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# *Growth and nutritional quality of leafy vegetables in soilless culture: effect of nutritional factors*


Tutor: Prof. Youssef Rouphael

PhD student:
Francesco Napolitano

Co-tutor : Prof. Stefania De Pascale


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## Preface

This thesis is submitted for the degree of Doctor of Philosophy in Agricultural and Food Science at the University of Naples Federico II. The thesis has been written on the basis of experiments conducted at the Departement of Agricultural Sciences, University of Naples Federico II and also at the Agricultural University of Athens from 2013-2016 under the supervision of Prof. Youssef Rouphael who have made substantial intellectual contributions to all the experiments. Many inspiring people have been involved in the work leading to my PhD thesis. The first two experiments were conducted under cosupervison of Prof. Stefania De Pascale, University of Naples Federico II. Dr. Maria Giordano, a post-doc supervised the laboratory activities including quality analysis of all the first two experiments. The work related to mineral analysis was performed under the supervision of Prof. Giuseppe Colla University of Tuscia, Viterbo. The third experiment was conducted at the Agricultural University of Athens under the co-supervision of Prof. Dimitrios Savvas. The third experiment was porposed and designed by both Prof. Rouphael and Prof. Savvas. The first two experiments proposed and designed by Prof. Rouphael will be submitted by the end of 2017 to two international scientific journals for publication. The third experiment has been published recently in ***Environmental and Experimental Botany 2017: 141, 113-123***.

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Francesco Napolitano

# *Chapter 1*

# *Introduction*

## 1.1 Baby leaf

At present there is an important culinary trend for the consume of salads consisting of baby leaf vegetables. In fact there is a high profit for the growers, because baby leaf need a shorter cultivation period, and there is also an increasing demand among consumers in higher nutritional baby leaf product (Kim et al., 2016; Neocleous et al., 2013; Manzocco et al., 2011). Consumers also require a fresh-cut product with a good color, brightness, and a long shelf-life (Martinez-Sanchez et al., 2012). Quality of the product is highlight by: its resistance to oxidation processes after cutting; the absence of soil or other solid material, residues of pesticides; nitrate content within the limits of tolerance; limited microbial load; high dry-matter content; long shelf life (Alvino and Barbieri, 2016). Baby leaf vegetables are young edible plants with a few true leaves. The floating system is considered ideal for growing small-sized vegetables (Neocleous et al., 2013). The maturity of the plants is defined by their height, which, at harvest depending on the species and the customer demands. At harvest, growers apply a cut that excludes most of the petiole and includes the major part of lamina. The most popular leafy vegetables are lettuce, spinach, chicory, rocket and Swiss chard (Fallovo et al., 2009¸ Martinez-Sanchez et al., 2012), and other species that are of lesser importance, but which are often used in order to vary the appearance and taste, especially in mixtures (e.g., watercress, dandelion, mizuna, purslane, tatsoi, mustard, Russian kale, pacchoi, etc.) (Alvino e Barbieri, 2016). Nitrate content of baby leaves can be far from the maximum level established by European legislation, and could reflect the influence of microclimatic conditions, cultural practices and water uptake (Neocleous et al., 2013). Similar to mature leaves, baby leaf provides several dietary minerals, bioactive compounds such as vitamin C, E and carotenoids (Kim et al., 2016; Neocleous et al., 2013). Samuoliene et al. (2013) reported for β-carotene and total phenolic contents, in baby leaf lettuces, values comparable or lower than mature lettuce. Santos et al. (2014) and Neocleous et al. (2014), reported for baby leaf lettuce an K, Mg and Fe content similar or lower than values of mature lettuce reported by USDA (2015). In the case of spinach, Pandjaitan et al. (2005) found that the intermediate maturity leaves have a higher content of bioactive compounds, while Zhao et al. (2007) showed that mature leaves exhibited a higher antioxidant content when compared with baby-leaves.

Twelve days is the limit of marketability for green baby cultivars, examined by Fadda et al. (2015), because at this time correspond a general decrease of phenolic concentration. As mature leaves, quality of baby leaf depend on cultivars, pre-harvest and post-harvest factors. For example, Fadda et al. (2015) showed as red baby leaf lettuce cultivars have a higher storability and nutritional quality than green cultivars. In particular, the antioxidant activity was 11-fold higher in red than in green baby leaf lettuce, due to high concentration of flavonoid glycosides (quercetin, luteolin and cyanidin conjugates). The authors identify some baby leaf cultivars as the most appropriate as read-to eat food thanks to their genotype traits, such as a resistant cell membrane limiting ion leakage, low respiration rate and a reduced ammonium accumulation, that depend on the nitrogen uptake and utilization of each cultivar, namely all factors that show a good visual appearance during storage. Besides, the authors show as the wastes of some baby leaf cultivars (at 20 days of storage) have a high phenolic content, that could be used as a source of natural additives for plants, also reducing the environmental impact. This conclusion are in common with the results of Martinez-Sanchez et al. (2012). Also fresh-cut carrots, examined by Alarcón-Flores et al. (2014), have the highest content of total phytochemicals after the expiration date, probably due to the stress conditions during cold storage.

### 1.2 Genotype

Overtime consumer interest in the quality and safety of food, particularly of vegetables, is steadily increasing, so the research is aimed to meet this demand. Many pre- and post-harvest factors (as agricultural practices, processing, and storage conditions, irrigation and soil composition, temperature, light intensity) influence the quality and shelf life of vegetables, but their role depends on specific cultivar characteristics (Selma et al., 2012; Mai et al., 2013; Baslam et al., 2013; Perez-Lopez et al., 2015). In the scientific literature, the effects of genetic factors are well documented for several crops. Kim et al. (2016), highlights as lettuce exists as many different types and cultivars, and these differences are reflected on different health-beneficial bioactive compounds content. Phenols are important for nutrient uptake, protein synthesis, enzyme activity, photosynthesis, structural components, and allelopathy. But more interest has been addressed to phenolic compounds for their role in determining food

quality, in fact they have been associated with color, sensory qualities and nutritional properties of foods. Di-caffeoyltartaric acid; chlorogenic acid; chicoric acid, are the main compounds phenolics found in green lettuces (Nicolle et al., 2004). Lorach et al. (2008) show among three green varieties of lettuce (Iceberg, Romaine and continental) significant differences on total phenolic content and antioxidant activities. Similar differences were also detected between eight lettuce varieties analyzed by Nicolle et al. (2004). Liu et al. (2007) assess and compare phenolic compounds and antioxidant capacity of 25 cultivars of lettuce and they show that cultivar may alter the phenols and antioxidant activities. Phenolic content varied greatly among five lettuce examined by Bunning et al., (2010), so they conclude that quantification of phenolic content in lettuce can be used to identify specific cultivars that exhibit superior attributes and may improve market competitiveness of various types and particular cultivars of lettuce. Bystrická et al. (2015), found significant differences in the content of total polyphenols and in the value of antioxidant activity among four spinach cultivars analysed by them.

Nitrate is more important for plant growth, and plant yield is closely related to the N supply, compared to other nutrients limiting growth (Pinto et al., 2014; Hernandez et al., 2016). Nitrate accumulation can occur in leaf crops such as lettuce and spinach and it can be harmful to human health. In fact its reaction products and metabolites (e.g., $NO_2^-$, NO and nitrosamines) having adverse effect on human health. Significant genotypic variations in nitrate accumulation by lettuce, independently if it was grown in soil or solution culture, was demonstrated from Burns et al.(2011, 2012) and from Lopez et al. (2014).

Umar and Iqbar, (2007), indicate that different locations of nitrate reductase activity, differences in photosynthetic capacity, or differences in capacity to translocate the nitrate from roots to leaves, are some genetic factors that can influence nitrate accumulation in plants and they highlight as numerous genes are involved in nitrate accumulation, such as genes encoding nitrate reductase, glutamine synthetase, and ferredoxin-dependent glutamate synthase and genes encoding nitrate transporters.

As indicated by Santamaria, (2006), $NO_3^-$ varies belong different families of vegetables but nitrate content can vary also within species, cultivars and even genotypes with different ploidy, and it also differs in the various parts of a plant.

Correira et al. (2010), report nitrate content of 34 different vegetables, and they found for different varieties of spinach a minimum of nitrate of 797 mg/kg fresh weight and a maximum of 1427 mg/kg fresh weight. Chung et al. (2005), report nitrate content

in ten cultivars of spinach and lettuce grown in greenhouse conditions, and they were able to demonstrate that a large and significant genotypical variations in the nitrate content. This variation could be attributed to the different optimum nitrogen level required by cultivars and to differences in their photosynthetic capacity. Differences of shelf life, structure and quality was also demonstrated in different genotypes of basil (Bekhradi et al., 2015).

### 1.3 Soilless systems

Soilless cultivation systems for horticultural crops represents a recent innovation to traditional agriculture because it permits a higher water-use efficiency, an aspect particularly important where water is becoming an economically scarce resource (Gottardi et al., 2012). Soilless cultivation provides plant grow in absence of soil and a supply of water and minerals by nutrient solution. Soilless cultivation systems can be divided depending on support to the plants, in systems with liquid medium, as the case of hydroponic system, or systems with the solid medium. The substrate, organic or inorganic, alone or in a mixture, has different functions, such as to support the plant and provide air, water and nutrients to the roots; it must not contain pathogens; and should not be phytotoxic. Pratically, characteristics of the substrates must correlate with water and fertilizer supply, climate conditions, and plant needs (Gruda, 2009). Substrates used in hydroponics are rockwool, peat, perlite, pumice, coconut fibre, etc. The substrate material can be selected according to the species to be cultivated, cultivation phase (germination, rooting of cuttings, plant production, plant breeding) and the system of cultivation. A good culture medium is characterized by a good water retention capacity, ensure sufficient aeration to the roots. (Di Lorenzo et al., 2013).

The main objective of soilless culture is to provide optimal conditions that improve the growth and quality of plants reducing problems that normally are related soil cultivation, such as salinization caused by the excessive input of fertilisers and lack of rainfall. With soilless culture is possible to reduce pollution caused by pesticides, and fertilisers. There is a better control of plant growth due to the absence of the different limitations of the soil, such as reactions chemicals, nutrients availability, density and structure of the soil, water retention, and presence of pathogens. (Di Lorenzo et al., 2013).

An important characteristic of nutrient solution in the soilless system is the nutrients ionic concentration, that is usually much greater than that of the circulating solution of the soil (Gottardi et al., 2012). This is is attributed to the fact that the solutions of the soil are buffered by reactions of ion exchange, absorption-deabsorption, and precipitation, as well as by the cycle of nutrients and mineralization of organic matter. This buffering capacity is absent in systems of soilless culture, so it needs to use high concentrations of nutrients. Higher nutrient concentrations ensures a good nutrient reserve to the plants and less energy to actively remove nutrients from the environment. Another characteristic of nutrient solutions in the soilless systems are their easy preparation because they require four to five salts to satisfy the need the macroelements (Di Lorenzo et al., 2013).

In Italy, the first studies on soilless cultivation were started in the 1960s and 1970s, and in 1990 only 40-50 ha the surface area were dedicate to soilless cultivation, to reach 400 ha ten years later (Di Lorenzo et al., 2013). In Italy today, soilless cultivation is dedicate in particular to ornamental crops and cut flowers, to whom normally it is necessary to operate in difficult pedoclimatic conditions or in the presence of species that are difficult to cultivate, or during the multiplication and reproduction phase (Di Lorenzo et al., 2013).

Among different soilless culture systems, the floating growing system is a system that consists of trays floating on a water bed or hydroponic nutrient solution (Nicola et al., 2005). The floating system is an important soilless system used for leafy vegetable production that permits the precise management of the salt concentration in the nutrient solution and represent an effective system to improve quality aspects of products. For example, increasing salinity in the nutrient solution decreased the plant growth parameters (leaf dry biomass and number) but increased the antioxidant activity, total polyphenol, chlorogenic acid, cynarin and luteolin levels of leaves of artichoke and cultivated cardoon grown in a floating system (Colla et al., 2013). An increasing interest in the floating raft system is due to the advantage of cultivation of leafy vegetables for salads with very short cycles. In fact, it permits higher sanitary quality than conventional soil-based culture, and usually reduces nutrients and water use. It is the easiest and cheapest system among hydroponic methods to produce baby leaf vegetables because of it requires low installation and manpower costs, weeds are avoided and harvesting is straightforward. In addition, plants are grown at high densities and the

resulting products are almost clean and practically ready to be packed (Rodriguez-Hidalgo et al., 2010).

Fertilizer concentration and water supply for horticultural crops in a soilless system also depend on the environmental conditions, such as different temperatures and solar radiation conditions. Yield and quality of leafy lettuce grown in a floating system were significantly affected by the growing season (Fallovo et al., 2009).

When the quality of products grown in soilless cultivation is compared to that of soil culture, it possible to have contradictory results. However it is not a general phenomena that soilless culture result in high-quality products. In fact, Gruda (2009) reports as tomato fruits of soil culture have a better size, dry matter, fiber, carotenoids content and acidity, than those cultivated in soilless system. Furthermore, Rouphael et al. (2004) show that no differences were observed in dry matter or total protein content, while carbohydrate concentration (glucose, fructose, sucrose, and starch) was higher in soilless cultivation zucchini (*Cucurbitapepo* L.) cv. 'Afrodite' with respect to soil culture.

### 1.4 Nutrient solution management

Several properties of the nutrient solution can effectively modify products quality, for instance, electrical conductivity (EC) or nutrient concentration, chemical forms of the elements, nutrient management, temperature of the nutrient solution, as well as nutrient solution pH (Gruda, 2009). Generally, high levels of nutrients induces osmotic stress, ion toxicity and nutrient imbalance, and low levels generally lead to nutrient deficiencies. Increasing the fertilizer concentration in the nutrient solution increase plant growth parameters, but the quality of products can be reduced (Fallovo et al., 2009). The primary irrigation water used to prepare nutrient solutions frequently contains high concentrations of salt ions such as Na and Cl (Neocleous et al., 2013). Water availability and its salt concentration affect the synthesis and/or accumulation of health promoting compounds. De Pascale et al. (2001) found significant increases in fruit quality parameters and lycopene content when tomatoes were irrigated with moderately saline solutions, in terms of NaCl or nutrients. Sato et al. (2006) also found an increase not only in sugar content, but also in the organic matter and some amino acids of tomato fruits, due to a NaCl application in the nutrient solution. Proietti et al. (2008) found that

fruit quality of mini-watermelon plants was affected by drought with an increase in K, Mg, and spermine concentration. Changes of flavonols can occur due to the activities of the enzymes involved in the biosynthesis of phenolic compounds, such as L-phenilalanine ammonia-lyase which is more active under higher water stress (Tovar et al., 2002). Water stress is known to increase glucosinolate content in watercress (Ciska et al., 2000). EC regulation of nutrient solution can also enhance health promoting substances like vitamin C, lycopene, ß-carotene, phenols, carotenoids, and antioxidative capacity, in cucumber (Trajkova et al., 2006) and watermelon (Colla et al., 2006 b). Rouphael et al. (2006) reported that increasing salinity from 2.0 to 4.1 dS $m^{-1}$ improved fruit quality of zucchini squash with regard to a higher content of dry matter, reduced sugars, starch, total carbohydrates, and vitamin C.

Long-term irrigation with relatively low salt concentration can increase phenolic and carotenoid content in green romaine lettuce (Kim et al., 2008),while salinity decreased nitrate content in green romaine lettuce (Scuderi et al., 2009). Salinity was able to reduce K, while it was able to enhance Zn and Cu in green and red baby lettuce grown in floating systems. Salinity was even able to decrease Ca in green lettuce and to increase Fe, Mn and B in red lettuce. (Neocleous et al., 2013). Neocleous et al. (2014) reported that the reduced yield of green and red leaf baby lettuce, grown under saline water, could be at least partly compensated by the improved anthocyanin content and coloration in the red cultivar and enhanced freshness in green lettuce.

With soilless cultivation it is possible to enhance the secondary metabolites in plants with a good management of the nutrient solution. For example, lettuce and spinach have more iron in their leaves if near harvest the concentration of this nutrient is increased. Furthermore, leaves of endive and celery had lower nitrate accumulation when water was replaced the nutrient solution a few days before harvesting (Alvino and Barbieri, 2016). It was demonstrated that the use of silicon (Si) in the nutrient solution can improve productivity and quality of baby leaf vegetable corn salad (*Valerianella locusta* (L.). The ability of Si is to reduce the root assimilation of nitrate from the outer medium, changing the root activity of nitrate and Fe uptake, and adjusting gene expression levels of the proteins involved in this phenomenon. Furthermore, Si improves the shelf life of these edible tissues because reduce the chlorophyll degradation, thus delaying leaf senescence (Gottardi et al., 2012).

Vegetable quality can be improved also changing macro-cations composition. For example, a high content of K in the nutrient solution increases fruit dry matter, total

soluble solids content and lycopene of tomatoes grown in soilless culture, and a high content of Mg in the solution improved the total antioxidant activity of tomatoes cv. 'Lunarossa' (Fanasca et al. 2006 a, b). Increasing the Fe concentration in the nutrient solution 6 hours before harvest enhanced foliar Fe content in lettuce from 0.8 to 3.0 mg per 100 g of fresh weight without phytotoxicity symptoms (Inoue et al., 2000).

Nitrogen (N) fertilisation promotes plant growth and increases both crop production and quality, but excessive and/or inappropriate N use induces accumulation of derived compounds, like nitrates and oxalates, in the edible products which may be harmful to humans, and causes environmental pollution and economic losses (Santamaria, 2006; Chen et al., 2004; Stagnari et al., 2007, Citak and Sahriye, 2010). The maintenance of nitrate concentrations within the foliage at levels below EU maxima is an indicator of the nutritional quality of leaf vegetables (Konstantopoulou et al. 2010). There are many nitrate-accumulating vegetables, in particular those belonging to the families of *Brassicaceae* (rocket, radish, musterd), *Chenopodiaceae* (beetroot, swiss chard, spinach), *Amarantaceae*, *Asteraceae* (lettuce) and *Apiaceae* (celery, parsely) (Santamaria 2006).

Management of nutrient solution in soilless systems can regulate nitrate concentration in the products. For example, nitrate concentration was reduced in leaves of lamb's lettuce replacing the nutrient solution with water three days before harvesting (Gonnella et al., 2004). Furthermore, the addition of K decreased nitrate concentrations in cabbage by 26% compared to the control treatment in hydroponic systems. In contrast, the addition of K increased nitrate concentrations by 8.2% in spinach (Gao et al. 1989). Moreover, the modification of nitrate:ammonium ratio can modulate the relative uptake of anions and cations, changing primary and secondary metabolism, and consequently influencing the vegetable quality (Sonneveld, 2002).

### 1.5 Environmental factors

Nutritional quality of vegetables is also influenced by environmental factors in particular light and air temperature (Rouphael et al., 2012). A reduction in the light level was associated with reduced nitrate reductase activity and increased nitrate accumulation in lettuce and spinach (Rouphael et al., 2012). Furthermore, in spinach, low levels of light radiation determine the accumulation of nitrate in the leaves and an

increase in the content of oxalic acid (antinutritional factor that limits the bioavailability of Ca in the human body) and a decrease in vitamin C, reducing the nutritional quality of the product. Also high temperature can enhance accumulation of nitrate in lettuce (Alvino and Barbieri, 2016). Spinach plants grown at a photon flux density of 200 μmol $m^{-2}$ $s^{-1}$ exhibited higher amount of oxalate as well as more nitrates and less ascorbate in comparison with plants grown at 800 μmol $m^{-2}$ $s^{-1}$(Proietti et al., 2004).

Nitrate concentration in the leaves of Asian *Brassica* species increases when grown under low daily photosynthetically active radiation (Fallovo et al., 2009). The ratio between red and far-red wavelengths, increase the content of nitrates and antioxidants in rocket leaves, and blue light can enhance antioxidant compounds in lettuce (Alvino and Barbieri, 2016).

In greenhouse environment it is possible to control microclimatic parameters (light, temperature, humidity, and $CO_2$) but the reduction of light intensity, due to the covering material, can enhance concentration of nitrate in leaves of vegetables, as lettuce reducing the nutritional quality.

The reduction of UVB radiation (280–320 nm) in glasshouses has a negative effects on the biosynthesis of polyphenols and flavonoids. In fact, tomatoes field-grown in Spain and South Africa contain four- to five-fold more flavonolsthat those in the United Kingdom, where glasshouses are used for plant cultivation (Stewart et al., 2000).

Climatic factors such as temperature, light radiation, stimulate secondary metabolism and the biosynthesis of biologically active substances. For example, total phenolic content, plant secondary metabolites with health effect, have higher value in lamb's lettuce, mizuna, red chard, and red lettuce when plants were harvested at high light intensity (Alvino and Barbieri, 2016). Low light conditions, determine a reduction of sugars content in fruits melon (Pardossi et al., 2000) in tomatoes as well as in strawberries (Caruso et al., 2004).

Tomato, lettuce, sweet pepper and strawberry produce less ascorbic acid at low light intensity (Lee et al., 2000). The color of vegetables is a direct signal of nutritional compounds and it depends on light intensity (Schreiner et al., 2002). Non optimal temperatures can also reduce product quality of vegetable crops. For example, tomato cultivated in cold greenhouses will result in less juicy and aromatic fruit, with low acidity content, and thinner skin with lower quality (Proietti et al., 2004).

Temperature seems have a principal role in the biosynthesis of lycopene and carotenoids in tomatoes, in particular temperatures between 18 and 26°C, and

temperature lower 10°C could inhibit lycopene production (Rouphael et al., 2012). However, low temperatures have an important role determining quality of products. For example, bitter fruits in cucumber (*Cucumissativus* L.) plants enhance when grown at low temperatures (Kano et al., 2003), tomatoes harvested in cool seasons have higher carbohydrate content than in warm season (Islam et al. 2001), leaves of *Brassica oleracea* present higher concentration of total glucosinolates at 12°C than at 22°C (Charron and Sams, 2004).

Excessive temperatures are more susceptible to physiological disorders. For example, high temperature determine damage to cellular membranes, proteins, and nucleic acids. Temperature has also an effect on the nitrate content of vegetables. Higher temperatures result in higher nitrate contents in lettuce and radish, because high temperature reduce shoot nitrate reductase activity leading to nitrate accumulation. (Rouphael et al., 2012).

### 1.6 Aim and outline of the research

Lettuce (*Lactuca sativa* L.) and Spiny chicory (*Cichorium spinosum L.*) were selected as experimental crops. This choice was based on horticultural argument. Lettuce is widely distributed under greenhouse conditions in the Mediterranean region, especially in Italy. In the last decade lettuce in particular baby lettuce has gained popularity in Southern Italy and occupied the first place in the protected cultivation. To our knowledge, no research has been reported on how pre harvest factors such as the genetic materials, nutrient solution management and the number of cuts could affect growth and nutritional quality of leafy vegetables.

In Chapter 2, crop performance under an increasing level of NaCl in terms of growth, physiological responses and bioactive compounds is presented. In Chapter 3, we reported the effect of different salinity sources, genotypes and cut number on growth and quality of baby lettuce. Moreover the aim of Chapter four was to comparably evaluate the effects of four sodium and chloride salts ($Na_2SO_4$, NaCl, KCl or $CaCl_2$) on growth, mineral composition and metabolic profiling of *C. spinosum* grown in a closed soilless cultivation system. The four salts were tested at two different iso-osmotic concentration levels, in order to assess the ionic effects of the four salinity sources on

the metabolome. In the general conclusions chapter an attempt is made to provide an overall picture of the current research.

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# *Chapter 2*

## *Influence of salinity on yield, leaf gas exchange and quality of two baby lettuce cultivars grown in a floating system*

## 2.1 INTRODUCTION

In recent years high interest has been giving to the consumption of fruits and vegetables due to their significant role as promoter of human health. In fact, several scientific studies demonstred that a vegetable consumption can reduce some human diseases, such as specific forms of cancer and cardiovascular disorders (Rouphael et al., 2017a). The beneficial properties of vegetables could be associated to the important presence of macronutrients, micronutrients and bioactive compounds (Kim et al., 2016). Italy, with its 15,000 ha of cultivation and about 140 Kt of leafy vegetables production, is the European leader in this sector (Colonna et al., 2016). Among leafy vegetables, the production and consumption of lettuce is highly significant. The healthy properties of lettuce are related to the content of minerals, antioxidant compounds such as vitamin C and polyphenols, and to the low content of dietary fats (Llorach et al., 2008; Kim et al., 2016). Lettuce is consumed as fresh-cut or as so-called baby leaf vegetables (Alvino and Barbieri, 2016). Lettuce baby leaf is gaining popularity among growers thanks to the short cycle of cultivation, high percentage of usable product, little or no oxidation due to small stem diameter and because the product does not require many steps of processing but the entire leaf is harvested.

It is well established that pre-harvest factors can significantly influence the quality of leafy vegetables. The floating system can provide an alternative cultivation techniques to traditional soil cultivation for leafy vegetable production that permits the precise management of the salt concentration in the nutrient solution and represents an effective system to improve quality aspects for salads with very short cycles (Borgognone et al., 2013). Increasing salinity in the nutrient solution generally decreases the plant growth parameters (leaf dry biomass and number) but increase the quality in terms of antioxidant activity or concentration of bioactive molecules (Rouphael et al., 2006; Colla et al., 2013; Fanasca et al., 2006a,b; Manzocco et al., 2011; Chisari et al., 2010; Fallovo et al., 2009). In fact, increasing salinity in the nutrient solution generates in plants an oxidative stress and the formation of reactive oxygen species (ROS) that are responsible for damage to membrane structure, photosynthetic pigments, proteins, nucleic acids and lipids. ROS also stimulate the synthesis of antioxidant compounds such as phenylpropanoid derivatives (Borgognone et al., 2013).

The quality of vegetables depend on specific cultivar characteristics. In literature, the effects of genetic factors are well documented for several crops (Selma et al., 2012; Mai et al., 2013; Baslam et al., 2013; Perez-Lopez et al., 2015). Kim et al. (2016), highlight that lettuce exists as many different types and cultivars, and these differences are reflected based the on different health-beneficial bioactive compounds.

Oour hypothesis was that applying moderate salinity stress in the nutrient solutions can modulate target compounds such as phytochemicals and antioxidants. Therefore, the aim of this study was to evaluate the responses of two baby leaves lettuce cultivars ("Red and Green salad bowl"), grown in a floating system, to four salinity levels of NaCl (1, 10, 20 e 30 mM) on biomass production, mineral composition, phenolic composition and bioactive compounds.

## 2.2 MATERIALS AND METHODS

### 2.2.1. Plant material, growing conditions and experimental design

The experiment was carried out in a cold greenhouse located at the experimental station 'Torre Lama' of the University of Naples Federico II, South Italy (Bellizzi (SA) 40°37′00″N 14°57′00″E). Light was provided only by natural solar radiation. Seeds of lettuce (*Lactuca sativa* L. var. *acephala* cvs. 'Green Salad Bowl' and 'Red Salad Bowl' SAIS seed company, Cesena, Italy) were sown on 22 March 2015 in a floating raft growing system, with a planting density of 1025 plants /$m^2$. The system consisted of polystyrene plug trays floating in wood tanks with a constant volume (150 L) of stagnant nutrient solution, which was continuously aerated with an air compressor in order to maintain the oxygen content above 6 mg $L^{-1}$.

The treatments were arranged in a randomized complete block design with three replicates. A factorial combination of four nutrient solutions (non-salt control and three saline solutions with 10, 20 e 30 mM of NaCl) and two cultivars (Green or Red Salad Bowl) were compared. The composition of the basic nutrient solution was 12.0 mM $NO_3^-N$, 1.0 mM $NH_4^+N$, 1.75 mM S, 1.5 mM P, 5.0 mM K, 4.0 mM Ca, 1.5 mM Mg, 1.0 mM Na, 1.0 mM Cl, 20 µM Fe, 9 µM Mn, 0.3 µM Cu, 1.6 µM Zn, 20 µM B, e 0.3 µM Mo, with an electrical conductivity (EC) of 1.9 dS $m^{-1}$. The three saline solutions had the same basic composition plus an additional of 10 mM NaCl, 20 mM NaCl and 30

mM NaCl, with an EC of 2.8, 4.0 e 5.1 dS $m^{-1}$, respectively. The pH of the nutrient solution in all treatments was 6.0 ± 0.2.

### 2.2.2 Collection of samples and analysis

Plants of both cultivars were harvested at the same physiological stage and leaf tissues were dried in a forced air oven at 80 °C for 72 h for dry biomass determination and for mineral analysis. At harvest, 30 fresh plants were collected for further analysis, and leaf area was measured using an imaging analysis system (Delta-T Devices Ltd., Cambridge, UK).

***Mineral content analysis*:** The dried leaf tissues were ground in a Wiley mill to pass through a 20-mesh screen, then 0.5 g samples were analyzed for the following nutrients: N, P, K, Ca, Na, Cl and nitrate. Total nitrogen was determined by the Kjeldahl method (Bremner et al. 1965), after mineralization with $H_2SO_4$. Phosphorus, $NO_3$, K, Ca, Mg, Na and Cl concentrations were obtained by ion chromatography (ICP 3000 Dionex, Thermo Fisher Scientific Inc., MA USA).

***Gas exchanges*:** Photosynthesis (Pn), stomata resistance (rs) and transpiration (E) measurements were performed in a sunny day on 18 fully expanded leaves per treatment using a portable system IRGA *(infra red gas analyzer*, LCA4, ADC Bioscientific Ltd, Hoddesdon, UK). It reveals the Photosynthesis ($P_n$ in µmol $CO_2$ $m^{-2}$ $s^{-1}$), stomata resistance ($r_s$ in $m^2$ $s^1$ $mol^{-1}$) and transpiration (E, mol $H_2O$ $m^{-2}$ $s^{-1}$) by measuring the $CO_2$ removed from leaves and $H_2O$ released. Water use efficiency (WUE) was calculated as $P_n$/E ratio

***SPAD index and color parameters*:** A chlorophyll meter SPAD-502 (Konica-Minolta corporation, Ltd., Osaka, Japan) was used for greenness readings (i.e. light transmittance) from the fully expanded leaves. The leaf color was measured as reflected in the CIELAB ($L^*$ $a^*$ $b^*$) color space using a Minolta CR-300 Chroma Meter (Minolta Camera Co. Ltd, Osaka, Japan). The observations were made on twenty randomly selected leaves per plot. The measuring aperture diameter was 8 mm, and the instrument was calibrated with Minolta standard white reflector plate, before sampling baby leaves. $L^*$ (lightness ranging from 0 = black to 100 = white), $a^*$ (ranging from green [-] to red [+]), $b^*$ (ranging from blue [-] to yellow [+]) were read and transformed to those of the

L, a, b color space (Fallovo, et al., 2009). Chroma, C* represents color saturation and was calculated using the following formula $(a^2 + b^2)^{1/2}$.

***Determination of antioxidant activity*:** freeze-dried leaves (0.2 g) was used to determine hydrophilic (HAA) and lipophilic (LAA) antioxidant activity, with the N,N-dimethyl-p-phenylenediamine (DMPD) method (Fogliano et al. 1999) and the 2,2′-azinobis 3-ethylbenzothiazoline-6-sulfonic acid ABTS method (Pellegrini et al. 1999), respectively. In the first assay hydrophilic antioxidant molecules are extracted in distilled water, and they transfer a hydrogen atom to the coloured radical cation (DMPD.+). In this way the solution change color proportionaly to the quantity of antioxidant compounds.

In the second assay, methanol is used to extract lipofilic molecules that reduce ABTS radical cataion proportionaly to their concentration. After a time point is measured the absorbance of solutions by UV–Vis spectrophotometry at 505 and 734 nm, respectively. HAA and LAA were expressed as mmol ascorbic acid (AA) and as mmol of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) per 100 g of dry weight, respectively (Fogliano et al., 1999).

***Determination of ascorbic acid*:** The total ascorbic acid was detected according with protocol of Kampfenkel et al. (1995). The total ascorbic acid reduces $Fe^{3+}$ to $Fe^{2+}$. $Fe^{2+}$ forms a complex with 2,2-dipyridyl, and the absorbance of the solution was measured at 525 nm. Data were expressed as mg ascorbic acid on 100 g fresh weight.

***Determination of phenols acid and total phenols*:** One gram of materials was extracted by 30 mL of methanol/water (70:30, v/v) and sonicated at room temperature for 30 min. The extraction procedure was repeated twice for each sample. The mixtures were centrifuged at 14.800g, filtered through a Whatman filter paper, and then used for LC-MS/MS analysis using a method previously described by Ferracane et al. (2010). Chromatographic separation was performed using an HPLC apparatus equipped with two micro-pumps series 200 (Norwalk, CT, USA), a UV−Vis series 200 (PerkinElmer) detector set at 280 nm, and a Prodigy ODS3 100 Å column (250 mm × 4.6 mm, particle size = 5 μm) (Phenomenex, Torrance, CA USA). The eluents were (A) water containing 0.2% formic acid and (B) acetonitrile/methanol (60:40, v/v). The gradient program was as follows: 20−30% B (6 min), 30−40% B (10 min), 40−50% B (8 min), 50−90% B (8 min), 90−90% B (3 min), and 90−20% B (3 min) at a constant flow of 0.8 mL/min. The LC flow was split, and 0.2 mL/min was sent to the mass spectrometer. The injection volume was 20 μL. Two injections were performed for each sample. MS and MS/MS

analyses of extracts were performed on an API 3000 triple-quadrupole mass spectrometer (Applied Biosystems, Canada) equipped with a TurboIonSpray source working in the negative ion mode. Six phenolic acids Caffeoyltartaric acid, chlorogenic acid, caffeoylmalic acid, cichoric acid, caffeoyltartaric acid, isochlorogenic acid and total phenols content were identified with LC-MS/MS.

### 2.2.3 Statistical analysis

All data were subjected to analysis of variance (ANOVA) using the SPSS software package (SPSS 10 for Windows, 2001). Duncan's multiple range test was performed for mean comparisons on each of the significant ($p < 0.05$) variables measured.

## 2.3 RESULTS

### 2.3.1 Growth parameters

Leaf area, fresh and dry biomass were significantly influenced by salinity (S) and cultivar (C), with no significant S ×C interaction (**Table 2.1**). All measured parameters showed higher values in green respect to the red cultivar. In particular, an increase in leaf area, fresh and dry yield by 11%, 14% and 33% respectively was recorded in green cultivar.

The effect of different saline concentrations was also evident in the present study. In fact, increasing the saline concentration from 1 to 30 mM NaCl induced a reduction of all measured parameters respect to the control treatment. In particular, leaf area and fresh biomass decreased meanly by 14.7% when the salinity increase from 1 to 30mM of NaCl, while dry biomass decreased by 11.0 % in the same conditions. No significant difference in dry biomass was observed between no-salinized and 10 and 20 mM NaCl treatments (**Table 2.1**).

**Table 2.1** Effects of salinity and cultivar on leaf area, fresh and dry biomass of baby lettuce plants grown in a floating raft culture.

| Salinity (mM NaCl) | Cultivar | Leaf Area ($cm^2$ $plant^{-1}$) | Fresh Biomass ($kg\ m^{-2}$) | Dry Biomass ($g\ m^{-2}$) |
|---|---|---|---|---|
| 1 | Red | 90.0 | 5.6 | 220.4 |
| | Green | 99.8 | 6.4 | 294.2 |
| | *mean* | *94.9 a* | *6.0 a* | *257.1 a* |
| 10 | Red | 85.9 | 5.4 | 217.3 |
| | Green | 99.0 | 6.0 | 267.5 |
| | *mean* | *92.5 a* | *5.7 ab* | *242.4 ab* |
| 20 | Red | 83.2 | 5.3 | 211.0 |
| | Green | 93.6 | 5.9 | 258.3 |
| | *mean* | *88.4 ab* | *5.6 ab* | *234.6 ab* |
| 30 | Red | 78.1 | 4.9 | 209.1 |
| | Green | 83.9 | 5.4 | 248.1 |
| | *mean* | *80.9 b* | *5.1 b* | *228.9 b* |
| Significance | | | | |
| Salinity (S) | | * | * | * |
| Cultivar (C) | | * | ** | *** |
| S × C | | NS | NS | NS |

Means within each column and main effect followed by different letters are significantly different ($p \leqslant 0.05$) according to Duncan's multiple-range test.
NS, *, **, *** =non significant or significant at $P < 0.05$, 0.01 e 0.001, respectively

### 2.3.2 Mineral composition

The results reported in **Table 2.2** showed that the mineral composition was strongly influenced by salinity and cultivar. Respect to the green cultivar, red salad bowl showed a significant higher accumulation of N. The content of P was reduced by salinity in both cultivars. Red cultivar showed significant higher content of K by 45 % respect to green cultivar. Ca concentration was significantly reduced by salinity (**Table 2.2**).

No significant differences were observed for Mg content between the two cultivars. Mg decrease when salinity concentration increase from 1 to 30 mM NaCl, with lower values observed with 30 mM NaCl (**Table 2.2**).

Green salad bowl accumulatd more Na in leaf tissue respect to the red cultivar. Increasing the NaCl concentration in the nutrient solution increased linearly the accumulation of both toxic ions Na and Cl (**Table 2.2**).

**Table 2.2** Effects of salinity and cultivar on leaf mineral composition of baby lettuce plants grown in a floating raft culture.

| Salinity (mM NaCl) | Cultivar | Mineral Composition (g $kg^{-1}$ dry weght) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | N | P | K | Ca | Mg | Na | Cl |
| 1 | Red | 42.6 | 15.9 | 62.7 | 6.0 | 2.6 | 2.8 | 14.6 |
| | Green | 40.1 | 16.4 | 43.1 | 9.3 | 3.3 | 4.2 | 9.6 |
| | *mean* | *41.3* | *16.1 a* | *52.9* | *7.6 a* | *2.9 a* | *3.5 c* | *12.1 d* |
| 10 | Red | 42.1 | 15.5 | 56.1 | 5.3 | 2.4 | 7.1 | 26.4 |
| | Green | 41.7 | 15.0 | 41.5 | 7.3 | 2.6 | 9.6 | 21.0 |
| | *mean* | *41.8* | *15.3 ab* | *48.8* | *6.3 ab* | *2.5 ab* | *8.4 b* | *23.7 c* |
| 20 | Red | 43.9 | 14.1 | 53.0 | 5.0 | 2.3 | 10.6 | 31.5 |
| | Green | 39.7 | 14.8 | 32.6 | 4.6 | 1.7 | 10.4 | 25.1 |
| | *mean* | *41.9* | *14.5 bc* | *42.8* | *4.8 bc* | *2.0 bc* | *10.6 b* | *28.3 b* |
| 30 | Red | 42.8 | 14.1 | 52.8 | 4.7 | 2.1 | 15.7 | 35.4 |
| | Green | 41.9 | 13.2 | 30.8 | 4.3 | 1.5 | 13.0 | 29.1 |
| | *mean* | *42.4* | *13.6 c* | *41.8* | *4.5 c* | *1.8 c* | *14.3 a* | *32.2 a* |
| Significance | | | | | | | | |
| Salinity (S) | | NS | *** | NS | ** | ** | *** | *** |
| Cultivar (C) | | * | NS | *** | * | NS | NS | *** |
| S × C | | NS | NS | NS | * | NS | NS | NS |

Means within each column and main effect followed by different letters are significantly different ($p \leqslant 0.05$) according to Duncan's multiple-range test.
NS, *, **, ***= No significative or significative at $P < 0.05$, 0.01 e 0.001, respectively.

### 2.3.3 Leaf gas exchange

**Table 2.3** represent the effects of cultivar and salinity on net photosynthesis, stomata resistance, transpiration and intrinsic water use efficiency (WUE). The effects of salinity and cultivar on net photosynthesis and stomata resistance and transpiration were highly significant on almost all physiological parameters (**Table 2.3**).

Green cultivar exhibited higher value of photosynthesis, stomata resistance, transpiration and intrinsic water use efficiency (WUE) respect to red cultivar.

Increasing salinity in the nutrient solution from 1 to 30 mM NaCl decreased the net photosynthesis by 10.1%, 20.0% and 37.1% in 10, 20 and 30 mM treatments, resepcetively (**Table 2.3**). Moreover, stomatal resistance increased significantly by increasing salinity concentration in the nutrient solution, with the highest values recorded under severe salt stress conditions (i.e. 30 mM NaCl). Similarly, transpiration rate was significantly affected by both treatments cultivars and salinity, with the lowest values observed under 30 mM NaCl. Finally, the WUE was significantly higher in green compared to the red cultivar, whereas no significant effect was observed with increasing NaCl concentration in the nutrient solution (**Table 2.3**).

**Table 2.3** Effects of salinity and cultivar on net photosynthesis ($P_n$), stomata resistance ($r_s$), transpiration rate (E) and water use efficiency (WUE) of baby lettuce grown in a floating raft culture.

| Salinity (mM-NaCl) | Cultivar | $P_n$ ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$) | $r_s$ ($m^2\ s^1\ mol^{-1}$) | E ($mol\ H_2O\ m^{-2}\ s^{-1}$) | WUE ($\mu mol\ CO_2\ mol\ H_2O$) |
|---|---|---|---|---|---|
| 1 | Red | 5.96 | 3.50 | 5.40 | 1.10 |
| | Green | 9.31 | 3.53 | 6.49 | 1.43 |
| | *mean* | *7.63 a* | *3.51 b* | *5.94 a* | *1.27* |
| 10 | Red | 5.78 | 3.99 | 5.21 | 1.11 |
| | Green | 8.11 | 4.16 | 6.00 | 1.35 |
| | *mean* | *6.94 a* | *4.07 b* | *5.60 ab* | *1.23* |
| 20 | Red | 4.95 | 4.83 | 4.91 | 1.01 |
| | Green | 6.50 | 5.86 | 5.80 | 1.12 |
| | *mean* | *5.72 b* | *5.34 a* | *5.35 ab* | *1.06* |
| 30 | Red | 4.47 | 5.35 | 4.51 | 0.99 |
| | Green | 5.12 | 7.13 | 5.34 | 0.96 |
| | *mean* | *4.79 b* | *6.24 a* | *4.92 b* | *0.97* |
| Significance | | | | | |
| Salinity (S) | | * | *** | ** | NS |
| Cultivar (C) | | *** | *** | *** | * |
| S × C | | NS | ** | NS | NS |

Means within each column and main effect followed by different letters are significantly different ($p \leqslant 0.05$) according to Duncan's multiple-range test.
NS, *, **, ***= No significative or significative at $P < 0.05$, 0.01 e 0.001, respectively.

### 2.3.4 SPAD index and leaf color parameters

Hunter color parameters (L*. a*. b*), Chroma and SPAD index were only affected by cultivar, with no effect of NaCl concentration in the nutrient solution and the interaction between the two factors (**Table 2.4**). When averaged over salinity concentration in the nutrient solution the SPAD index was higher in red salad bowl compared to the green salad bowl. Similarly to the SPAD index, the lightness (L*) yellowness (b*) as well as chroma were higher in the green pigmented cultivar, whereas an opposite trend was observed for the redness parameter (a*) with the highest values observed in the red pigmented cultivar (**Table 2.4**).

**Table 2.4** Effects of salinity and cultivar on SPAD Index and color parameters of baby lettuce grown in a floating raft culture.

| Salinity (mM NaCl) | Cultivar | | SPAD Index | L* | a* | b* | Chroma |
|---|---|---|---|---|---|---|---|
| 1 | Red | | 21.29 | 50.09 | -6.04 | 23.24 | 24.02 |
| | Green | | 17.58 | 53.73 | -7.25 | 26.16 | 27.15 |
| | | *mean* | *19.44* | *51.92* | *-6.64* | *24.70* | *25.59* |
| 10 | Red | | 20.67 | 48.01 | -6.70 | 22.86 | 23.83 |
| | Green | | 17.32 | 54.87 | -7.60 | 27.53 | 28.56 |
| | | *mean* | *19.00* | *51.44* | *-7.15* | *25.20* | *26.20* |
| 20 | Red | | 19.75 | 48.93 | -6.02 | 23.06 | 23.84 |
| | Green | | 16.73 | 53.59 | -7.49 | 27.66 | 28.66 |
| | | *mean* | *18.24* | *51.26* | *-6.75* | *25.36* | *26.25* |
| 30 | Red | | 19.43 | 50.43 | -6.12 | 21.67 | 22.52 |
| | Green | | 15.97 | 55.14 | -7.30 | 26.58 | 27.58 |
| | | *mean* | *17.70* | *52.79* | *-6.71* | *24.13* | *25.05* |
| Significance | | | | | | | |
| Salinity (S) | | | NS | NS | NS | NS | NS |
| Cultivar (C) | | | ** | *** | ** | *** | *** |
| S × C | | | NS | NS | NS | NS | NS |

Means within each column and main effect followed by different letters are significantly different ($p \leqslant 0.05$) according to Duncan's multiple-range test.
NS, **. *** No significative or significative at $P < 0.01$ and 0.001, respectively.

### 2.3.5 Nutritional and qualitative parameters

Green lettuce cultivar exhibited a significant higher dry matter percentage than red lettuce cultivar (**Table 2.5**). Increasing the NaCl concentration in the nutrient solution from 1 to 30 mM increased linearly the dry matter percentage with the highest value recorded with 30 mM NaCl, with an increased of 6.6% respect to the control treatment (**Table 2.5**).

Lypophilic antioxidant activity was significantly affected by cultivar. In particular it was observed an increase of 56.6 % in red salad bowl compared to green cultivar. Furthermore, total ascorbic acid content was significantly affected by both factors salinity and cultivar. Red salad bowl showed a higher content of total ascorbic acid compared to green pigmented cultivar (**Table 2.5**). Increasing salinity in the nutrient solution from 1 to 30 mM NaCl enhanced the biosynthesis and accumulation of total ascorbic acid content with the highest value recorded in 20 mM NaCl treatment. Finally, nitrate content decreases when salinity concentration increase from 1 to 30 mM NaCl, but the differences were not significant (**Table 2.5**).

**Table 2.5** Effects of salinity and cultivar on dry matter content (DM, %), lipophilic (LAA, mmol Trolox 100 $g^{-1}$ d.m.) and hydrophilic (HAA, mmol ascorbic acid 100 $g^{-1}$ d.m.) antioxidant activity, total ascorbic acid content (AA, mg $100^{-1}$ g fw) and nitrate (mg $kg^{-1}$ fw) of baby lettuce grown in a floating raft culture.

| Salinity (mM NaCl) | Cultivar | | DMC | LAA | HAA | AA | Nitrate |
|---|---|---|---|---|---|---|---|
| 1 | Red | | 3.9 | 7.83 | 1.57 | 19.10 | 1719.5 |
| | Green | | 4.6 | 5.00 | 1.54 | 18.77 | 1893.3 |
| | | *mean* | *4.2 b* | *6.41* | *1.55* | *18.56 b* | *1806.3* |
| 10 | Red | | 4.1 | 7.62 | 1.51 | 25.91 | 1648.6 |
| | Green | | 4.4 | 6.11 | 1.54 | 19.79 | 1817.9 |
| | | *mean* | *4.2 b* | *6.87* | *1.52* | *22.81 ab* | *1733.2* |
| 20 | Red | | 4.0 | 7.35 | 1.66 | 43.18 | 1615.1 |
| | Green | | 4.3 | 6.16 | 1.59 | 24.31 | 1749.0 |
| | | *mean* | *4.2 b* | *6.76* | *1.62* | *33.75 a* | *1682.1* |
| 30 | Red | | 4.3 | 8.11 | 1.67 | 31.56 | 1564.5 |
| | Green | | 4.6 | 5.59 | 1.56 | 19.36 | 1655.9 |
| | | *mean* | *4.4 a* | *6.85* | *1.62* | *25.47 ab* | *1610.2* |
| Significance | | | | | | | |
| Salinity (S) | | | * | NS | NS | * | NS |
| Cultivar (C) | | | *** | ** | NS | * | NS |
| S × C | | | NS | NS | NS | NS | NS |

Means within each column and main effect followed by different letters are significantly different ($p \leqslant 0.05$) according to Duncan's multiple-range test.
NS, *, **, ***= No significative or significative at $P < 0.05$, 0.01 and 0.001, respectively.

### 2.3.6 Phenol acids and total phenols

HPLC analysis revealed six phenolic acids: Caffeoyltartaric acid, 5-*O*-caffeoylquinic acid (chlorogenic acid), caffeoylmalic acid, di-*O*-caffeoyltartaric acid (cichoric acid), meso-di-*O*-caffeoyltartaric acid, 3,5-di-*O*-caffeoylquinic Acid (isochlorogenic acid). Among others, the chlorogenic acid was the dominant phenolic acid present in all treatments. Others phenolic acids were found in the following descending order: cichoric acid > isochlorogenic acid > caffeoylmalic acid > meso-di-*O*-caffeoyltartaric acid > caffeoyltartaric acid (**Table 2.6**).

Green cultivar has higher values of caffeoyltartaric acid, whereas red salad bowl showed an increase of 123% for chlorogenic acid, 24% for caffeoylmalic acid and 47% for total phenols (**Table 2.6**). Interestingly, chlorogenic acid, caffeoylmalic acid, cichoric acid, meso-di-*O*-caffeoyltartaric acid and isochlorogenic acid as well as total phenolic contents were significantly higher in 20 mM NaCl saline treatment compared to the non-salinized and the other saline treatements (**Table 2.6**).

**Table 2.6** Effects of salinity and cultivar on phenol acids and total phenols of baby lettuce grown in a floating raft culture.

| Salinity (mM NaCl) | Cultivar | CTA (mg 100 $g^{-1}$ dm) | 5-CQA (mg 100 $g^{-1}$ dm) | CMA (mg 100 $g^{-1}$ dm) | DCTA (mg 100 $g^{-1}$ dm) | m-DCTA (mg 100 $g^{-1}$ dm) | 3.5-DCTA (mg 100 $g^{-1}$ dm) | Total phenols (mg 100 $g^{-1}$ dm) |
|---|---|---|---|---|---|---|---|---|
| 1 | Red | 27.0 | 1094.3 | 85.0 | 561.0 | 53.3 | 126.6 | 1947.6 |
| | Green | 31.3 | 489.7 | 68.7 | 569.3 | 52.6 | 117.3 | 1327.6 |
| | *mean* | *29.2 a* | *792.0ab* | *76.8ab* | *565.1ab* | *53.1 a* | *122.1b* | *1637.1ab* |
| 10 | Red | 17.0 | 875.0 | 69.3 | 460.7 | 37.0 | 109.6 | 1569.3 |
| | Green | 27.3 | 481.0 | 62.0 | 519.0 | 41.1 | 127.0 | 1257.6 |
| | *mean* | *22.1 b* | *678.1bc* | *65.7bc* | *489.8 b* | *39.1 b* | *118.3b* | *1413.5 b* |
| 20 | Red | 24.7 | 1059.3 | 88.3 | 636.6 | 46.6 | 164.1 | 2020.0 |
| | Green | 31.0 | 664.6 | 80.0 | 639.0 | 47.6 | 133.0 | 1595.3 |
| | *mean* | *27.8 a* | *862.0 a* | *84.1 a* | *637.8 a* | *47.2 a* | *148.6a* | *1807.6 a* |
| 30 | Red | 20.0 | 859.7 | 79.3 | 446.0 | 37.3 | 100.3 | 1543.1 |
| | Green | 19.3 | 265.1 | 46.3 | 337.1 | 24.6 | 83.0 | 776.3 |
| | *mean* | *19.6 a* | *562.3 c* | *62.8 c* | *391.5 c* | *31.0 c* | *91.6 c* | *1159.2 c* |
| Significance | | | | | | | | |
| Salinity (S) | | ** | *** | ** | *** | *** | ** | *** |
| Cultivar (C) | | ** | *** | *** | NS | NS | NS | *** |
| S × C | | NS | NS | NS | NS | NS | NS | NS |

Means within each column and main effect followed by different letters are significantly different ($p \leqslant 0.05$) according to Duncan's multiple-range test.
NS, **, *** =non significant or significant at $P < 0.01$ and 0.001, respectively

**CTA**: Caffeoyltartaric acid; **5-CQA**: 5-O-caffeoylquinic acid (chlorogenic acid); **CMA**: caffeoylmalic acid **DCTA**: di-O-caffeoyltartaric acid (cichoric acid) ; **m-DCTA**: meso-di-O-caffeoyltartaric acid **3.5-DCQA**: 3.5-di-O-caffeoylquinic Acid (isochlorogenic acid).

## 2.4 DISCUSSION

It is well established that excess of sodium chloride (NaCl) in soil and water could create stress at plant level leading to a gradual reduction in plant growth and consequently crop productivity (Parihar et al., 2015). In the first phase an osmotic or water-deficit effect appeared leading to a significant decrease in water uptake. During this phase neither Na nor Cl inhibits growth because these are excluded from phloem and when they arrived in the xylem, meristematic cells can accumulate the toxic elements in their vacuoles. If salt continues to enter, the plant cells lose the ability to compartmentalize salts in the vacuole. Salts wil go to cytoplasm and inhibit enzyme activity and they can invade the cell walls and dehydrate the cell. This will lead to toxic effect, also called the salt-specific or ion-excess effect of salinity (Parihar et al., 2015).

In our study we have analyzed the response of two baby leaf lettuce cultivars red and green grown in a floating raft system under four increasing NaCl concentrations (1, 10, 20, 30 mM) in the nutrient solution by examining morphological, physiological and qualitative traits. Increasing the salt concentration from 1 to 30 mM NaCl was accompanied with a reduction of morphological traits such as leaf area, dry and fresh biomass in both cultivars respect to the control treatment. Interestingly, the significant reduction in yield and biomass was only observed under severe stress conditions, whereas no reduction was recorded at 10 or 20 mM NaCl. In both cultivars, increasing salt concentration, were accompained with a significant increase of toxic elements Na and Cl as well as significant reduction of macronutrients such as P, Ca and Mg. The reduction of growth in response to excessive salts could be attributed to the competition of Na and/or Cl with macronutrients that are essential to plants, such as phosphorus, potassium, nitrogen, magnesium and calcium. It is well established that high concentrations of Na reduce the K and Ca uptake, decrease photosynthesis, by reducing stomatal conductance. Instead, high Cl concentrations reduce photosynthesis rate through chlorophyll degradation. Many authors reported that salt stress decrease growth by reducing net photosynthetic rate, stomatal conductance, performance of PS II, electron transport rates and assimilation rate of $CO_2$ (Parihar et al., 2015). A reduced $CO_2$ assimilation rate leads to a decrease of the Rubisco large and small subunits, OEE proteins (components of oxygen evolving), carbonic anhydrase, and Rubisco activase and an enhanced degradation of Rubisco subunits. Sodium and chloride can reduce growth and photosynthesis in additive way and with different mechanisms in several

genotypes (Parihar et al., 2015). Our results are in line with those conducted on baby leaf lettuce by several authors such as Kim et al. (2008), Scuderi et al. (2011) and Neocleous et al. (2014).

Concerning the effect of genotypes, significant differences were observed between the green and red cultivars. For instance, the green cultivar was characterized by a high biomass production and Ca content, whereas the red cultivar was characterized by a higher nutritional profile (i.e. N and K). Perez-Lopez et al. (2015) also observed differences in the mineral profile among different lettuce cultivars. Furthermore, in our experiment, green cultivar had higher value of photosynthesis, stomatal resistance, transpiration and intrinsic water use efficiency (WUE) compared to red cultivar. As showed by Colla et al. (2006a, b, c) and Rouphael et al. (2012), higher concentration of NaCl in nutrient solution causes a decrease of the water potential and reduce the water flux in aerial part by increasing dry matter content in leaves.

All color parameters (L*, a*, b*), Chroma and SPAD index were only affected by cultivar. As we reported earlier, red cultivar had higher values of SPAD index and a* color parameter while green cultivar has higher values of L* and b* color parameter and Chroma values.

Concerning the undesirable components such as nitrates, irrespective of NaCl concentrations in the nutrient solution the average nitrate concentrations of red and green lettuce were 1719.5 and 1893.3 mg $Kg^{-1}$ fw, resepectively. The nitrate content found in the current study were below the maximum limit imposed by the European Community for lettuce (Commission Regulation No 1258/2011). As indicated by Neocleous et al. (2014), nitrate content in baby leaves was not affected by salinity in either the green or the red lettuce grown under 5, 10 or 20 mM NaCl.

Phenols are important for nutrient uptake, protein synthesis, enzyme activity, photosynthesis, structural components, and allelopathy. But more interestingly, phenolic compounds have a crucial role in determining food quality; in fact they have been associated with color, sensory qualities and nutritional properties of foods. According to Bunning et al. (2010), the quantification of phenolic content in lettuce can be used to identify specific cultivars that exhibit superior attributes and may improve market competitiveness of various types and particular cultivars of lettuce.

In the current study, increasing salinity enhanced the total ascorbic acid content with the highest values recorded in both 20 and 30 mM NaCl. The effect of genetic material was also very pronounced with the red cultivar exhibiting highest bioactive compounds

compared to the green pigmented cultivar. In fact, the accumulation of the antioxidant activity, total ascorbic acid, phenolic acids and total phenols was much more higher in red compared to green cultivar. Serafini et al. (2002), showed an increase of the plasma total radical-trapping antioxidant potential (TRAP), that coincided with an increase of vitamin C, and plasma circulating phenols (caffeic acid, p-coumaric acid and quercetin), after ingestion of 250 g of fresh lettuce.

In line with our findings, Chisari et al. (2010), reported that the main polyphenol compounds found in baby lettuce were O-caffeoyltartaric acid, 5-O-caffeoylquinic acid (chlorogenic acid), 3,5-di-O-caffeoylquinic acid (isochlorogenic acid) and dicaffeoyltartaric acid (chicoric acid). Furthermore, it has been also demonstrated that increasing levels of salinity up to 4.8 dS $m^{-1}$ was accompanied with a reduction of phenolics degradation, preserving then the antioxidant capacity of the product. In our work salinity affect significantly acids phenolic content. Caffeoyltartaric acid, chlorogenic acid, caffeoylmalic acid, cichoric acid, meso-di-*O*-caffeoyltartaric acid, isochlorogenic acid and total phenols contents were significantly higher in 20 mM NaCl saline treatment respect to the control and to the others salinity levels. Red salad bowl showed an increase of 123 % for chlorogenic acid, of 24% for caffeoylmalic acid and of 47 % for phenols total. It is well known, that phenol acids content are secondary metabolites with beneficial health effects, which can be influenced by genetic, climatic and cultural factors (Rouphael et al. 2012a). The increase in phenolic acids and total phenols recorded in the current experiment could be expected since polyphenols are known to play an important role in the neutralization of free radicals or in the decomposition of peroxides. The biosynthesis of these antioxidant molecules (i.e. phenolic compounds) has been described as actively involved in plants' response to various stressors, including salt toxicity (Perihar et al., 2015, Mahmoudi et al., 2012, Chisari et al., 2010, Kim et al., 2008).

## 2.5 CONCLUSIONS

Yield and quality of baby leaf lettuce grown in floating system was influenced by cultivar and salinity concentration in nutrient solution. In particular the green salad bowl exhibited higher marketable fresh and dry biomass production, leaf area, Ca and caffeoyltartaric acid content compared to the red pigmented cultivar. On the other, the

red pigmented cultivar was characterized by high nutritional factor such as antioxidant activity, vitamin C, minerals and phenolic acids. Our results also demonstrated that the manipulation of target compounds such as phytochemicals and antioxidants in baby lettuce by management of the nutrient solution is possible using floating system as a tool. Adding 20 mM NaCl to the nutrient solution improve vegetable quality of both lettuce cultivars without any significant reduction in fresh marketable yield.

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# *Chapter 3*

# *Influence of salinity source and cut number on yield and quality of two baby lettuce cultivars grown in a floating system*

## 3.1 INTRODUCTION

Lettuce is a popular consumed vegetable that includes 16 species in Europe, 51 in Asia, 43 in Africa and 12 in America (mostly North American subcontinent) (Lebeda et al., 2004). In 2013, 3 million tons of lettuce were produced in the EU, mostly in Mediterranean countries (Spain, Italy and France in this order) (Hernandez et al., 2016; FAOSTAT, 2014). There are six main types: crisphead lettuce, butterhead lettuce, romaine or cos lettuce, leaf or cutting lettuce, stem or stalk lettuce and Latin lettuce (Kim et al., 2016). More interest in lettuce production is due to the significant consumption as fresh-cut or minimally processed product or as so-called baby leaf vegetables (Alvino and Barbieri, 2016). In this form lettuce is appreciated by both growers and consumers due to the short cycle of cultivation, higher percentage of usable product, little or no oxidation due to smaller stem diameter and because the product do not require many steps of processing but the entire leaf is harvested. Baby leaf lettuces contain phytochemicals that protect human health (Kim et al., 2016). Phytochemicals could be phenolic compounds with a simple structure as phenolic acids, or a complex structure as phenylpropanoids or more polymerized compounds such as flavonoids (Santos et al., 2014). Phenolic compounds are important for nutrient uptake, protein synthesis, enzyme activity, photosynthesis, and as structural components. But more interest has been addressed to phenolic compounds for their role in determining food quality; in fact they have been associated with color, sensory qualities and nutritional properties of foods. They determine food organoleptic properties (flavor, astringency, and hardness). Moreover, they play a central role as antioxidants through hydrogen atom donation, electron donation and singlet oxygen quenching (Llorach et al., 2008; Baslam et al., 2011). Many factors influence phenolic content of a plant such as species, cultivar, water availability, maturity, environmental conditions (Santos et al., 2014). Soilless systems are considered an effective tool to improve vegetables quality in particular baby leaf vegetables because they permit to control plant nutrition and to avoid soil contamination (Selma et al., 2012). Among these the floating system is a an important soilless system used for leafy vegetable production that permits the precise management of the salt concentration in the nutrient solution and represents an effective system to improve quality attributes. Rouphael et al. (2006) showed that increasing salinity of nutrient solution from 2.0 to 4.1 dS $m^{-1}$, improves dry matter and total

carbohydrates content of zucchini squash. Similarly, Colla et al. (2013) demonstrated that increasing salinity in the nutrient solution decreases plant growth parameters (leaf dry biomass and number) but on the other hand increases antioxidant activity, total polyphenol, chlorogenic acid, cynarin and luteolin levels of leaves of artichoke and cultivated cardoon. Fanasca et al. (2006 a,b), observed that changing macrocation composition in the nutrient solution it was possible to increase fruit dry matter, total soluble solids content, lycopene content as well as antioxidant activity of tomato fruit.

Our hypothesis is that the application of saline or nutrient stress in the nutrient solution triggers defense mechanisms that contribute to the formation of secondary metabolites that improve the nutritional content of the plants. All these bioactive compounds are not only thought to contribute to the mechanisms of plants defense against biotical and abiotical stresses but they are also important to human health, recommended to reduce risk of oxidative stress-related diseases, giving an extra value to basic nutritional properties of vegetables (Rouphael et al., 2012a ; Kim et al., 2016). Many studies have shown the increase of carotenoids, phenols, ascorbic acid, amino acids, glycine betaines, and sugars in vegetables and baby lettuces in response to salt stress induced by NaCl (Manzocco et al., 2011; Mahmoudi et al., 2012; Borgognone et al., 2013; Neocleous et al., 2014), whereas information is lacking concerning the influence of other salt types on yield and quality of an important leafy vegetables such as baby lettuce.

In addition to nutrient solution management, another cultural practices that may affect the quality of leafy vegetables is the number of cuts, especially that baby lettuce is cut several times (2 or 3) during the growing cycle leading to physiological changes that affect the plant and obviously the product obtained.

The aim of this study is to evaluate the effect of three chloride salts (NaCl, KCl and $CaCl_2$) and cut number (first and second) on biomass production, mineral composition and bioactive compounds of two baby lettuce cultivars (red and rreen) grown in a floating system. The three different chloride salts were tested at equimolar concentrations in order to evaluate the ionic effects of the three salinity sources on productivity and leaf quality of baby lettuces.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Plant materials, treatments and growth conditions

The experiment was carried out in a unheated greenhouse located at the experimental station 'Torre Lama' of the University of Naples Federico II, South Italy (Bellizzi, SA) 40°37′00″N 14°57′00″E). Inside the greenhouse light was provided only by natural solar radiation.Seeds of lettuce (*Lactuca sativa* L. var. *acephala* cvs. 'Green Salad Bowl' and 'Red Salad Bowl') were sown on May in a floating raft growing system. The system consisted of polystyrene plug trays floating in wood tanks with a constant volume (150 L) of stagnant nutrient solution, which was continuously aerated with an air compressor in order to maintain the oxygen content above 6 mg $L^{-1}$. The planting density was 1149 plants $m^{-2}$,as used commercially for similar leafy vegetables in floating systems.

The experiment was designed as a factorial combination of four nutrient solutions (non-salt control and three saline solutions with NaCl, KCl or $CaCl_2$), two cultivars (Green or Red), and cut numbers (first or second). The treatments were arranged in a randomized complete block design with three replications. Each experimental unit consisted of a 1 $m^2$ (1149 plants) container filled with 150 L of aerated nutrient solution.

### 3.2.2 Nutrient solution management and salt treatments

The composition of the basic nutrient solution was: 13.0 mM $NO_3$-N, 1.0 mM $NH_4$-N, 1.75 mM S, 1.5 mM P, 5.0 mM K, 4.5 mM Ca, 2 mM Mg, 1.0 mM Na, 1.0 mM Cl, 20 µM Fe, 9 µM Mn, 0.3 µM Cu, 1.6 µM Zn, 20 µM B, e 0.3 µM Mo, with an electrical conductivity (EC) of 2.0 dS $m^{-1}$. The three saline nutrient solutions had the same control nutrient composition plus an additional 20 mmol $L^{-1}$ NaCl or KCl or 13.3 mmol $L^{-1}$ $CaCl_2$. The nutrient solutions were completely renewed weekly and prepared using deionised water.

### 3.2.3 Recording, sampling and analysis

***Plant growth measurements*:** Baby lettuce were harvested when the control treatment reached the commercial stage (5-6 leaves; 10-12 cm in height), 25 and 29 days after sowing for green and red lettuce, for the first cut. The second harvest occurred 43 and 45 DAS, for the green and red cultivar, respectively. At each harvest time, thirty plants per plot were separated into shoots and roots to determine marketable fresh yield. The marketable yield was expressed at fresh basis in g $m^{-2}$. Leaf tissues were dried in a forced-air oven at 80 °C for 72 h for biomass determination. Leaf area (LA) was measured on 30 plants per treatment using an electronic area meter (Delta-T Devices Ltd., Cambridge, UK).

***Mineral analysis*:** The dried leaf tissues were ground separately in a Wiley mill to pass through a 20 mesh screen, then 0.5 g of the dried plant tissues were analyzed for the following macro and micronutrients: N, P, K, Ca, Mg, and also for Na and Cl. Nitrogen concentration in the plant tissues was determined after mineralization with sulfuric acid by "Kjeldahl method" (Bremner, 1965). To briefly describe the method, 1 g of the dried samples was digested with sulfuric acid ($H_2SO_4$, 96%, Carlo Erba Reagents, Cornaredo, Milan, Italy) in the presence of potassium sulfate ($K_2SO_4$) and a low concentration of copper (Cu) catalyst, and nitrogen was liberated and retained as ammonium sulfate $(NH_4)_2SO_4$. The digestion occurred at 420 °C for about 45 min. Potassium sulfate was used to elevate the boiling point of $H_2SO_4$ and to increase the oxidizing power of the digestion mixture. Ammonia was released from the acid digest by the addition of sodium hydroxide (NaOH). The ammonia was distilled, collected in a boric acid ($H_3BO_3$), 4% solution, and titrated with standardized sulfuric acid. The amount of sulfuric acid used is proportional to the amount of nitrogen originally present in the sample.

Phosphorus, K, Ca, Mg, Na and Cl were extracted from 250 mg samples with deionized water at 80 ºC in a shaking water bath for 10 min (ShakeTemp SW22, Julabo, Seelbach, Germany). The resulting solution was filtered, diluted, and analysed by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA). A conductivity detector with IonPac CG12A guard column and IonPac CS12A analytical column were used for the analysis of K, Ca, Mg and Na whereas for nitrate, P and Cl an IonPac AG11-HC guard column and IonPac AS11-HC analytical column were used (Dionex Corporation).

***Leaf anatomical analysis*:** Nine representative fully expanded leaf samples per each combination of treatments were analyzed for anatomy. Samples were put for a week in a fixative solution of 5 ml formaldehyde 40%, 5ml glacial acetic acid and 90ml ethyl alcohol 50% in water (FAA). Then, lower epidermis was peeled off to analyse stomata features through transmitted light microscopy (Olympus BX60, Germany). Digital images were captured by digital camera (Olympus, Camedia C4040) and subjected to digital image analysis by software AnalySIS 12.0 (Olympus) to analyze stomata frequency (n/mm$^2$) and guard cells size.

***SPAD index and color measurements*:** The leaf colour was analyzed in the CIELAB (L∗a∗b∗) colour space using a Minolta CR-300 Chroma Meter (Minolta Camera Co. Ltd, Osaka, Japan) immediately after harvest. The measuring aperture diameter was 8 mm, and the instrument was calibrated with Minolta standard white plate before sampling baby leaves. Whole leaf samples were placed on a white background and single readings were taken with the hand-held unit on the upper surface of each leaf midway between the apical and basal ends. L* (lightness ranging from 0 = black to 100 = white), a∗ (ranging from green [−60] to red [+60]), b∗ (ranging from blue [−60] to yellow [+60]) readings were transformed to those of the L, a, b colour space (Fallovo et al., 2009).

SPAD index was measured at the mid point of twenty fully expanded leaves per plot using a chlorophyll meter (SPAD-502, Konica Minolta Sensing, Japan). Measurements were made at a central point on the leaflet between the midrib and the leaf margin. The meter was shielded from direct sunlight by the operator during each measurement. Fifteen leaves were measured randomly per plot and averaged to a single SPAD value for each treatment.

***Leaf dry matter, soluble solids content and juice pH*:**The leaf dry matter (DM) was determined following official method 934.01 of the Association of Official Analytical Chemists (AOAC, 2005). Briefly, triplicates of leafy vegetable samples were oven dried at 95 °C until reaching a constant weight, transferred to a desiccator, and allowed to cool at room temperature. The total soluble solids (or the TSS) value in fresh leaves samples was determined using a digital refractometer (Atago N1, Japan) (Matsumoto et al., 1983), whereas, juice pH was determined using a pH Meter (HI-9023; Hanna Instruments, Padova).

***Analysis of hydrophilic and lipophylic antioxidant activities*:** In order to assess comprehensively the antioxidant activity of baby lettuce, samples underwent two

different extraction procedures and two different methods of analysis were subsequently performed. The hydrophilic fraction (HAA) from freeze-dried leaves (0.2 g) was extracted with distilled water and its antioxidant activity was measured with 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 and colored radical cation ($DMPD^{\cdot+}$). Antioxidant compounds (AO) which are able to transfer a hydrogen atom to $DMPD^{\cdot+}$ quench the color and produce a decoloration 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 lipophilic fraction (LAA) was also extracted from freeze-dried leaves (0.2 g) with methanol and antioxidant activity of this extract was measured with the 2,2′-azinobis 3-ethylbenzothiazoline-6-sulfonic acid ABTS method (Pellegrini, 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 HAA and LAA were determined by UV–Vis spectrophotometry. The absorbance of the solutions was measured at 505 and 734 nm, respectively. HAA and LAA were expressed as mmol ascorbic acid (AA) and as mmol of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) per 100 g of fresh weight, respectively (Fogliano et al., 1999).

***Analysis of total ascorbic acid*:** The total ascorbic acid defined as ascorbic acid (ASA) and dehydroascorbate (DHA) acid was assessed by spectrophotometric detection on fresh plant tissues. The assay is based on the reduction of $Fe^{3+}$ to $Fe^{2+}$ by ASA and the spectrophotometric detection of $Fe^{2+}$ complexes with 2,2-dipyridyl (Kampfenkel et al., 1995). 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 on 100 g fresh weight.

***Determination of phenols acids and total phenols*:** One gram of dry materials was extracted by 30 mL of methanol/water (70:30, v/v) and sonicated at room temperature for 30 min. The extraction procedure was repeated twice for each sample. The mixtures were centrifuged at 14,800g, filtered through a Whatman filter paper, and then used for LC-MS/MS analysis using a method previously described by Ferracane et al. (2010). Chromatographic separation was performed using an HPLC apparatus equipped with two micro-pumps series 200 (Norwalk, CT, USA), a UV−Vis series 200 (PerkinElmer) detector set at 280 nm, and a Prodigy ODS3 100 Å column (250 mm × 4.6 mm, particle

size = 5 μm) (Phenomenex, Torrance, CA USA). The eluents were (A) water containing 0.2% formic acid and (B) acetonitrile/methanol (60:40, v/v). The gradient program was as follows: 20−30% B (6 min), 30−40% B (10 min), 40−50% B (8 min), 50−90% B (8 min), 90−90% B (3 min), and 90−20% B (3 min) at a constant flow of 0.8 mL/min. The LC flow was split, and 0.2 mL/min was sent to the mass spectrometer. The injection volume was 20 μL. Two injections were performed for each sample. MS and MS/MS analyses of extracts were performed on an API 3000 triple-quadrupole mass spectrometer (Applied Biosystems, Canada) equipped with a TurboIonSpray source working in the negative ion mode. Six phenolic acids Caffeoyltartaric acid, chlorogenic acid, caffeoylmalic acid, cichoric acid, caffeoyltartaric acid, isochlorogenic acid and total phenols content were identified with LC-MS/MS.

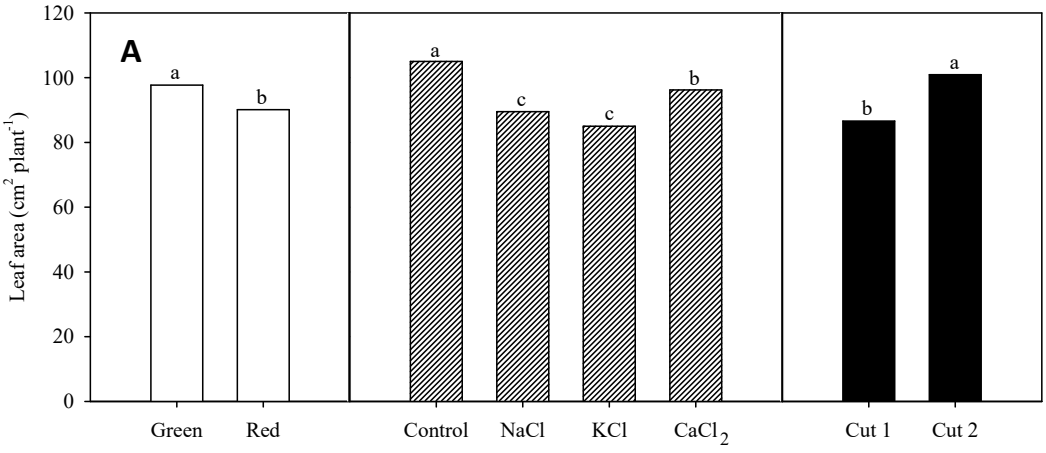
### 3.2.4 Statistical analysis

All data were subjected to analysis of variance (ANOVA) using the SPSS software package (SPSS 10 for Windows, 2001). Duncan's multiple range test was performed for mean comparisons on each of the significant ($p < 0.05$) variables measured.

## 3.3 RESULTS

### 3.3.1 Growth parameters and marketable yield

Cultivar, salinity source and cut had a significant effect on leaf area, fresh and dry biomass measurements (**Fig. 3.1 A, B, C**). Green cultivar showed higher leaf area, fresh and dry biomass values (by 8%, 16% and 21%) than red cultivar. When averaged over treatments the leaf area decreased by 15%, 19% and 9% in NaCl, KCl and $CaCl_2$ treatments compared to the control, with no significant differences between the control and the $CaCl_2$ treatments (**Fig. 3.1 A**). Fresh (**Fig. 3.1 B**) and dry biomass (**Fig. 3.1 C**) decreased in the same magnitude. Finally, all parameters were significantly higher in second cut (**Fig. 3.1 A, B, C**).



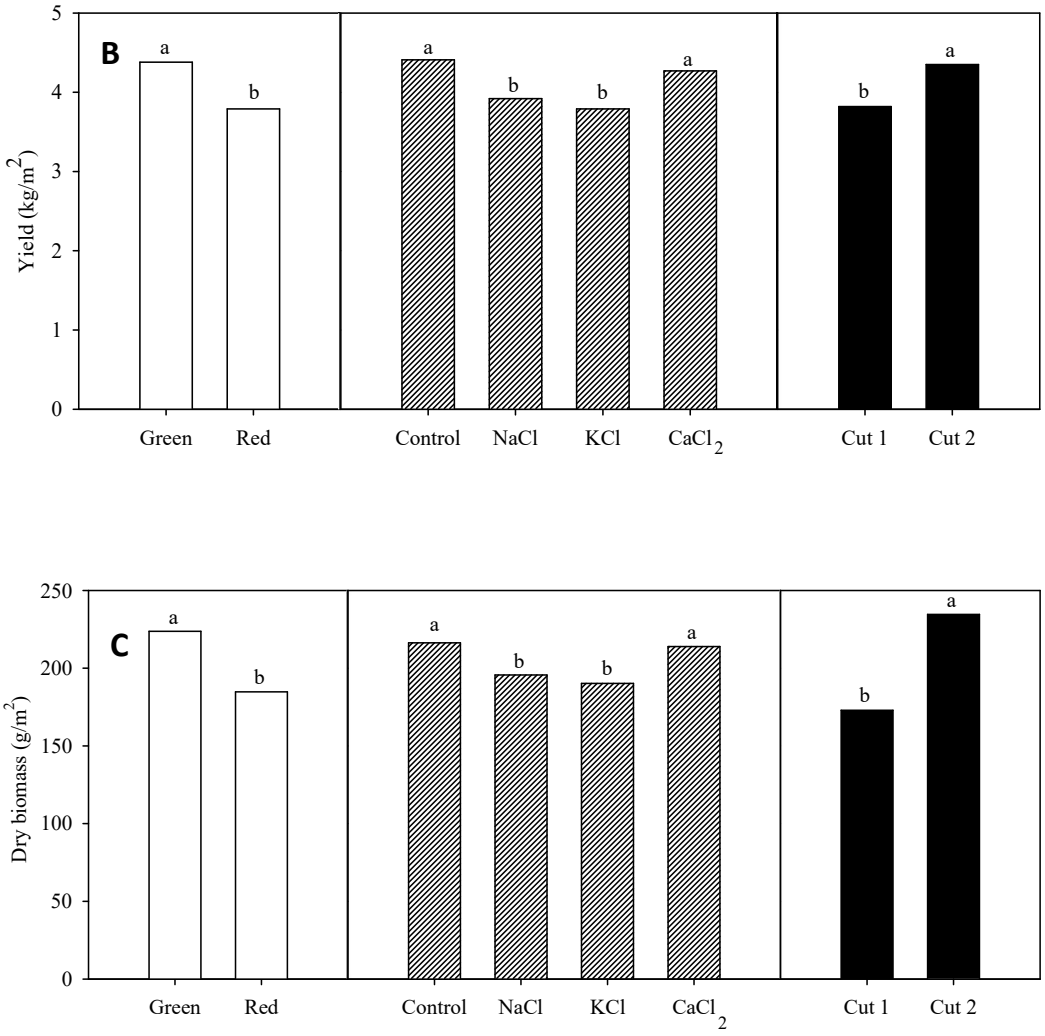



**Figure 3.1.** Effects of cultivar, salinity source and cut number on (A) leaf area ($cm^2$ $plant^{-1}$), (B) marketable yield (kg $m^{-2}$ fw) and (C) dry biomass (g $m^{-2}$ fw) of baby lettuce plants grown in a floating raft culture. Values are mean of three replicates. Different letters indicate significant differences according to Duncan's test ($p \leqslant 0.05$).

### 3.3.2 Mineral composition

The leaf mineral composition of baby lettuce was significantly affected by cultivar, salinity, cut and their interactions as reported in **Table 3.1**. The Ca and Mg content recorded in green cultivar were significantly higher by 35% and, 23% respectively in comparison to the red cultivar. There was a significant interaction CV × CUT for K content.

The monovalent and bivalent cations were negatively affected by NaCl and KCl treatments, whereas an opposite trend was observed for $CaCl_2$, where K and Mg content were similar to those recorded in non-salt control treatment. When averaged over salinity sources and cultivars the second cut enhances P, Ca, and Mg concentrations compared to those recorded in the first cut (**Table 3.1**).

**Table 3.1** Effects of cultivar, salinity source, and cutnumber on leaf mineral composition (g kg-[1] dw) of baby lettuce plants grown in a floating raft culture.

| | Mineral composition (g $kg^{-1}$ dw) | | | | | | |
|---|---|---|---|---|---|---|---|
| | N | P | K | Ca | Mg | Na | Cl |
| Cultivar (CV) | | | | | | | |
| Green Salad Bowl | 44.9 | 3.1 | 50.5 | 7.3 a[2] | 2.1 a | 5.6 a | 23.4 |
| Red Salad Bowl | 43.9 | 3.0 | 51.1 | 5.4 b | 1.7 b | 4.5 b | 25.3 |
| Salinity (S) | | | | | | | |
| Control | 44.3 | 3.3 | 48.7 b | 6.2 b | 2.2 a | 2.0 b | 11.5 c |
| NaCl | 45.0 | 3.0 | 40.1 c | 4.6 c | 1.7 b | 14.6 a | 27.2 b |
| KCl | 44.4 | 3.2 | 68.0 a | 3.9 c | 1.5 b | 1.7 b | 33.5 a |
| $CaCl_2$ | 43.8 | 2.8 | 46.3 b | 10.8 a | 2.1 a | 2.0 b | 25.3 b |
| Cut (CUT) | | | | | | | |
| CUT 1 | 46.0 a | 2.8 b | 48.9 b | 4.9 b | 1.5 b | 1.5 b | 15.3 b |
| CUT 2 | 42.8 b | 3.3 a | 52.7 a | 7.9 a | 2.2 a | 2.2 a | 33.4a |
| Significance[a] | | | | | | | |
| CV | NS | NS | NS | *** | *** | * | NS |
| S | NS | NS | *** | *** | *** | *** | *** |
| CUT | *** | *** | NS | *** | *** | * | *** |
| CV × S | NS | NS | NS | NS | NS | NS | NS |
| CV × CUT | NS | NS | * | NS | NS | NS | NS |
| S × CUT | NS | NS | ** | * | * | * | *** |
| CV × S × CUT | NS | NS | NS | NS | NS | NS | ** |

Different letters within each column indicate significant differences according to Duncan's multiple-range test ($p \leqslant 0.05$). [a] Significance: *$P<$ 0.05; **$P<0.01$; *** $P<0.001$; NS, not significant.

### 3.3.3 Sodium and chloride content

A significant interaction S × CUT for Na and Cl contents in leaves has been recorded in the current experiment. NaCl treatment increased the accumulation of Na content in leaves in comparison to the control and the other two chloride salt treatments in both first and second cut, with the highest value recorded during the second cut (**Fig. 3.2 A**).

KCl treatment increased the accumulation of Cl content in leaves respect to control and the others two saline treatments in the first cut. In the second cut the accumulation of Cl was much more pronounced with the higher values recorded in both NaCl and KCl in comparison to control and $CaCl_2$ treatments (**Fig. 3.2 B**).

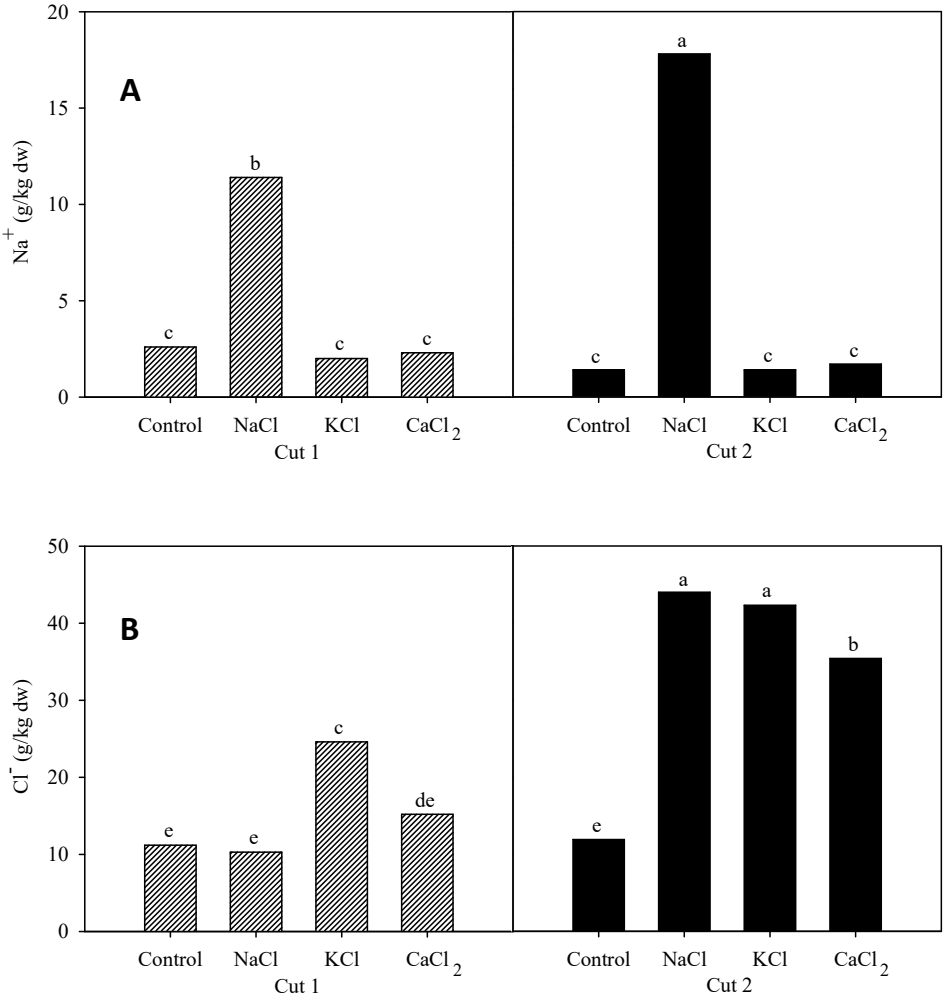



**Figure 3.2.** Effects of salinity source and cut number on Na (g $kg^{-1}$ dw) (A) and Cl (g $kg^{-1}$ dw) (B) contents in leaves of baby lettuce plants. Values are mean of three replicates. Different letters indicate significant differences according to Duncan's test ($p \leqslant 0.05$).

### 3.3.4 Leaf anatomy

**Table 3.2** reported the effects of salinity source and cultivars on stomata frequency and guard cells size. Green cultivar showed higher values of stomata frequency (+14%) while red cultivar exhibited higher values of guard cells width.

NaCl, $CaCl_2$, KCl saline treatment, respect to the control treatment, caused an increase of stomata per unit area by 6%, 7%, 2% respectively, and a significant reduction of guard cells length by 2%, 5%, 3%.

**Table 3.2** – Effects of cultivar, salinity source, on stomata frequency, length and width of guard cells in leaves of baby lettuce plants grow in a floating raft culture.

| | Stomata frequency (n/mm²) | Length of guard cells (μm) | Width of guard cells (μm) |
|---|---|---|---|
| Cultivar (CV) | | | |
| Green Salad Bowl | 90.57 a[2] | 32.44 | 9.81 b |
| Red Salad Bowl | 68.70 b | 32.35 | 10.40 a |
| Salinity (S) | | | |
| Control | 73.11 b | 33.23 a | 10.02 |
| NaCl | 83.56 ab | 32.55 ab | 9.96 |
| KCl | 74.99 ab | 32.37 b | 10.00 |
| $CaCl_2$ | 84.53 a | 31.43 c | 10.42 |
| Significance[a] | | | |
| CV | *** | NS | *** |
| S | * | *** | NS |
| CV × S | ** | *** | *** |

Different letters within each column indicate significant differences according to Duncan's multiple-range test ($p \leqslant 0.05$).
[a] Significance: *$P$< 0.05; **$P$<0.01; *** $P$<0.001; NS, not significant.

### 3.3.5 SPAD index and color parameters

SPAD index and Hunter color parameters (L*, a*, b*) were significantly influenced by cultivar (C). The red pigmented cultivar exhibited a higher SPAD index (9%) and redness-a* (-3,6) compared to the green pigmented cultivar (-7,8). Moreover, L* e b* parameters were significantly higher by 22% and 49% respectively in green compared to the red cultivar.

SPAD index and a* and b* parameters were significantly affected by the cut number. In the first cut we observed a higher SPAD index and b* color parameter by 6% and of 9% respectively, compared to the second cut. (**Table 3.3**). Finally, in the current study no significant effects among the four salinity treatments were observed for the leaf colorimetry parameters.

**Table 3.3** Effects of cultivar, salinity source, and cut on Soil Plant Analysis Development (SPAD) index and Hunter color parameters L∗ (brightness), a∗ (+a∗ = red; −a∗ = green) and b∗ (+b∗ = yellow; −b = blue) in leaves of baby lettuce plants grown in a floating raft system.

| | SPAD | L* | a* | b* |
|---|---|---|---|---|
| Cultivar (CV) | | | | |
| Green Salad Bowl | 18. b[2] | 50.2 a | -7.8 b | 24.5 a |
| Red Salad Bowl | 20.2 a | 41.2 b | -3.6 a | 16.4 b |
| Salinity (S) | | | | |
| Control | 20.2 | 45.4 | -5.1 | 20.5 |
| NaCl | 19.5 | 46.7 | -6.3 | 21.6 |
| KCl | 19.0 | 45.7 | -5.4 | 19.6 |
| $CaCl_2$ | 19.1 | 45.1 | -5.9 | 20.5 |
| Cut | | | | |
| CUT 1 | 20.0 a | 45.9 | -6.1 b | 21.4 a |
| CUT 2 | 18.8 b | 45.6 | -5.2 a | 19.5 b |
| Significance[a] | | | | |
| CV | ** | *** | *** | *** |
| S | NS | NS | NS | NS |
| CUT | * | NS | * | ** |
| CV × S | NS | * | NS | NS |
| CV × CUT | * | NS | NS | NS |
| S × CUT | NS | NS | NS | NS |
| CV × S × CUT | NS | NS | NS | NS |

Different letters within each column indicate significant differences according to Duncan's multiple-range test ($p \leqslant 0.05$).

[a] Significance: *$P$< 0.05; **$P$<0.01; *** $P$<0.001; NS, not significant.

### 3.3.6 Leaf dry matter, total soluble solids, juice pH and EC

Statistical analysis showed a significant increment (+7%) of dry matter content in 'Green salad bowl' respect to the 'Red salad bowl', whereas no significant effects between the two cultivars were observed for the total soluble solids (TSS) content, juice pH and electrical conductivity (EC) (**Table 3.4**).

Adding NaCl, KCl and $CaCl_2$ to the basic nutrient solution improved the TSS contents by 16%, 16% and 9% respectively, compared to the control treatment.

In the second cut the dry matter content, TSS and juice EC were significantly higher by 18%, 31% and 13% respectively, compared to the first cut (**Table 3.4**).

**Table 3.4** Effects of cultivar, salinity source, and cut on dry matter content, total soluble solids (TSS), juice EC and juice pH in leaves of baby lettuce plants grown in a floating raft culture.

| | DM (%) | TSS (ºBrix) | EC (dS $m^{-1}$) | pH |
|---|---|---|---|---|
| Cultivar (CV) | | | | |
| Green Salad Bowl | 5.1 a[2] | 4.7 | 4.8 | 6.0 |
| Red Salad Bowl | 4.8 b | 4.8 | 4.7 | 6.0 |
| Salinity (S) | | | | |
| Control | 4.9 | 4.3 b | 4.2 b | 5.9 b |
| NaCl | 4.9 | 5.0 a | 4.9 ab | 6.0 a |
| KCl | 4.8 | 5.0 a | 4.9 ab | 6.0 a |
| $CaCl_2$ | 5.0 | 4.7 ab | 5.1 a | 5.9 b |
| Cut (CUT) | | | | |
| CUT 1 | 4.5 b | 4.1 b | 4.5 b | 6.0 |
| CUT 2 | 5.3 a | 5.4 a | 5.1 a | 6.0 |
| Significativity[a] | | | | |
| CV | * | NS | NS | NS |
| S | NS | * | * | *** |
| CUT | *** | *** | * | NS |
| CV × S | NS | NS | NS | NS |
| CV × CUT | NS | NS | NS | NS |
| S × CUT | NS | NS | NS | NS |
| CV × S × CUT | NS | NS | NS | NS |

Different letters within each column indicate significant differences according to Duncan's multiple-range test ($p \leqslant 0.05$).

[a] Significance: *$P$< 0.05; **$P$≤0.01; *** $P$<0.001; NS, not significant.

### 3.3.7 Nitrate content

No significant differences in the nitrate content were recorded between the two cultivars. The three chloride salts decreased the nitrate content, particularly $CaCl_2$ treatment showed the lowest content of nitrate in leaves. Finally, the second cut determined an effective reduction of nitrate content respect to the first cut (**Fig. 3.3**).

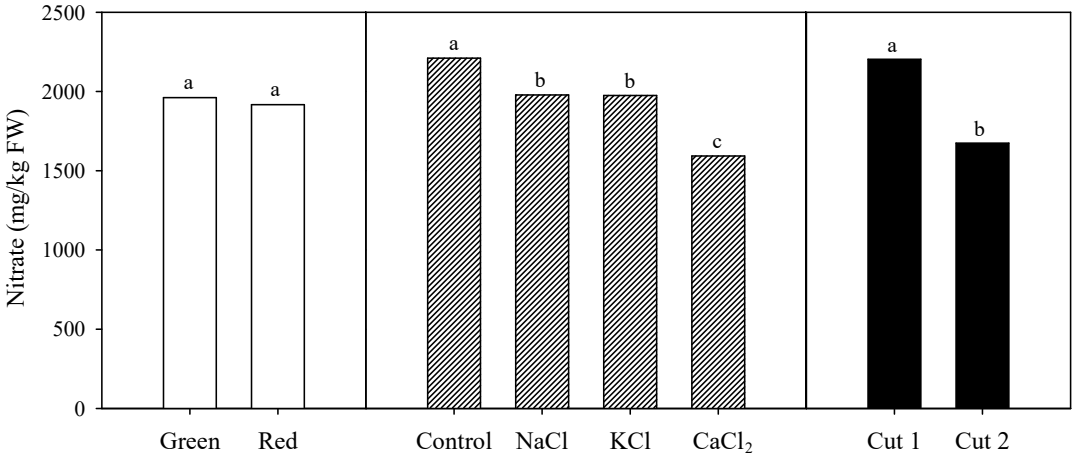



**Figure 3.3** Effects of cultivar, salinity source and cut on nitrate (mg $kg^{-1}$ fw) content in leaves of baby lettuce plants. Values are mean of three replicates. Different letters indicate significant differences according to Duncan's test ($p \leqslant 0.05$).

Concerning the cut × salinity source interaction, we clearly observed that in the first cut the nitrate content was reduced in both KCl and $CaCl_2$ treatments, whereas in the second cut the nitrate content was reduced in all saline treatments in comparison to the control with the lowest values recorded in the nutrient solution containing $CaCl_2$ (**Fig. 3.4**).

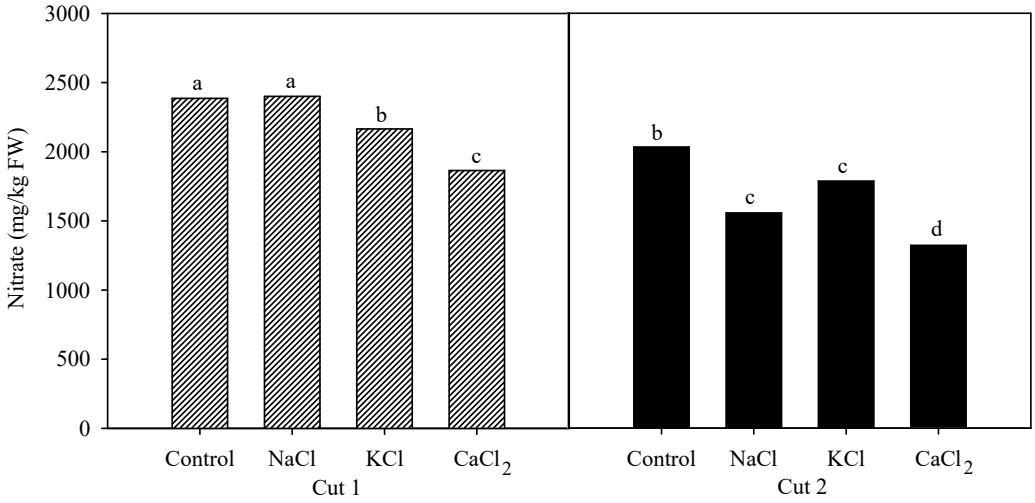



**Figure 3.4** Effects of salinity source and cut interaction on nitrate (mg $kg^{-1}$ fw) content in leaves of baby lettuce plants. Values are mean of three replicates. Different letters indicate significant differences according to Duncan's test ($p \leqslant 0.05$).

### 3.3.8 Lipophilic and hydrophilic antioxidant activity

Red cultivar exhibited the higher lipophilic antioxidant activity (+15%, **Fig. 3.5 A**) than green cultivar; whereas the green cultivar had higher hydrophilic antioxidant activity than red cultivar (+16%, **Fig. 3.5 B**). LAA enhances in all saline treatments respect to control with the highest values observed in the first cut. HAA had higher values in the control than saline treatments and was higher in the second than in the first cut.

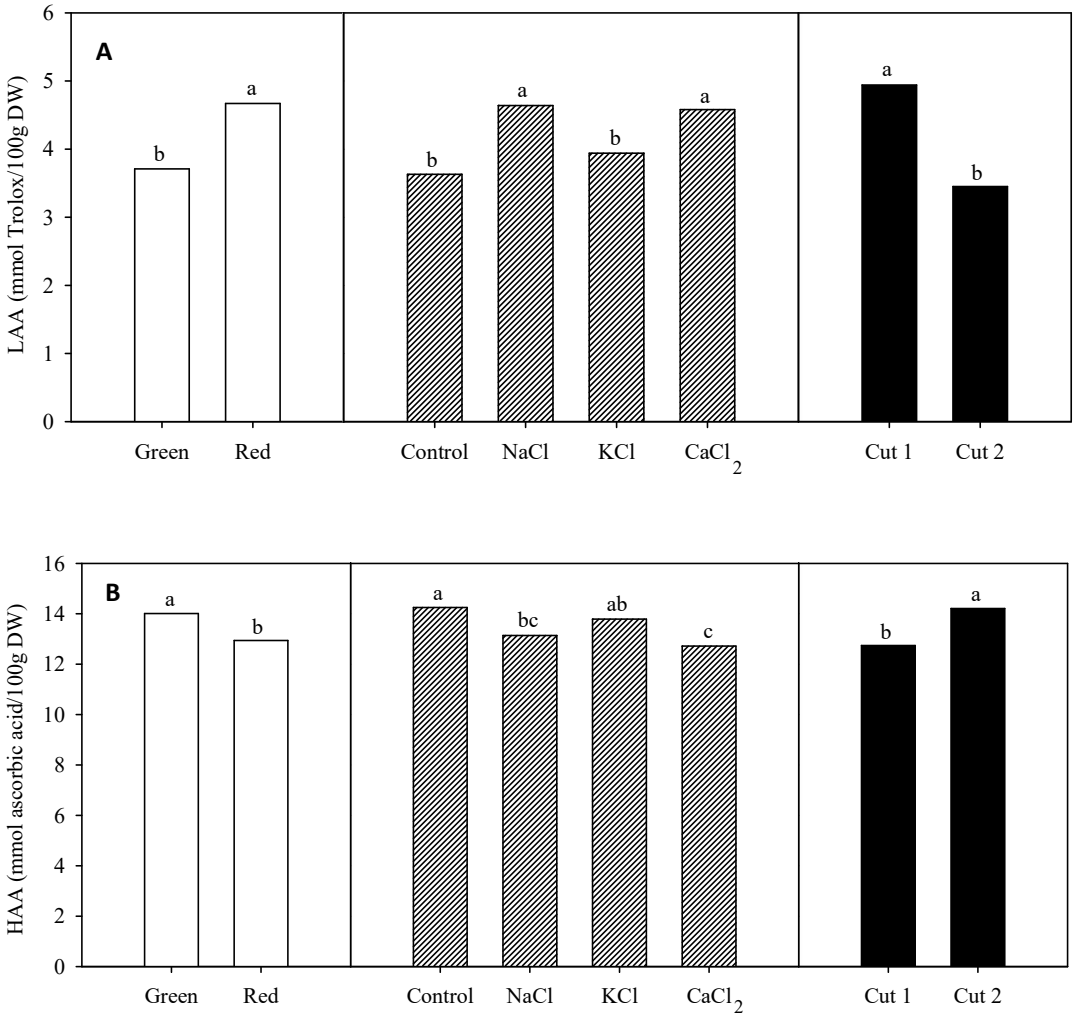



**Figure 3.5** Effects of cultivar, salinity source and cut on Lipophilic (LAA) (A) and Hydrophilic (HAA) (B) antioxidant activities in leaves of baby lettuce plants. Values are mean of three replicates. Different letters indicate significant differences according to Duncan's test ($p \leqslant 0.05$).

### 3.3.9 Total ascorbic acid

Red salad bowl cultivar showed a higher ascorbic acid content respect to the green cultivar; addiction of $CaCl_2$ to the nutrient solution determined a significant increase of ascorbic acid content in lettuce leaves (**Fig. 3.6**).

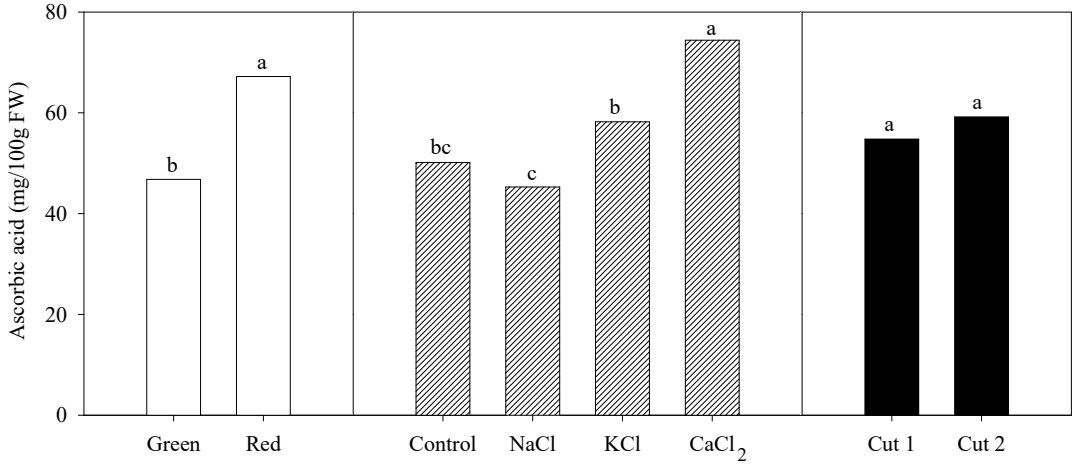



**Figure 3.6** Effects of cultivar, salinity source and cut on ascorbic acid content (mg 100 $g^{-1}$ fw) in leaves of baby lettuce plants grown in a floating raft culture. Values are mean of three replicates. Different letters indicate significant differences according to Duncan's test ($p \leqslant 0.05$).

KCl and $CaCl_2$ treatments enhanced ascorbic acid content in the first cut. In the second cut ascorbic acid content was reduced in NaCl and KCl treatments and with the highest values recorded in $CaCl_2$ treatment (**Fig. 3.7**).

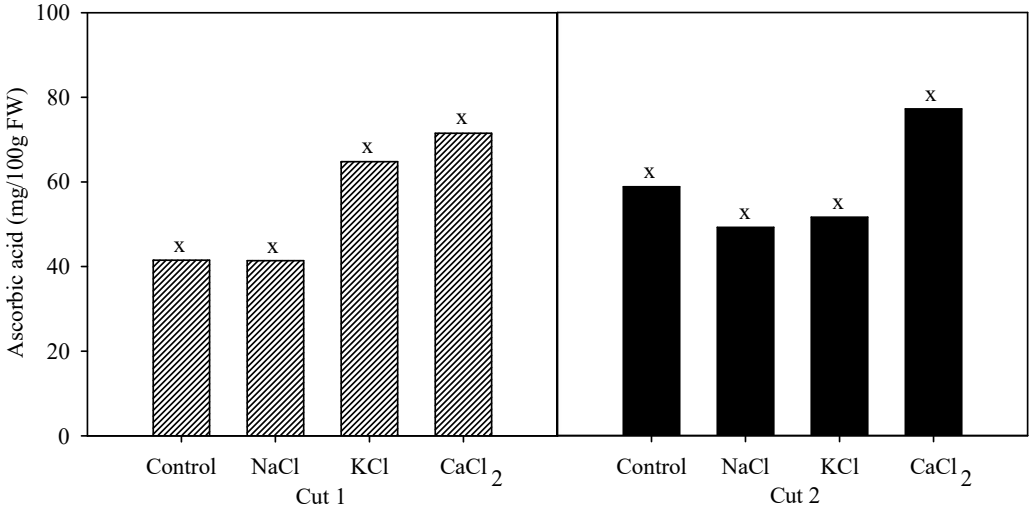
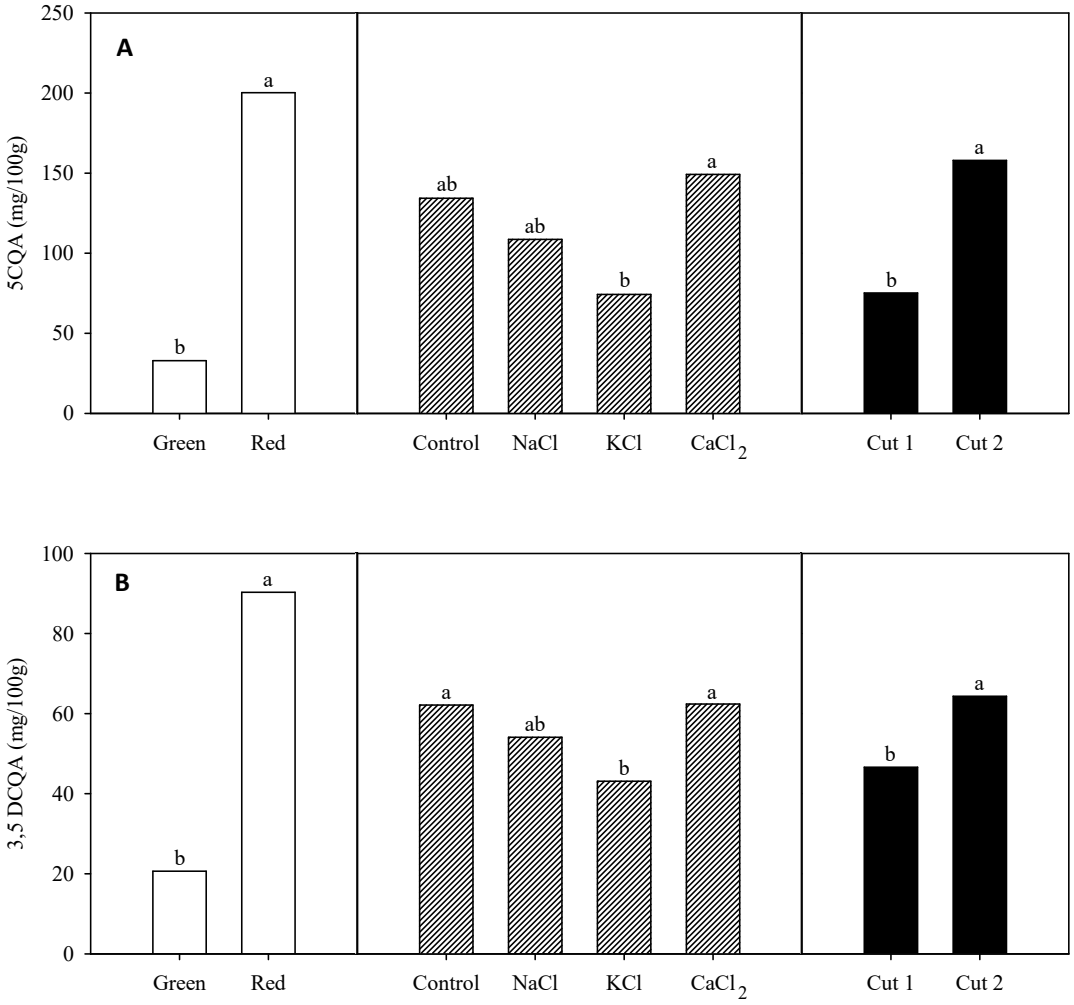



**Figure 3.7** Effects of salinity source and cut interaction on total ascorbic acid (mg 100 $g^{-1}$ fw) content in leaves of baby lettuce plants grow in a floating raft culture. Values are mean of three replicates. Different letters indicate significant differences according to Duncan's test ($p \leqslant 0.05$).

### 3.3.10 Phenolic acids profile

Analysis of phenolic acids demosntrated that red cultivar had higher values of 5-O-caffeoylquinic acid (chlorogenic acid) (5CQA); 3,5-di-*O*-caffeoylquinic acid (isochlorogenic acid) (3,5 DCQA); Caffeoyltartaric acid (CTA); di-*O*-caffeoyltartaric acid (cichoric acid) (DCTA); meso-di-O-caffeoyltartaric acid (mDCTA), than green cultivar (**Fig. 3.8 A,B,C,D,E**).

Saline treatment significantly influenced the content of the five phenolic acids. For instance, the highest content has been recorded in $CaCl_2$ saline treatment, whereas the lowest values were recorded in KCl saline treatment. Second cut affected positively all phenolic acids examined.



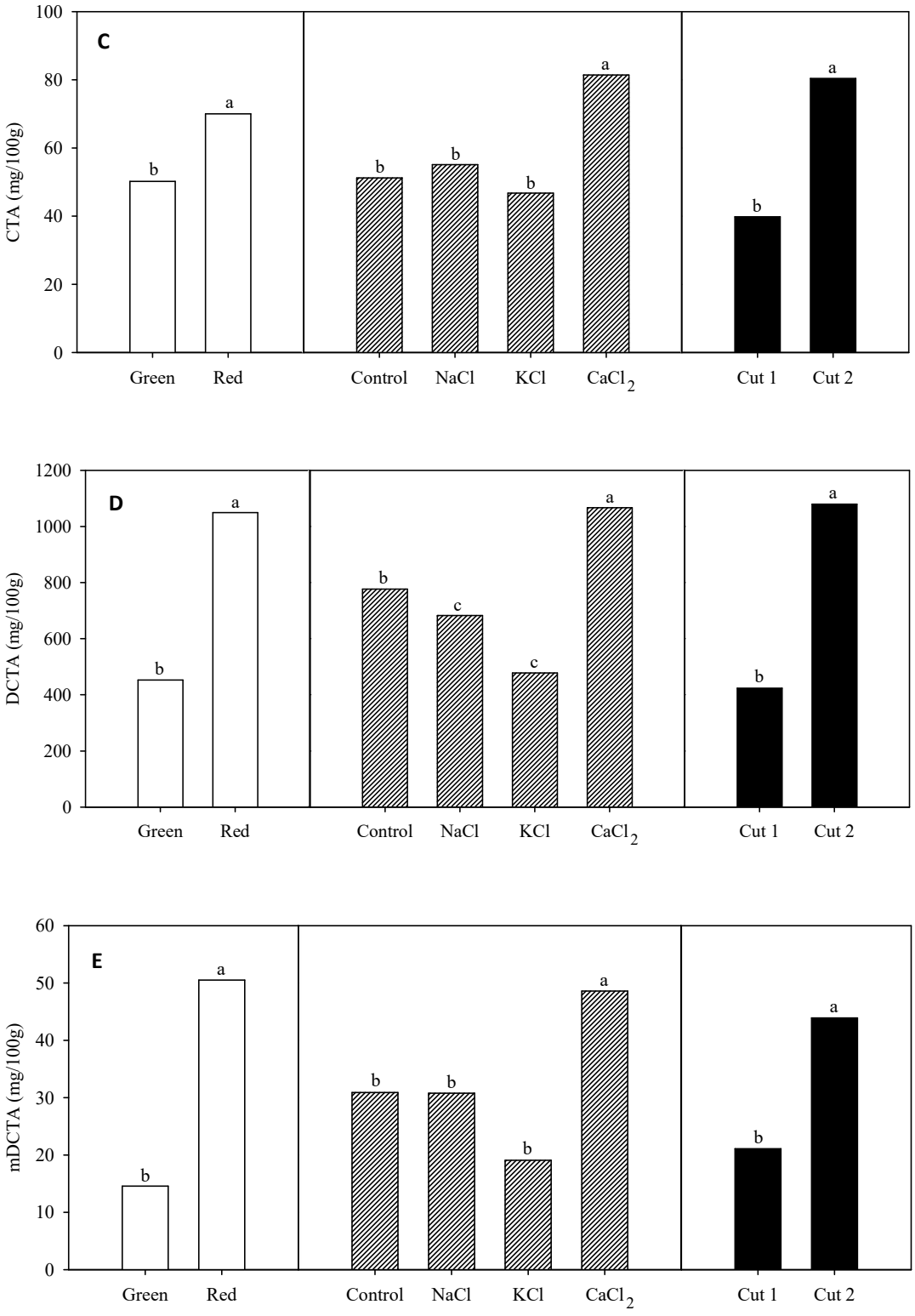



**Figure 3.8** Effects of cultivar, salinity source and cut on 5CQA (mg $100g^{-1}$ dw) (A), 3,5 DCQA (mg $100g^{-1}$ dw) (B), CTA (mg $100g^{-1}$ dw) (C), DCTA (mg $100g^{-1}$ dw) (D) and mDCTA (mg $100g^{-1}$ dw) (E) content in leaves of baby lettuce plants grown in a floating raft culture. Values are mean of three replicates. Different letters indicate significant differences according to Duncan's test ($p \leqslant 0.05$).

### 3.3.11 Total phenols

Red cultivar showed a higher content of total phenols in comparison to the green cultivar (**Fig. 3.9**). $CaCl_2$ treatment significantly increases total phenols respect to the control and to the others two saline treatments. Finally, the second cut enhanced the total phenols content in baby lettuce.

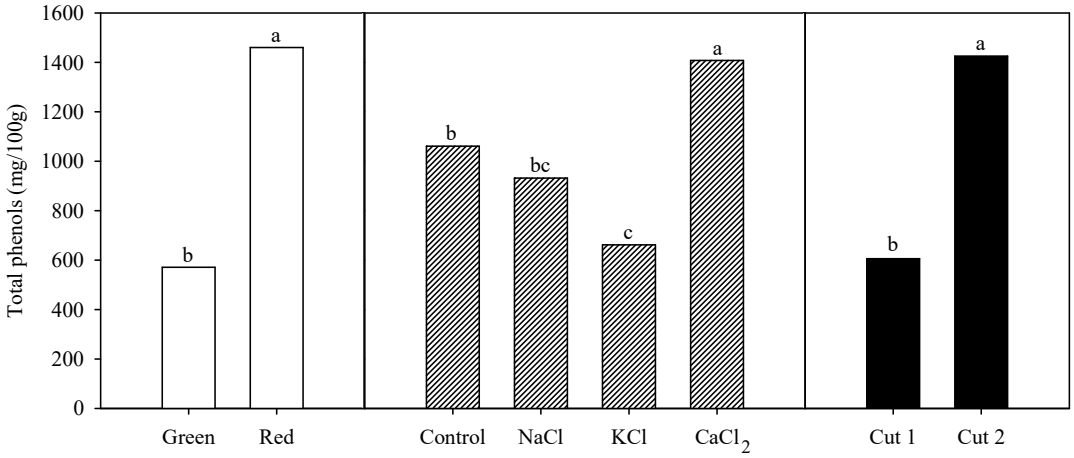



**Figure 3.9** Effects of cultivar, salinity and cut on total phenols content (mg $100g^{-1}$ dw) in leaves of baby lettuce plants grown in afloating raft culture. Values are mean of three replicates. Different letters indicate significant differences according to Duncan's test ($p \leqslant 0.05$).

## 3.4 DISCUSSION

Salinity can mainly affect plants with an osmotic or water-deficit effect that reduces the water potential of the nutrient solution and the water transition in plants. Then, if salts enter the plant these will cause an ion imbalance and toxicity known as ionic effect. The initial symptom of salinity is a growth reduction of plant but the persistence of salt stress can lead to reduced productivity and to death of plants (Parihar et al., 2015). At physiological level salinity generates reactive oxygen species (ROS), including superoxide radical ($O2^{\bullet-}$), hydrogen peroxide ($H_2O_2$), hydroxyl radical ($HO^{\bullet}$), and singlet oxygen ($^1O_2$) in plant that may damage macromolecules such as DNA, proteins, and membrane lipids. Plant defense is the removal of ROS or the production of various secondary metabolites, such as hydrophilic phenolics, lipophilic α-tocopherols and carotenoids, and water-soluble ascorbate and glutathione (Mahmoudi et al., 2012).

In our study, a reduction in leaf area has been found in response to the salts application and a reduction in marketable yield and total dry biomass has been found in response to the NaCl and KCl applications. This is probably related to a reduction of K, Ca and Mg up take and to the accumulation of Na and Cl content in leaf tissue in the presence of NaCl and KCl. The highest Ca content in $CaCl_2$ treatment seems to have contrasted the deleterious effect of Na and Cl. Furthermore, in the present research the green cultivar that has higher Ca content is the one that exhibited the highest values of leaf area, yield and total dry biomass. The highest marketable yield and dry biomass that we observed earlier in the second cut compared to the first cut could be associated to the better nutritional status even if Na and Cl concentrations were higher, but could not be considered critical since no visual symptoms of damage were observed.

It is well-established that crop growth and yield are negatively affected by salinity (Colla et al., 2010). Also Dashti et al. (2009) found a low growth rate and a reduced absorption of Ca in *Sorghum bicolor* (L.) treated with NaCl and KCl, and Cramer et al. (1991) recorded a less Ca absorption in leaves of *Hordeum vulgare* by KCl and NaCl salinity. Our experiment demonstrated that salinity by NaCl reduce concentration of K in leaf, probably due to an intracellular competition with Na (Cerda et al, 1995; Parida and Das 2005), affecting consequently the K/Na ratio (Colla et al, 2012). In our experiment KCl saline treatment increased the K concentration, respect to the no-salinized treatment, and was responsible for an excessive accumulation of $Cl^-$, which led probably to a lower growth rate and photosynthetic activity. The competition between

different ions cause a nutritional deficit which led to a less growth rate (Grattan and Grieve, 1999). Ca is essential for the plants because it is involved in the stabilization of cell membranes and in the formation of ion channels across membrane. It is involved in stomata opening and closing mechanism and it plays a key role as secondary messenger in many physiological and cellular processes (Nedjimi and Daoud, 2009). According to Zhao et al. (2005), an increased flow of Ca ions into the cell seems can induce the production of secondary metabolites in the plant. In contrast with NaCl and KCl, saline treatment $CaCl_2$ led to a higher yield and total biomass. Another study confirmed the increment of plant growth in *Cassia angustifolia* Vahl. adding $CaCl_2$ to the nutrient solution (Arshi et al., 2006). In recent years, several studies are focusing on searching species that can develop tolerance mechanisms or adaptation to abiotic stress, taking care that plants belonging to the same genus and species may develop different tolerance mechanisms (Barbieri et al., 2012). This supports the result of our experiment, because in saline treatments, in particular in $CaCl_2$, a higher stomata density per unit area and a smaller cell guard length were found. A smaller stomata is a sign of adaptation to the new conditions of cultivation, since stomata of small size are characterized by an high speed of opening and closing as a result of changes in turgidity of the guard cells (Drake et al, 2003). The speed of closing of stomata in stress condition is an important adaptive mechanism which allow a better gas exchange control and water use optimization. Many other mechanisms were described that increase salinity tolerance, as maintenance of integrity and function of cell membranes (Mancuso and Rinaldelli, 1996), resulting in better compartmentalization of potentially phytotoxic ions (Na, Cl) in vacuoles and selective absorption of ions. Furthermore, usually salt stress leads to an increase in the root / shoot ratio which should allow to the plant to have a relatively more developed root system in order to increase the absorption of water and nutrients (Colla et al, 2012).

Concerning the effect of cultivar, the green pigmented genotype exhibited the higher leaf area, yield and dry biomass production. This could be attributed to a better nutritional status of baby lettuce in particular Ca and Mg. Our results were in line with those observed by Neocleous et al. (2014).

Several previous studies showed that under mild to severe salt stress conditions plants accumulate high quantity of antioxidant since they play an important role of defense and tolerance against salt stress (Borgognone et al, 2013) and may contribute to ROS detoxification in plants exposed to salinity (Pardo, 2010).

In the current experiment, the saline treatments positively affected nutritional and qualitative parameters in both cultivars, in particular soluble solids, antioxidant activity and vitamin C. Kim et al. (2007) showed that antioxidant activity in lettuce can be related to the phenols content. The highest nutritional quality, in particular vitamin C content (Fig 3.6), phenolic acids content (Fig. 3.8 A-E), known to possess a wide range of therapeutic uses (Subhasree et al., 2009), have been recorded in 'Red Salad Bowl' treated with $CaCl_2$ during the second harvest (Fig 3.8). Also Neocleous et al. (2014), reported a higher content of phenols in red pigmented cultivar. Finally, the nitrate content was significantly reduced with the three chloride salts in comparison to the control with the lowest values recorded in the $CaCl_2$ treatment, probably to the antagonism effect between nitrate and chloride. Nitrate accumulation in leafy vegetables is of high interest to governments and regulators owing to the possible implications for health and to check that controls on the content are effective. Nitrate itself is relatively non-toxic but its metabolites may produce a number of health effects. Until recently the nitrate was perceived as a purely harmful dietary component which causes infantile methaemoglobinaemia, carcinogenesis and possibly even teratogenesis (Addiscott and Benjamin, 2004; Santamaria, et al, 2006). European maximum limit of nitrate concentration in lettuce of 3000-5000 mg $kg^{-1}$ FW. In our experiment, according with results of Hu and Schmidhalter (2005), all treatments did not exceed this limit. In particular, in NaCl, KCl and $CaCl_2$ treatments the nitrate contents were 1978, 1975, 1593 mg $kg^{-1}$ FW, respectively and lower than the non-salinized treatment (2210 mg $kg^{-1}$ FW). Moreover, from cut1 to cut2, a decrease in nitrate concentration was observed. This could be linked mainly to environmental factors. These include the increasing in light intensity that certainly played an important role in reducing the nitrate content by promoting the activity of nitrate reductase during May compared to April.

The concentration of phenolic acids vary as a function of many factors such as genotype, cultural practices, environment and biotic/abiotic stresses. This was the case in the current experiment since the phenolic acids and total phenols were higher in the red cultivar. This may be because red color (red pigmentation) of lettuce is primarily due to anthocyanins, a subgroup of phenolic compounds. The highest phenolic acids concentration was observed mainly with $CaCl_2$ and with NaCl treatment for target phenolic acids. Finally, in terms of cuts, the phenolic acids increased from cut 1 to cut 2. These responses are linked to the considerable stress due to cuts.

## 3.5 CONCLUSIONS

To summarize, our results confirmed that foating raft culture are a promising tool to obtain valuable baby lettuce production as well as improving quality aspects of leaf through proper management of salts in the nutrient solution. Green salad bowl cultivar exhibited higher yield, whereas the red salad bowl cultivar which was characterized by high content in AA, Vitamin C and phenolic acids. Our findings also demonstrated that green salad bowl exhibited higher yield, whereas the red pigmented lettuce was characterized by high content in AA, Vitamin C and phenolic acids. Finally, the second cut influenced positively the mineral composition and nutritional quality in both green and red pigment cultivars.

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# *CHAPTER 4*

## *Salinity source alters mineral composition and metabolism of Cichorium spinosum*

## 4.1 INTRODUCTION

Saline soils represent 20% of the earth's irrigated arable land (FAO, 2015). In saline soils, osmotic stress and/or ion toxicities caused mainly by excessive $Na^+$ and $Cl^-$ concentrations in the root environment are the major factors limiting crop productivity (Colla et al., 2010). High salinity, frequently caused by excess of sodium chloride (NaCl) can induce several morphological, physiological and metabolic changes leading to stunted growth (Rouphael et al., 2016a). Particularly, in vegetable crops, excessive concentration of NaCl causes chlorophyll and carotenoid degradation (Colla et al., 2013a), limitation of photosynthetic capacity (Rouphael et al., 2017) as well as restriction of macro- and micronutrient uptake and translocation (Grattan and Grieve, 1999), leading to significant yield and quality loses (Munns, 2005).

The decline of productivity has been always associated to osmotic (i.e., water deficit stress) or ion-specific (i.e., $Na^+$ and $Cl^-$) effects (Tester and Davenport, 2003). However, the water deficit and ion excess effects cannot be fully discriminated. Salt stress will always cause an osmotic effect, and its magnitude (i.e. intensity) will always be directly proportional to salt concentrations (Colla et al., 2013b). Therefore, the application of iso-osmotic salt solutions obtained by using different salinity sources could be a meaningful approach to discriminate the effects of specific ion toxicities under salt stress conditions (Navarro et al., 2003; Pagter et al., 2009). Most studies dealing with salt tolerance of vegetables have been carried out considering NaCl as the predominant salt (Colla et al., 2012, 2013b). Limited number of studies were conducted with the aim to assess the effect of other types of salinity ($Na_2SO_4$, KCl or $CaCl_2$) on plant growth, nutritional status and metabolic profiling; even though $Na_2SO_4$ and $CaCl_2$ are present at higher concentrations than sodium chloride in soils, groundwater and surface water in many areas worldwide (Banuelos et al., 1993; Marschner, 2012).

Most of the cultivated vegetables are salt sensitive, growing poorly in salinized soils (Colla et al., 2010). Therefore, an efficient strategy to assure productivity of vegetables under salinity conditions would be the selection of salt-tolerant genotypes and/or landraces (Rouphael et al., 2012a). In a Mediterranean environment, spiny chicory (*Cichorium spinosum* L.; *Asteraceae* family), also known in Greek language as *stamnagathi*, provides a *niche* product combining unique taste and fortified phytonutrient content, i.e. vitamins C and K1, lutein, β-carotene, tocopherols, phenolic

acids, fatty acids, minerals and glutathione (Zeghichi et al., 2003; Klados and Tzortzakis, 2014; Petropoulos et al., 2016). *Stamnagathi* is a native plant of the Mediterranean area, which abounds in the coastal areas of Greek islands (Crete) as well as in Cyprus, Italy (Sicily), Malta and Spain (Brieudes et al., 2016). The fact that *stamnagathi* usually grows in coastal areas characterized by sea water intrusion, indicates a potential tolerance to salt stress (Petropoulos et al., 2017). Despite the increasing economic importance of *stamnagathi* as a newly introduced vegetable crop, which is fostered by the increasing demand from consumers, information on its responses to salinity is largely fragmented. High salinity reduces significantly the fresh yield and protein content of *stamnagathi*, but increases total phenols, bitterness, antioxidant activity and mineral composition (Klados and Tzortzakis, 2014;Petropoulos et al., 2016). However, these studies have been conducted by exposing the plants to nutrient solutions containing NaCl, whereas nothing is known about the effect of other types of salinity ($Na_2SO_4$, KCl, $CaCl_2$) on *stamnagathi*. In addition, the previous studies on *stamnagathi* have focused on target compounds (i.e. phenols, proteins, sugars, ascorbic acid, tocopherols), with limited information on the full set of metabolites recorded under salt stress conditions. In this regard, metabolomics provides a powerful tool for investigating and quantifying the full suite of small molecules or metabolites of a given biological system (Aliferis et al., 2014; Farag, 2014; Aliferis et al., 2015; Lucini et al., 2016; Rouphael et al., 2016b). The monitoring of the complete set of metabolites under salt stress conditions could improve our understanding to the specific physiological mechanisms underlying plant growth suppression imposed by salinity (Lucini et al., 2015; Tian et al., 2016). Moreover, global profiling of crop metabolome could also contribute to the identification of genetic manipulations necessary to enhance the nutraceutical value of crops (Rouphael et al., 2016a; Tian et al., 2016).

Taking this background into consideration, the aim of the current study was to comparably evaluate the effects of four sodium and chloride salts ($Na_2SO_4$, NaCl, KCl or $CaCl_2$) on growth, mineral composition and metabolism of *C. spinosum* grown in a closed soilless cultivation system. The four salts were tested at two different iso-osmotic concentration levels, to assess the ionic effects of the four salinity sources on plant metabolism.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Plant material, growth conditions and treatments

The experiment was conducted in autumn 2015 in a glasshouse at the Agricultural University of Athens (N 37°59′10′′, E 23°42′29′′, altitude 24 m). Seeds of stamnagathi (*Cichorium spinosum* L.) originating from reproduction of seeds collected from wild plants growing at a mountainous location in Crete, were sown in seed trays containing peat on 2 September 2015. On 28 October 2015, seedlings at the stage of three true leaves were transferred into 36 closed-loop hydroponic circuits (experimental plots). Each circuit comprised one individual supply tank, a pump, irrigation pipes and one channel, 3.0 m in length, 0.025 m in width, and 0.03 m in height, which accommodated 32 plants. The plant density was 9.2 plants $m^{-2}$. *C. spinosum* plants were grown under natural light conditions. Inside the glasshouse, the daily air temperature was always maintained below 25ºC while the night temperature was always higher than 12ºC.

The *C. spinosum* plants were supplied with nutrient solution which was constantly recirculating at a flow rate of 0.6 $m^3$ $h^{-1}$. The composition of the basic nutrient solution (NS) used to replenish nutrients and water absorbed by plants (replenishment NS) was as follows: 15.2 mM $NO_3^-$, 1.2 mM $H_2PO_4^-$, 2.9 mM $SO_4^{2-}$, 8.0 mM $K^+$, 5.2 mM $Ca^{2+}$, 1.5 mM $Mg^{2+}$, 0.9 mM $NH_4^+$, 15.0 μM Fe, 8.0 μM Mn, 6.0 μM Zn, 0.7 μM Cu, 30.0 μM B and 0.5 μM Mo. The electrical conductivity (EC) and the pH of the replenishment NS were 2.5 dS $m^{-1}$ and 5.6, respectively. The total volume of recirculating NS in each experimental unit amounted to 0.8 L per plant, i.e. 26 L totally. In each unit, the replenishment NS was automatically supplied from an individual tank using a floater to maintain a constant NS level in the supply tank. The NS consumed by the plants was recorded daily and replaced by refilling the replenishment tank. The pH in the recirculating NS was adjusted once per day to 5.6 by adding nitric acid (1 N) or potassium hydroxide (1 N) to the supply tank. All channels were covered with black-white polyethylene sheets to avoid water evaporation. Furthermore, no drainage water was discharged and losses due to technical failures were negligible.

The experiment treatments consisted of nine NS, particularly a basic NS used as control, and eight saline NSs with two different levels of total molar concentrations, obtained by adding to the replenishment NS different amounts of NaCl, KCl, $Na_2SO_4$ or $CaCl_2$. Two addition dosages were applied for each salt resulting in a “low salinity” and a “high salinity” level. At each salinity level, the salt concentrations in the four different NSs were isosmotic, i.e. properly selected to achieve the same osmotic potential (**Table**

**4.1**). The levels of the osmotic potential ($\Psi_s$) at 20 °C (-0.286 and -0.480 MPa in the low- and high-salinity level, respectively) were calculated using the Van't Hoff's equation (Abebe et al., 2003):

$$\Psi_s = \sum CiRT$$

where, $C$ is the concentration (mol $L^{-1}$) of each solute including nutrients and salts used to induce salinity $i$ is the number of ions per molecule of solute, $R$ the perfect gas constant (0.00831 L MPa $mol^{-1}$ $K^{-1}$), and $T$ the temperature (°K). Treatments were commenced two weeks after transplanting. All experimental units were arranged in a randomized complete block design with three replicates per treatment. Each experimental unit accommodated 15 plants.

**Table 4.1.**Addition of different salts (mmol $L^{-1}$) to a basic nutrient solution aiming at establishing eight salinity treatments differing in the osmotic potential level (-0.286 and -0.480 MPa at 20 °C, referred to as low and high salinity level) and the salinity source: 1: Control (standard nutrient solution); 2: low NaCl-salinity; 3: high NaCl-salinity; 4: low KCl-salinity; 5: high KCl-salinity, 6: low $CaCl_2$-salinity; 7: high $CaCl_2$-salinity; 8: low $Na_2SO_4$-salinity; 9: high $Na_2SO_4$-salinity.

| Treatments | NaCl | KCl | $CaCl_2$ | $Na_2SO_4$ | Total ionic conc. | ECdS/m |
|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 0 | 2.10 |
| 2 | 40 | 0 | 0 | 0 | 80 | 6.45 |
| 3 | 80 | 0 | 0 | 0 | 160 | 10.27 |
| 4 | 0 | 40 | 0 | 0 | 80 | 6.45 |
| 5 | 0 | 80 | 0 | 0 | 160 | 10.27 |
| 6 | 0 | 0 | 26.7 | 0 | 80 | 7.44 |
| 7 | 0 | 0 | 53.3 | 0 | 160 | 12.17 |
| 8 | 0 | 0 | 0 | 26.7 | 80 | 7.44 |
| 9 | 0 | 0 | 0 | 53.3 | 160 | 12.17 |

### 4.2.2 Biomass determination and growth analysis

Ten plants were sampled to estimate the fresh and dry weight of the plants at the beginning of the experiment. At the end of the experiment (56 days after transplanting) plants were separated into leaves and roots. Both plant tissues were dried at 80 ºC for 72 h until they reached a constant weight and weighed again to determine the corresponding dry biomasses. The root-to-shoot ratio and the dry matter percentage were also calculated.

The relative growth rate (RGR) expressed as g $g^{-1}$ $day^{-1}$ was calculated using the equation reported by De Groot et al. (2001):

$$RGR = (\ln W_2 - \ln W_1)/(t_2 - t_1)$$

where $W_1$ and $W_2$ are the fresh masses (g) of the above-ground plant part (shoot) at times $t_1$ and $t_2$ (days), corresponding to the beginning and to the end of the experiment, respectively.

### 4.2.3 Mineral analysis

The dried tissue samples were powdered using a blade mill, and passed through a 40-mesh sieve. Sub-samples of all dried plant tissue samples were used for chemical analysis to determine the following elements: N, P, K, Ca, Mg, S, Na, and Cl concentrations. Total nitrogen was determined following the Dumas combustion technique using a C/N analyzer (Elementar, Hanau, Germany). Phosphorus, K, Ca, Mg, and Na were determined by dry ashing at 550 $^{o}$C for 5 h, dissolving ash in 1 N HCl. The concentrations of K, Ca, and Mg in aqueous tissue extracts were measured using an atomic absorption spectrophotometer (Perkin Elmer 1100A, Waltham, MA, USA). P was measured photometrically as phosphomolybdate blue complex at 880 nm using a 96-position microplate spectrophotometer (Anthos Zenyth 200; Biochrom, USA). The Na concentrations in aqueous extracts were determined by flame photometry using a Sherwood Model 420 (Sherwood Scientific, Cambridge, UK). The S concentration was extracted from 250 mg samples with deionized water at 80 ºC in a shaking water bath for 10 min (ShakeTemp SW22, Julabo, Seelbach, Germany). The resulting solution was filtered, diluted, and analyzed by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA). A conductivity detector with IonPac AG11-HC guard column and IonPac AS11-HC analytical column (Dionex Corporation) was used for the analysis of S. The determination of Cl in the plant-tissue extracts and nutrient solutions was performed by titration with $AgNO_3$ in the presence of $K_2CrO_4$ (Eaton et al., 1995).

### 4.2.4 Leaf $^{1}$H NMR metabolomics in *Cichorium spinosum*

#### 4.2.4.1 Sampling and metabolite extraction

At the end of the experiment, the top five fully expanded leaves of three different plants of each experimental unit were collected in falcon tubes (50 mL, Sigma-Aldrich) and immediately frozen in liquid nitrogen for metabolism quenching. Samples were stored at −80°C until further processing.Leaves were pulverized to a fine powder in a mortar using a pestle inliquid nitrogen. The pulverized tissues wereplaced in falcon tubes (50 mL) and immediately immersed in liquid nitrogen. Sample extraction and processing for$^{1}$H NMR metabolomicswas performed as previously described (Aliferis et al., 2015). Briefly, the pulverized leaf tissues (100 ± 2 mg) were transferred into Eppendorf tubes (2 mL, Sigma-Aldrich) and for the water removal, theywerelyophilized for 24 h. The extraction of polar compounds was performed by adding to the dried extracts 1 mL deuterium oxide ($D_2O$)containing 0.05% trimethylsilyl-2,2,3,3-d4-propionic acid sodium salt (TSP) (Sigma-Aldrich Chemie GmbH, Munich, Germany) intoEppendorf tubes (2 mL). Initially, extracts were sonicated for 25 min and then they were kept under continuous agitation (150 rpm) for 1 h at 24°C. For the removal of debris, samples were centrifuged (12,000 × *g*) for 1 h at 4°Cand the supernatants were subjected to a second centrifugation (12,000 × g) for 30 minat 4°C. Supernatants were then collected and kept in Eppendorf tubes at −80°C until the acquisition of $^{1}$H NMR spectra.

#### 4.2.4.2 $^{1}$H NMR analyses

Extracts were placed in NMR tubes (5 mm Thin Wall Precision NMR Sample Tubes 8" L, Wilmad, Vineland, NJ, USA) for the recording of $^{1}$H NMR spectra. $^{1}$H NMR spectra were recorded using a Bruker Avance spectrometer at 500 MHz equipped with a 5 mm inverse detection probe. A total of 128 transients of 64 K data points were acquired per sample (90$^{o}$ pulse angle, 2 s acquisition time and 2 s recycle delay) with presaturation of $H_2O$ during the recycle delay.

#### 4.2.4.3 Data pre-processing and biomarker discovery

The pre-processing and deconvolution of the obtained spectra, multivariate analyses and biomarker discoverywere performed as previously described (Aliferis et al., 2015) with minor modifications. Initially, spectra were Fourier transformed, their phase and

baseline were automatically corrected, and offsets of chemical shifts were corrected based on the signal of TSP at 0.00 ppm using the software Spectrus (ACD Labs, Toronto, Canada). Metabolite identification was based on chemical shifts, coupling constants (*J*) and comparisons to $^{1}$H NMR spectra of analytical standards in $D_2O$.

The spectral region between 0.70 and 8.80 ppm was integrated after the removal of regions such as, the one that corresponds to the water signal (4.70-4.90 ppm), using the "intelligent bucketing" option of the software. The obtained matrix was subjected to multivariate data analyses for the discovery of trends and biomarkers using the software SIMCA-P 13.0 (Umetrics, MKS Instruments Inc,USA) based on orthogonal projections to latent structures-discriminate analysis(OPLS-DA) (Aliferis et al., 2015). Data were pareto-scaled (1/HSD) and cross validation was performed based on the default software settings and the corresponding values of the explained variation ($R^2X$ and $R^2Y$) and predictive ability, $Q_{(cum)}^2$. The discovery of biomarkers of salinity was based on scaled OPLS regression coefficients (Efron and Gong, 1983).

In addition to OPLS-DA for the visualization of the fluctuations in the plant's metabolome in response to the various treatments, a cluster heat map in combination with two-dimensional (2D) hierarchical cluster analysis (HCA) was constructed using the software Matlab R2016a (MathWorks, Natick, MA, USA). HCA was performed applying the Ward's linkage method.

### 4.2.5 Statistical analysis

Analysis of variance (one way-ANOVA) of the experimental data (plant biomass and mineral composition) was performedusing the software package Statistica for Windows 9.0 (Tulsa, OK, USA). To separate treatment means within each measured parameter, the Duncan's Multiple Range Test was performed at $P \leq 0.05$

## 4.3 RESULTS

### 4.3.1 Biomass production and partitioning

The shoot and root biomass, leaf dry matter content, relative growth rate (RGR) and root-to-shoot (R/S) ratio were affected by salinity but the effects were depending on both the level and the source of salinity (**Fig. 4.1**). The leaf fresh biomass decreased with low $CaCl_2$- and $Na_2SO_4$-salinity, while low NaCl- and KCl-salinity at isosmotic concentrations had no effect on this growth parameter. At high salinity, all salts were detrimental to the production of fresh plant biomass without any significant differences

due to the source of salinity. The salinity-induced reductions in fresh shoot biomass resulted from commensurate reductions in the rates of fresh biomass accumulation, as indicated by the estimated RGRs.

In contrast to the fresh shoot biomass, the dry shoot biomass was not impaired by the low-salinity treatments, regardless of the salt species, while at high salinity only $CaCl_2$ was detrimental to the accumulation of dry shoot biomass in comparison with the control (**Fig. 4.1**). Nevertheless, the highest performance it terms of dry biomass accumulation was observed in the low KCl-salinity treatment; the dry shoot biomass in the latter was significantly higher not only than that measured in the high $CaCl_2$-salinity treatment but also in comparison with those recorded at high NaCl-, high KCl-, and low $CaCl_2$-salinity. The much smaller impairment of the dry shoot biomass by the tested salt treatments than that imposed to the fresh shoot biomass was due to a significant increase of the dry matter content by salinity, which tended to be stronger with increasing salinity level.

With respect to the root growth, the low NaCl-salinity increased the root dry biomass production in comparison with the non-salinized NS and the $CaCl_2$-salinity, regardless of the $CaCl_2$-salinity level (**Fig. 4.1**). The root to shoot ratio in terms of dry biomass was increased by both levels of NaCl-salinity, while the other three salinity sources increased this ratio only at the highest level.

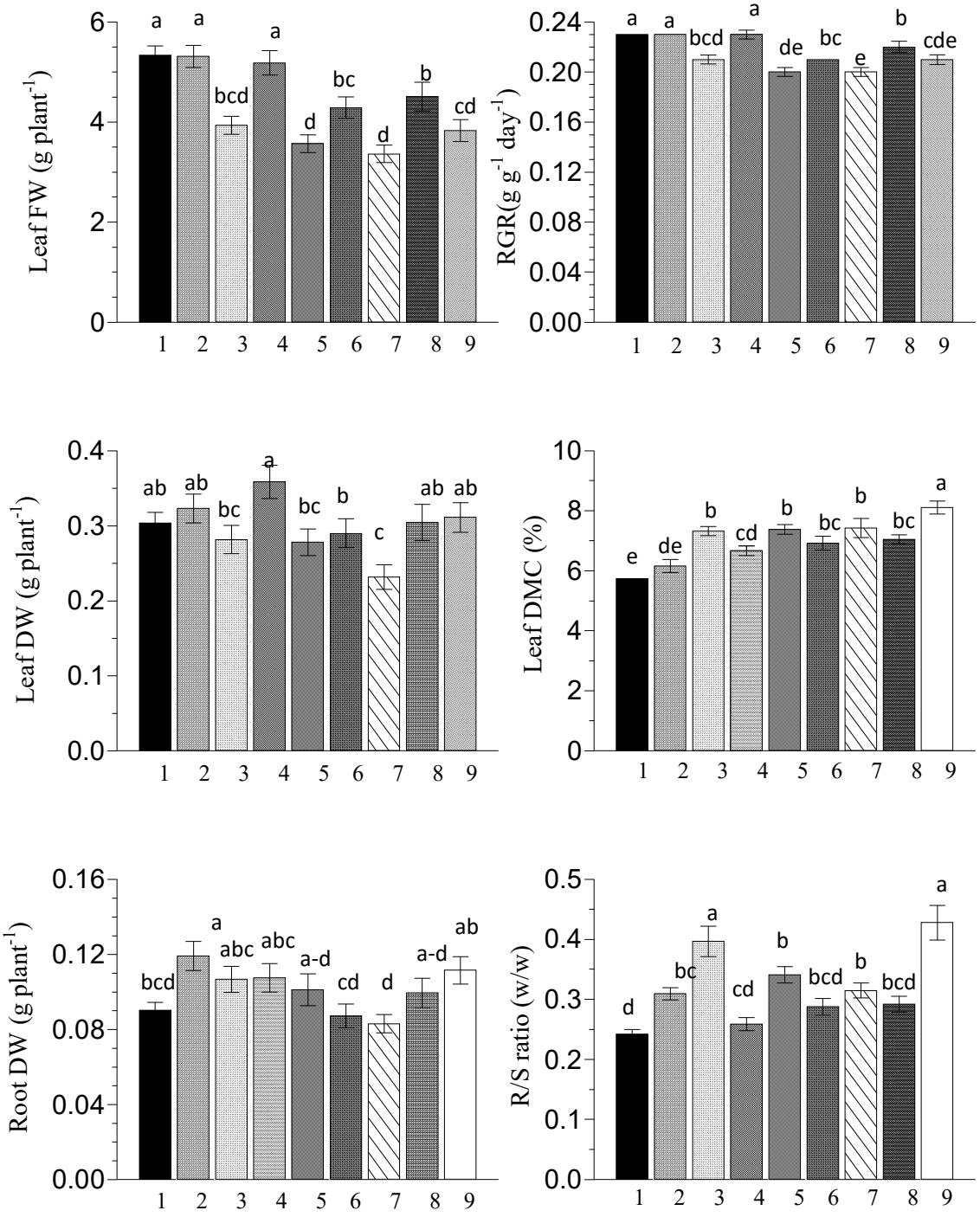



**Figure 4.1**. Leaf fresh weight (FW), leaf and root dry weight (DW), relative growth rate (RGR) of the fresh shoot, leaf dry matter content (DMC), and root to shoot (R/S) ratio (DW basis) in Cichorium spinosum plants grown in recirculating nutrient solution, as influenced by eight salinity treatments differing in the osmotic potential level (-0.286 and -0.480 MPa at 20 oC, referred to as low and high salinity level, respectively) and the salinity source: 1: Control (standard nutrient solution); 2: low NaCl-salinity; 3: high NaCl-salinity; 4: low KCl-salinity; 5: high KCl-salinity, 6: low CaCl2-salinity; 7: high CaCl2-salinity; 8: low Na2SO4-salinity; 9: high Na2SO4-salinity. Vertical bars indicate ± standard errors of means of four measurements. Similar letters indicate non-significant differences at $P \leq 0.05$

### 4.3.2 Mineral composition and partitioning

The leaf K concentration was reduced by the high NaCl-salinity and by both levels of the $Na_2SO_4$-salinity, while it was not affected by the $CaCl_2$ salinity and increased by the KCl-salinity (**Fig. 4.2)**. A similar effect was observed also in the roots with the exception that the root K was restricted also by low NaCl-salinity.

The leaf Ca concentration was reduced only by low $Na_2SO_4$-salinity, while it was enhanced by $CaCl_2$-salinity to levels related to the external $CaCl_2$ concentration (**Fig. 4.2)**. In the roots, the Ca concentration was not reduced by any salinity treatment while it increased significantly only by high $CaCl_2$-salinity.

Both NaCl- and KCl-salinity reduced moderately the leaf Mg concentration to levels not influenced by their external concentration, while the $Na_2SO_4$ concentration imposed a much stronger suppression of the leaf Mg concentration (**Fig. 4.2)**. The $CaCl_2$-salinity had no impact on the leaf Mg concentration. In contrast to the leaf Mg concentration, the root Mg concentration was not significantly influenced by any salinity treatment, regardless of salt species and concentration level.

Both the leaf and root Na concentrations were increased when Na salts were used to impose salinity, to levels related to the external Na concentration (**Fig. 4.2**).

The total-N concentration was slightly reduced by KCl- and $CaCl_2$-salinity at both concentration levels both in leaves and roots, while it was not influenced by $Na_2SO_4$-salinity (**Fig. 4.3)**. The NaCl-salinity reduced the total-N concentration at both concentration levels in the roots but only at the high level in the leaves.

The leaf P concentration tended to increase by the low-salinity treatments regardless of the salt species, while at the high-salinity level, it was enhanced only KCl- and $Na_2SO_4$-salinity (**Fig. 4.3)**. The root P concentration was also enhanced by salinity but the differences between the control and the $Na_2SO_4$-salinity were insignificant.

The leaf sulphur concentration was reduced by KCl and $CaCl_2$-salinity but only at the high concentration level, while it was not influenced by NaCl-salinity and was raised by $Na_2SO_4$ salinity (**Fig. 4.3)**. In the roots, the S concentration was reduced only by the high KCl-salinity treatment.

The concentration of Cl was raised by salinity imposed by chloride salts (NaCl, $CaCl_2$ and KCl) in both leaves and roots, to levels related to the Cl concentration in the root zone, while it was not influenced by $Na_2SO_4$-salinity (**Fig. 4.3)**.

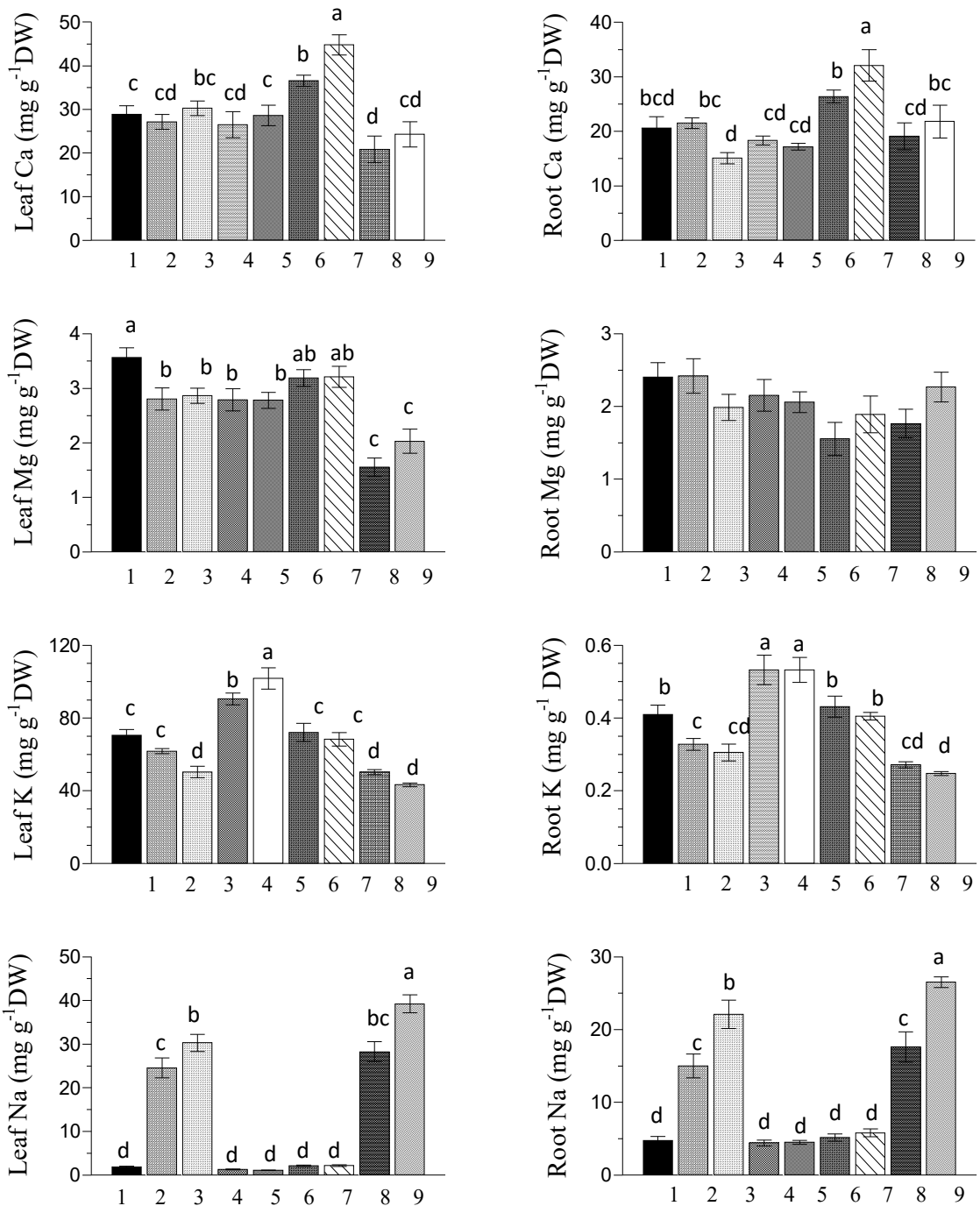



**Figure 4.2.**Concentrations of Ca, Mg, K and Na in the leaves (left) and roots (right) of *Cichorium spinosum* plants grown in recirculating nutrient solution, as influenced by eight salinity treatments differing in the osmotic potential level (-0.286 and -0.480 MPa at 20 °C, referred to as low and high salinity level, respectively) and the salinity source: 1: Control (standard nutrient solution); 2: low NaCl-salinity; 3: high NaCl-salinity; 4: low KCl-salinity; 5: high KCl-salinity, 6: low $CaCl_2$-salinity; 7: high $CaCl_2$-salinity; 8: low $Na_2SO_4$-salinity; 9: high $Na_2SO_4$-salinity. Vertical bars indicate ± standard errors of means of four measurements. Similar letters indicate non-significant differences at $P \leq 0.05$.

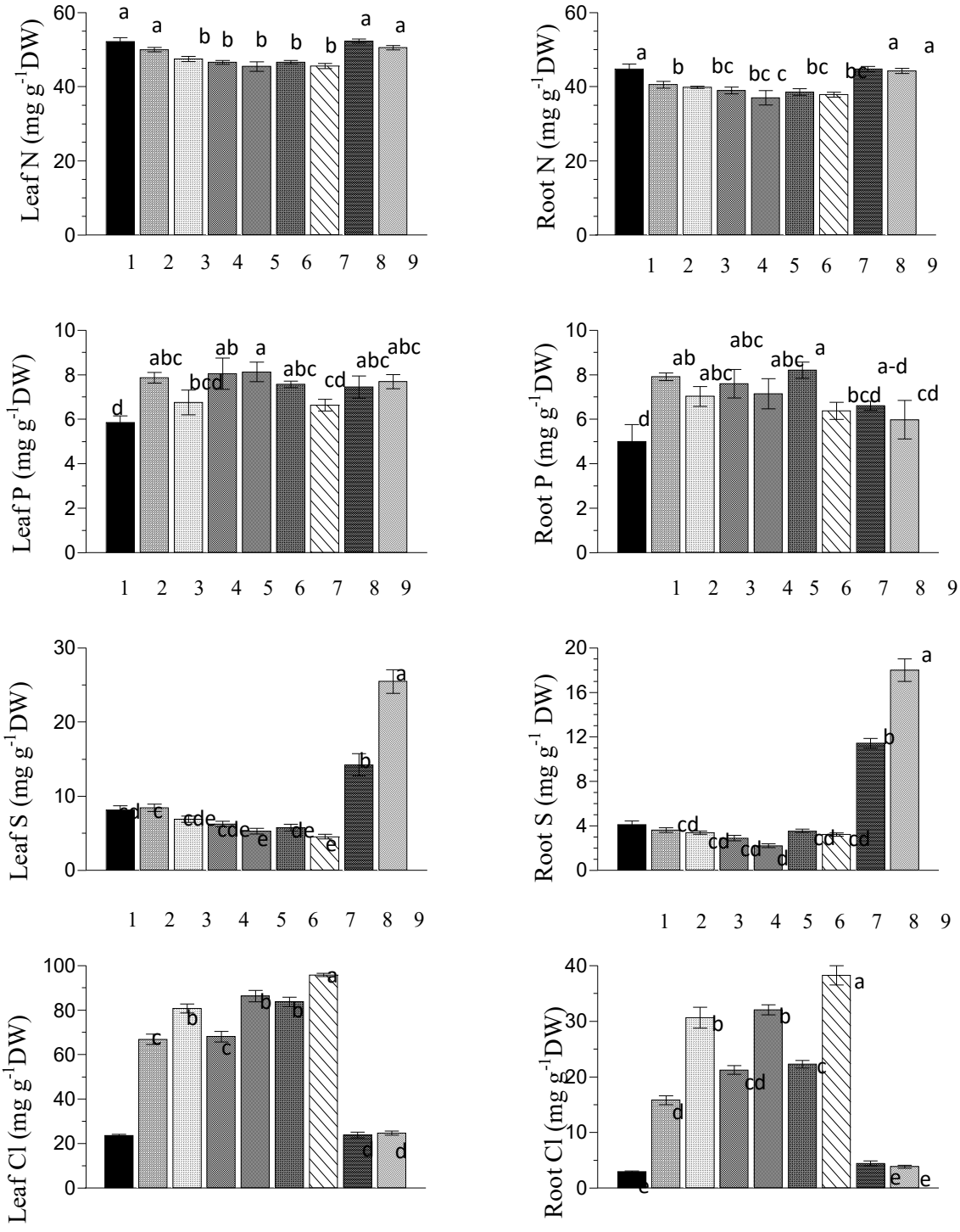



**Figure 4.** Cl in the leaves (lef

*spinosum* plants grown in recirculating nutrient solution, as influenced by eight salinity treatments differing in the osmotic potential level (-0.286 and -0.480 MPa at 20 °C, referred to as low and high salinity level) and the salinity source: 1: Control (standard nutrient solution); 2: low NaCl-salinity; 3: high NaCl-salinity; 4: low KCl-salinity; 5: high KCl-salinity, 6: low $CaCl_2$-salinity; 7: high $CaCl_2$-salinity; 8: low $Na_2SO_4$-salinity; 9: high $Na_2SO_4$-salinity. Vertical bars indicate ± standard errors of means of four measurements. Similar letters indicate non-significant differences at $P \leq 0.05$.

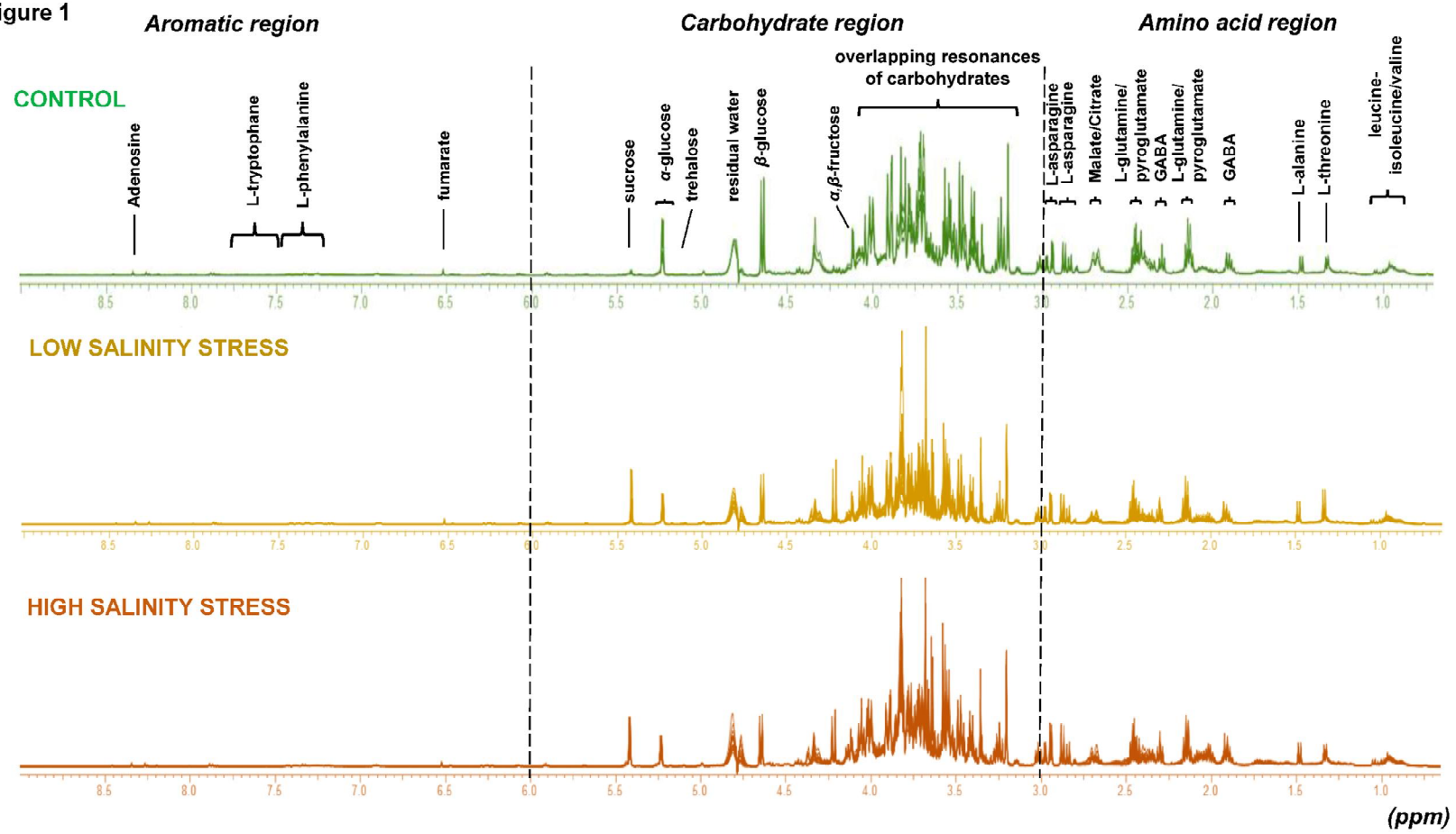
### 4.3.3 Effect of salinity on leaf metabolism of *Cichorium spinosum*

#### 4.3.3.1 Overview of the $^{1}$H NMR metabolomics analyses

$^{1}$H NMR metabolomics revealed the reproducibility and robustness of the applied experimental and bioanalytical protocols, as it is confirmed by the quality of the obtained spectra (**Supplementary Fig. 4.1**), the tight grouping between replications of the various treatments in the OPLS-DA score plots and the absence of outliers (**Fig. 4.4**), and the tight clustering performing HCA (**Fig. 4.5**). The distances between the various points in the OPLS-DA score plots and the cluster distances performing HCA are proportional to the differences between the recorded metabolic profiles of the plants. Additionally, both the level and the sources salinity had a major impact on the observed discriminations as it is indicated by the tight grouping that was achieved performing OPLS-DA and setting either to define classes (**Supplementary Fig. 4.2** and **4.3**).

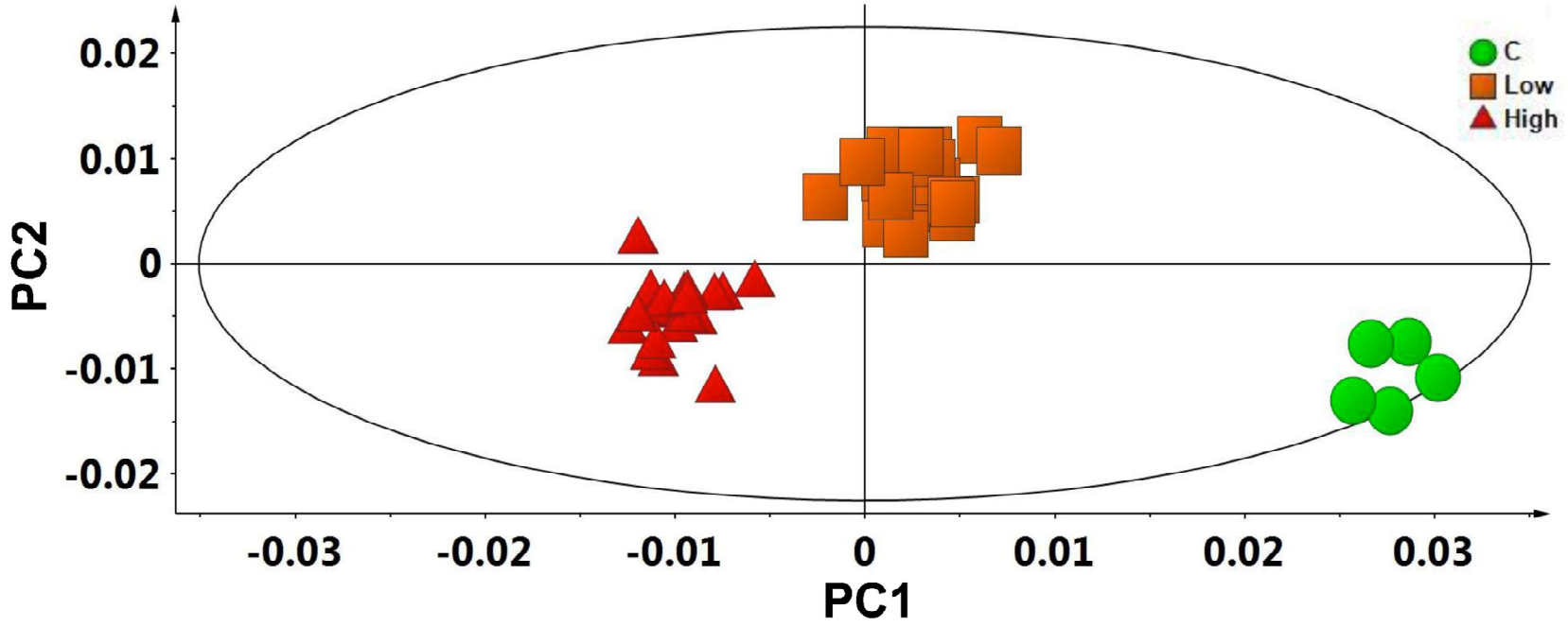
Analyses were performed on the obtained NMR matrix following pre-processing of the data, which is composed of 254 buckets of 0.02 *ppm* width and contains information on the identified metabolites. In total 37 metabolites were identified and annotations of representative identified metabolites are displayed in **Fig. 4.6** and **4.7** and in the **Supplementary Fig. 4.1**.

Suppl Figure 1



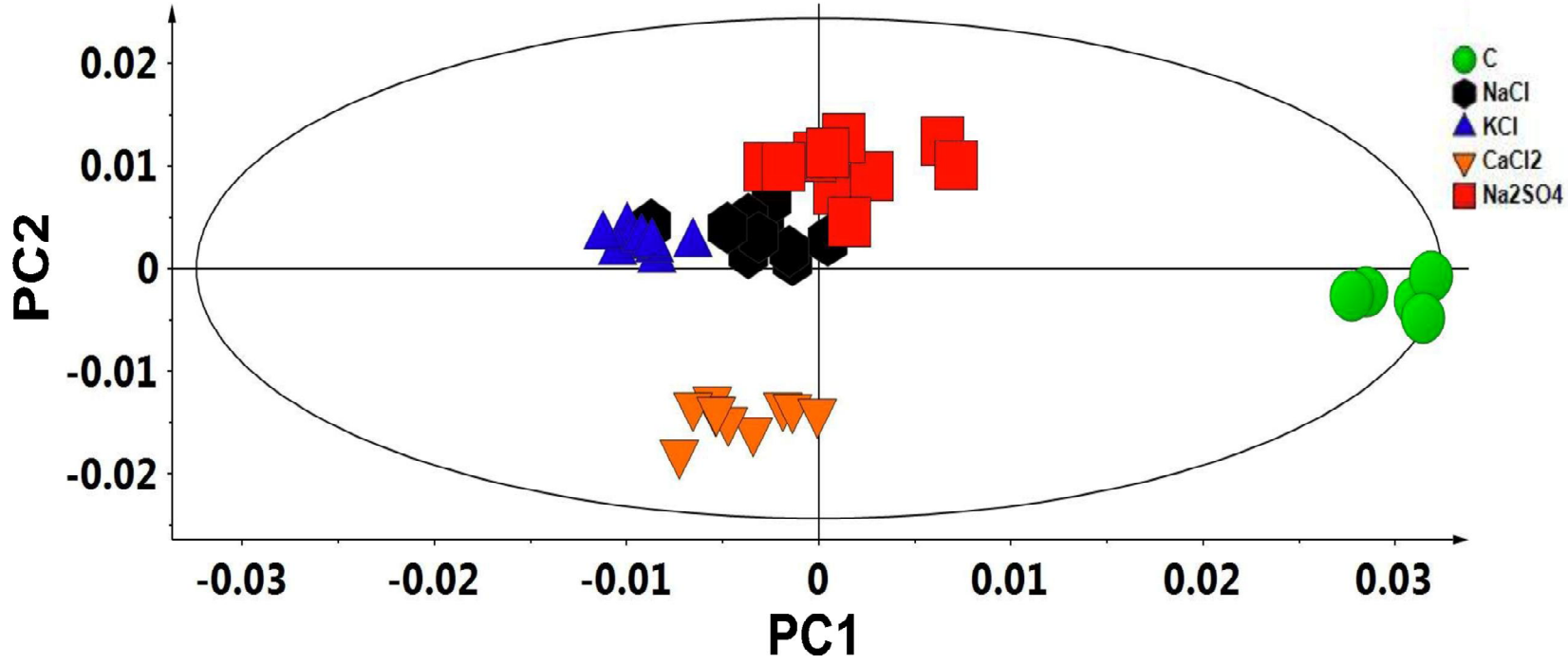

**Supplementary Figure 4.1s.** Representative $^{1}$H NMR spectra of *Cichorium spinosum* (Stamnagathi) leaves after exposure or not to salinity stress. Annotations for selected identified metabolites are displayed.

**Suppl Figure 2**




**Supplementary Figure 4.2s.** Orthogonal partial least squares-discriminant analysis (OPLS-DA) PC1/PC2 score plot of $^{1}$H NMR profiles of stamnagathi plants. Grouping was based on the level of stress. The ellipse represents the Hotelling $T^2$ with 95% confidence interval.

**Suppl Figure 3**




**Supplementary Figure 4.3s.** Orthogonal partial least squares-discriminant analysis (OPLS-DA) PC1/PC2 score plot of $^{1}$H NMR profiles of stamnagathi plants. Grouping was based on treatment irrespective of the level of stress. The ellipse represents the Hotelling $T^2$ with 95% confidence interval.

Figure 4

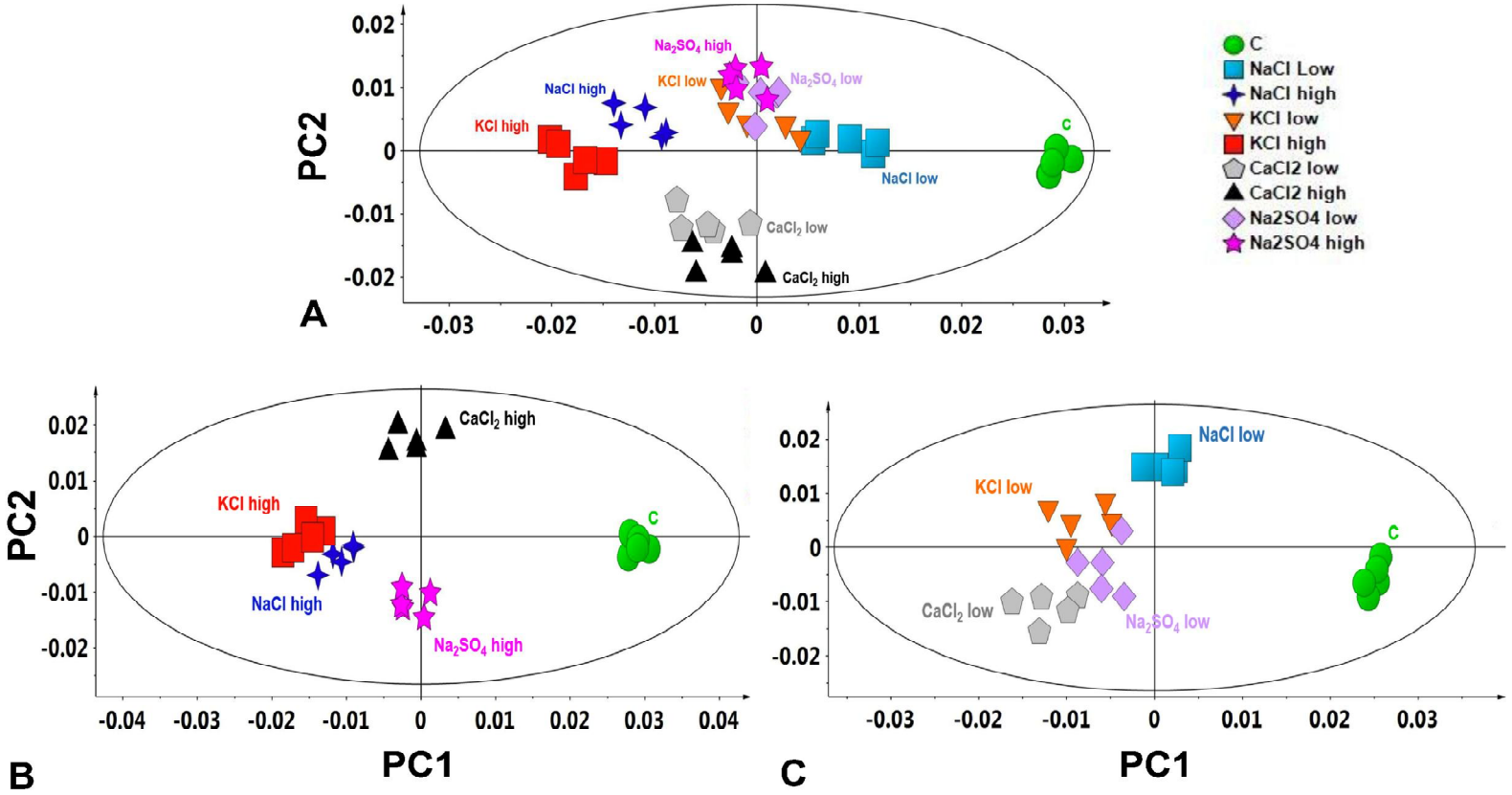



**Figure 4.4.** Orthogonal partial least squares-discriminant analysis (OPLS-DA) PC1/PC2 score plot of $^{1}$H NMR profiles of non-salinized (control) and exposed to low and high levels of salinity stress *Cichorium spinosum* (stamnagathi) plants (**A**), non-salinized (control) and exposed to high levels of salinity stress plants (**B**), and non-salinized (control) and exposed to low levels of salinity stress plants (**C**). The ellipse represents the Hotelling $T^2$ with 95% confidence interval.

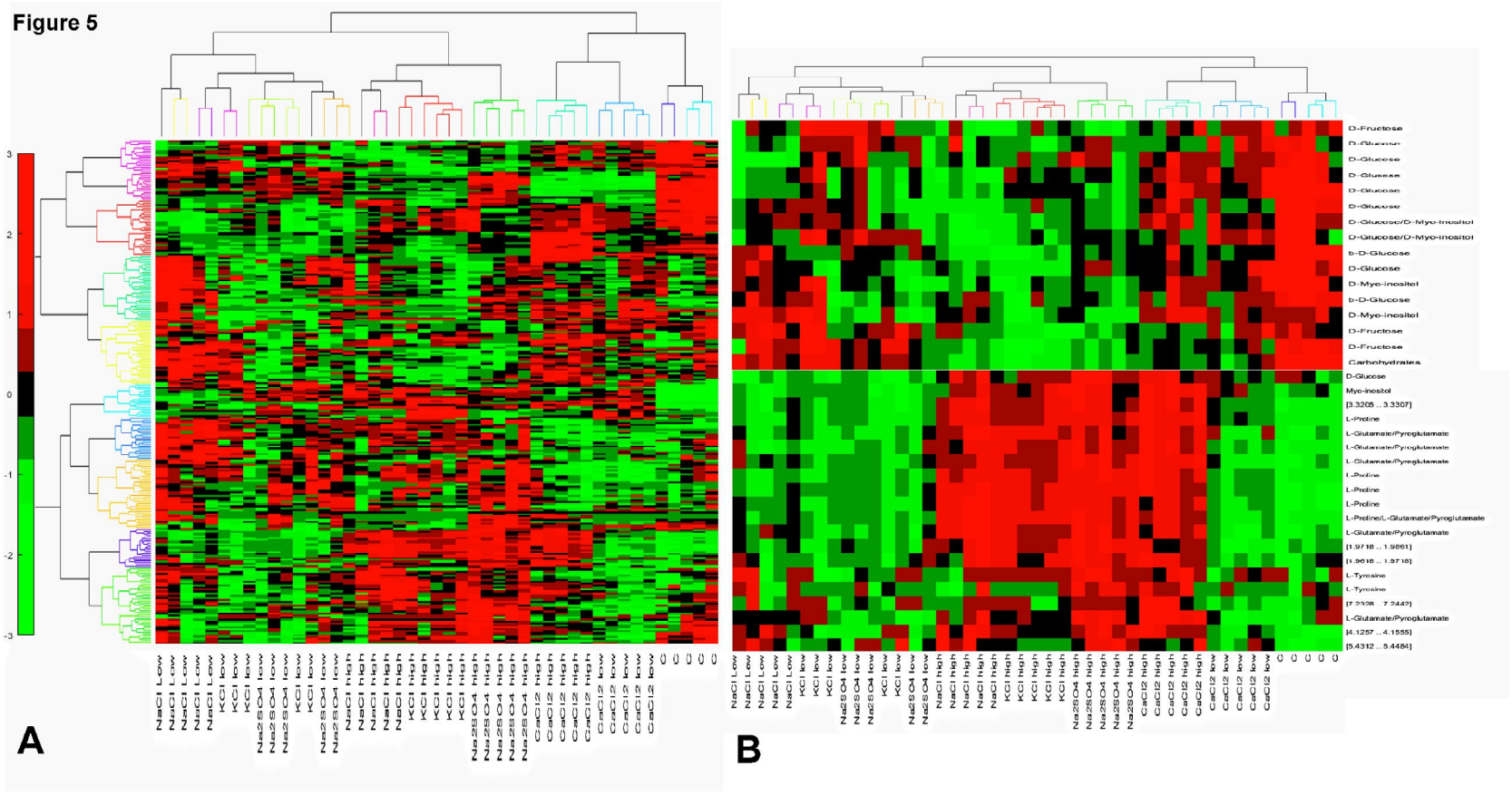



**Figure 4.5.** Cluster heat maps of the recorded $^{1}$H NMR profiles of non-salinized and exposed to low and high levels of salinity stress *Cichorium spinosum* (stamnagathi) plants (**A**) and selected region of the heat map with metabolite annotations (**B**). Two-dimensional (2D) hierarchical cluster analysis (HCA) was performed applying the linkage method of Ward. Rows represent metabolites or metabolic features and columns represent the various treatments being performed. Each cell is colorized based on the relative concentration of the corresponding metabolite in the sample using a color-scale ranging from -3 (light green) indicating low values to 3 (light red) indicating high values.

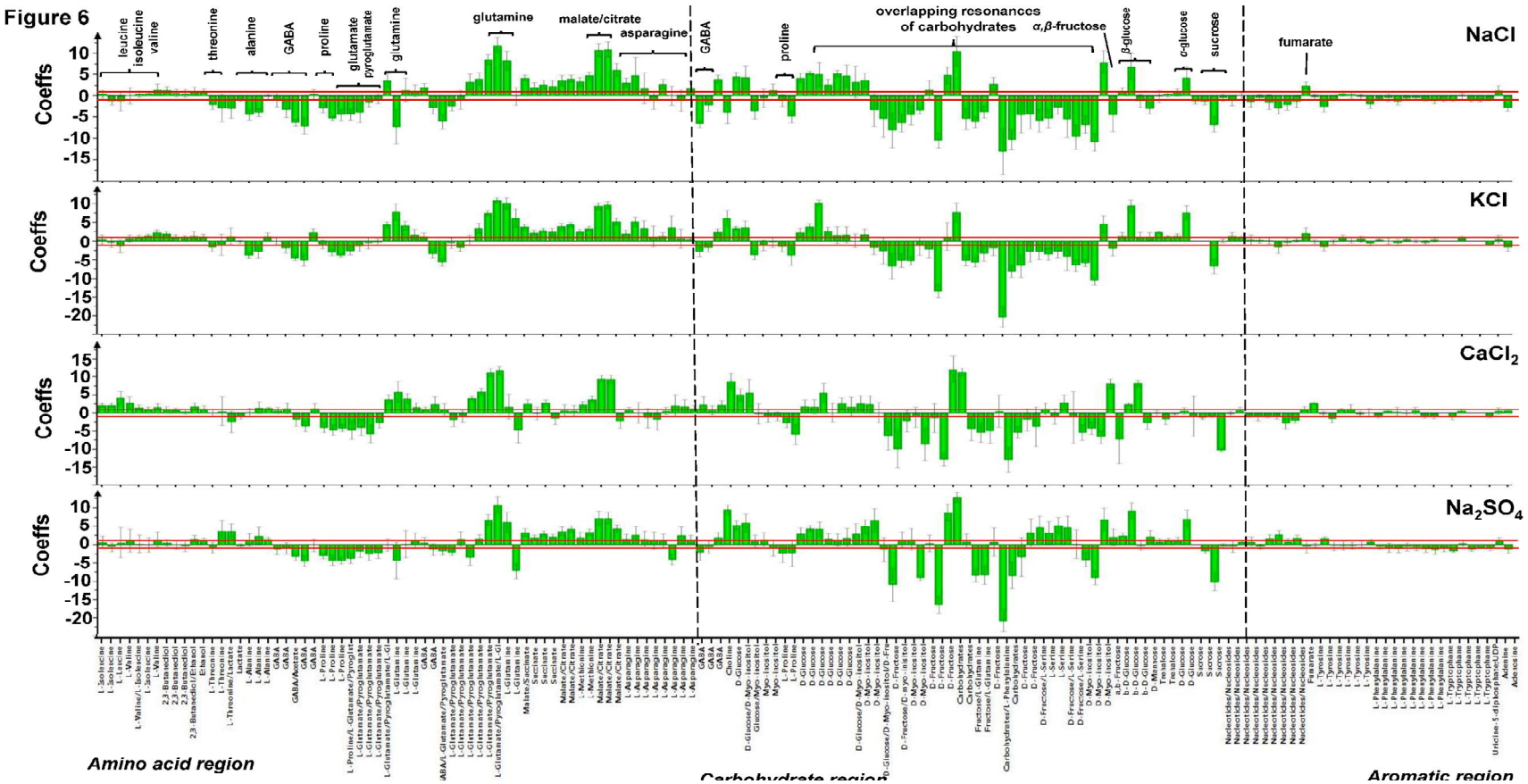



**Figure 4.6.** Orthogonal partial least squares (OPLS) coefficient plots for the $^{1}$H NMR metabolic profiles of *Cichorium spinosum* (stamnagathi) leaves performing pairwise comparisons between control plants and low level-stressed plants treated with the various stressors. Annotations for selected metabolites are being displayed. Influential metabolites for the observed discriminations between the metabolomes of control and salinity-stressed plants are displayed with Jack-knifed confidence intervals ($P$<0.05). Negative values of CoeffCS denote metabolites with higher concentration in stressed plants whereas positive values those with higher concentration in controls. Horizontal lines denote the level of CoeffCS for substantial differences (CoeffCS<-1 and CoeffCS>1).

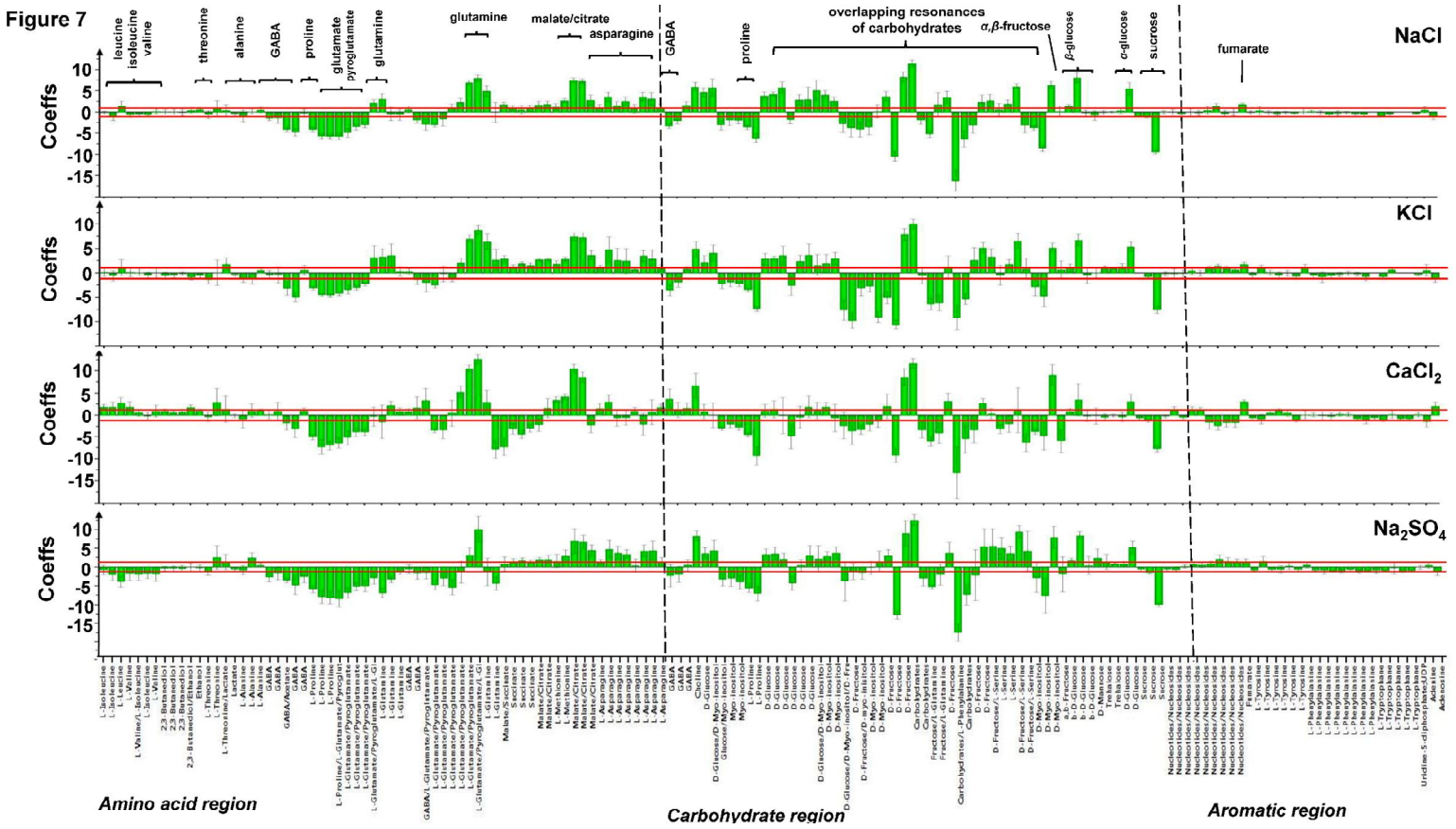



**Figure 4.7.** Orthogonal partial least squares (OPLS) coefficient plots for the $^{1}$H NMR metabolic profiles of *Cichorium spinosum* (stamnagathi) leaves performing pairwise comparisons between control plants and high level-stressed plants treated with the various stressors. Annotations for selected metabolites are being displayed. Influential metabolites for the observed discriminations between the metabolomes of control and salinity-stressed plants are displayed with Jack-knifed confidence intervals ($P$<0.05). Negative values of CoeffCS denote metabolites with higher concentration in stressed plants whereas positive values those with higher concentration in controls. Horizontal lines denote the level of CoeffCS for substantial differences (CoeffCS<-1 and CoeffCS>1).

#### 4.3.3.2 Effect of different salinity sources and levels on leaf metabolism

Both low and high levels of salinity stress caused distinct changes in the plant metabolism (**Fig. 4.4** and **4.5**). These observations agree with the results concerning the effect of salinity stress on plant biomass production and mineral composition and partitioning shown in **Fig. 4.1**, **4.2** and **4.3**, confirming the intimate link of plant growth and mineral composition with plant metabolism.

Although distinct from the non-salinized control, salinity stress caused by $CaCl_2$ and $Na_2SO_4$, resulted in minor changes in plant's metabolic profiles, as indicated by the proximity between their corresponding groups (**Fig. 4.4A** and **4.5**). On the other hand, exposure of plants to low and high levels of KCl- and NaCl-salinity resulted in substantial metabolic changes as indicated by the large distances between their corresponding groups (**Fig. 4.4A** and **4.5**). At the high salinity level, the effect of KCl on plant metabolism follows a similar pattern to that of NaCl, with $CaCl_2$ and $Na_2SO_4$ forming two clearly distinct groups (**Fig. 4.4B**). This grouping is altered when plants were exposed to low salinity stress, with NaCl forming a distinct group (**Fig. 4.4C**).

The fluctuations in the levels of the recorded metabolites or metabolic features that are responsible for the observed grouping and clustering of treatments, and were used for the dissection of the salinity effect on plant metabolism, are displayed in the cluster heat map of **Fig. 4.5** and the coefficient plots in **Fig. 4.6** and **4.7**.

In the cluster heat maps, clustering patterns within the data are observed, which can be used for the interpretation of the obtained phenotypes based on the metabolic changes (**Fig. 4.5**). It is evident that the various treatments cause distinct changes in the recorded metabolite profiles, and patterns can be discovered. For example, in **Fig. 4.5B**, it is evident that the relative concentrations of various carbohydrates [e.g., fructose, glucose ($\alpha$ and$\beta$)], including sugar alcohols [e.g., myo-inositol] were higher in non-salinized plants than in plants subjected to the low level of salinity stress.

The results shown in the heat maps are confirmed by results of multivariate analyses in the coefficient plots (**Fig. 4.6** and **4.7**). In these plots, the fluctuations of metabolites are displayed for the pairwise comparisons between control plants and plants subjected to various salinity stresses. Among the identified metabolites, $\gamma$-aminobutyric acid (GABA), glutamate, pyroglutamate, L-proline, and sucrose were discovered as major biomarkers of the plant response to salinity stress (**Fig. 4.6** and **4.7**). Interestingly, the GABA levels were raised by all salinity sources, at both salinity levels, with the exception of $CaCl_2$-salinity, which reduced its levels. On the other hand, the leaves of

salt-stressed *C. spinosum* plants contained substantially less glutamine, asparagine, fumarate, fructose, and glucose ($\alpha$ and$\beta$). Additionally, the levels of the amino acids L-leucine, L-isoleucine, and L-valine were lower in all salt-stressed plans but not in the plants exposed to $CaCl_2$-salinity, which exhibited higher levels. Trehalose did not play a key-role in plant responses to salinity stress. Finally, based on the recorded metabolite profiles, aromatic compounds had a minor leverage on the observed discriminations, which implies that they do not play a key role in the responses of *C. spinosum* to salinity stress.

## 4.4 DISCUSSION

Stamnagathi (*C. spinosum*) proved to be a highly tolerant plant species to salinity stress, given that at external salt concentrations as high as 53.6 mmol $L^{-1}$ $CaCl_2$ or $Na_2SO_4$, and 80 mmol $L^{-1}$ NaCl or KCl, the shoot fresh and dry weights were reduced by maximum 25% and 20%, respectively. Additionally, at 40 mmol $L^{-1}$ NaCl or KCl neither the fresh nor the dry shoot biomasses were impaired by salinity. These findings corroborate a previous report pointing to a high salt tolerance of stamnagathi when exposed to NaCl salinity (Klados and Tzortzakis, 2014). Our results showed that stamnagathi is tolerant not only to NaCl but also to other sources of salinity, although the tolerance to salts comprising only monovalent ions tends to be higher, particularly at lower salinity levels. The higher tolerance of stamnagathi to monovalent salt ions at low but not at high salinity, as indicated by the biomass data, is in agreement with the substantial metabolic changes observed at low compared to high levels of KCl- and NaCl-salinity, as indicated by the large distances between their corresponding groups. A higher susceptibility of cultivated vegetables to moderate salinity caused by salts containing divalent ions has been also reported by Sonneveld (1988). Nevertheless, at the high salinity level, all sources of salinity reduced similarly the fresh and dry biomass of the shoot, with the exception of $CaCl_2$ which reduced the dry biomass more markedly than the other three salinity sources.

Combined consideration of the plant biomass data indicates that the osmotic potential level is the dominant factor for the impact of salinity on stamnagathi, but the salinity source may also play a role, especially at lower salinity levels. Nevertheless, the dominating impact of the external osmotic potential level on the effects of salinity does not indicate that under saline conditions stamnagathi suffers merely from osmotic stress. Indeed, the plants in all treatments had a normal appearance without any wilting symptoms, which pointed to full osmotic adaptation. It is well-known that, under salinity conditions, the plants with a certain tolerance to salinity can decrease the leaf water potential by synthesizing compatible solutes in the cytosol and compartmentalizing the excess salts to the vacuoles to avoid cell dehydration (Shabala, 2013; Bassil and Blumwald, 2014). Thus, the plants can maintain a sufficiently high water potential gradient between cells and the external solution, despite the salinity-induced decrease in the external water potential (Greenway and Munns, 1983; Mansour and Ali, 2017). In agreement with this consideration, the shoot concentrations of $K^+$,

$Ca^{2+}$, $Na^{+}$, $SO_4^{2-}$ and $Cl^{-}$ increased appreciably in the corresponding salinity treatments in comparison with the control when the concentrations of these ions in the external medium were high. This finding indicates that stamnagathidoes not rely on ion exclusion to combat salinity. As stated by Munns and Gilliham (2015), the vast majority of plants with a moderate to high level of salt tolerance are salt includers characterized by efficient salt compartmentalization into the cells.

Since stamnagathi did not seem to suffer from lack of osmotic adaptation, while the external osmotic potential but not the salinity source proved to be the dominating factor for the observed salinity effects, it is plausible to suggest that the cost of osmotic adaptation was the causal factor for the observed decreases in dry biomass at high salinity. Indeed, the increased accumulation of compatible solutes in the cytosol has an energy-cost for synthesis of such compounds, for maintenance of the synthesizing machinery, and for its operation, as has been shown by several investigators (Parida and Das, 2005; Munns and Gilliham, 2015). Thus, the availability of metabolites for leaf area expansion is being decreased resulting in a gradual restriction of the whole-plant photosynthetic capacity in comparison with non-salinized plants, despite the maintenance of normal net assimilation rates per leaf area unit (Chaves et al., 2009).

Compatible solutes in plants exposed to salinity or drought stress not only contribute to osmotic adjustment in the cytoplasm but also improve the tolerance to osmotic stress by stabilizing membrane lipids, proteins and other cellular structures (Munns and Tester, 2008; Slama et al., 2015). Compatible solutes used as osmoprotectants include mainly amino acids and their derivatives such as proline and glycinebetaine (Chen et al., 2007; Mansour and Ali, 2017), carbohydrates such as sucrose (Juan et al., 2005) and mannitol (Khalid and Cai, 2011; Slama et al., 2015), and organic acids such as citrate, formate, lactate, acetate, succinate, and oxalate (Liu and Shi, 2010; Wang et al., 2011). The analysis of the metabolite profiles of stamnagathi under salinity stress imposed by various salts indicates that the metabolites γ-aminobutyric acid (GABA), glutamate, pyroglutamate, L-proline, and sucrose are key components of the osmoprotection mechanisms in this plant species. The $CaCl_2$ salinity seems to be an exception with respect to GABA, and this may be associated with its lower tolerance to high salinity in comparison with the other three salinity sources in terms of dry biomass production. The increased levels of the amino acids L-leucine, L-isoleucine, and L-valine in the $CaCl_2$-treated plants in comparison with the controls was presumably associated with impairments in their metabolism and not with an adaptation aiming at increased salt

tolerance. This result demonstrates that calcium accelerates the conversion of soluble proteins to amino acids and the conversion of glutamic acid to GABA or other amino acids (Gao et al., 2011).

In contrast to the levels of glutamate and proline, those of glutamine and asparagine were reduced by salinity regardless of the salt source, indicating that these amino acids do not function as osmoprotectants in stamnagathi. Furthermore, the reduced levels of fumarate and the lack of any impact of salinity on the levels of organic acids indicates that osmotic adjustment in response to salinity is not based on enhanced biosynthesis of organic acids in stamnagathi. According to Sanchez et al. (2007), the reduced content of organic acids under salt stress may be due to increased contribution of inorganic cations to ionic balance in the vacuoles.

In agreement with the results of the present study, Wu et al., (2013) found that the relative concentration of sucrose was enhanced in roots of barley, while those of fructose-6-P, glucose-6-P and 3-PGA decreased. The most important compatible solutes in barley were proline, sugars (sucrose, raffinose and trehalose), mannitol and inositol in roots, and raffinose, proline and some amino acids in leaves. The reduced levels of glucose and fructose in salt-stressed stamnagathi plants may be associated with the increased sucrose level, since these two hexoses are components of the latter. Thus, salinity may stimulate the utilization of glucose and fructose for sucrose biosynthesis aiming at osmotic adaptation in the cytoplasm, thereby lowering the levels of these two reducing sugars in the leaves. In a study of Fernandes et al. (2004), the sucrose content was almost three times higher in plants treated with 150 mM NaCl, while the glucose content decreased with salt stress, and the fructose content did not change significantly. These results indicate that the role of sucrose, glucose, and fructose to osmotic adaptation under salt stress conditions may exhibit some peculiarities in different plant species, although the increased sucrose levels seem to be a common response (Sanchez et al., 2007).

In plants, GABA metabolism has different functions including osmotic and pH regulation (Kinnersley and Turano, 2000), nitrogen metabolism (Barbosa et al., 2010), and salt stress tolerance (Renault et al., 2010; Akçay et al., 2012). According to Xiang et al. (2015), exogenous GABA accelerated the ROS metabolism in chloroplasts, promoted the recycling of AsA-GSH and maintained the permeability of cell membranes thereby improving the defense efficiency of melon chloroplasts against salinity-alkalinity stress. Xiang et al. (2016) found that exogenous GABA alleviated

stress-related damage on the acceptor side of PSII in muskmelon seedlings exposed to salinity-alkalinity stress. Moreover, exogenous GABA supply altered the gene expression in roots under NaCl stress and the activation of multiple mechanisms involved in ROS production, regulation of protein degradation, hormone biosynthesis and PA metabolism (Shi et al., 2010).

Reduced translocation of $K^+$, $Ca^{2+}$ and/or $Mg^{2+}$ to leaves under salt stress conditions has been also reported as one of the causal factors of salinity-induced growth restriction (Yao et al., 2010; Shoresh et al., 2011; Wang et al., 2013). In the present study, significant reductions in leaf $K^+$ concentrations were recorded only in plants exposed to high NaCl-salinity, and to $Na_2SO_4$-salinity at both salinity levels. However, the fresh and dry biomass in plants subjected to these salt treatments were not significantly lower than in those exposed to isosmotic levels of KCl- and $CaCl_2$-salinity. Similarly, the leaf $Ca^{2+}$ and $Mg^{2+}$ levels were reduced only by $Na_2SO_4$-salinity but this salt did not result in a stronger reduction of shoot biomass than the other three salts. These results indicate that the adverse effects of salinity on stamnagathi were not due to antagonistic impairment of nutrient cation uptake.

The decreased leaf $K^+$ concentrations in plants exposed to NaCl- and $Na_2SO_4$- salinity, but not in those exposed to $CaCl_2$- and KCl-salinity is associated with partial substitution of $Na^+$ for $K^+$, a response observed in tolerant plant species to salinity, such as sugar beet (Lessani and Marschner, 1978; Marschner, 2012). The reduction of the leaf $Ca^{2+}$ and $Mg^{2+}$ concentrations by $Na_2SO_4$-salinity but not by the other three salts tested in this study suggests that their uptake is affected not only by $Na^+$ but also by an interaction of $Na^+$ with the accompanying anion. According to Pagter et al. (2009), reduced tissue concentrations of $Ca^{2+}$ and $Mg^{2+}$ in plants exposed to Na-salinity may be due either to interference with their uptake by $Na^+$, or due to reduction of their activity in the external solution caused by changes in ionic strength, ion-pair formation and precipitation. However, the effects of $Na^+$ on $Ca^{2+}$ and $Mg^{2+}$ uptake may be offset when the accompanying cation is $Cl^-$, as indicated by previous studies which showed that high external $Cl^-$ concentrations improve $Ca^{2+}$ uptake (Nukaya et al., 1991; Voogt and Sonneveld, 2004). In agreement with the results of the present study, Reich et al., (2017) found a stronger decrease of $Ca^{2+}$ and $Mg^{2+}$ levels in *Brassica rapa* plants exposed to $Na_2SO_4$ salinity than in those exposed to NaCl-salinity.

The increased P concentration in the leaves and roots of stamnagathi exposed to salinity agrees with a report of Gunes et al. (2007). On the other hand, Reich et al.

(2017) found decreased phosphorus concentrations in salt-stressed *Brassica rapa* plants exposed to NaCl-, KCl-, $Na_2SO_4$, and $K_2SO_4$-salinity. Phosphorus is involved in energy transfer in plant metabolism (Marschner, 2012) and this contrast in the response of plant P to salinity between *C. spinosum* and *B. rapa* may point to deployment of different salt tolerance mechanisms by these two species. Further studies are needed to elucidate these mechanisms and the involvement of P in salt tolerance. The slight decreases of shoot total-N in some salinity treatments does not seem to correlate with the salinity-induced biomass reduction. Thus, it can be concluded that the growth reduction imposed by salinity was not a result of shortages in N and P uptake. Finally, the growth restriction imposed by salinity to stamnagathi does not seem to be related with specific $Na^+$ or $Cl^-$ toxicity. Indeed, the restrictions in dry biomass accumulation were not associated with the presence of one of these ions to the salt used to impose salinity.

## 4.5 CONCLUSIONS

This study was commissioned to investigate whether salt effects on the salinity-tolerant *C. spinosum* are predominantly imposed by the level of the external osmotic potential or by specific toxicities of salt ions. To monitor fluctuations in plant metabolism caused by different salinity levels and sources, $^1$H NMR metabolomics was applied which was successful in monitoring the undergoing metabolic changes. The obtained results indicate that the osmotic potential level is the dominant factor for the impact of salinity on *C. spinosum*, but the salinity source may also play a role. Although differences in dry biomass accumulation due to the salt species were observed only between $CaCl_2$ and the other three salinity sources at the highest salinity level, the recorded metabolic profiles following salt stress exhibited distinct differences depending on both the level and source of salinity. Glutamate, pyroglutamate, L-proline, $\gamma$-aminobutyric acid (GABA), and sucrose were signatory metabolites enhanced by salinity stress in *C. spinosum*, which implies that they may be involved in intracellular osmoprotection mechanisms. Reductions in fresh and/or dry plant biomass did not seem to be related to salt-induced changes in tissue mineral levels.

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# *CHAPTER 5*

# *General conclusions*

The configuration of the two leafy vegetables: lettuce (*Lactuca sativa* L.) and spiny chicory (*Cichorium spinosum* L.) was analyzed in a multi-factorial approach accounting for the effects of cultivars, salinity sources and number of cut. Soilless culture in particular floating raft culture appears to be a promising tool to obtain valuable baby lettuce and spiny chicory production as well as improving quality aspects of leaf through proper management of salts in the nutrient solution.

Under increasing level of NaCl in the nutrient solution the yield and growth of baby lettuce decreased with more detrimental effects on the green variety. However, the overall quality of baby lettuce increased at 20 mM NaCl whereas a significant decrease of the nutritional value was recorded at 30 mM NaCl. The $CaCl_2$ treatment adopted in the second experiment was able to increase the nutraceutical properties of baby lettuce in particular the mineral and phenolic profile along with the anitoxidant capacity without a significant decrease crop productivity. The second cut inccured a significant increase in total phenols, vitamin C and anitoxidant activities.

The results of the third experiment indicate that the osmotic potential level is the dominant factor for the impact of salinity on *C. spinosum*, but the salinity source may also play a role. Although differences in dry biomass accumulation due to the salt species were observed only between $CaCl_2$ and the other three salinity sources at the highest salinity level, the recorded metabolic profiles following salt stress exhibited distinct differences depending on both the level and source of salinity. Glutamate, pyroglutamate, L-proline, $\gamma$-aminobutyric acid (GABA), and sucrose were signatory metabolites enhanced by salinity stress in *C. spinosum*, which implies that they may be involved in intracellular osmoprotection mechanisms.

For a pratical point of view, the results obtained in the present PhD thesis can help the growers in the crop management of these potential leafy vegetables; they can help the consumer in the knowledge of the overall quality of leafy vegetables under differeent preharvest factors; and they are a good basement for other researches. This can be helpful to understand some unknown mechanisms that lead to the biosynthesis of phytochemical compounds in these two target leafy vegetables.