass and dry matter content were significantly higher in green than in red Salanova®. However, no significant difference was found in terms of fresh biomass on the average effect of the cultivar (Table 1).

By analyzing the mean effect of selenium application, the dry biomass decrease significantly at the lowest dose (8 μM) and at the last two higher doses (32 and 40 μM) compared to the dose 24 μM Se; conversely, the dry matter percentage is higher in all Se treatments than the control (5.06%), although the highest value is recorded at the most concentrated Se dose (5.71%). However, the leaf number per plant is not affected by selenium applications.

Leaf area and fresh biomass were influenced by cultivar and Se treatments with significant cultivar × Se interaction (C × S). The effect of selenium dose on these parameters is dependent on the cultivar. In the red cultivar, a reduction of the leaf area was observed with the increase of Se dose, decreasing by about 12% in the range of 8-32 μM up to 20% at the higher Se dose compared to the control, while no significant differences were recorded in the green cultivar for this measurement. In the red Salanova®, Se increase in the nutrient solution induced a reduction in the fresh biomass of 11% and 21% at the dose 32 and 40 μM, respectively, compared to the control; whereas in the green Salanova® a significant decrease in fresh biomass (about 10%) is observed in all Se treatments (Table 1).

The beneficial or toxic effect of selenium on plant growth depends not only on Se concentration, but also on the species as well as on the cultivar (Pedrero and Madrid, 2009). In a work on 30 lettuce accessions grown hydroponically in a nutrient solution with 0 or 15 μM of selenate, it was found that just 5 of 30 accessions showed an increase in fresh biomass compared to control (Ramos et al., 2011). In our work, we observed in the green cultivar a reduction of fresh biomass in all Se treatments, but no significant effect on leaf area, leaf number, dry biomass and dry matter. In contrast, in a study on green lettuce cv Justyna grown hydroponically at different doses of Se (0, 2, 4,

6, 10, 20, 30, 40 and 60 μM), a significant decrease in both leaf area and fresh biomass was found at selenate concentrations up to 10 μM (Hawrylak-Nowak, 2013), while in another similar work on green lettuce cv Vera, a reduction of dry biomass was observed only at 8 μM selenate dose (Ramos et al. 2010). However, in several studies on the Philipus green lettuce cultivar, grown hydroponically at different concentrations of Se (0, 5, 10, 20, 40, 60, 80 and 120 μM), it was highlighted that selenate application at dose of 80 μM or higher significantly reduced dry biomass compared to the control (Rios et al., 2008, 2010a). Regarding fresh biomass, in our work, the red cultivar showed a better tolerance to selenate compared to the green one, this result is relatively comparable with a study on red lettuce cv Veneza Roxa where there were no significant reductions in shoot fresh weight with selenate concentrations ranging from 10 to 40 μM (Silva et al., 2018). With respect to the percentage of dry matter, our results are in agreement to those found by Businelli et al. (2015), who reported an increase in this parameter in lettuce with increased selenate applications.

Table 1. Analysis of variance and mean comparisons for leaf area, leaf number, fresh biomass, dry biomass and leaf dry matter percentage for green and red Salanova butterhead lettuce grown under increasing selenium concentration in the nutrient solution.

Source of variance	Leaf area (cm^2 $plant^{-1}$)	Leaf number (no. $plant^{-1}$)	Fresh biomass (g $plant^{-1}$)	Dry biomass (g $plant^{-1}$)	Dry matter (%)
Cultivar (C)					
Green Salanova	1193 ± 16.5 a	59 ± 0.79 a	78.55 ± 1.13	4.32 ± 0.05 a	5.48 ± 0.06 a
Red Salanova	1147 ± 21.8 b	55 ± 0.69 b	76.95 ± 1.65	3.96 ± 0.06 b	5.19 ± 0.06 b
Selenium (µM Se) (S)					
0	1253 ± 27.8 a	57 ± 1.26	84.33 ± 1.71 a	4.26 ± 0.15 ab	5.06 ± 0.07 d
8	1141 ± 18.0 b	56 ± 1.37	76.69 ± 1.47 bc	4.04 ± 0.06 b	5.28 ± 0.06 bc
16	1192 ± 25.6 ab	57 ± 1.46	80.04 ± 0.95 b	4.15 ± 0.08 ab	5.18 ± 0.10 cd
24	1186 ± 8.3 ab	57 ± 1.02	80.46 ± 1.84 ab	4.37 ± 0.06 a	5.33 ± 0.08 bc
32	1121 ± 37.7 b	56 ± 2.15	74.87 ± 1.46 c	4.03 ± 0.13 b	5.44 ± 0.06 b
40	1127 ± 49.8 b	60 ± 2.23	70.09 ± 2.35 d	4.01 ± 0.19 b	5.71 ± 0.09 a
C x S					
Green Salanova x 0 µM Se	1207 ± 29.6 ab	59 ± 1.02	86.29 ± 1.47 a	4.48 ± 0.12	5.19 ± 0.07
Green Salanova x 8 µM Se	1126 ± 21.2 bcd	58 ± 0.85	75.72 ± 2.88 cd	4.07 ± 0.10	5.38 ± 0.07
Green Salanova x 16 µM Se	1236 ± 22.6 ab	59 ± 1.76	79.30 ± 1.85 bcd	4.26 ± 0.10	5.38 ± 0.09
Green Salanova x 24 µM Se	1201 ± 6.2 ab	57 ± 1.02	78.08 ± 1.71 bcd	4.48 ± 0.02	5.50 ± 0.02
Green Salanova x 32 µM Se	1169 ± 66.9 bc	58 ± 3.00	76.90 ± 2.42 bcd	4.23 ± 0.20	5.53 ± 0.10
Green Salanova x 40 µM Se	1219 ± 59.8 ab	64 ± 0.93	74.99 ± 0.97 cd	4.41 ± 0.09	5.88 ± 0.12
Red Salanova x 0 µM Se	1299 ± 29.5 a	55 ± 1.19	82.37 ± 2.93 ab	4.05 ± 0.23	4.94 ± 0.08
Red Salanova x 8 µM Se	1157 ± 30.4 bc	53 ± 1.47	77.67 ± 1.26 bcd	4.01 ± 0.08	5.17 ± 0.06
Red Salanova x 16 µM Se	1147 ± 27.5 bc	55 ± 1.35	80.78 ± 0.76 abc	4.03 ± 0.11	4.99 ± 0.09
Red Salanova x 24 µM Se	1172 ± 9.3 bc	57 ± 2.04	82.84 ± 2.87 ab	4.27 ± 0.08	5.17 ± 0.09
Red Salanova x 32 µM Se	1074 ± 18.1 cd	53 ± 2.92	72.84 ± 0.86 d	3.82 ± 0.05	5.35 ± 0.05
Red Salanova x 40 µM Se	1036 ± 21.8 d	55 ± 1.11	65.20 ± 1.67 e	3.61 ± 0.07	5.54 ± 0.03
Significance					
Cultivar (C)	*	***	NS	***	***
Selenium (S)	**	NS	***	*	***
C x S	**	NS	*	NS	NS

NS,*,**, *** Non-significant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test ($P = 0.05$). All data are expressed as mean ± standard error, n = 3.

7.4.2 Nitrate content, mineral composition and selenium biofortification

Nitrate content in plants grown for human consumption is extremely important, since a high intake of this nutrient can be harmful to human health, because after ingestion, it is rapidly transformed into nitrites and nitrogenous compounds that can cause serious pathological disorders such as methaemoglobinaemia and blue baby syndrome (Mensinga et al., 2003; Santamaria, 2006). In addition, it should be taken into account that lettuce is considered a nitrates hyperaccumulator and in this sense, the European Commission (Commission Regulation n° 1258/2011) has set as maximum limit of this anion concentration in lettuce at 4000-5000 mg kg^{-1} fw for harvest occurring from April 1 to September 30 and from October 1 to March 31, respectively. In our study, the green cultivar has a higher nitrate content (1810 mg kg^{-1} fw) than the red one (1272 mg kg^{-1} fw) and in both cases these values are below EU regulations limits (Table 2). As found in literature, nitrate accumulation in lettuce is independent from the cultivation management, but mainly depends on genotypic factors (Burns et al., 2011, 2012; Lopez et al., 2014). Nitrate content was influenced by cultivar and Se treatments with significant cultivar × Se interaction. In green Salanova® a significant reduction of nitrate content was observed at Se doses 8 (15%), 32 (16%) and 40 μM (32%) compared to the control, while no significant selenium effect was found regarding this parameter in red Salanova® (Table 2). The nitrates reduction prompted by selenate could be due to an antagonistic effect of these two anions (Rios et al., 2010). Other studies by Nowak et al. (2002, 2004) have demonstrated that Se affect the nitrate reductase and nitrites reductase enzymes, diminishing their activity. Our data reflect a nitrate reduction observed in other works where selenate has been applied on green lettuce at different concentrations (Lee et al., 2008; Rios et al., 2010a, 2010b).

The growth and development of plants depends on the equilibrium of the mineral elements, as stress occurs in the presence of nutritional imbalances (Salt et al., 2008). Minerals are also essential for human health and lettuce is considered a good source of them (Baslam et al., 2013; Kim et al., 2016). In our work, in the comparison between the cultivars, green Salanova® recorded the highest phosphorus, potassium and calcium content, while red Salanova® showed the highest quantity of magnesium and sulphur (Table 2). As reported in literature, lettuce mineral content is quite variable depending on head type and cultivar (Rouphael et al., 2017).

The mean effect of selenate application shows that calcium content at 40 μM Se was significantly lower than the control (9%), while phosphorus content at selenate concentrations ranging from 24 to 40 μM decrease significantly (about 18%) compared to other Se doses (Table 2). The lowest calcium content at the highest selenate dose is in agreement with Rios et al. (2013) who found a 9% reduction of this element at a Se dose of 40 μM compared to the control. Regarding the results of phosphorus content, our findings suggest a possible antagonistic relationship between Se and P, as confirmed in some works on different species (Hopper and Parker, 1999; Liu et al., 2004). In addition, our results seem consistent with those of Rios et al. (2013) that showed a decrease of P content at the Se range of 20-120 μM with respect to control.

Leaf content of potassium, magnesium, sodium and sulphur were influenced by cultivar and Se treatments with significant C × S interaction. In the green cultivar, a significant reduction of K content is observed at Se doses 8 μM (10%) and 40 μM (17%) compared to the control (Table 2). Likewise, a 10% decrease in Mg content was noted with respect to the control, both at dose 8 and 40 μM. On the contrary, in the red cultivar a potassium rise (9%) was registered at Se dose of 32 μM and about a 12% increase in magnesium content was observed at the Se range of 16-40 μM compared to control (Table 2). The lowest K and Mg content resulted in green Salanova® with Se application of 40 μM coincide with the results obtained by Rios et al. (2013) at the same dose of selenate on Philipus green lettuce cultivar. On the other hand, even if the increase of K and Mg recorded in red Salanova® treated with Se was in disagreement with literature, other authors have not found variation in these two elements content after selenate applications (Wu and Huang, 1992; Silva et al., 2018). With regard to sodium in our study, the green cultivar does not present significant differences, while the red one shows a higher content of this element at the two most concentrated Se doses compared to the control (Table 2). This latter result disagree with Silva et al. (2018) findings, who found no difference in the Na content after application of selenate up to 40 μM. Furthermore, sulphur content increased significantly and linearly in both cultivars with selenate concentration ranging from 0.70 to 4.10 mg kg^{-1} dw in the green Salanova® and from 1.21 to 9.20 mg kg^{-1} dw in the red Salanova® (Table 2). These data imply a synergic relationship between selenate and sulphur. Our results are confirmed by several authors (Ramos et al. 2011; Hawrylak-Nowak, 2013; Rios et al., 2013; Silva et al., 2018), and in particular Rios et al. (2008) who found an increase in S

content with Se concentrations up to 40 μM. Similarly, Lyons et al. (2005) suggested that low concentration of Se in the culture medium resulted in an accumulation of S in the plants. The first stage in the S-assimilation process consists of the activation of the enzyme ATP-sulphurylase, which produces adenosine phosphosulphate from sulphate and ATP (Pilon-Smits et al., 1999). In this sense, selenate applications can increase the ATP-sulphurylase activity and consequently a greater presence of selenate could imply increased production of Se and S end products (Rios et al., 2008).

The effectiveness of a selenium biofortification program is strongly correlated with the capacity of the candidate crop to assimilate and accumulate this element in the edible parts of the plant. As expected, with the same trend of sulphur (Table 2), in our work Se leaf content increased with selenate application rate (Figure 1). Comparing between cultivars, red leaf lettuces accumulated 57% more Se than green ones. Selenium leaf content was influenced by cultivar and Se treatments with highly significant interaction. In particular, Se concentration peaking in green Salanova® at 40 μM dose (128.43 mg kg^{-1} dw), while in red Salanova® it reached the highest values at the last two most concentrated doses (116.67 and 128.20 mg kg^{-1} dw respectively). Anyhow, selenium leaf content was significantly higher than the control starting from dose 16 μM for both cultivars. Our results are in agreement with previous studies on red and green-pigmented lettuce (Ramos et al., 2010; Hawrylak-Nowak, 2013; Silva et al., 2018) and demonstrate the actual feasibility of using lettuce crops in Se biofortification programs.

Table 2. Analysis of variance and mean comparisons of leaf mineral composition in green and red Salanova butterhead lettuce grown under increasing selenium concentraton in the nutrient solution.

Source of variance	NO_3 (mg kg^{-1} fw)	P (g kg^{-1} dw)	S (g kg^{-1} dw)	K (g kg^{-1} dw)	Ca (g kg^{-1} dw)	Mg (g kg^{-1} dw)	Na (g kg^{-1} dw)
Cultivar (C)							
Green Salanova	1810 ± 69 a	4.85 ± 0.12 a	1.90 ± 0.31 b	59.50 ± 1.19 a	6.13 ± 0.09 a	2.25 ± 0.03 b	0.36 ± 0.012
Red Salanova	1272 ± 25 b	4.68 ± 0.12 b	4.92 ± 0.77 a	54.81 ± 0.67 b	5.21 ± 0.11 b	2.62 ± 0.04 a	0.39 ± 0.029
Selenium (µM Se) (S)							
0	1660 ± 175 a	5.32 ± 0.18 a	0.96 ± 0.12 d	58.57 ± 3.00 a	5.73 ± 0.35 a	2.41 ± 0.06 ab	0.37 ± 0.039
8	1480 ± 112 b	5.05 ± 0.07 ab	1.30 ± 0.21 d	54.75 ± 1.12 b	5.62 ± 0.14 ab	2.31 ± 0.06 b	0.32 ± 0.010
16	1680 ± 149 a	5.06 ± 0.02 ab	2.17 ± 0.46 c	58.71 ± 1.72 a	6.00 ± 0.29 a	2.52 ± 0.10 a	0.36 ± 0.013
24	1704 ± 168 a	4.80 ± 0.05 b	3.49 ± 0.89 b	60.04 ± 1.56 a	5.68 ± 0.18 a	2.47 ± 0.11 a	0.35 ± 0.011
32	1487 ± 111 b	4.28 ± 0.14 c	5.92 ± 1.38 a	58.18 ± 1.19 a	5.80 ± 0.23 a	2.51 ± 0.13 a	0.44 ± 0.076
40	1234 ± 64 c	4.07 ± 0.09 c	6.65 ± 1.04 a	52.69 ± 0.84 b	5.21 ± 0.28 b	2.39 ± 0.13 ab	0.42 ± 0.033
C x S							
Green Salanova x 0 µM Se	2011 ± 168 a	5.51 ± 0.33	0.70 ± 0.08 f	63.49 ± 4.54 a	6.34 ± 0.37	2.40 ± 0.11 bc	0.44 ± 0.043 bc
Green Salanova x 8 µM Se	1718 ± 68 b	5.00 ± 0.12	0.84 ± 0.02 f	56.89 ± 0.69 cd	5.86 ± 0.14	2.17 ± 0.03 d	0.34 ± 0.005 cd
Green Salanova x 16 µM Se	2011 ± 30 a	5.07 ± 0.02	1.14 ± 0.06 ef	62.52 ± 0.36 ab	6.49 ± 0.23	2.31 ± 0.06 bcd	0.37 ± 0.023 bcd
Green Salanova x 24 µM Se	2074 ± 46 a	4.85 ± 0.10	1.51 ± 0.03 e	63.38 ± 0.94 a	6.04 ± 0.10	2.22 ± 0.04 cd	0.35 ± 0.017 cd
Green Salanova x 32 µM Se	1681 ± 148 b	4.45 ± 0.24	3.12 ± 0.15 d	57.83 ± 1.56 bcd	6.29 ± 0.08	2.22 ± 0.02 cd	0.31 ± 0.011 d
Green Salanova x 40 µM Se	1366 ± 36 c	4.22 ± 0.07	4.10 ± 0.35 c	52.91 ± 1.15 d	5.74 ± 0.07	2.15 ± 0.03 d	0.36 ± 0.016 bcd
Red Salanova x 0 µM Se	1309 ± 36 cd	5.12 ± 0.11	1.21 ± 0.06 ef	53.66 ± 0.39 cd	5.11 ± 0.30	2.42 ± 0.07 bc	0.29 ± 0.010 d
Red Salanova x 8 µM Se	1242 ± 41 cd	5.09 ± 0.11	1.77 ± 0.05 e	52.62 ± 1.10 d	5.37 ± 0.12	2.44 ± 0.04 b	0.30 ± 0.012 d
Red Salanova x 16 µM Se	1349 ± 10 cd	5.05 ± 0.03	3.20 ± 0.05 d	54.89 ± 0.29 cd	5.51 ± 0.37	2.73 ± 0.05 a	0.35 ± 0.015 bcd
Red Salanova x 24 µM Se	1334 ± 54 cd	4.76 ± 0.02	5.46 ± 0.17 b	56.70 ± 0.31 cd	5.32 ± 0.17	2.72 ± 0.01 a	0.36 ± 0.016 bcd
Red Salanova x 32 µM Se	1293 ± 47 cd	4.10 ± 0.09	8.71 ± 0.13 a	58.53 ± 2.14 abc	5.30 ± 0.11	2.80 ± 0.03 a	0.57 ± 0.112 a
Red Salanova x 40 µM Se	1103 ± 45 d	3.92 ± 0.13	9.20 ± 0.52 a	52.47 ± 1.48 d	4.68 ± 0.33	2.64 ± 0.15 a	0.48 ± 0.038 ab
Significance							
Cultivar (C)	***	*	***	***	***	***	NS
Selenium (S)	***	***	***	***	*	*	NS
C x S	*	NS	***	*	NS	**	***

NS,*,**, *** Non-significant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (P = 0.05). All data are expressed as mean ± standard error, n = 3.

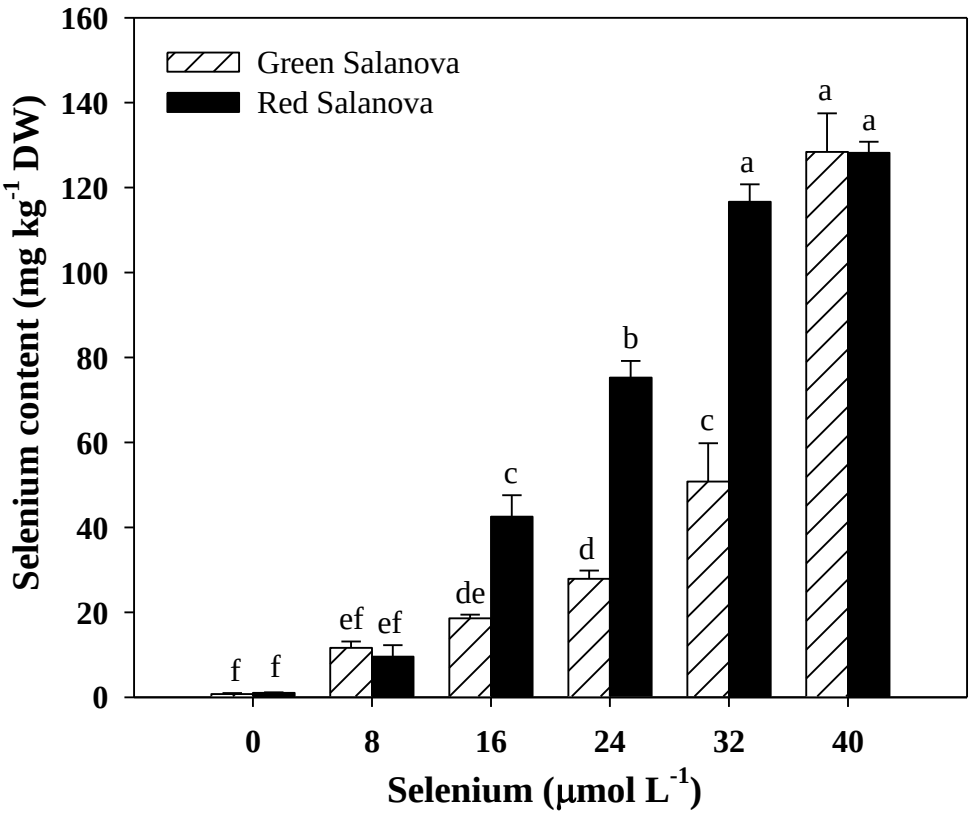

Figure 1. Selenium leaf accumulation in lettuce leaves. Different letters indicate significant differences according to Duncan's test ($P < 0.05$). The values are means of three replicates. Vertical bars indicate ± SE of means

7.4.3 Hydrophilic antioxidant activity, phenolic acids and carotenoids profiles

In our work, the HAA was found to be significantly higher in the green cultivar than in the red one (Figure 2). In a study on seven different iceberg lettuce cultivar by Rouphael et al. (2017), it was demonstrated that the HAA was strongly influenced by genetic factors. Regarding the effect of selenate concentration on hydrophilic antioxidant activity, in red Salanova® a significant reduction of HAA was observed (about 28%) at Se doses ranging from 24 to 40 μM compared to the control, while no significant selenium effect was found on this parameter in green Salanova® (Figure 2). During oxidative stress, plants generates reactive oxygen species (ROS) that damage numerous macromolecules and cell structures, as well as negatively affect growth and development (Das et al., 1992; Fu and Huang, 2001). In order to protect against ROS, plants contain antioxidant enzymes such as CAT, SOD, APX and GSH-Px (Blokhina et

al., 2003). Se application can affect the activity of redox enzymes and consequently change the oxidation reduction status of the leaves in stress tolerance (Vikhreva et al., 2002). However, Ramos et al. (2010) has found in a work on lettuce with selenium applied at different doses that the catalase (CAT) activity significantly decreased with selenate concentrations above 16 μM. This finding can probably explain the HAA reduction found in red Salanova® treated with selenate doses above 16 μM, to which is also associated, as previously seen, a decrease in fresh yield. As found by Ramos et al. (2011) on thirty Se biofortified lettuce cultivars, CAT activity is strongly influenced by genetic factors and, in particular, in our case a greater tolerance to hydrophilic antioxidant activity was found in green Salanova® compared to red Salanova®.

HPLC analysis revealed in both cultivars the presence of four main caffeic acid derivatives (Table 3). Chicoric acid was the most abundant phenolic acid detected in both cultivars (101.44 and 105.99 mg 100 g^{-1} dw, respectively for the green and the red cultivar), chlorogenic acid (88.02 mg 100 g^{-1} dw) and caffeoyl-meso-tartaric acid (41.08 mg 100g^{-1} dw) were higher in red Salanova®, while caffeoyl-tartaric acid (17.77 mg 100 g^{-1} dw) was higher in green Salanova® compared to the red cultivar (Table 3). Anyhow, the sum of detected phenolic acids was higher in the red cultivar with respect to the green one (239.52 and 139.10 mg 100 g^{-1} dw, respectively). The content of phenolic acids varies according to the type of lettuce (Kim et al., 2016). Our results are consistent with the literature in which red cultivars have more phenolic acids than green ones (Llorach et al., 2008; Kim et al., 2016). The presence of chlorogenic acid, chicoric acid and caffeoyl tartaric acid was also detected in seven different lettuce cultivars studied in Rouphael et al. (2017). All phenolic acids were affected by cultivar and Se treatments with significant cultivar × Se interaction (Table 3). In green Salanova®, caffeoyl-tartaric acid increases by 69% and 46% respectively at Se doses 16 and 24 μM, but decreases by 75% at dose 32 μM, while in red Salanova® the higher content is obtained at a dose of 16 μM (105%) compared to the control. Chorogenic acid, in green cultivar, decreased by 57% at Se dose 32 μM, but increased by 143% at the most concentrated dose, while in red one the content increased at doses 8, 16, 24 and 40 μM with the highest value recorded at dose 16 μM (191.64 mg 100 g^{-1} dw). Similarly, chicoric acid, in green cultivar, increased at Se doses 8, 16, 24 and 40 μM with the highest value recorded at dose 16 μM (148.53 mg 100g^{-1} dw), but decreased by 67% at dose 32 μM, conversely in the red one chicoric acid content increased by 32% at dose

16 μM, but decreased at Se doses 8, 24, 32 and 40 μM. In red Salanova®, caffeoyl-meso-tartaric acid increases by 270%, 84% and 89% respectively at Se doses 16, 24 and 40 μM with respect to the control, while no significant differences were found for this phenolic acid in green Salanova®. In green cultivar, the sum of detected phenolic acids was significantly higher at doses 8, 16, 24 and 40 μM with the highest value recorded at dose 24 μM (194.55 mg 100 g^{-1} dw), but decreased by 67% at dose 32 μM, while in red one the sum of phenolic acids increased by 112 % at dose 16 μM, but decreased at Se doses 8, 32 and 40 μM compared to the control (Table 3). Our results showed irregular variations of phenolic acids content in both cultivars, where the concentrations of these antioxidant compounds varied with selenium level without a clear trend. Furthermore, this pattern is consistent with what was found by Schiavon et al. (2016) in radish and by D'Amato et al. (2018) in rice sprouts, but is in disagreement with Rios et al. (2008), which has found a rise in the total phenol content in lettuce as the applied selenium concentration increased. On the other hand, the presence of selenium represent an abiotic stress similar to that caused by other heavy metals where the plant reacts by activating the phenylpropanoid pathway (Wang et al., 2016) for producing phenolic compounds, which can act as metal chelators and inhibitors of enzymes such as oxidase xanthine, and accordingly prevent the ROS production (Rios et al., 2008).

Anthocyanins are one of the phenolic phytochemicals subclasses (Harborne and Williams, 2001). They are coloured water-soluble pigments responsible for red pigmentation in lettuces (Kim et al., 2016). Therefore, these pigments were not detected in green Salanova®, but exclusively in the red cultivar with an average concentration of 13.28 μg g^{-1} dw (Table 3). Anthocyanin has many physiological effects on plants and humans, such as antioxidation, protection against ultraviolet damage and the prevention and treatment of various diseases (Hamilton, 2004). Anthocyanins in red Salanova®, were found to be significantly affected by selenate applications, in particular they increased by 184%, 84% and 31% respectively at Se doses 16, 24 and 32 μM with respect to the control (Table 3). Our results are in accordance with Liu et al. (2017), where anthocyanins in red lettuce cv "Purple Rome" increased significantly at moderate doses of selenium, while they where lower and comparable to the control at higher Se doses. In this study it was demonstrated that the selenium influence on accumulation and molecular regulation of anthocyanins synthesis was mainly due to the expression levels of the F3H and UFGT genes (Liu et al., 2017).

Carotenoids are essential lipid-soluble pigments that have antioxidant properties and are found in all photosynthetic organisms (Gross, 2012). These compounds play significant roles in the prevention of chronic diseases, such as cancer, cardiovascular disease, diabetes and osteoporosis, owing to their potent antioxidant, immunomodulatory, gap-junction communication, photoprotective, neuroprotective and Vitamin A activity (Saini et al., 2015). Carotenoids are classified into two groups, xanthophylls which include neoxanthin, violaxanthin, lutein, zeaxanthin, and β-cryptoxanthin, and carotenes which include β-carotene, α-carotene and lycopene. In human diet, neoxanthin, violaxanthin, lutein and β-carotene are primarily obtained from dark green or red vegetables; in particular, it has been found in lettuce higher carotenoids content in red leaf cultivar compared to green ones (Nicolle et al., 2004). This finding is in agreement with our results where red Salanova® has a significant higher content of all the detected carotenoids compared to green Salanova®. In particular, the sum of the detected carotenoids was 133% higher in the red cultivar compared to the green one (Table 4). Carotenoids content was affected by cultivar and Se treatments with significant cultivar × Se interaction. In green Salanova®, all detected carotenoids decreased following selenate applications with respect to the control (Table 4), a similar trend was noted in red Salanova® except for dose 16 μM where a slight increase was noted. On the other hand, violaxanthin + neoxanthin, lutein and β-cryptoxanthin increased in red Salanova® with selenate application levels, reaching the highest content at Se dose 32 μM, while the level of β-carotene was on average 23% lower than the control with Se applications above 16 μM. Regarding the green cultivar, our results are in agreement with what has been found in the literature on lettuce (Hawrylak-Nowak, 2013), rice (D'Amato et al., 2018) and arabidopsis (Sams et al., 2011), where a reduction of the carotenoids total content was found following the application of sodium selenate.

With respect to these results, it has been demonstrated in arabidopsis that the presence of selenate may down-regulate the phytoene synthase (PSY) which is the major enzyme involved in the biosynthesis of carotenoids (Sams et al., 2011). Regarding the increase in xanthophylls (violaxanthin, neoxanthin, lutein and β-cryptoxanthin) found at the 32 μM Se dose in the red Salanova, this could be associated to a dissimilar activation of molecular and physiological mechanisms in this cultivar, which have differently influenced the biosynthesis and accumulation of secondary metabolites, such as xanthophylls. Moreover, in our experiment, it is noted that the presence of selenate has

an opposite effect on the various secondary metabolites, since overall the increase in phenolic acids is almost always associated with a decrease in the carotenoids

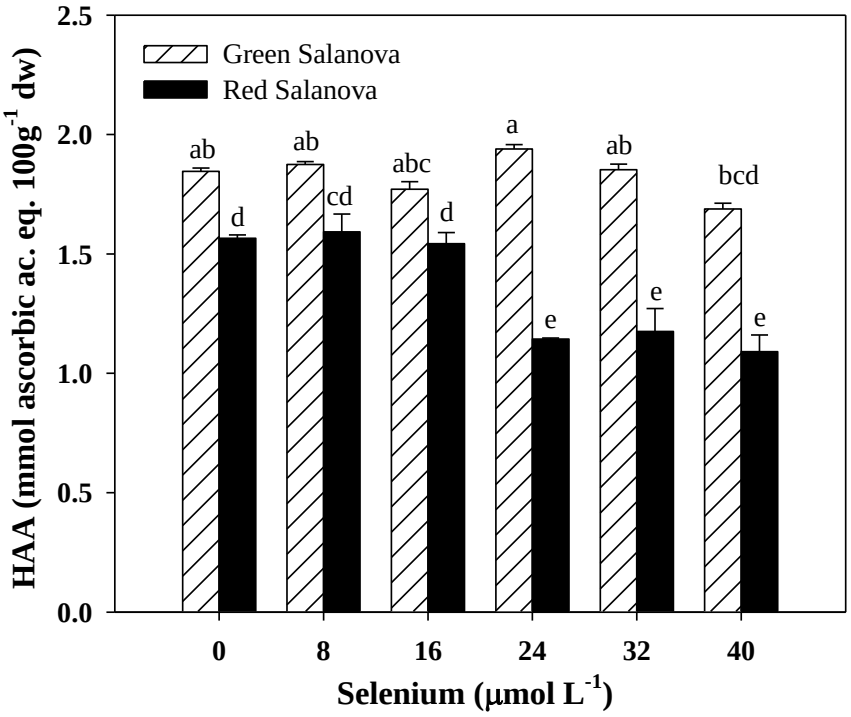

Figure 2. Effects of cultivar and selenium concentration in the nutrient solution on hydrophilic antioxidant activity in lettuce leaves. Different letters indicate significant differences according to Duncan's test ($P < 0.05$). The values are means of three replicates. Vertical bars indicate ± SE of means.

Table 3. Analysis of variance and mean comparisons for target phenolic acids, total phenolic acids and anthocyanins in green and red Salanova butterhead lettuce grown under increasing selenium concentration in the nutrient solution.

Source of variance	Caffeoyl tartaric acid (mg 100 g^{-1} dw)	Chlorogenic acid (mg 100 g^{-1} dw)	Chicoric acid (mg 100 g^{-1} dw)	Caffeoyl meso tartaric acid (mg 100 g^{-1} dw)	∑ phenolic acids (mg 100g^{-1} dw)	Anthocyanins (µg cyanidin eq. g^{-1} dw)
Cultivar (C)						
Green Salanova	17.77 ± 1.86 a	13.94 ± 1.51 b	101.44 ± 9.27	5.96 ± 0.49 b	139.10 ± 12.42 b	n.d.
Red Salanova	4.43 ± 0.42 b	88.02 ± 11.71 a	105.99 ± 12.20	41.08 ± 5.11 a	239.52 ± 26.73 a	13.28 ± 1.45
Selenium (µM Se) (S)						
0	9.99 ± 2.76 c	30.76 ± 9.46 c	116.65 ± 16.75 b	14.59 ± 3.50 c	171.99 ± 25.77 c	n.a.
8	11.79 ± 3.48 bc	45.34 ± 14.22 b	92.41 ± 8.03 c	16.16 ± 4.47 cd	165.70 ± 8.33 c	n.a.
16	17.56 ± 4.43 a	103.47 ± 39.47 a	160.34 ± 15.88 a	44.45 ± 17.67 a	325.81 ± 68.1 a	n.a.
24	13.97 ± 4.48 b	51.68 ± 15.67 b	114.71 ± 15.29 b	23.31 ± 8.09 bc	203.68 ± 6.37 b	n.a.
32	3.42 ± 0.34 d	28.67 ± 11.01 c	45.83 ± 7.90 d	17.21 ± 6.82 cd	95.13 ± 25.33 d	n.a.
40	9.88 ± 2.89 c	45.96 ± 10.37 b	92.33 ± 9.29 c	25.39 ± 7.73 b	173.56 ± 8.48 c	n.a.
C x S						
Green Salanova x 0 µM Se	16.15 ± 0.27 c	9.76 ± 0.97 g	85.40 ± 3.40 d	6.85 ± 0.23 d	118.17 ± 3.82 g	n.d.
Green Salanova x 8 µM Se	19.30 ± 1.98 c	13.71 ± 1.46 g	109.83 ± 4.00 c	6.36 ± 0.19 d	149.19 ± 6.72 f	n.d.
Green Salanova x 16 µM Se	27.23 ± 2.09 a	15.30 ± 1.18 fg	124.90 ± 1.53 c	6.33 ± 0.70 d	173.75 ± 2.52 def	n.d.
Green Salanova x 24 µM Se	23.60 ± 2.67 b	16.99 ± 0.64 fg	148.53 ± 4.47 b	5.43 ± 0.70 d	194.55 ± 7.59 cd	n.d.
Green Salanova x 32 µM Se	4.00 ± 0.37 e	4.18 ± 0.66 h	28.35 ± 1.47 f	2.21 ± 0.41 d	38.74 ± 2.31 h	n.d.
Green Salanova x 40 µM Se	16.32 ± 0.45 c	23.73 ± 0.62 f	111.63 ± 7.62 c	8.55 ± 0.39 d	160.23 ± 8.46 ef	n.d.
Red Salanova x 0 µM Se	3.84 ± 0.06 e	51.76 ± 2.26 e	147.89 ± 20.38 b	22.32 ± 1.10 c	225.82 ± 20.25 b	8.76 ± 0.23 d
Red Salanova x 8 µM Se	4.27 ± 0.12 de	76.98 ± 2.90 c	75.00 ± 1.79 de	25.96 ± 1.93 c	182.21 ± 5.41 de	8.73 ± 0.37 d
Red Salanova x 16 µM Se	7.89 ± 0.63 d	191.64 ± 3.96 a	195.78 ± 1.65 a	82.57 ± 10.34 a	477.87 ± 7.83 a	24.85 ± 2.58 a
Red Salanova x 24 µM Se	4.34 ± 0.72 de	86.38 ± 4.79 b	80.90 ± 2.22 de	41.18 ± 2.69 b	212.80 ± 7.87 bc	16.10 ± 0.96 b
Red Salanova x 32 µM Se	2.84 ± 0.32 e	53.16 ± 2.48 e	63.31 ± 2.10 e	32.21 ± 2.69 bc	151.52 ± 4.75 f	11.48 ± 0.56 c
Red Salanova x 40 µM Se	3.43 ± 0.19 e	68.18 ± 6.56 d	73.04 ± 1.02 de	42.24 ± 3.80 b	186.89 ± 10.49 cde	9.78 ± 0.39 cd
Significance						
Cultivar (C)	***	***	NS	***	***	-
Selenium (S)	***	***	***	***	***	-
C x S	***	***	***	***	***	***

NS,*,**, *** Non-significant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (P = 0.05). n.d. not detected, n.a. not applicable. All data are expressed as mean ± standard error, n = 3.

Table 4. Analysis of variance and mean comparisons for target phenolic acids, total phenolic acids and anthocyanins in green and red Salanova butterhead lettuce grown under increasing selenium concentration in the nutrient solution.

Source of variance	Violaxanthin + neoxanthin (μg violaxanthin eq. g^{-1} dw)	Lutein (μg eq. g^{-1} dw)	β-Cryptoxanthin (μg g^{-1} dw)	β-carotene (μg g^{-1} dw)
Cultivar (C)				
Green Salanova	507.39 ± 14.1 b	207.62 ± 8.55 b	370.60 ± 13.8 b	165.62 ± 6.53 b
Red Salanova	993.13 ± 28.8 a	600.36 ± 15.3 a	989.43 ± 26.4 a	337.14 ± 11.8 a
Selenium (μM Se) (S)				
0	733.14 ± 53.0 c	421.04 ± 62.6 b	717.66 ± 107 b	296.43 ± 37.0 a
8	633.57 ± 95.3 d	357.59 ± 81.6 d	587.32 ± 127 d	252.25 ± 51.7 c
16	774.82 ± 117 b	421.51 ± 101 b	699.87 ± 165 b	272.02 ± 57.1 b
24	762.72 ± 123 b	385.30 ± 88.9 c	645.43 ± 138 c	215.09 ± 29.6 e
32	850.46 ± 148 a	461.27 ± 113 a	784.17 ± 176 a	239.98 ± 33.2 cd
40	746.85 ± 118 bc	377.20 ± 81.1 c	645.67 ± 119 c	232.51 ± 23.2 d
C x S				
Green Salanova x 0 μM Se	614.93 ± 5.54 d	282.15 ± 3.01 e	478.51 ± 3.85 e	214.60 ± 5.39 e
Green Salanova x 8 μM Se	421.46 ± 7.09 f	175.52 ± 3.87 g	305.07 ± 5.49 h	136.91 ± 2.42 h
Green Salanova x 16 μM Se	513.05 ± 3.29 e	195.75 ± 4.01 fg	331.35 ± 6.79 gh	145.04 ± 3.10 gh
Green Salanova x 24 μM Se	489.24 ± 7.10 e	186.75 ± 2.57 fg	337.96 ± 8.31 gh	149.09 ± 2.93 gh
Green Salanova x 32 μM Se	520.97 ± 4.26 e	209.40 ± 5.19 f	390.71 ± 2.76 f	166.05 ± 4.61 fg
Green Salanova x 40 μM Se	484.69 ± 2.68 e	196.11 ± 3.01 fg	379.99 ± 6.92 fg	182.06 ± 2.73 f
Red Salanova x 0 μM Se	851.34 ± 6.70 c	559.94 ± 17.4 cd	956.81 ± 21.7 c	378.27 ± 10.1 ab
Red Salanova x 8 μM Se	845.68 ± 19.1 c	539.67 ± 10.4 d	869.57 ± 32.3 d	367.60 ± 8.28 b
Red Salanova x 16 μM Se	1036.59 ± 11.4 b	647.27 ± 15.1 b	1068.38 ± 25.7 b	399.01 ± 13.9 a
Red Salanova x 24 μM Se	1036.19 ± 17.1 b	583.85 ± 7.42 c	952.89 ± 8.83 c	281.09 ± 3.13 d
Red Salanova x 32 μM Se	1179.95 ± 20.8 a	713.14 ± 0.18 a	1177.62 ± 26.2 a	313.91 ± 4.53 c
Red Salanova x 40 μM Se	1009.02 ± 26.4 b	558.28 ± 10.9 cd	911.34 ± 16.9 cd	282.95 ± 11.8 d
Significance				
Cultivar (C)	***	***	***	***
Selenium (S)	***	***	***	***
C x S	***	***	***	***

NS,*,**, *** Non-significant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (P = 0.05). All data are expressed as mean ± standard error, n = 3.

7.5 Conclusion

Nowadays, the demand for functional foods with beneficial effects on human health is increasingly sought. Selenium biofortification of lettuce grown in closed soilless cultivation has been proved to be an effective method to produce Se-enriched foods with a high nutritional value. Our findings indicate that shoot dry biomass, mineral composition, hydrophilic antioxidant activity as well as phenolic acids and carotenoids were strongly affected by genotype, where the red cultivar proved to have a highest nutritional and functional quality than the green one. Our results demonstrated that the application of 16 μM Se in the nutrient solution improved the phenolic acids content in both cultivars, especially in red Salanova®, which was also distinguished by a substantial increase in anthocyanins content (184%). In green Salanova®, selenium applications have slightly reduced the overall carotenoids content, while in the red cultivar the doses 16 and 32 μM Se triggered an increase in violaxanthin, neoxanthin, lutein and β-cryptoxanthin. Therefore, we can deduce that the best Se dose is 16 μM, as it improves the nutraceutical characteristics in both cultivars with a slight reduction in fresh marketable yield (8%) recorded only in green Salanova®. Se leaf content increased significantly with the sodium selenate application rate in both cultivars. Moreover, at dose 16 μM, Se leaf content is sufficient to satisfy the recommended daily amount (RDA) of this trace element stipulated for adults (55 μg day^{-1}) by consuming 171g fw $week^{-1}$ of red Salanova® or 391g fw $week^{-1}$ of green Salanova®, without any toxic effect to humans, since the amount does not exceed the maximum dietary dose of 400 μg day^{-1} (Johnson et al., 2003).

Overall, our findings support the actual feasibility of using lettuce crops in Se biofortification programs and that the closed soilless cultivation system can be used as an effective and sustainable tool to produce functional Se-enriched food.

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8 Chapter 8: Final conclusion

Cultivation of higher plants under space conditions entails consideration of several parameters with contrasting effects on plant growth and physiology and produce quality as well. For example, space limitations and low light intensity in space shuttles or space stations requires high light use efficiency without compromising fresh biomass yield and quality of the final product. The results of the second chapter supported the existing research reports that suggest lettuce as a candidate crop for space farming. The great variation among the existing cultivars allows the selection of genotypes with highly efficient light harvesting mechanisms in order to provide sufficient fresh biomass yield, while at the same time quality may increase through the accumulation of antioxidant compounds such as soluble pigments, ascorbic acid and phenolic compounds and the decrease of nitrates. The six lettuce cultivars behaved differently under optimal and low light. Red and green Salanova, Lollo Verde and Lollo Rossa where the best in terms of yield under optimal light. As for light use efficiency (LUE), red Salanova was one of the best under optimal light. Instead, baby Romaine had the highest LUE under low light (the best agronomic performance in terms of fresh biomass and physiologic parameters), and an increased content of P and Ca under this light regime without an alteration of nitrate content. Moreover, red-pigmented cultivars had less nitrate accumulation in comparison to green cultivars, which is a crucial point in leafy vegetables especially when health of space colonists is taken into consideration. When it comes to bioactive properties, red Salanova was the richest in phenolic acids under optimal light followed by red oak leaf under low light, and both of them showed the highest lipophilic antioxidant activity, regardless of light regime. The content of bioactive compounds in lettuce cultivars is strongly influenced by genetic material and light intensity.

Based on these results, red Salanova was chosen to carry out the rest of the experiments as a suitable candidate cultivar for BLSS. On the other hand, green Salanova was inserted in order to compare between two cultivars of butterhead with different pigmentation and depict their physiological and qualitative behaviour during the growing cycle of 19 days after transplanting and their adaptation and quality improvement under diverse nutrient solutions.

In the third chapter, we highlighted that red Salanova reached maturity faster than green Salanova, implying a shorter harvest cycle to attain target weight and maturity stage requirement as well, for maintaining better quality attributes after harvest in case storage of the commodity is an option in human life support systems. This red cultivar have better water and nutrient use efficiency than his green counterpart, higher lipophilic antioxidant activity, total phenols and ascorbic acid and contemporarily around 37 % less nitrate content.

Fourth and fifth chapters dealt with nutritional eustress as treatment in order to optimise certain qualitative aspects of both cultivars. Macronutrient deprivation eustress facilitated the strategic accumulation of bioactive compounds, a method that could substitute expensive breeding programs. We demonstrated in the fourth chapter that a 50 % decrease of macronutrient input caused a marginal decrease in yield of both Salanova cultivars reaching 14 %, but on the other side outstandingly improved qualitative characteristics, pronouncedly in Red Salanova where an increase of 266 % in ascorbic acid, 162 % in total phenolic acids, 381 % in anthocyanins, and an increase in lipophilic antioxidant activity were registered in comparison to the control treatment. These results display how such this strategy can modulate plants secondary metabolism to improve the functional quality of red butterhead lettuce and green butterhead but in a lower extent. While in the fifth chapter, we presented how the alteration of macrocation proportions in the nutrient solution (K/Ca/Mg proportions) can increase the concentrations of the respective macrominerals in lettuce (biofortification). Moreover, nutrient solution composition had an effect on bioactive properties and yield attributes. For instance, elevated K levels had a beneficial effect on fresh weight and colour attributes, especially in green Salanova plants. While elevated levels of Ca and Mg caused higher ascorbic acid, chicoric and chlorogenic acid and lower nitrate content in both cultivars. Finally, the pigmentation of red lettuce increased under elevated levels of Mg accompanied by higher content of anthocyanins, xanthophylls and β-carotene.

In the sixth and seventh chapters, we evaluated Selenium and Iron biofortification of lettuce.

.Our findings in chapter 6 proved the possibility of producing Fe-enriched lettuce (by 21-54 %) by adding 1 mM and especialy 2.0 mM Fe, but with a quite acceptable reduction in fresh marketable yield (13.5 % and 25.2 %, respectively). The addition of Fe in the nutrient solution differentially modulate the functional quality in green and

red-pigmented lettuce. The application of 0.5 and 1.0 mM Fe in the nutrient solution improved the target phenolic acids and total phenolics, and the application of 2.0 mM Fe enhanced the carotenoids profile in red Salanova, whereas no significant improvement in quality traits were observed in green Salanova.

As for selenium biofortification in closed soilless cultivation, it proved to be an effective method to produce Se-enriched food with high nutritional values. Our findings indicate that shoot dry biomass, mineral composition, hydrophilic antioxidant activity as well as phenolic acids and carotenoids were strongly affected by genotype, where the red cultivar proved to have a highest nutritional and functional quality than the green one. Our results demonstrated that the application of 16 µM Se as sodium selenate in the nutrient solution improved the phenolic acids content in both cultivars, especially in red Salanova, which was also distinguished by a substantial increase in anthocyanins contents (184%) and an increase in violaxanthin, neoxanthin, lutein and β-cryptoxanthin. We can deduce that the best Se dose is 16 µM, as it improves the nutraceutical characteristics in both cultivars with a slight reduction in fresh marketable yield only in green Salanova. Se leaf content increased significantly with Se application rate in both cultivars.

Eventually, specific cultivars and light conditions combinations could be applied in separate modules to obtain the desired profile of functional compounds and the adequate amounts of fresh produce to support human life in prolonged space missions or space stations. Our work highlighted that fresh yield as well as hydrophilic (ascorbic acid, chlorogenic, chicoric, caffeoyl meso tartaric acids, total phenolics and anthocyanins) and lipophilic (violaxanthin + neoxanthin, lutein + zeaxanthin, β-cryptoxanthin and β-caroten) antioxidant molecules were strongly affected by genotype with the higher nutritional and functional quality traits recorded in red-pigmented Salanova. These latter traits showed better elasticity for increasing under nutrition eustress and biofortification and better accumulation of biofortified Se and Fe in red Salanova. These qualitative findings along the horticulture requirements (short growing cycle) represent red Salanova as a new potential candidate cultivar for BLSS. Moreover, closed soilless cultivation could be used as an effective and sustainable tool to produce functional food and manipulate target bioactive compounds by simple manoeuvring of the nutrient solution composition or concentration. Yet, further experiments should be

held in order to determine the contribution of this cultivar in air regeneration and water and waste recycling.