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MECHANISMS OF ACTION OF BIOSTIMULANTS IN CROPS

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SUMMARY

ABSTRACT

Abiotic stresses can affect plant growth and lead to great losses in yield and quality. Among them water availability is a current issue and intensive agriculture has now reached a critical point in the negative effects on natural resources. Hence, the main challenge is to achieve an "ecological intensification" of agriculture minimizing negative externalities. To cope with this situation, in recent years, a notable diffusion of biostimulant products has been observed. Biostimulants are complex mixtures of compounds and substances able to promote plants growth, improving stress tolerance, quality, and yield. However, this variability in composition creates difficulties in the comprehension of the mode of action and the effectiveness of these products.

The purpose of this Ph.D. research project is to study the effect of different biostimulants prototypes on leafy vegetables subjected to abiotic stresses. The attention is focused on physiological and molecular changes induced by the stress and on the potential biostimulant effect of treatments. Qualitative and physiological parameters such as photosynthetic pigments, polyphenols, chlorophyll *a* fluorescence, transcription factors, and genes involved in plant stress responses have been analysed.

The first activity regarded the preparation and the evaluation of aqueous extracts obtained from leaves and flowers of *Borago officinalis* L. grown in a greenhouse. The extracts were prepared with different times of maceration and were applied on rocket and lettuce crops as foliar spray two times during the growing cycles. Results showed that the maceration time affected the efficacy of the products and different trends emerged from their applications on the two species.

Moreover, about twenty transcription factors (TFs) from the *NAC, MYB, bZIP* and other families have been chosen as potential stress markers and primers for qRT-PCR analysis have been designed. A borage extract was tested to evaluate the transcriptional changes induced in rocket grown under salt stress. The physiological responses linked to the primary and secondary metabolism of the leaves subjected to high salinity were monitored by measuring the changes in chlorophyll content, carotenoids, anthocyanins, lipid peroxidation, and in chlorophyll *a* fluorescence-related indices. At molecular level, the stress responses were studied by measuring the changes in the expression of the selected TFs within 24 hours after the beginning of the stress application. Results obtained showed that the treatment affects the gene expression in different ways. *DtRd29a*, a stress-responsive gene, was generally more expressed in stressed plants treated with borage extract. In general, salt stress induced the expression of all the TFs examined. Results obtained have allowed to point out the complex plant response to a sudden exposition to high level of salinity, to the treatment with borage extract and to the interaction between these two factors. Moreover, it has been possible to get information on different gene expression patterns during time. Some of these transcription factors were involved in the regulation of several pathways including sugars metabolism (*DtbZIP63*), cuticular wax

biosynthesis (*DtMYB30*), brassinosteroids signaling (*DtMYB30, DtbHLH122, DtBEE2, DtHBI1, DtIBH1, DtWRKY54, DtNAC72*) and intercellular transport (*DtRABC2B*).

At the same time a collaboration with a private company was carried out with the aim to evaluate the effectiveness of a biostimulant prototype against water and salt stress conditions. In order to better understand the mode of action, the experimental plan included the study of the plant responses to the application of the prototype alone, in combination with proline and glutamic acid solutions. Treatments were applied on lettuce plants (*Lactuca sativa* var. acephala 'Chiara') subjected to a period of water deprivation of 30%.

Results obtained showed that the addition of proline to the formulation did not affect the efficacy of the product in a significant way. Some interesting results were obtained after the application of glutamic acid. An increase of chlorophyll and carotenoids content and a higher water use efficiency was observed in plants grown with a lower water availability. Based on the above-mentioned results, the prototype formulation was slightly changed and the new product was tested on romaine lettuce (*Lactuca sativa* var. longifolia) grown under water stress and salt stress. Physiological and biochemical traits of plant responses to stressful environments and biostimulant treatment were investigated. Moreover, the study of the plant response at molecular level, focusing in particular on the genes involved in oxidative stress and antioxidant defence was performed. The expression of catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDAR), and glutathione reductase (GR) was examined. Generally, the results obtained showed that stress conditions had a more significant effect than treatments and the expression levels of selected genes significantly decreased in response to stressful conditions.

The last part of the research project was carried out at Cardiff University aiming to evaluate the effect of a treatment with glutamic acid on the aroma of rocket salad subjected to a period of salt stress. Volatile organic compound (VOC) profiles have been assessed using a thermal desorption gas chromatography time-of-flight mass spectrometry (TD-GC–TOF-MS). Data were processed using MSD ChemStation software deconvoluted and integrated with AMDIS (NIST14) using a retention-indexed mass spectral library. The identification of each peak has been performed comparing the mass spectrum against a rocket library and a NIST database. PerMANOVA analysis indicated that the aroma of rocket was significantly affected by salt stress. About 190 compounds were identified from all the rocket salad samples. 3-Hexen-1-ol, acetate, (E)-, 3-Hexen-1-ol, (Z)-, 1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester, Butanoic acid, 3-hexenyl ester, (Z)-, "1-Penten-3-ol, Dimethyl sulfone, Sulfur dioxide, Acetic acid, hexyl ester, 3-Pentanone and an undefined branched alkane were the most abundant compounds found in control plants.

INTRODUCTION

Biostimulants Application in Horticultural Crops under Abiotic Stress Conditions

1. Abiotic Stresses

Plants are continuously subjected to a multitude of stressful events, from seed germination through to the whole life cycle. These stresses are commonly divided into two categories - biotic and abiotic stresses - depending on the nature of the trigger factor. The former are caused by other living organisms including insects, bacteria, fungi, and weeds that affect plant development and productivity. The latter are generally linked to the climatic, edaphic, and physiographic components of the environment, when they are limiting factors of plant growth and survival. The most important abiotic stresses limiting agricultural productivity almost all over the world are: drought, salinity, non-optimal temperatures, and low soil fertility. Among these, drought, and nutrient deficiencies are the major problems mostly in developing countries where the incomes of rural people depend on agriculture [1]. Actually, in "The State of Food and Agriculture 2007", FAO reported that only 3.5% of the global land area is not affected by some environmental constraints. In 1982, Boyer estimated that yield losses caused by unfavourable environments were as much as 70% [2,3]. Farooq et al. [4] reported that drought induced a reduction of yield between 13% and 94% in several crops, depending on the intensity and duration of the stress. Afterwards, Cramer et al. [5] estimated the impacts of different abiotic stresses on crop production in terms of the percentage of global land area affected, considering the 2000 and 2007 FAO reports. They also referred to the increasing number of publications focused on this topic between 2001 and 2011. The exact impact of these changes on agricultural systems is extremely difficult to predict and it depends on numerous parameters that are not always included in predictive models. Even if some projections show that positive and negative outcomes on crop production could be balanced in the medium term, several studies agree that in long term, the negative ones will prevail [6,7]. Based on future scenarios, adaptation and mitigation strategies are essential to increase the resilience capacity of agricultural systems and to ensure crops yield and quality. Since environmental conditions cannot be controlled, several strategies on different levels are required, such as agronomical techniques or breeding for more tolerant cultivars [8].

In 2010, at the society's annual conference, Vegetable Breeding and Stress Physiology working groups of the American Society for Horticultural Sciences focused particularly on the "Improvement of Horticultural Crops for Abiotic Stress Tolerance" considering the effects of climate change [9]. Up to now, most of the studies on climate change impacts are focused on the major crops, and only few papers pay attention to fruit and vegetable in terms of production, quality, and supply chain [10,11]. An important aspect to take into consideration is the

effect of the combination of different stressful factors. Most of the time, crops are subjected to several abiotic stresses that occur simultaneously. In these situations, the study of each stress separately is not enough because plant response is unique and cannot be predicted by the reply obtained when each factor is applied individually [12–14]. Moreover, biotic and abiotic components typically interact in an ecosystem. For instance, environmental conditions affect plant-pest interaction in different ways, by decreasing plant tolerance or increasing the risk of pathogen infection [15,16].

Focusing on horticultural species, the tolerance to abiotic stresses is an important trait because their cash value is usually higher than field crops, they require more resources for farming and because they provide a source of many nutrients, fibre, minerals, and carbohydrates which are essential in a healthy diet [17]. Food and Agriculture Organization (FAO) reports that about 90% of essential vitamin C and 60% of vitamin A for human comes from vegetables. Indeed, low fruit and vegetable intake is a major contributing risk factor to several widespread and debilitating nutritional diseases. According to the Global Burden of Disease Study 3.4 million deaths can be attributed to low consumption of fruit and 1.8 million to low vegetables diets worldwide [18]. Therefore, growing high-quality vegetables becomes one of the most important goals of current agriculture, in order to meet the needs of the population and the increasing demand for fruit and vegetables. Abiotic stresses do not only affect the yield but also the quality of these products, triggering morphological, physiological and biochemical changes that can alter the visual appearance and/or the nutraceutical value in a way that the product could become unmarketable [19]. Bisbis et al. [11] investigated the double effect of elevated temperature and increased CO_2 on the physiology of different vegetables. They observed several responses according to the plant species and the severity of the stress, taking into consideration the possible adaptation strategies that could be implemented in order to mitigate the effects of climate change. Nonetheless these mechanisms are still under research and they should be studied in depth, because not only different species but also different cultivars could respond differently to the same environmental stress. For example, cultivars with low levels of antioxidants are particularly vulnerable to oxidative stress compared to those with high antioxidant activity [20–23]. This aspect has a particular importance as selection criterion in the choice of appropriate cultivars for a specific situation. Oxidative stress is a common phenomenon caused by several adverse conditions; it generally occurs when the balance between the production of reactive oxygen species (ROS) and the quenching activity is upset by a stressful event [24]. Low levels of ROS are normally produced by different reactions during physiological metabolisms like the photosynthesis or respiration, and they play an important signaling role in plant growth and development. Their amount dramatically increases under abiotic stress conditions and, if not controlled could results in cellular damage and death. Besides their toxicity to proteins, lipids or nucleic acids, the increased production of ROS under stressful conditions plays a key role in the complex signaling network of plants stress responses. Their concentration is maintained at non-toxic levels by the activity

of the antioxidant system: a wide range of enzymatic or non-enzymatic antioxidant molecules are accumulated in plant tissues to quench ROS induced by stress [25–28]. Moreover, the maintenance of this equilibrium is also dependent on numerous factors, such as the timing of stress application, its intensity and duration. Indeed, moderate or controlled stress conditions could have a positive effect on quality traits of several crops [29]. For example, water deprivation might be a useful crop management strategy to improve the quality of lettuce and fleshy fruits in terms of nutritive and health-promoting value and taste, by stimulating the secondary metabolism and the concentration of different phytochemicals such as α-tocopherol, β-carotene, flavonoids and so on [30,31]. Besides the production of ROS scavenging compounds, the biosynthesis and accumulation of compatible solutes with an osmoprotective role, like sugars and proline increase.

Plants generally reply to non-optimal environmental conditions both with short- and long-term adaptation strategies, by the activation and regulation of the expression of specific stress associated genes [32,33].

Since plants are sessile organisms and they have to cope with adverse external conditions all these mechanisms are essential for their survival. These strategies are effective if they are activated in time, in order to set a defence response and anticipate the environmental changes that might irreversibly affect the plant growth. The trade-off between growth and acclimation metabolisms results in a sort of fitness cost for plants since the energy and nutrients normally destined to growth and production are intended for stress responsive mechanisms [34].

Agronomic management conducted in order to enhance plant tolerance towards abiotic stresses evolved over the centuries due to the technologic progress, the climate change, the scientific knowledge, and the farmers experiences. The choice of the correct cultivar, the best growing period, the sowing density, and the amount of water or fertilizers are some of the most common strategies applied to mitigate the negative effects of abiotic stresses [8]. Protected cultivation is a cropping technique adopted to preserve plants from unfavourable outdoor conditions. It is mainly suited to vegetables and floriculture production in a non-optimal environment, through the control of temperatures, radiation or atmospheric composition. Another agronomical strategy, especially applied in vegetable crops, is soilless cultivation. This approach allows the control of water and nutrients, avoiding the use of soil for cultivation and all the problems related to it, like poor quality or soil contamination.

Grafting is an additional tool adopted to counteract environmental stresses and to increase tolerance in vegetable crops. This technique is especially applied to high-yielding fruits and vegetables such as cucurbits and solanaceous to enhance tolerance against saline soil, nutrient or water deficiency, heavy metals or pollutants toxicity [35–37].

Agronomical strategies are essential in mitigating the negative effects of abiotic stresses but sometimes their application is not enough. Moreover, current experiments aim to transfer one or more genes involved in signaling or regulatory pathways, or genes encoding to molecules, such as osmolytes and antioxidants,

conferring tolerance to a specific abiotic stress [38]. Several functional and regulatory genes involved in abiotic stress tolerance have been identified and studied. The results of these studies can be exploited for genetic improvement aiming to introduce tolerance traits in cultivated crops. Since different physiological traits related to stress tolerance are under multigenic control, the manipulation of a single gene generally is not enough. Hence, scientists have paid more attention to regulatory genes including transcription factors due to their ability to regulate a vast array of downstream stress-responsive genes at a time [39–41].

However, the huge existing genetic variability among vegetable species, the lack of knowledge about minor cultivars genome, the complex responses triggered by abiotic stress conditions and the limited strategies currently available make genetic improvement really difficult and often inefficient. Moreover, besides the wide diversity of germplasm available, plant tolerance to stress depends both on the stress features such as duration, severity, and frequency, as well as the affected tissues and the crops development stages [24,42–44].

Additionally, the increase of crops tolerance through genetic improvements requires many years of work and different cultivation environments that cannot be always taken into consideration. As a result, several new cultivars that can be used by the growers are released each year.

Another technique widely used for developing stress tolerance in plants is in vitro selection. This culture-based tool allows a better understanding of several plants' physiological and biochemical responses to adverse environmental conditions. This technique has been applied specially to obtain salt/ and drought/tolerant lines in a wide range of plant species, including vegetables [45]. In vitro selection is based on the induction of a genetic variation among cells, tissues or organs, their exposure to a stressor, and the subsequent regeneration of the whole organism starting from the surviving cells [46]. Even if in vitro selection is a less expensive and time saving approach compared with classic molecular engineering, some limitations, mostly concerning the stability of the selected traits and epigenetic adaptation, still exist.

In addition to these strategies, it has been observed that stress tolerance can also be induced by biostimulants or specific bioactive compounds, if they are applied on vegetable crops when they really need to be protected [47–49]. Biostimulants application on horticultural crops under environmental stress conditions will be discussed in detail below.

2. Biostimulants

Biostimulant products have been considered innovative agronomic tools as demonstrated by the increase of scientific publications and by the constant expansion of their market [46]. France, Italy, and Spain are the leading EU countries in the production of biostimulants [47]. According to a new report by Grand View Research, Inc., the biostimulants market size is expected to reach USD 4.14 billion by 2025 [48]. The complex nature of the

composition of these products and the wide range of molecules contained make it complicated to understand and define which compounds are the most active. The isolation and study of a single component is almost impossible and the efficacy of a biostimulant is not due to a single compound but is the consequence of the synergistic action of different bioactive molecules. Moreover, the application rules and timing are not always clear. For all these reasons, the European Commission developed a proposal for a new regulatory framework and a draft for a new fertilizer regulation was prepared in 2016. The amendments to the proposal of the European Commission were adopted by the European Parliament in October 2017, while the legislative resolution on the proposal was approved on 27 March 2019 [49-51].

Plant biostimulants are defined as products obtained from different organic or inorganic substances and/or microorganisms, that are able to improve plant growth, productivity and alleviate the negative effects of abiotic stresses [52,53]. Mineral elements, vitamins, amino acids, and poly- and oligosaccharides, trace of natural plant hormones are the most known components. However, it is important to underline that the biostimulant activity must not depend on the product's nutrients or natural plant hormones content. The mechanisms activated by biostimulants are often difficult to identify and they are still under investigation [54]. High-throughput phenotyping and omic technologies seem to be useful approaches to understand biostimulants activity and hypothesize a mode of action [55–57]. They can act directly on plant physiology and metabolism by improving soil conditions [58,59]. They are able to modify some molecular processes that allow to improve water and nutrient use efficiency of crops, to stimulate plant development, and to counteract abiotic stresses [47] by enhancing primary and secondary metabolism [51,57,59].

One of the key points of the discussion is about the application of these products in stressful condition and their role as nutrients, not with a curative function. In particular, if a product has a direct effect against biotic stresses, it should not be included in the biostimulant category but should be registered as plant protection products.

2.1. Classification of Biostimulants in Categories

During the years, different authors proposed several categorizations of biostimulant products on the basis of their main component or mode of action. In many countries outside the European Union both information must be reported on the label in order to register these products [51]. The current classification is based on the source of the raw material, even if this choice does not always provide the correct information about the biological activity of the product [52]. Thus, biostimulants are classified as these major groups:

Humic substances (*HSs*): they include humic acids, fulvic acids and humins. *HSs* are natural constituents of soil organic matter, resulting from the decomposition processes of plants, animals, and microbial residues,

but also from the metabolic activity of soil microbes [53]. It has been observed that treatments with humic substances stimulate plants roots growth and development [64,65]. This is reflected in a better uptake of nutrients and water, and in an enhanced tolerance to environmental stresses [66,67]. How the *HSs* affect plant physiology is not fully understood. This is due to the molecular complexity of these substances and to the abundance and diversity of plants responses altered by their application. Moreover, a strong relationship between medium properties and *HSs* bioactivity has been reported [68]. The positive effects exerted by these complex aggregates could be ascribed both to the hormone-like activity of some of their components and also to IAA independent mechanisms [69]. For example, like auxins, *HSs* are able to promote plant growth and induce H^+ATPase activity in plasma membrane [70–72].

Seaweed extracts: seaweeds are a vast group of macroscopic, multicellular marine algae that can be brown, red, and green. They are an important source of organic matter and fertilizer nutrients. Seaweed extracts have been used in agriculture as soil conditioners or as plant stimulators. They are applied as foliar spray and they are able to enhance plant growth, abiotic stresses tolerance, photosynthetic activity, and resistance to fungi, bacteria and virus, improving yield and productivity of several crops [73–75]. Seaweeds used for biostimulants production contain cytokinins and auxins or other hormone-like substances [76]. They also contain many active mineral and organic compounds, including complex polysaccharides such as laminarin, fucoidan, alginates and plant hormones that contribute to plant growth [77]. Recently the potential application of micro-algae as plant biostimulants has been considered too [78–80].

Hydrolysed proteins and amino acids containing products: hydrolysed proteins are a mixture of amino acids, peptides, polypeptides and denatured proteins that can be obtained by chemical, enzymatic and thermal hydrolysis of proteins (or by combining these different hydrolysis types) from both plant and animal sources [67,81]. Studies reported that the applications of some commercial protein hydrolysate products from animal origin were phytotoxic having negative effects on plant growth when compared to a commercial protein hydrolysate of plant origin [82,83]. In another study, Botta et al. [84] observed that lettuce plants treated with an animal-based protein hydrolysed had a higher fresh and dry weight compared with the control. Generally, they can induce plant defence responses and increase plant tolerance to many abiotic stresses as reported by several authors [85–88].

Microorganisms: this group includes bacteria, yeast, filamentous fungi, and micro-algae. They are isolated from soil, plants, water, and composted manures or other organic materials. They are applied to soil to increase crop productivity through metabolic activities. They enhance the uptake of nutrients through nitrogen fixation and the solubilization of nutrients, they modify a hormonal status by inducing plant hormones biosynthesis such as auxins, cytokinins, etc.; they also enhance tolerance to abiotic stresses and produce volatile organic compounds (VOCs), which may also have a direct effect on plants. Plant growth-promoting

rhizobacteria (PGPR) are able to ameliorate plant responses to abiotic stresses stimulating physical, chemical and biological activities [89,90]. Positive effects are given by microorganisms that form a protective biofilm on root surface enhancing nutrient and water uptake.

Another category of biostimulants includes those derived from extracts of food waste or industrial waste streams, composts and compost extracts, manures, vermicompost, aquaculture residues and waste streams, and sewage treatments among others [91]. Biostimulants derived from agro-industrial by-products were reported to be effective in improving plant productivity, increasing the synthesis of secondary compounds involved in several plant physiological responses, and enhancing the activity of the enzyme phenylalanine ammonia lyase (PAL E.C. 4.3.1.5) [92]. The effect of biostimulant application on PAL activity and on the expression of genes encoding for this enzyme was observed by several authors [56,88,89 and references therein], even if at present it is not possible to define if this is a direct or indirect effect. Because of the diversity of source materials and extraction technologies, the mode of action of these products is not easily determined [55]. The use of by-products as raw material that can be transformed into fertilizing products is the idea underlying the new fertiliser regulation and the Circular Economy Action Plan, which is focused on reaching a sustainable agriculture. The guidelines for fertiliser regulation, the need to produce in a more environmentally friendly cultivation system maintaining good crop yield and quality, the increase in price of synthetic fertilizer, the withdrawn of several agrochemicals and the multifaceted effects on plants or soil of biostimulants are favouring the expansion of this market.

A new category of biostimulant product, including nanoparticles and nanomaterials, has been recently proposed by Juárez-Maldonado et al. [93]. Nanoparticles and nanomaterials are usually defined as particles with dimensions between about 1 nm and 100 nm that show properties that are not found in their bulk form. They are able to modify the quality of the production and the tolerance to abiotic stresses when applied in small quantities as foliar spray or in nutrient solution, also in vegetable crops [94–97]. Their biostimulant properties seems to be associated with the structure and nature of the materials. The interaction between plant and nanoparticles and nanomaterials surfaces can positively affect ions and metabolites transport and receptors activity by modifying the surrounding environment in terms of energy and charges. This activity is not dependent one chemical composition. Moreover, nanoparticles and nanomaterials release chemical elements like iron or carbon that could be useful for plant when are metabolised.

A study showed that application of zinc oxide nanoparticles on tomato as soil amendment or by foliar spray increased plant height, chlorophyll and total soluble protein content [98].

2.2. Effect of Biostimulants on Chlorophyll Content, Photosynthesis and Growth in Vegetables

Biostimulants can be used in vegetables cultivation to improve productivity and yield, and to enhance plant health and tolerance to stress factors. Indeed, they have positive effects on plant metabolism both in optimal and sub-optimal environmental conditions.

Many authors have observed that plant based biostimulants and seaweed extracts often increase the colour of leaves by stimulating chlorophyll biosynthesis or reducing its degradation [99,100]. Leaf colour is an important quality parameter in vegetable crops because it contributes to the visual appearance of the product, especially in leafy vegetables for which the greenness influences the consumer's appeal. In addition, a higher chlorophyll content also allows for a greater photosynthetic activity of leaves. High concentration of leaf pigments (chlorophyll and carotenoids) has been observed after biostimulant treatments in rocket [101,102], in lettuce, and endive by Bulgari et al. [103]. Amino acids or seaweed extract application had positive effects on photosynthetic pigments, P and K content, fresh and dry weight of celeriac leaves [104]. Similar results have been observed after root inoculation with several plant growth promoting bacteria (PGPR) in broccoli (*Brassica oleracea* 'italica') using *Bacillus cereus*, *Brevibacillus reuszeri*, and *Rhizobium rubi* [105], and tomato under non-stressful conditions treated with PGPRs belonging to the genera *Bacillus*, *Pseudomonas* and *Azotobacter* [106], in strawberry (*Fragaria ananassa*) with five PGPRs (*Bacillus subtilis, Bacillus atrophaeus, Bacillus spharicus* subgroup, *Staphylococcus kloosii*, and *Kocuria erythromyxa*) [107] and also in lettuce grown under salt stress after inoculation with *Serratia* sp., *Rhizobium* sp., and *Azospirillum* [108,109]. Brown seaweeds are widely used as a biostimulant products to improve plant growth, and recently a phenolic compound isolated from *Ecklonia maxima* showed stimulatory effects in cabbage plants, improving photosynthetic pigments concentration, phytochemicals and myrosinase activity [110].

Abdalla [111] reported that moringa leaf extracts increased vegetative growth, chlorophyll content, total sugars, phenols, ascorbic acid, and photosynthetic rate of rocket salad. Similar effects have been observed in fennel [112,113] and squash under water stress condition (plants under a deficit irrigation of 80% or 60% ETc) [114]. In tomato plants it led to a greater fruit weight, volume and firmness, and enhanced titratableacidity, chlorophyll and ascorbic acid content [115].

Luziatelli et al. [116] recently found that different vegetal-derived bioactive compounds significantly increased the chlorophyll content and fresh weight of lettuce. Kulkarni et al. [117] investigated the promoting effect of bioactive molecules derived from smoke and seaweed in spinach and they observed that morphological, physiological and biochemical parameters including growth, chlorophyll and carotenoids content were positively improved.

Broccoli plants were significantly affected by two different products: Goe¨mar BM86 and Seasol. The content of micro- and macro-nutrients increased, and also the leaf area, stem diameter and biomass, as reported by Gajc-Wolska et al. [74] and Mattner et al. [118].

Paradikovic´ et al. [119] studied the effect of four different commercial biostimulants (Radifarm, Megafol, Viva, and Benefit), containing amino acid, polysaccharides and organic acids as active compounds, on pepper plants and observed an increase both in yield and fruits quality. Radifarm and Viva treatments also affected tomato plants, stimulating the root apparatus in optimal and drought condition respectively [120,121].

Recently, a sago bagasse hydrolysate was tested on tomato plants. The product showed a growth promoting ability as observed by the higher seed germination and protein and sugar content compared to the control. Moreover, the expression of the genes related to carbon and nitrogen metabolisms increased [122].

2.3. Biostimulants and Crop Tolerance to Abiotic Stresses

Table 1 is a summary of biostimulant products or bioactive molecules from different origins that have been evaluated for amelioration of abiotic stresses in several vegetables species. The biostimulants effectiveness to counteract the stressful condition depends on several factors, such as timing of application and their mode of action. The application of biostimulants can be carried out with different timings: before the stress affects the cultivation, during the stress, or even after. They could be applied on seeds, when plants are in early stages of growth, or when crops are fully developed, depending on the desired results [123]. As general consideration, biostimulants that contain anti-stress compounds, such as proline or glutamic acid, can be applied when the stress occurs or during stress conditions. On the contrary, those that are involved in the activation of bioactive compounds biosynthesis must be applied before the stress occurs. Proper timing of application during crop development differs from species to species and it also depends on the most critical phases for crop productivity. Thus, the identification of the right time of biostimulant application is as important as the determination of the exact dose, in order to avoid waste of product, high production costs, and unexpected results. Biostimulants can be applied as foliar spray or to the roots, at sowing for protecting the seedling in the early development stages, in floating system nutrient solution or during blooming or fruit setting. There is not general recipe that works in any crop species and in every stress situation.

The protective role of biostimulants on plants has been increasingly studied. These products are able to counteract environmental stress such as water deficit, soil salinization, and exposure to sub-optimal growth temperatures in several ways [47,56,124,125] They improve plant performance, enhance plant growth and productivity, interact with several processes involved in plant responses to stress, and increase the accumulation of antioxidant compounds that allow a decrease in plant stress sensitivity.

More recent results of interest on vegetable crops tolerance have been obtained after the application of different exogenous treatments. Cao et al. [126] reported that a lower red to far-red ration improved tomato seedling tolerance to salt stress, acting on phytochrome activity. Mertinez et al. [127] showed positive results obtained after the application of exogenous melatonin in tomato plants grown under a combination of salinity and heat. Another interesting approach to induce tolerance to abiotic stresses is soaking plant seeds with different compounds, synthetic or natural. This strategy is generally called seed priming and has been deeply reviewed by Asharaf et al. [128].

2.4. Biostimulants and cold or chilling stress

Low temperatures reduce plant metabolism and delay physiological responses. A reduced metabolism, consequent to cold stress leads to an inhibition of the activity of photosystem II, called photoinhibition. Cold induces damages to cell membranes with destabilization of the phospholipid layers.

In tomato, cold tolerance has been enhanced by the application of psychrotolerant soil bacteria. Several strains have been isolated from soil during winter conditions and used as a cold protectant. Tomato treated with these psychrotolerant bacteria showed higher seeds germination, reduced membrane damage, and antioxidant systems activation when exposed to chilling temperatures [129,130]. These soil bacteria can be considered as putative biostimulants for protecting plants against cold stress. Since low temperature causes stress to plant, especially during transplant, Marfà et al. [131] studied the effect of an enzymatic hydrolysates obtained from animal haemoglobin on strawberry plants in the firsts growing stages. They observed an increase in roots biomass and in the early production of fruit. The same product was also tested on lettuce plants subjected to cold stress and an increase in fresh weight, dry weight, specific leaf area, and relative growth rate was observed [132].

External applications of an amino acid biostimulant (Terra-Sorb® Foliar) on lettuce plants grown in different cold situations led to an increase in fresh weight and to an higher stomatal conductance [84]. A typical plants response to stress is the accumulation of compatible osmolytes, such as amino acids, which confer tolerance. The exogenous application of amino acids has the benefit of avoiding protein breakdown and saving energy resources in plants, even if the exact mechanism of action is not fully understood. Pepper (*Capsicum annuum*) seedlings were treated with 5-aminolevulinic acid in order to improve chilling tolerance through three different methods - soaking the seeds, spraying the leaves or drenching the soil. All the applications showed good effects in terms of stress tolerance. Fresh biomass, proline, sucrose, and water content were significantly higher while membrane permeability was reduced [133].

Positive effects on coriander plant grown in cold vegetative chambers have been observed in response to Asahi SL or Goemar Gateo (Arysta Life Science) treatments [124]. Results obtained by the study of stress indicators such as antioxidant activity, photosynthetic pigment concentration and activity, hydrogen peroxide and malondialdehyde amount showed that biostimulant application affected different metabolic pathways in a positive way, leading stressed plants to a phase of acclimation to low temperature. The biostimulant action against cold stress usually increases the accumulation of osmotic molecules by stimulating the biosynthetic pathways that lead to the cold protectant substances. These biostimulants also increase membrane thermostability, reducing the chilling injury.

2.5. Biostimulants and Heat Stress

Global warming and the projection of a rising temperature have a negative impact on agriculture [134,135]. High temperatures could induce several damages to plant cells, disturbing proteins synthesis and activity, inactivating enzymes and damaging membranes. The range between 30 °C and 45 °C is the optimal for structural integrity and enzymes activity, which are irreversibly denatured when temperature increases above 60 °C. As a consequence, physiological activities like photosynthesis or respiration are affected. An overproduction of toxic compounds, like reactive oxygen species, causing oxidative stress, is one of the most frequent throwbacks [136]. As response, plants start synthesizing compatibles solutes in order to maintain cell homeostasis and turgor, organize proteins, and cellular structures. Moreover, they generally close stomata and increase the number of trachomatous, in order to prevent water loss. Also, at the molecular level there is a variation of the expression of genes involved in the synthesis or activity of antioxidant enzymes related to ROS scavenging, osmolytes or transporters. Temperature above optimum inhibits seeds germination and retards plant growth. Heat stress could negatively affect the yield by interfering with the reproductive phase, decreasing pollen vitality and germination, inhibiting flower differentiation and development and reducing fruit set, which ultimately reduces growth and yield.

Tomato is considered one of the most sensitive species to non-optimal temperatures, and heat stress often results in long style lengths and in a decreased fruit set [137]. There is little information in the literature about treatments specifically applied to vegetable crops exclusively against high temperature since, most of the time, heat stress is combined with drought or salinity. The application of brassinosteroids on tomato [138] and snap bean [139] has resulted in a higher biomass accumulation and net photosynthesis rate, increased growth and quality of snap bean pod in terms of NPK content and the total free amino acids levels in leaves. This might be due to the protective role of brassinosteroids on the photosynthetic apparatus from oxidative stress, increasing the ability to regenerate RuBP and carboxylation efficiency.

Nahar et al. [140] investigated the effect of exogenous application of glutathione against heat stress. Mung bean seedlings treated before their exposition to high temperature, showed a reduced oxidative stress and methylglyoxal content, a reactive compound that damages cells. This results in a more efficient antioxidant defense system. Pre-treatment with glutathione enhanced tolerance to short term heat stress, improving plant physiological adaptation. For example, leaf relative water content and turgidity, which usually decreases under high temperature, were protected. Positive effect on mung bean has been observed in response to the application of nitric oxide [141] and ascorbic acid [142]. Nitric oxide treatment resulted in a promotion of photosynthetic activity, increasing the quantum maximum efficiency of PS2. It also affected electrolyte leakage, leading to a better cell membrane integrity. Oxidative stress, lipid peroxidation, and H_2O_2 content were decreased and antioxidant enzyme activity was restored. Similar results have been obtained after the application of proline and abscisic acid on chickpea [143,144]. Chickpea is sensitive to high temperature that generally leads to yield and quality losses. After treatments, membrane damage, measured as electrolyte leakage, MDA and H_2O_2 levels, was decreased while leaf water content was increased. These effects might be related with the osmoprotectant role of proline and with the accumulation of osmolytes after ABA treatments. Treated plants also showed a high chlorophyll content and this result, which has been already seen in other experiment with exogenous proline, could be related to membrane stability. The activity of oxidative metabolism was enhanced in treated plants, as expected also by the less oxidative damage of cells.

As discussed above, melatonin treatment exerts positive effect to counteract chilling stress in coriander plants, otherwise, Martinetz et al. [127] found that melatonin treatments also have a protective role against the combination of heat and salt stress in tomato plants. Biostimulant treatments used against heat stress protect cell membranes by increasing their stability and reduce or avoid the accumulation of ROS.

2.6. Biostimulants and Salinity Stress

Among abiotic stresses, salinity is one of the main damaging factors affecting plant growth and metabolism as an effect of osmotic stress caused by salt. Sodium chloride (NaCl) is the more abundant salt presents in saline environments and is toxic in higher concentrations [145]. It happens especially near the coasts, where crops are frequently irrigated with saline water [85,146]. In many Mediterranean areas the problem of seawater intrusion may cause a reduction of 50% of yield in lettuce cultivation, as reported by Miceli et al. [147]. A significant reduction of both fresh weight and chlorophyll content is a typical effect of salinity condition on plants and was observed also in spinach [148], in bean [149] and other crops [150]. Besides, chlorophyll content is a central parameter of the product quality particularly in green leafy vegetable, not only in terms of plant physiology status but also from a market point of view. This is a huge problem for vegetable crops where the edible parts

are leaves, sprouts or flower buds. Consumers choices, in fact, are guided mostly by the visual appearance of products, hence a less green leafy vegetable or a malformed fruit are generally not accepted.

Salt stress causes a nutrient imbalance due to the limited uptake of the nutrients from the soil, threatening the nutritional quality of horticultural crops. Nutrient availability is compromised by salinity that causes several disorders such as competitive uptake with other ions like Ca^{2+}, P and K, mobility problems within the plant and a reduced water potential [151–155]. The solubility of micronutrients such as Cu, Fe, Mn, Mo and Zn is also affected by the pH of the soil solution, and in saline condition their availability is very low. Bano et al. [156] reported an important reduction of total phenolics, total soluble proteins and a suppressed activity of catalase, superoxide dismutase and peroxidase in carrot under saline condition. Salt stress could also alter several metabolic processes in plants, such as photosynthesis [157,158], respiration [159], phytohormone regulation, protein biosynthesis, nitrogen assimilation [160], and can also generate secondary oxidative stress [146,161]. It generally leads to a decrease of production and to a lower quality of the final product, due to an inhibition of leaves and roots growth and a change in leaves colour [17]. To verify the effects deriving from the applications of biostimulants, several trials on lettuce plants under salt stress were performed, since this crop is considered moderately sensitive to salinity.

Lucini et al. [85] showed that a plant-derived protein hydrolysates improved tolerance to salinity in lettuce plants, increasing yield and dry weight. Treated plants also have a higher performance and an increased maximum quantum efficiency of PS2 compared to the control. Similar results have been recently observed in lettuce plants in response to the application of an organic commercial biostimulant named Retrosal® [162].

Several experiments have been carried out using different PGPR that are able to enhance abiotic stress tolerance. Inoculation with *Azospirillum brasilense* showed positive results on lettuce [163,164], sweet pepper [165], chickpea and faba beans [166] grown under salty environment. Lettuce fresh weight, dry weight, ascorbic acid content, and germination percentage were increased; also, the visual appearance of the final product was better because of higher chlorophyll levels. In chickpeas and faba beans the inoculation relieved the stress caused by salinity, increasing the root and shoot growth compared with the non-inoculated plants. Sweet pepper is a salt-sensitive crop and inoculation showed positive effect mitigating deleterious effects of NaCl. Dry weight, indeed, was higher than non-inoculated plants under several salt concentrations. Moreover, the inoculation also increased the CO_2 assimilation rate. A similar result has been obtained by Cordovilla et al. [167] applying two different *Rhizobium* strain on faba bean and pea plants. Pea plants inoculated with tolerant strain showed no reduction by salt stress condition in shoot and roots dry weight. However, the same strain was not effective on faba beans. These results highlight the variation existing inter and intra species, and the difficulty in improving tolerance through selection and breeding. A comparable experiment has been carried out by Mayak et al. [168] on tomato seedling. They tested several strains of *rhizobacterium* and found that plants inoculated

with *Achromobacter piechaudii* and irrigated with saline water had a higher fresh and dry weights and an increased water use efficiency. Yildirim et al. [169] obtained similar results in squash with the application of several biological products based on the *Bacillus* and *Trichoderma* species.

It is known that humic acids have a lot of beneficial effect stimulating shoot and root growth and improving environmental stress tolerance even if the exact mechanism of action is not completely clear. These activities were confirmed in several vegetable crops like sweet pepper [170], beans [171] and cucumber [172] grown under different salt stress conditions.

Bioactive compounds present in seaweed extracts are able to improve plant tolerance against abiotic stresses too. Two seaweed-based plant biostimulants containing *Ascophyllum nodosum* named Super Fifty® and Acadian were applied respectively on lettuce [173] and strawberry [174] and were associated with a significant increase in yield and root dry weight despite the adverse salinity condition.

Sulphated exopolysaccharides extracted from the microalgae *Dunaliella salina* were applied on tomato plants to investigate their potential effect alleviating salt stress damages. Results obtained showed that treatment enhance plant growth, antioxidant enzymes activities and several metabolic mechanisms related to jasmonic acid pathway [175].

The application of seaweed extracts from *Sargassum muticum* and *Jania rubens* significantly alleviated the negative effects of salt through regulation of amino acids metabolism, ionic content balanced and improved antioxidant defence in chickpeas plants. Amino acids such as serine, threonine, proline and aspartic acid were identified in roots as responsible for salt stress amelioration [176].

Besides lettuce and pepper, bean is also considered a salt sensitive plant but in most developing countries it is cultivated in saline condition. Several plant extracts based on licorice root, *Moringa oleifera* or maize grain have been tested on common bean by Egyptian researchers [177–181]. They observed that soaking seeds in propolis or maize grain extract improves seed germination percentage, stability of cell membrane and relative water potential under saline condition. Antioxidant system activity was increased while lipid peroxidation and electrolyte leakage were reduced compared with the control plants. *Moringa oleifera* leaf extract, used alone or in combination with salicylic acid, and administered as foliar spray or as seed soaking, improved several physiochemical parameters as chlorophyll and carotenoids concentration, total soluble sugars and ascorbic acid content. A very similar trial has been carried out with licorice root extract and best results have been recorded integrating seed soaking and foliar spray applications.

A recent study highlighted the ability of a bee-honey based biostimulant to improve the tolerance of onion plants to salinity stress. Indeed, treated plants showed higher biomass, bulb yield, and photosynthetic pigments. Moreover, the osmoprotectans content as proline, soluble sugars and total free amino acids, the membrane stability index and the enzymatic and non-enzymatic antioxidant activity were enhanced [182]. Hence,

biostimulants applied in case of salinity stress induce the accumulation of osmolytes, in order to enhance the cell osmotic potential and the level of protective molecules against oxidative stress.

2.7. Biostimulants and Drought Stress

Abiotic stresses are closely connected with the problem of resources availability and farmers are frequently forced to work in suboptimal conditions. A more sustainable use of resources also concerns water availability, a critical growing factor. The increasing use of aquifer-based irrigation by farmers worldwide poses a serious threat to the long-term sustainability of the agricultural system. Over-utilization of this dwindling water supply is leading to an ever-enlarging area in which productive farming itself has ceased or is threatened. Moreover, the increase of irrigation leads to a higher risk of soil salinization. Scientists generally agree with the perspective that several regions could become arid due to the negative impacts of global climate change on water resources [183]. Since one of the main effects of biostimulants is to improve water use efficiency, their application could be a possible strategy to reduce the amount of water added to crops [184]. Drought stress strongly influences plant gas exchange changing photosynthetic and transpiration rates, which are directly linked to yield. Application of Ascophyllum nodosum on broccoli [185] and spinach [186] enhanced gas exchange through the reduction of stomatal closure resulting in increased plant resistance to water stress. Leaf yellowing is another common symptom of drought stress due to chlorophyll degradation during leaf senescence and is used as reliable indicator of metabolic and energetic imbalance in plants under stress. Biostimulant treatments with A. nodosum increased total chlorophyll content in tomato leaves [187]. A reduction of water loss, wilting damages and 3-carbon dialdehyde MDA after biostimulant applications were observed. Similar results have been obtained by Petrozza et al. [188] in responses to Megafol treatments in tomato plants. The results revealed that treated plants were healthier than non-treated ones in terms of biomass and chlorophyll fluorescence. Moreover, plants treated with the biostimulant product were able to recover more quickly when they had access to water. The expression of two drought stress marker genes was analysed and the results obtained showed that treated plants were experiencing a low level of water stress.

Sometimes, water stress in plants is caused by bacterial infection clogging xylem vessels and preventing water flow. Romero et al. [189] demonstrated that treatments with Azospirillum brasilense, a strain isolated in arid environments, delayed wilting of tomato plants. Treated plants, indeed, showed a high xylem vessels area resulting in a more efficient water transport from the soil to the leaves. On the other hand, there are several strains of bacteria populating soil promoting plant growth through its metabolic activities and plant interactions. They produce exopolysaccharides, phytohormones, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, volatile compounds, inducing several metabolic plant responses as accumulation of osmolytes and antioxidants,

or up or down regulation of stress responsive genes and alteration in root morphology leading to a tolerance of water stress [190,191]. Some examples are reported below. Tomato seedlings treated with Achromobacter piechaudii were stimulated to accumulate biomass during the stress period and, the amount of ethylene that usually has negative effects on membrane status was lower than control [168].

Arshad et al. [192] investigated the growth of two plants promoting rhizobacteria on pea (Pisum sativum) crop grown under drought stress condition in different phenological phases. They observed that PGPR containing ACC-deaminase, a precursor of ethylene, significantly decreased the stress effects on growth and yield too. Positive results in terms of antioxidant and photosynthetic pigments activity have been collected in basil plants treated with Pseudomonas sp. under water stress conditions [193].

Seaweed extracts are already largely used for cultivated plant treatments and most of them contain plant growth hormones, auxins, abscisic acid, cytokinins, gibberellins, polyamines, oligosaccharides, betaines and brassinosteroids. A micro-algae-based biostimulan, with known composition was tested on water stressed tomato plants. Results revealed that biostimulant application reduced the damaging effects of stress, increased plant height, root length and enhanced the number and the area of the leaves [78]. Biostimulants are capable to reduce drought injures, are able to enhance the biosynthesis of osmolytes and antioxidants against ROS, such as observed for salinity stress, and of plant hormones, like abscisic acid, regulating transpiration and avoiding excessive water losses.

2.8. Biostimulants and Nutrient Deficiency

One of the roles ascribed to biostimulant products is the ability to increase nutrient uptake [53] through different strategies. For instance, they are able to change soil structure or nutrient solubility, modify roots morphology directly or ameliorate nutrient transport in plants [194]. Their application might be really useful in poor soil conditions and in low input horticultural cultivation systems [195]. Indeed, soil nutrient imbalance is an increasing problem for farmers that spend a lot of money every year on fertilizers to resume soil fertility. All these mechanisms result in a better nutrient use efficiency for both micro- and micro-nutrients.

Several experiments have been performed to investigate if the application of biostimulants allows a reduction of fertilizers without affecting crop yield and quality.

Koleška et al. [196] showed that the application of a biostimulant product named Viva® on tomato plants, growing under reduced NPK nutrition, help counteract the negative effects of nutrient deficiency. For example, lycopene and chlorophyll content that is usually affected by the availability of macronutrients was preserved in treated plants grown with NPK reduction. Moreover, biostimulant application helped maintaining cell homeostasis and preventing oxidative stress. A similar experiment was performed by Anjum et al. [197] on garlic

plants grown with half of the recommended dose of nutrients. Garlic growth and yield were positively affected by the biostimulant application in combination with a low dose of macronutrients.

A seaweed-based product (Kelpak®) has been tested on okra seedlings grown with different nutrient deficiencies [198]. Treatments were applied three times a week and were compared with a polyamine solution treatment. Plants treated with the biostimulant showed an increase in growth parameters, such as shoot length, stem thickness, leaves and roots numbers and fresh weight under phosphorous and potassium deficiency. Kelpak® efficacy might be due to the combination of auxins, cytokinins and polyamines contained in the product.

Spinelli et al. [199] measured the effects of another commercial seaweed extract, named Actiwave®, on the vegetative and productive performance of strawberry plants grown on an iron deficient substrate. They found that vegetative growth, chlorophyll content, stomatal density and photosynthetic rate were enhanced after biostimulant treatment. Fruit production and weight were also increased. Nutrient uptake might have been positively influenced by the more developed root system of treated plants. Treatment also contrasted the negative effects of iron chlorosis and this could be linked to betaine contained in this product.

The positive effects of seaweed extracts are usually ascribed to their polysaccharides content that helps soil structure; nevertheless, Vernieri et al. [102] obtained good results by applying Actiwave in a hydroponic system with different concentrations of nutrient solutions. Yield and leaf area were higher in rocket plants grown with the lowest nutrient concentration, indicating a better nutrient use efficiency.

Most of the biostimulant contains a mixture of different amino acids and short peptides that are usually called protein hydrolysates. They have a positive effect on plant growth and protection against several stresses. The Cerdán et al. [200] study showed that amino acids origin might influence the efficacy of the product. Tomato plants grown under iron deficiency conditions and treated with two products containing amino acids from plant and animal origin showed different responses. Plants-derived amino acids promoted growth and chlorophyll content both in controlled and iron deficiency conditions. This effect might be ascribed to glutamic acids content. Indeed, this amino acid plays an important role in nitrogen metabolism [201] and chlorophyll biosynthesis [202].

Nutrient imbalance might be the cause of several disorders during plants growth and development. Blossom-end rot in pepper is usually caused by a local calcium deficiency in young fruits. Parađiković et al. [203] tested four different biostimulant products for their effects on yield and BER incidence on pepper. They also evaluated the application as foliar spray or in a nutrient solution of the same products. The results obtained revealed that biostimulants applications helped to reduce the occurrence of BER and increase yield. Moreover, nutrient accumulation in fruits and leaves was promoted by the treatments.

These experiments revealed that biostimulant products cannot totally replace fertilizers but they could be really useful to reduce the amount of mineral nutrition or help in nutrient deficiency and imbalanced situation. For example, in the floating system cultivation of baby leaf such as rocket, the nutrient solution can be reduced by 75% of the Hoagland's solution [101].

The biostimulants that help to reduce the nutrient deficiencies usually improve the crops nutrient uptake by increasing roots biomass, nutrient transport/translocation and enzyme activities involved in nutrients assimilation.

Table 1. Examples of biostimulant products or substances with a biostimulant effect on horticultural crops to counteract abiotic stress conditions. Abbreviation: Fv/Fm maximum quantum efficiency of Photosystem II; Pn net photosynthetic rate; E transpiration rate; gs stomatal conductance; Ci sub stomatal CO_2 concentration; SLA specific leaf area; RGR relative growth rate; RLWC relative leaf water content; RWC relative water content; WUE water use efficiency; PI performance index; MDA malondialdehyde; TTC 2,3,5-triphenyltetrazolium chloride; GSH reduced glutathione; GSSG oxidized glutathione; LOX lipoxygenase; CAT catalase; SOD superoxide dismutase; APX ascorbate peroxidase; POX peroxidase; GR glutathione reductase; HI harvest index; ABA abscisic acid; ETR electron transport rate

ABIOTIC STRESS	SEVERITY AND TIME OF EXPOUSURE	BIOSTIMULANT PRODUCT OR SUBSTANCES WITH A BIOSTIMULANT EFFECT	DOSE	APPLICATION METHODS	CROP	BENEFICIAL EFFECTS	REFERENCE
Chilling or cold stress	6 °c for 6 days	Asahi sl (sodium para-nitrophenolate, sodium ortho-nitrophenolate, sodium 5-nitroguaiacolate) / goëmar goteo (composition (w/v): organic substances 1.3–2.4%, phosphorus (p2o5) .24.8%, potassium (k2o) .4.75%)	0.1%	Foliar spray (3x)	*Coriandrum sativum l.*	↓electrolyte leakage ↑chlorophyll *a* and carotenoids ↑fv/fm ↑e ↑gs ↓ci	[124]
	10, 12 °c for 7 days / 15 °c for 7, 10 days	*Flavobacterium glaciei, pseudomonas frederiksbergensis, pseudomonas vancouverensis*	-	Seed inoculation	*Solanum lycopersicum*	↑shoot height ↑root length ↑biomass accumulation ↓electrolyte leakage ↓lipid peroxidation ↑proline accumulation ↑sod, cat, apx, pod, gr activity	[129,130]
	- 6 °c for 5 nights	Pepton 85/16 (enzymatic hydrolysates obtained from animal haemoglobin. L-α amino acids (84.83%) and free amino	2 l ha-1, 4 l ha^{-1}	Injection into the soil (5x)	*Fragaria × ananassa*	↑new roots ↑flowering ↑fruit weight	[131]

	acids (16.52%), organic-nitrogen content (12%), mineral-nitrogen content (1.4%), potassium content (4.45%), iron content (4061 ppm), very low heavy-metal content)					
- 3 °c for 4 hours	Pepton 85/16	0.4, 0.8, 1.6 g l^{-1}	Soil application (1x)	*Lactuca sativa l.*	↑fresh and dry weight ↑sla ↑rgr	[132]
4 °c for 8 days or nights /6 °c for 8 days only to the roots	Terra-sorb® foliar (free amino acids (asp, ser, glu, gly, his, arg, thr, ala, pro, cis, tyr, val, met, lys, ile, leu, phe, trp) 9,3% (w/w), total amino acids 12% (w/w), total nitrogen (n) 2,1% (w/w), organic nitrogen (n) 2,1 % (w/w), boron (b) 0,02 % (w/w), manganese (mn) 0,05 % (w/w), zinc (zn) 0,07 % (w/w), organic matter 14,8 % (w/w))	3 ml l^{-1}	Foliar spray (3x)	*Lactuca sativa l. Var. Capitata*	↑roots fresh weight ↑green cover %	[84]
3 °c for 48 hours	5-aminolevulinic acid	0, 1, 10, 25, 50 ppm (15 ml for seed soaking and 25 ml for soil drench)	Seed soaking/ foliar spray / soil drench (1x)	*Capsicum annuum*	↓visual injuring ↑chlorophyll ↑rwc ↑gs ↓membrane permeability ↑shoot and root mass ↑sod activity	[133]

Drought stress	Occlusion of xylem vessels	*Azospirillum brasilense (bnm65)*	-	Seed inoculation	*Solanum lycopersicum*	↑height plants ↑dry weight ↑xylem vessel area	[189]
	No irrigation for 5 days	Megafol® (composition (w/v): total nitrogen (n) 3.0% (36.6 g l^{-1}); organic nitrogen (n) 1.0% (12.2 g l^{-1}); ureic nitrogen (n) 2.0% (24.4 g l^{-1}); potassium oxide (k_2o) soluble in water 8.0% (97.6 g l^{-1}); organic carbon (c) of biological origin 9.0% (109.8 g l^{-1}))	2 ml l^{-1}	Foliar spray (1x)	*Solanum lycopersicum*	↑leaf area ↑rlwc	[188]
	50% et	*Ascophyllum nodosum*	0.50%	Foliar spray and drench	*Spinacia oleracea*	↑rlwc ↑leaf area ↑fresh and dry weight ↑sla ↑gas exchange	[186]
	No irrigation until symptoms of wilting appear	Pseudomonas spp. (*p. Putida p. Fluorescens*)	-	Seed inoculation	*Pisum sativum*	↑grain yield ↑root growth ↑shoot length ↑number of pods per plant ↑chlorophyll	[192]
	No irrigation for 12 days	*Achromobacter piechaudii* (arv8)	-	Seedling inoculation	*Solanum lycopersicum*	↑fresh and dry weight of seedling ↑plant growth ↓ethylene	[168]
	No irrigation for 12 days	*Achromobacter piechaudii* (arv8)	-	Seedling inoculation	*Capsicum annuum*	↑ fresh and dry weight of seedling ↑plant growth	[168]
	No irrigation for 7 days	*Ascophyllum nodosum*	0.33%	Foliar spray (2x)	*Solanum lycopersicum*	↑rwc ↑plant growth ↑foliar density ↑chlorophyll ↓lipid peroxidation ↑proline ↑soluble sugars	[187]

	No irrigation for 2 days	*Ascophyllum nodosum* + amino acids	-	Soil application (1x)/ foliar spray (3x)	*Brassica oleracea var. Italica*	↑pn ↑gs ↑chlorophyll	[185]
	40, 70% field capacity	Gibbrellic acid and titanium dioxide	250, 500 ppm (ga3) 0.01, 0.03% (titanium nanoparticles)	Stems and foliar spray (2x)	*Ocimum basilicum*	↑cat activity ↓lipid peroxidation ↑lrwc	[95]
	No irrigation	Viva®	-	2x	*Solanum lycopersicum*	↑plant biomass ↑roots biomass	[120]
	60, 40% field capacity	*Pseudomonades, bacillus lentus, azospirillum brasilens*	-	Seed inoculation	*Ocimum basilicum*	↑cat, gpx activity ↑chlorophyll	[193]
	60, 40% et	Moringa leaf extract	3%	Foliar spray (2x)	*Cucurbita pepo*	↑growth ↑hi ↑wue ↑fv/fm ↑pi ↑soluble sugars ↑free proline ↓electrolyte leakage ↑membrane stability	[114]
Heat stress	35 °c	Nano-tio_2	0.05, 0.1, 0.2 g l^{-1}	Foliar spray (1x)	*Solanum lycopersicum*	↑gs ↑e ↑ pn	[94]
	40/30 °c for 8 days	Brassinosteroids	0.01, 0.1, and 1.0 mg l^{-1}	Foliar spray (1x)	*Solanum lycopersicum*	↑antioxidant enzyme activities ↓h_2o_2 ↓mda ↑shoot weight	[138]
	35.2 °c (tmax)	Brassinosteroids	25, 50, 100 ppm	Foliar spray (2x)	*Phaseolus vulgaris*	↑plant length ↑number of leaves, branches and shoots per plant ↑fresh and dry weight ↑pod weight ↑n, p, k in bean pods	[139]
	45 °c for 90 min	Nitric oxide	150 µm	Immersion of leaf disks	*Phaseolus radiatus*	↑fm ↓electrolyte leakage	[141]

	35/25 40/30 45/35 °c	Ascorbic acid	50 µm	In the nutrient solution	*Phaseolus radiatus*	↑% germination ↑seedling growth ↓electrolyte leakage ↑ttc reduction ability ↑rlwc ↓mda ↓h_2o_2 ↑antioxidant activity ↑ascorbic acid ↑gsh ↑proline	[142]
	35/25 40/30 45/35 °c	Proline	5, 10, 15 µm	In the nutrient solution	*Cicer arietinum*	↑% germination ↑shoot and root length ↓electrolyte leakage ↑chlorophyll ↑rlwc ↓lipid peroxidation ↓h_2o_2 ↑gsh ↑proline	[143]
	35/25 40/30 45/35 °c for 10 days	Abscisic acid	2.5 µm	In the nutrient solution	*Cicer arietinum*	↑shoot length ↑osmolytes ↑chlorophyll ↑cellular oxidizing ability	[144]
	42 °c for 48 hours	Glutathione	0.5 mm	-	*Vigna radiata l.*	↑rlwc ↑chlorophyll ↑proline ↓mda ↓h_2o_2 ↓o_2^- ↓lox activity ↑ascorbate ↓gssg	[140]
Heat and salt stress	35 °c and 75 mm nacl for 15 days	Melatonin	100 µm	Foliar spray (5x)	*Solanum lycopersicum*	↑biomass ↑pn ↑gs ↑e ↑chlorophyll a ↑carotenoids ↑fv/fm ↑efficiency of psii ↑etr ↑antioxidant capacity ↓h_2o_2 ↓lipid peroxidation ↓protein oxidation	[127]
Iron deficiency	-	Actiwave® (*ascophyllum nodosum*)(composition (w/v): total nitrogen (n) 3.0% (38.7 g l^{-1}); organic nitrogen (n) 1.0% (12.9 g l^{-1}); ureic nitrogen (n) 2.0% (25.8 g l^{-1}); potassium oxide (k_2o) soluble	10 ml in 20 ml tap water	In the nutrient solution	*Fragaria ananassa*	↑vegetative growth ↑chlorophyll ↑stomatal density ↑photosynthetic rate ↑ fruit production ↑berry weight	[199]

		in water 7.0% (90.3 g l^{-1}); organic carbon (c) of biological origin 12% (154.8 g l^{-1}); iron (fe) soluble in water 0.5% (6.45 g l^{-1}); iron (fe) chelated by ethylenediaminedi (2-hydroxy-5-sulfophenylacetic) acid (eddhsa) 0.5% (6.45 g l^{-1}); zinc (zn) soluble in water 0.08% (1.03 g l^{-1}); zinc (zn) chelated by ethylenediaminetetraacetic acid (edta) 0.08% (1.03 g l^{-1}))					
	-	Amino acids	0.1, 0.2 ml l^{-1} / 0.2, 0.7 ml l^{-1}	Root application / foliar spray (4x)	*Solanum lycopersicum*	↑plant growth ↑root and leaf ferrum chelate reductase activity ↑chlorophyll ↑leaf fe ↑fe_2:fe ratio	[200]
Reduced npk	Npk reduced of 40%	Viva® (composition (w/v): total nitrogen (n) 3.0% (37.2 g l^{-1}); organic nitrogen (n) 1.0% (12.4 g l^{-1}); ureic nitrogen (n) 2.0% (24.8 g l^{-1}); potassium oxide (k_2o) soluble in water 8.0% (99.2 g l^{-1}); organic carbon (c) of biological origin 8.0% (99.2 g l^{-1}); iron (fe) soluble in water 0.02% (0.25 g l^{-1}); iron (fe) chelated by eddhsa 0.02% (0.25 g l^{-1}))	10.5 ml /plant	Foliar spray	*Solanum lycopersicum*	↑yield ↑ascorbic acid ↑lycopene ↑chlorophyll ↑carotenoids	[196]

	Npk deprivation	Kelpak (*ecklonia maxima,* containing polyamine, cytokinins and auxins, putrescine, spermine)	0.40%	In the nutrient solution (twice per week for 8 weeks)	*Abelmoschus esculentus*	↑number of leaves ↑number of roots ↑stem thickness ↑shoot weight ↑root weight ↑leaf area	[198]
	Npk reduced of 50%	Bio-cozyme (concentrated micro-biological biostimulant and soil inoculants. Total nitrogen (n) 0.20%, soluble potash (ko) 5.00%, magnesium (mg) 1.40%, boron (b) 0.20%, copper (cu) 0.50%, iron (fe) 3.00%, manganese (mn)1.00%, molybdenum (mo) 0.0.25%, zinc (zn) 2.00%, humic acid, humates & derivatives 8.00%, vitamins, e, c, b complex, organic acids, Natural sugars carbohydrates, amino acids 1.40%)	2 kg ha^{-1}	Foliar application (4x)	*Allium sativum*	↑bulb yield ↑plant height ↑npk in leaves	[197]
Salt stress	30, 50, 80 mol m^{-3} nacl for 30 days / 40, 80, 120 mol m^{-3} nacl	*Azospirillum brasilense*	-	Seed inoculation	*Lactuca sativa*	↑germination % ↑total fresh and dry weight ↑biomass partition ↑plantlets number ↑plantlets dry weight ↑total leaf fresh weight ↑leaf area ↑leaves number ↑chlorophyll ↑root dry weigh ↑ascorbic acid ↑plant survival after transplant	[163,164]

40, 80, 120 mm nacl	*Azospirillum brasilense/pantoea dispersa*	-	Inoculation	*Capsicum annuum*	↑plant dry weight ↑k^+:na^+ratio ↑gs ↑relative growth rate ↑net assimilation rate ↓ cl^- accumulation ↑no_3^- concentration ↑co_2 assimilation	[165]
714 $mg \cdot l^{-1}$ nacl	*Azospirillum brasilense (atcc 29729)*	-	Soil inoculation	*Cicer arietinum*	↑nodule formation ↑shoot dry weight	[166]
100 mmol l^{-1} nacl	*Rhizobium leguminosarum (gra19 - grl19)*	-	Seedling inoculation	*Vicia faba / pisum sativum*	↑plant growth	[167]
50, 100 mm nacl	*Bacillus species, bacillus pumilis, trichoderma harzannum, paenibacillus azotoformans and polymyxa*	-	Seed treatment/ watering	*Cucurbita pepo*	↑fresh weight ↑potassium uptake ↓sodium uptake ↑ k^+:na^+ ratio	[169]
30, 60, 120 mm (nacl, na_2so_4, $cacl_2$, $caso_4$, kcl, k_2so_4, $mgcl_2$, $mgso_4$) for 60 days	Humic acid	0.05, 0.1%	Soil application	*Phaseolus vulgaris*	↑plant nitrate, nitrogen and phosphorus ↓soil electricity conductivity ↓proline ↓electrolyte leakage ↑plant root and shoot dry weight	[171]
-	Acadian (*ascophyllum nodosum*)	-	Soil application	*Fragaria ananassa*	↑yield ↑growth ↑root length ↑surface area, volume and number of tips ↑numbers of crowns	[174]
80 mm nacl	Super fifty® (*ascophyllum nodosum*)	0.4, 1, 2.5, 10 ml l^{-1}	In the nutrient solution	*Lactuca sativa*	↑root, stem, total plant weight	[173]

25 mm nacl	Protein hydrolysates	2.5 ml l^{-1}	Foliar spray / soil application	*Lactuca sativa*	↑fresh yield ↑dry biomass ↑root dry weight ↑plant nitrogen metabolism ↑fv/fm ↓oxidative stress ↑osmolytes ↑glucosynolates	[85]
0.8, 1.3, and 1.8 ds/m nacl	Retrosal® (organic mix with high concentration of carboxylic acids, containing calcium oxide (cao) 8.0% (w/w) soluble in water and 1.4% complexed by ammonium ligninsulfonate, zinc (zn) 0.2% (w/w) soluble in water and 0.2% (w/w) chelated by edta.)	0.1 or 0.2 ml/plant	Soil application (4x)	*Lactuca sativa*	↑fresh weight ↑chlorophyll pn ↑ gas exchange ↓proline ↓aba	[162]
43, 207 mm nacl for 7 weeks	*Achromobacter piechaudii*	-	Seedling inoculation	*Solanum lycopersicum*	↑fresh and dry weights of tomato seedlings ↓ethylene ↑uptake phosphorous and potassium ↑wue	[204]
200 mm nacl	Nano-tio_2	5, 10, 20 and 40 mg l^{-1}	Foliar spray	*Solanum lycopersicum*	activities of carbonic anhydrase, nitrate reductase, sod and pox ↑proline ↑glycinebetaine ↑growth ↑yield	[97]
28, 56 mmol kg^{-1}	*Ascophyllum nodosum*	1, 2 g kg^{-1}	Soil application	*Cucumis sativus*	↑fruit yield ↑pn	[172]

7.15, 7.2 dsm^{-1}	Licorice root extract	0.50%	Seed soaking /foliar spray	*Phaseolus vulgaris*	↑plant growth ↑yield ↑rwc ↑chlorophylls ↑free proline ↑total soluble carbohydrates ↑total soluble sugars ↑nutrients ↑selenium ↑k^+:na^+ ratio ↑membrane stability index ↑activities of all enzymatic antioxidants ↓electrolyte leakage ↓mda ↓na^+ ↓h_2o_2 ↓o_2^-	[181]
100 mm nacl	Propolis and maize grain extract	1, 2%	Soaking seed	*Phaseolus vulgaris*	↑% germination ↑seedling growth ↑cell membrane stability index ↑rwc ↑free proline ↑total free amino acids ↑total soluble sugars ↑indole-3-acetic acid ↑gibberellic acid ↑activity of the antioxidant system ↓lipid peroxidation ↓electrolyte leakage ↓aba	[178]
6.23 – 6.28 ds m^{-1}	Salycilic acid and *moringa oleifera*	0.30%	Seed soaking /foliar spray	*Phaseolus vulgaris*	↑shoot length ↑number and area of leaves ↑ plant dry weight ↑rwc ↑chlorophyll ↑carotenoid ↑total soluble sugars ↑free proline ↑ascorbic acid ↑n, p, k and ca, ↑ratios of k/na and ca/na ↑green pod and dry seed yields	[179]
100 mm nacl	*Moringa oleifera*	Crude extract	Soaking seed	*Phaseolus vulgaris*	↑shoot and root lengths ↑plant dry mass ↑total soluble sugars ↑proline ↑k^+, na^+ and cl^- ↑ascorbic acid ↑total	[177,180]

						glutathione ↓mda ↓ h_2o_2 ↓o_2^- ↑sod, apx, gr	
	50, 150 mm nacl	*Sargassum muticum* and *jania rubens*	1%	Foliar spray (2x)	*Cicer arietinum*	↑plant growth ↑chlorophyll ↑carotenoid ↑soluble sugars ↑phenols ↓na^+ ↑ k^+ ↓h_2o_2 ↑cat, sod, pod, apx activity ↓mda	[176]
	3, 6 g l^{-1}	*Dunaliella salina* exopolysaccharides	0.1 g l^{-1}	Foliar spray (2x)	*Solanum lycopersicum*	↑chlorophyll ↑protein ↓proline	[175]
	8.81 ds m^{-1}	Bee-honey based biostimulant	25–50 g l^{-1}	Foliar spray	*Allium cepa*	↑biomass ↑bulb yield ↑wue ↑photosynthetic pigments ↑osmoprotectants ↑membrane stability index ↑rwc ↑enzymatic and non-enzymatic antioxidants	[182]
	8 mm nacl	Phosphorus / humic acid	50, 100, 150 mg kg^{-1} (p) / 750, 1500 mg kg^{-1} (humic acid)	Soil application	*Capsicum annuum*	↑fresh and dry weight of shoot and root ↓membrane damage ↑nutrient uptake	[170]
Uv-stress	300–340nm illumination for 15 min	Nano-anatase	0.25%	Soaking seed and foliar spray	*Spinacia oleracea*	↓o_2^-↓h_2o_2 ↓mda ↑ sod, cat, apx, gpx activity	[96]

REFERENCES

1. Verma, A. Abiotic Stress and Crop Improvement: Current Scenario. *Adv. Plants Agric. Res.* **2016**, *4*.
2. Boyer, J.S. Plant Productivity and Environment. *Science (80-.).* **1982**, *218*, 443–448.
3. La Peña, R.D.; Hughes, J. Improving Vegetable Productivity in a Variable and Changing Climate. *J. SAT Agric. Res.* **2007**, *4*, 666–675.
4. Farooq, M.; Wahid, A.; Kobayashi, N.; S.M.A. Fujita, D.B. Plant drought stress : effects , mechanisms and management To cite this version : Review article. *Agron. Sustain. Dev.* **2009**, *29*, 185–212.
5. Cramer, G.R.; Urano, K.; Delrot, S.; Pezzotti, M.; Shinozaki, K. Effects of abiotic stress on plants: A systems biology perspective. *BMC Plant Biol.* **2011**, *11*, 163.
6. Global Warming of 1.5 °C Available online: https://www.ipcc.ch/sr15/.
7. FAO THE STATE OF FOOD AND AGRICULTURE (SOFA) Available online: http://www.fao.org/3/a-i6030e.pdf.
8. Mariani, L.; Ferrante, A. Agronomic Management for Enhancing Plant Tolerance to Abiotic Stresses—Drought, Salinity, Hypoxia, and Lodging. *Horticulturae* **2017**, *3*, 52.
9. Mou, B. Improvement of horticultural crops for abiotic stress tolerance: An introduction. *HortScience* **2011**, *46*, 1068–1069.
10. Parajuli, R.; Thoma, G.; Matlock, M.D. Environmental sustainability of fruit and vegetable production supply chains in the face of climate change: A review. *Sci. Total Environ.* **2019**, *650*, 2863–2879.
11. Bisbis, M.B.; Gruda, N.; Blanke, M. Potential impacts of climate change on vegetable production and product quality – A review. *J. Clean. Prod.* **2018**, *170*, 1602–1620.
12. Mittler, R. Abiotic stress, the field environment and stress combination. *Trends Plant Sci.* **2006**, *11*, 15–19.
13. RIVERO, R.M.; MESTRE, T.C.; MITTLER, R.; RUBIO, F.; GARCIA-SANCHEZ, F.; MARTINEZ, V. The combined effect of salinity and heat reveals a specific physiological, biochemical and molecular response in tomato plants. *Plant. Cell Environ.* **2014**, *37*, 1059–1073.
14. Choudhury, F.K.; Rivero, R.M.; Blumwald, E.; Mittler, R. Reactive oxygen species, abiotic stress and stress combination. *Plant J.* **2017**, *90*, 856–867.
15. Pandey, P.; Irulappan, V.; Bagavathiannan, M. V.; Senthil-Kumar, M. Impact of Combined Abiotic and Biotic Stresses on Plant Growth and Avenues for Crop Improvement by Exploiting Physio-morphological Traits. *Front. Plant Sci.* **2017**, *8*, 1–15.

16. Atkinson, N.J.; Urwin, P.E. The interaction of plant biotic and abiotic stresses: from genes to the field. *J. Exp. Bot.* **2012**, *63*, 3523–3543.
17. Shannon, M.C.; Grieve, C.M. Tolerance of vegetable crops to salinity. *Sci. Hortic. (Amsterdam).* **1998**, *78*, 5–38.
18. FRUIT AND VEGETABLES FOR HEALTH INITIATIVE Available online: http://www.fao.org/3/a-i6807e.pdf.
19. Rao, N.K.S.; Laxman, R.H.; Shivashankara, K.S. Physiological and Morphological Responses of Horticultural Crops to Abiotic Stresses. In *Abiotic Stress Physiology of Horticultural Crops*; Rao, N.K.S., Shivashankara, K.S., Laxman, R.H., Eds.; Springer India: New Delhi, 2016; pp. 3–17 ISBN 978-81-322-2723-6.
20. Sánchez-Rodríguez, E.; Rubio-Wilhelmi, Mm.; Cervilla, L.M.; Blasco, B.; Rios, J.J.; Rosales, M.A.; Romero, L.; Ruiz, J.M. Genotypic differences in some physiological parameters symptomatic for oxidative stress under moderate drought in tomato plants. *Plant Sci.* **2010**, *178*, 30–40.
21. Mittova, V.; Tal, M.; Volokita, M.; Guy, M. Up-regulation of the leaf mitochondrial and peroxisomal antioxidative systems in response to salt-induced oxidative stress in the wild salt-tolerant tomato species Lycopersicon pennellii. *Plant, Cell Environ.* **2003**, *26*, 845–856.
22. Abedi, T.; Pakniyat, H. Antioxidant enzyme changes in response to drought stress in ten cultivars of oilseed rape (Brassica napus L.). *Czech J. Genet. Plant Breed.* **2010**, *46*, 27–34.
23. Venkateswarlu, B.; Shanker, A.K.; Shanker, C.; Maheswari, M. *Crop stress and its management: Perspectives and strategies*; 2012; Vol. 9789400722; ISBN 9789400722200.
24. Kusvuran, S.; Kiran, S.; Ellialtioglu, S.S. Antioxidant Enzyme Activities and Abiotic Stress Tolerance Relationship in Vegetable Crops. In *Abiotic and Biotic Stress in Plants - Recent Advances and Future Perspectives*; InTech, 2016.
25. You, J.; Chan, Z. ROS Regulation During Abiotic Stress Responses in Crop Plants. *Front. Plant Sci.* **2015**, *6*, 1–15.
26. Suzuki, N.; Koussevitzky, S.; Mittler, R.; Miller, G. ROS and redox signalling in the response of plants to abiotic stress. *Plant, Cell Environ.* **2012**, *35*, 259–270.
27. Das, K.; Roychoudhury, A. Reactive oxygen species (ROS) and response of antioxidants as Reactive oxygen species (ROS) and response of antioxidants as ROS- Kaushik Das , Aryadeep Roychoudhury. **2014**, *2*, 1–13.
28. Mittler, R.; Vanderauwera, S.; Suzuki, N.; Miller, G.; Tognetti, V.B.; Vandepoele, K.; Gollery, M.; Shulaev, V.; Van Breusegem, F. ROS signaling: The new wave? *Trends Plant Sci.* **2011**, *16*,

300–309.

29. Wang, Y.; Frei, M. Stressed food - The impact of abiotic environmental stresses on crop quality. *Agric. Ecosyst. Environ.* **2011**, *141*, 271–286.
30. Oh, M.-M.; Carey, E.E.; Rajashekar, C.B. Regulated Water Deficits Improve Phytochemical Concentration in Lettuce. *J. Am. Soc. Hortic. Sci.* **2019**, *135*, 223–229.
31. Ripoll, J.; Urban, L.; Staudt, M.; Lopez-Lauri, F.; Bidel, L.P.R.; Bertin, N. Water shortage and quality of fleshy fruits-making the most of the unavoidable. *J. Exp. Bot.* **2014**, *65*, 4097–4117.
32. Hasanuzzaman, M.; Nahar, K.; Alam, M.M.; Roychowdhury, R.; Fujita, M. Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. *Int. J. Mol. Sci.* **2013**, *14*, 9643–9684.
33. Xu, Z.; Jiang, Y.; Zhou, G. Response and adaptation of photosynthesis, respiration, and antioxidant systems to elevated CO2 with environmental stress in plants. *Front. Plant Sci.* **2015**, *6*, 1–17.
34. Bechtold, U.; Field, B. Molecular mechanisms controlling plant growth during abiotic stress. *J. Exp. Bot.* **2018**, *69*, 2753–2758.
35. Schwarz, D.; Rouphael, Y.; Colla, G.; Venema, J.H. Grafting as a tool to improve tolerance of vegetables to abiotic stresses: Thermal stress, water stress and organic pollutants. *Sci. Hortic. (Amsterdam).* **2010**, *127*, 162–171.
36. Al-Harbi, A.R.; Al-Omran, A.M.; Alharbi, K. Grafting improves cucumber water stress tolerance in Saudi Arabia. *Saudi J. Biol. Sci.* **2018**, *25*, 298–304.
37. Kumar, P.; Rouphael, Y.; Cardarelli, M.; Colla, G. Vegetable Grafting as a Tool to Improve Drought Resistance and Water Use Efficiency. *Front. Plant Sci.* **2017**, *8*.
38. Wang, W.; Vinocur, B.; Altman, A. Plant responses to drought, salinity and extreme temperatures: Towards genetic engineering for stress tolerance. *Planta* **2003**, *218*, 1–14.
39. Zhuang, J.; Zhang, J.; Hou, X.L.; Wang, F.; Xiong, A.S. Transcriptomic, Proteomic, Metabolomic and Functional Genomic Approaches for the Study of Abiotic Stress in Vegetable Crops. *CRC. Crit. Rev. Plant Sci.* **2014**, *33*, 225–237.
40. Wang, H.; Wang, H.; Shao, H.; Tang, X. Recent Advances in Utilizing Transcription Factors to Improve Plant Abiotic Stress Tolerance by Transgenic Technology. *Front. Plant Sci.* **2016**, *7*, 1–13.
41. Singhal, P.; Jan, A.T.; Azam, M.; Haq, Q.M.R. Plant abiotic stress: a prospective strategy of exploiting promoters as alternative to overcome the escalating burden. *Front. Life Sci.* **2016**, *9*, 52–63.

42. Sade, N.; Del Mar Rubio-Wilhelmi, M.; Umnajkitikorn, K.; Blumwald, E. Stress-induced senescence and plant tolerance to abiotic stress. *J. Exp. Bot.* **2018**, *69*, 845–853.
43. Ahmad, P.; Prasad, M.N.V. *Abiotic stress responses in plants: metabolism, productivity and sustainability*; Springer Science & Business Media, 2011; ISBN 146140634X.
44. Shah, L.R.; Sharma, A.; Nabi, J.; Prasad, J. Breeding approaches for abiotic stress management in vegetable crops. *J. Pharmacogn. Phytochem.* **2018**, *7*, 1023–1028.
45. Mª Pérez-Clemente, R.; Gomez-Cadenas, A. In vitro Tissue Culture, a Tool for the Study and Breeding of Plants Subjected to Abiotic Stress Conditions. In *Recent Advances in Plant in vitro Culture*; InTech, 2012; p. 13.
46. Rai, M.K.; Kalia, R.K.; Singh, R.; Gangola, M.P.; Dhawan, A.K. Developing stress tolerant plants through in vitro selection-An overview of the recent progress. *Environ. Exp. Bot.* **2011**, *71*, 89–98.
47. Van Oosten, M.J.; Pepe, O.; De Pascale, S.; Silletti, S.; Maggio, A. The role of biostimulants and bioeffectors as alleviators of abiotic stress in crop plants. *Chem. Biol. Technol. Agric.* **2017**, *4*, 1–12.
48. Yamauchi, Y. Integrated Chemical Control of Abiotic Stress Tolerance Using Biostimulants. In *Plant, Abiotic Stress and Responses to Climate Change*; InTech, 2018; pp. 133–143.
49. Colla, G.; Rouphael, Y. Biostimulants in horticulture. *Sci. Hortic. (Amsterdam).* **2015**, *196*, 1–2.
50. Povero, G.; Mejia, J.F.; Di Tommaso, D.; Piaggesi, A.; Warrior, P. A Systematic Approach to Discover and Characterize Natural Plant Biostimulants. *Front. Plant Sci.* **2016**, *7*, 1–9.
51. Traon, D.; Amat, L.; Zotz, F.; du Jardin, P. A Legal Framework for Plant Biostimulants and Agronomic Fertiliser Additives in the EU, Report for the European Commission Enterprise & Industry Directorate - General. **2014**, 133.
52. Biostimulants Market Size Worth $4.14 Billion By 2025 | CAGR: 10.2% Available online: https://www.grandviewresearch.com/press-release/global-biostimulants-market.
53. EBIC WELCOMES COMPROMISE REACHED AT TRILOGUE MEETING ON FERTILISING PRODUCTS REGULATION, FIRST STEP TOWARDS EU-WIDE MARKET CREATION FOR BIOSTIMULANTS Available online: http://www.biostimulants.eu/2018/11/ebic-welcomes-compromise-reached-at-trilogue-meeting-on-fertilising-products-regulation-first-step-towards-eu-wide-market-creation-for-biostimulants/.
54. EP APPROVES FPR: A GIANT LEAP FOR BIOSTIMULANTS Available online: http://www.biostimulants.eu/2019/03/ep-approves-fpr-a-giant-leap-for-biostimulants/.

55. Yakhin, O.I.; Lubyanov, A.A.; Yakhin, I.A.; Brown, P.H. Biostimulants in Plant Science: A Global Perspective. *Front. Plant Sci.* **2017**, *7*.

56. du Jardin, P. Plant biostimulants: Definition, concept, main categories and regulation. *Sci. Hortic. (Amsterdam).* **2015**, *196*, 3–14.

57. Rouphael, Y.; Colla, G. Synergistic Biostimulatory Action: Designing the Next Generation of Plant Biostimulants for Sustainable Agriculture. *Front. Plant Sci.* **2018**, *9*, 1–7.

58. Paul, K.; Sorrentino, M.; Lucini, L.; Rouphael, Y.; Cardarelli, M.; Bonini, P.; Reynaud, H.; Canaguier, R.; Trtílek, M.; Panzarová, K.; et al. Understanding the Biostimulant Action of Vegetal-Derived Protein Hydrolysates by High-Throughput Plant Phenotyping and Metabolomics: A Case Study on Tomato. *Front. Plant Sci.* **2019**, *10*, 1–17.

59. Bulgari, R.; Morgutti, S.; Cocetta, G.; Negrini, N.; Farris, S.; Calcante, A.; Spinardi, A.; Ferrari, E.; Mignani, I.; Oberti, R.; et al. Evaluation of Borage Extracts As Potential Biostimulant Using a Phenomic, Agronomic, Physiological, and Biochemical Approach. *Front. Plant Sci.* **2017**, *8*, 1–16.

60. Dalal, A.; Bourstein, R.; Haish, N.; Shenhar, I.; Wallach, R.; Moshelion, M.; Sciences, P.; Sciences, W. A High-Throughput Physiological Functional Phenotyping System for Time- and Cost-Effective Screening of Potential. **2019**.

61. Nardi, S.; Carletti, P.; Pizzeghello, D.; Muscolo, A. Biological activities of humic substances. In *Biophysico-Chemical Processes Involving Natural Nonliving Organic Matter in Environmental Systems*; 2009; pp. 305–340 ISBN 0470494948, 9780470494943.

62. Bulgari, R.; Cocetta, G.; Trivellini, A.; Vernieri, P.; Ferrante, A. Biostimulants and crop responses: A review. *Biol. Agric. Hortic.* **2015**, *31*, 1–17.

63. Caradonia, F.; Battaglia, V.; Righi, L.; Pascali, G.; La Torre, A. Plant Biostimulant Regulatory Framework: Prospects in Europe and Current Situation at International Level. *J. Plant Growth Regul.* **2018**.

64. Canellas, L.P.; Olivares, F.L.; Okorokova-Facanha, A.L.; Facanha, A.R. Humic Acids Isolated from Earthworm Compost Enhance Root Elongation, Lateral Root Emergence, and Plasma Membrane H+-ATPase Activity in Maize Roots. *PLANT Physiol.* **2002**, *130*, 1951–1957.

65. Trevisan, S.; Francioso, O.; Quaggiotti, S.; Nardi, S. Humic substances biological activity at the plant-soil interface: From environmental aspects to molecular factors. *Plant Signal. Behav.* **2010**, *5*, 635–643.

66. Canellas, L.P.; Olivares, F.L.; Aguiar, N.O.; Jones, D.L.; Nebbioso, A.; Mazzei, P.; Piccolo, A. Humic and fulvic acids as biostimulants in horticulture. *Sci. Hortic. (Amsterdam).* **2015**, *196*,

15–27.

67. Nardi, S.; Pizzeghello, D.; Schiavon, M.; Ertani, A. Scientia Agricola Plant biostimulants: physiological responses induced by protein hydrolyzed-based. *Sci. Agric. v* **2016**, *73*, 18–23.
68. Canellas, L.P.; Dobbss, L.B.; Oliveira, A.L.; Chagas, J.G.; Aguiar, N.O.; Rumjanek, V.M.; Novotny, E.H.; Olivares, F.L.; Spaccini, R.; Piccolo, A. Chemical properties of humic matter as related to induction of plant lateral roots. *Eur. J. Soil Sci.* **2012**, *63*, 315–324.
69. Trevisan, S.; Botton, A.; Vaccaro, S.; Vezzaro, A.; Quaggiotti, S.; Nardi, S. Humic substances affect Arabidopsis physiology by altering the expression of genes involved in primary metabolism, growth and development. *Environ. Exp. Bot.* **2011**, *74*, 45–55.
70. Nardi, S.; Pizzeghello, D.; Reniero, F.; Rascio, N. Chemical and Biochemical Properties of Humic Substances Isolated from Forest Soils and Plant Growth. *Soil Sci. Soc. Am. J.* **2000**, *64*, 639.
71. Nardi, S.; Pizzeghello, D.; Muscolo, A.; Vianello, A. Physiological effects of humic substances on higher plants. *Soil Biol. Biochem.* **2002**, *34*, 1527–1536.
72. Dobbss, L.B.; Canellas, L.P.; Olivares, F.L.; Aguiar, N.O.; Peres, L.E.P.; Azevedo, M.; Spaccini, R.; Piccolo, A.; Façanha, A.R. Bioactivity of chemically transformed humic matter from vermicompost on plant root growth. *J. Agric. Food Chem.* **2010**, *58*, 3681–3688.
73. Norrie, J.; Keathley, J.P. Benefits of Ascophyllum Nodosum Marine-Plant Extract Applications To ´Thompson Seedless´ Grape Production. *Acta Hortic.* **2006**, 243–248.
74. Gajc-Wolska, J.; Spiewski, T.; Grabowska, A. The Effect of Seaweed Extracts on the Yield and Quality Parameters of Broccoli (Brassica oleracea var. cymosa L.) in Open Field Production. *Acta Hortic.* **2013**, *1009*, 83–90.
75. Sharma, H.S.S.; Fleming, C.; Selby, C.; Rao, J.R.; Martin, T. Plant biostimulants: A review on the processing of macroalgae and use of extracts for crop management to reduce abiotic and biotic stresses. *J. Appl. Phycol.* **2014**, *26*, 465–490.
76. Hamza, B.B.; Suggars, A. Biostimulants : Myths and Realities. *TurfGrass Trends* **2001**, 6–10.
77. Battacharyya, D.; Babgohari, M.Z.; Rathor, P.; Prithiviraj, B. Seaweed extracts as biostimulants in horticulture. *Sci. Hortic. (Amsterdam).* **2015**, *196*, 39–48.
78. Oancea, F.; Velea, S.; Mincea, C.; Ilie, L. Micro-algae based plant biostimulant and its effect on water stressed tomato plants. *Rom. J. Plant Prot.* **2013**, *VI*, 104–117.
79. Mógor, Á.F.; Ördög, V.; Lima, G.P.P.; Molnár, Z.; Mógor, G. Biostimulant properties of cyanobacterial hydrolysate related to polyamines. *J. Appl. Phycol.* **2018**, *30*, 453–460.
80. Chiaiese, P.; Corrado, G.; Colla, G.; Kyriacou, M.C.; Rouphael, Y. Renewable Sources of Plant

Biostimulation: Microalgae as a Sustainable Means to Improve Crop Performance. *Front. Plant Sci.* **2018**, *9*, 1–6.

81. Colla, G.; Nardi, S.; Cardarelli, M.; Ertani, A.; Lucini, L.; Canaguier, R.; Rouphael, Y. Protein hydrolysates as biostimulants in horticulture. *Sci. Hortic. (Amsterdam).* **2015**, *196*, 28–38.
82. Cerdán, M.; Sánchez-Sánchez, A.; Oliver, M.; Juárez, M.; Sánchez-Andreu, J.J. Effect of foliar and root applications of amino acids on iron uptake by tomato plants. *Acta Hortic.* **2009**, *830*, 481–488.
83. Lisiecka, J. The effect of animal protein hydrolysate on quantity and quality of strawberry daughter plants cv . ' Elsanta ' THE EFFECT OF ANIMAL PROTEIN HYDROLYSATE ON QUANTITY AND QUALITY OF STRAWBERRY DAUGHTER PLANTS CV . ' ELSANTA ' Jolanta Lisiecka , Miko ł aj Kn. **2011**, 30–40.
84. Botta, A. Enhancing plant tolerance to temperature stress with amino acids: An approach to their mode of action. *Acta Hortic.* **2012**, *1009*, 29–36.
85. Lucini, L.; Rouphael, Y.; Cardarelli, M.; Canaguier, R.; Kumar, P.; Colla, G. The effect of a plant-derived biostimulant on metabolic profiling and crop performance of lettuce grown under saline conditions. *Sci. Hortic. (Amsterdam).* **2015**, *182*, 124–133.
86. Rouphael, Y.; Cardarelli, M.; Bonini, P.; Colla, G. Synergistic Action of a Microbial-based Biostimulant and a Plant Derived-Protein Hydrolysate Enhances Lettuce Tolerance to Alkalinity and Salinity. *Front. Plant Sci.* **2017**, *08*, 1–12.
87. Colla, G.; Hoagland, L.; Ruzzi, M.; Cardarelli, M.; Bonini, P.; Canaguier, R.; Rouphael, Y. Biostimulant Action of Protein Hydrolysates: Unraveling Their Effects on Plant Physiology and Microbiome. *Front. Plant Sci.* **2017**, *8*, 1–14.
88. Trevisan, S.; Manoli, A.; Quaggiotti, S. A Novel Biostimulant, Belonging to Protein Hydrolysates, Mitigates Abiotic Stress Effects on Maize Seedlings Grown in Hydroponics. *Agronomy* **2019**, *9*, 28.
89. Ruzzi, M.; Aroca, R. Plant growth-promoting rhizobacteria act as biostimulants in horticulture. *Sci. Hortic. (Amsterdam).* **2015**, *196*, 124–134.
90. Turan, M.; Yildirim, E.; Kitir, N.; Unek, C.; Nikerel, E.; Ozdemir, B.S.; Güne\cs, A.; N.E.P, M. Beneficial Role of Plant Growth-Promoting Bacteria in Vegetable Production Under Abiotic Stress. In *Microbial Strategies for Vegetable Production*; Zaidi, A., Khan, M.S., Eds.; Springer International Publishing: Cham, 2017; pp. 151–166 ISBN 978-3-319-54401-4.
91. Xu, L.; Geelen, D. Developing Biostimulants From Agro-Food and Industrial By-Products. *Front. Plant Sci.* **2018**, *9*, 1–13.

92. Ertani, A.; Schiavon, M.; Altissimo, A.; Franceschi, C.; Nardi, S. Phenol-containing organic substances stimulate phenylpropanoid metabolism in Zea mays. *J. Plant Nutr. Soil Sci.* **2011**, *174*, 496–503.

93. Juárez-Maldonado, A.; Ortega-Ortíz, H.; Morales-Díaz, A.; González-Morales, S.; Morelos-Moreno, Á.; Cabrera-De la Fuente, M.; Sandoval-Rangel, A.; Cadenas-Pliego, G.; Benavides-Mendoza, A. Nanoparticles and Nanomaterials as Plant Biostimulants. *Int. J. Mol. Sci.* **2019**, *20*, 162.

94. Qi, M.; Liu, Y.; Li, T. Nano-TiO2 Improve the Photosynthesis of Tomato Leaves under Mild Heat Stress. *Biol. Trace Elem. Res.* **2013**, *156*, 323–328.

95. Kiapour, H.; Moaveni, P.; Habibi, D.; Sani, B. Evaluation of the application of gibbrellic acid and titanium dioxide nanoparticles under drought stress on some traits of basil (Ocimum basilicum L.). *Int. J. Agron. Agric. Res* **2015**, *6*, 138–150.

96. Lei, Z.; Mingyu, S.; Xiao, W.; Chao, L.; Chunxiang, Q.; Liang, C.; Hao, H.; Xiaoqing, L.; Fashui, H. Antioxidant Stress is Promoted by Nano-anatase in Spinach Chloroplasts Under UV-B Radiation. *Biol. Trace Elem. Res.* **2008**, *121*, 69–79.

97. Khan, M.N. Nano-titanium Dioxide (Nano-TiO2) Mitigates NaCl Stress by Enhancing Antioxidative Enzymes and Accumulation of Compatible Solutes in Tomato (Lycopersicon esculentum Mill.). *J. Plant Sci.* **2016**, *11*, 1–11.

98. Raliya, R.; Nair, R.; Chavalmane, S.; Wang, W.-N.; Biswas, P. Mechanistic evaluation of translocation and physiological impact of titanium dioxide and zinc oxide nanoparticles on the tomato (Solanum lycopersicum L.) plant. *Metallomics* **2015**, *7*, 1584–1594.

99. Abbas, S.M.; Akladious, S.A. Application of carrot root extract induced salinity tolerance in cowpea (Vigna sinensis L.) seedlings. *Pakistan J. Bot.* **2013**, *45*, 795–806.

100. Khan, W.; Rayirath, U.P.; Subramanian, S.; Jithesh, M.N.; Rayorath, P.; Hodges, D.M.; Critchley, A.T.; Craigie, J.S.; Norrie, J.; Prithiviraj, B. Seaweed Extracts as Biostimulants of Plant Growth and Development. *J. Plant Growth Regul.* **2009**, *28*, 386–399.

101. Vernieri, P.; Borghesi, E.; Ferrante, A. Application of Biostimulants in Floating System for Improving Rocket Quality Bioactive Compounds Biosynthesis and Metabolism in Fruit and Vegetables View project Plant responses to biostimulants application View project. **2005**, 1–4.

102. Vernieri, P.; Borghesi, E.; Tognoni, F.; Serra, G.; Ferrante, A.; Piaggesi, A. Use of biostimulants for reducing nutrient solution concentration in floating system. *Acta Hortic.* **2006**, *718*, 477–484.

103. Bulgari, R.; Podetta, N.; Cocetta, G.; Piaggesi, A.; Ferrante, A. The effect of a complete fertilizer for leafy vegetables production in family and urban gardens. *Bulg. J. Agric. Sci.* **2014**, *20*, 1361–1367.

104. Shehata, S.M.; Abou El-Yazied, A. Effect of Foliar Spraying with Amino Acids and Seaweed Extract on Growth Chemical Constitutes, Yield and its Quality of Celeriac Plant Effect of Bio-stimulants on Yield and Quality of Head Lettuce Grown Under Two Sources of Nitrogen View project. *Eur. J. Sci. Res.* **2011**, *58*, 257–265.

105. Yildirim, E.; Karlidag, H.; Turan, M.; Dursun, A.; Goktepe, F. Promotion of Broccoli by Plant Growth Promoting Rhizobacteria. *Hort Sci.* **2011**, *46*, 932–936.

106. Narendra Babu, A.; Jogaiah, S.; Ito, S.; Kestur Nagaraj, A.; Tran, L.-S.P. Improvement of growth, fruit weight and early blight disease protection of tomato plants by rhizosphere bacteria is correlated with their beneficial traits and induced biosynthesis of antioxidant peroxidase and polyphenol oxidase. *Plant Sci.* **2015**, *231*, 62–73.

107. Karlidag, H.; Yildirim, E.; Turan, M.; Pehluvan, M.; Donmez, F. Plant growth-promoting rhizobacteria mitigate deleterious effects of salt stress on strawberry plants (Fragaria × ananassa). *HortScience* **2013**, *48*, 563–567.

108. Han, H.S.; Lee, K.D. Plant Growth Promoting Rhizobacteria Effect on Antioxidant Status, Photosynthesis, Mineral Uptake and growth of lettuce under soil salinity. *Res. J. Agric. Biol. Sci. 1(3)* **2005**, *1*, 210–215.

109. Fasciglione, G.; Casanovas, E.M.; Yommi, A.; Sueldo, R.J.; Barassi, C.A. Azospirillum improves lettuce growth and transplant under saline conditions. *J. Sci. Food Agric.* **2012**, *92*, 2518–2523.

110. Rengasamy, K.R.R.; Kulkarni, M.G.; Pendota, S.C.; Van Staden, J. Enhancing growth, phytochemical constituents and aphid resistance capacity in cabbage with foliar application of eckol - a biologically active phenolic molecule from brown seaweed. *N. Biotechnol.* **2016**, *33*, 273–279.

111. Abdalla, M.M. The potential of Moringa oleifera extract as a biostimulant in enhancing the growth, biochemical and hormonal contents in rocket (Eruca vesicaria subsp. sativa) plants. *Int. J. Plant Physiol. Biochem.* **2013**, *5*, 42–49.

112. Abou-Sreea, A.I..; Matter, F.M.. . USING MORINGA LEAF EXTRACT AS BIOSTIMULANT AND GIBERRELLIC ACID FOR ENHANCING FENNEL (Foeniculum vulgare var . azoricum Mill .) GROWTH AND OIL YIELD. *Acta Sci. Intellectus* **2016**, *2*.

113. Abdel-Rahman, S.S.A.; Abdel-Kader, A.A.S. Response of Fennel (Foeniculum vulgare, Mill)

plants to foliar application of moringa leaf extract and benzyladenine (BA). *South African J. Bot.* **2019**, 1–10.

114. Abd El-Mageed, T.A.; Semida, W.M.; Rady, M.M. Moringa leaf extract as biostimulant improves water use efficiency, physio-biochemical attributes of squash plants under deficit irrigation. *Agric. Water Manag.* **2017**, *193*, 46–54.
115. Ullah, A.; Ali, S.; Shah, S.M. Influence of foliar application of bio-stimulants on growth, yield and chemical composition of tomato Influence of foliar application of bio-stimulants on growth , yield and chemical composition of tomato. *Int. J. Biosci.* **2019**, *14*, 309–316.
116. Luziatelli, F.; Ficca, A.G.; Colla, G.; Baldassarre Švecová, E.; Ruzzi, M. Foliar Application of Vegetal-Derived Bioactive Compounds Stimulates the Growth of Beneficial Bacteria and Enhances Microbiome Biodiversity in Lettuce. *Front. Plant Sci.* **2019**, *10*, 1–16.
117. Kulkarni, M.G.; Rengasamy, K.R.R.; Pendota, S.C.; Gruz, J.; Plačková, L.; Novák, O.; Doležal, K.; Van Staden, J. Bioactive molecules derived from smoke and seaweed Ecklonia maxima showing phytohormone-like activity in Spinacia oleracea L. *N. Biotechnol.* **2019**, *48*, 83–89.
118. Mattner, S.W.; Wite, D.; Riches, D.A.; Porter, I.J.; Arioli, T. The effect of kelp extract on seedling establishment of broccoli on contrasting soil types in southern Victoria, Australia. *Biol. Agric. Hortic.* 2013, *29*, 258–270.
119. Paradiković, N.; Vinković, T.; Vinković Vrček, I.; Žuntar, I.; Bojić, M.; Medić-Šarić, M. Effect of natural biostimulants on yield and nutritional quality: An example of sweet yellow pepper (Capsicum annuum L.) plants. *J. Sci. Food Agric.* **2011**, *91*, 2146–2152.
120. Petrozza, A.; Summerer, S.; Tommaso, G. Di; Tommaso, D. Di; Piaggesi, A. An evaluation of tomato plant root development and Morpho- Physiological response treated with VIVA® by image analysis. *Acta Hortic.* **2013**, *1009*, 155–160.
121. Petrozza, A.; Summerer, S.; Tommaso, G. Di; Tommaso, D. Di; Piaggesi, A. Evaluation of the Effect of RADIFARM® treatment on the Morpho - Physiological characteristics of root systems via image analysis. *Acta Hortic.* **2013**, *1009*, 149–154.
122. Kumar, S.; Chinnannan, K.; Thamilarasan, S.K.; Seralathan, M.; Shanmuganathan, R.; Padikasan, I.A. Enzymatically hydrolysed sago bagasse improves physiological, biochemical and molecular attributes of Solanum lycopersicum. *Biocatal. Agric. Biotechnol.* **2019**, *17*, 499–506.
123. Kunicki, E.; Grabowska, A.; Sękara, A.; Wojciechowska, R. The effect of cultivar type, time of cultivation, and biostimulant treatment on the yield of spinach (Spinacia oleracea L.). *Folia Hortic.* **2010**, *22*, 9–13.

124. Pokluda, R.; Sękara, A.; Jezdinský, A.; Kalisz, A.; Neugebauerová, J.; Grabowska, A. The physiological status and stress biomarker concentration of Coriandrum sativum L. plants subjected to chilling are modified by biostimulant application. *Biol. Agric. Hortic.* **2016**, *32*, 258–268.

125. Le Mire, G.; Nguyen, M.L.; Fassotte, B.; du Jardin, P.; Verheggen, F.; Delaplace, P.; Jijakli, M.H. Implementing plant biostimulants and biocontrol strategies in the agroecological management of cultivated ecosystems. A review. *Biotechnol. Agron. Soc. Environ.* **2016**, *20*, 299–313.

126. Cao, K.; Yu, J.; Xu, D.; Ai, K.; Bao, E.; Zou, Z. Exposure to lower red to far-red light ratios improve tomato tolerance to salt stress. *BMC Plant Biol.* **2018**, *18*, 10–15.

127. Martinez, V.; Nieves-Cordones, M.; Lopez-Delacalle, M.; Rodenas, R.; Mestre, T.; Garcia-Sanchez, F.; Rubio, F.; Nortes, P.; Mittler, R.; Rivero, R. Tolerance to Stress Combination in Tomato Plants: New Insights in the Protective Role of Melatonin. *Molecules* **2018**, *23*, 535.

128. Ashraf, M.A.; Akbar, A.; Askari, S.H.; Iqbal, M.; Rasheed, R.; Hussain, I. Recent Advances in Abiotic Stress Tolerance of Plants Through Chemical Priming: An Overview. In *Advances in Seed Priming*; Rakshit, A., Singh, H.B., Eds.; Springer Singapore: Singapore, 2018; pp. 51–79 ISBN 978-981-13-0032-5.

129. Subramanian, P.; Kim, K.; Krishnamoorthy, R.; Mageswari, A.; Selvakumar, G.; Sa, T. Cold stress tolerance in psychrotolerant soil bacteria and their conferred chilling resistance in tomato (solanum lycopersicum Mill.) under low temperatures. *PLoS One* **2016**, *11*, 1–17.

130. Subramanian, P.; Mageswari, A.; Kim, K.; Lee, Y.; Sa, T. Psychrotolerant Endophytic Pseudomonas sp. Strains OB155 and OS261 Induced Chilling Resistance in Tomato Plants (Solanum lycopersicum Mill.) by Activation of Their Antioxidant Capacity . *Mol. Plant-Microbe Interact.* **2015**, *28*, 1073–1081.

131. Marfà, O.; Cáceres, R.; Polo, J.; Ródenas, J. Animal protein hydrolysate as a biostimulant for transplanted strawberry plants subjected to cold stress. *Acta Hortic.* **2009**, *842*, 315–318.

132. Polo, J.; Barroso, R.; Ródenas, J.; Azcón-Bieto, J.; Cáceres, R.; Marfà, O. Porcine hemoglobin hydrolysate as a biostimulant for lettuce plants subjected to conditions of thermal stress. *Horttechnology* **2006**, *16*, 483–487.

133. Korkmaz, A.; Korkmaz, Y.; Demirkiran, A.R. Enhancing chilling stress tolerance of pepper seedlings by exogenous application of 5-aminolevulinic acid. *Environ. Exp. Bot.* **2010**, *67*, 495–501.

134. Challinor, A.J.; Watson, J.; Lobell, D.B.; Howden, S.M.; Smith, D.R.; Chhetri, N. A meta-

analysis of crop yield under climate change and adaptation. *Nat. Clim. Chang.* **2014**, *4*, 287–291.

135. Fahad, S.; Bajwa, A.A.; Nazir, U.; Anjum, S.A.; Farooq, A.; Zohaib, A.; Sadia, S.; Nasim, W.; Adkins, S.; Saud, S.; et al. Crop Production under Drought and Heat Stress: Plant Responses and Management Options. *Front. Plant Sci.* **2017**, *8*, 1–16.
136. Hasanuzzaman, M.; Nahar, K.; Fujit, M. Extreme Temperature Responses, Oxidative Stress and Antioxidant Defense in Plants. In *Abiotic Stress - Plant Responses and Applications in Agriculture*; InTech, 2013; pp. 169–205.
137. Camejo, D.; Rodríguez, P.; Morales, M.A.; Dell'Amico, J.M.; Torrecillas, A.; Alarcón, J.J. High temperature effects on photosynthetic activity of two tomato cultivars with different heat susceptibility. *J. Plant Physiol.* **2005**, *162*, 281–289.
138. Ogweno, J.O.; Song, X.S.; Shi, K.; Hu, W.H.; Mao, W.H.; Zhou, Y.H.; Yu, J.Q.; Nogués, S. Brassinosteroids Alleviate Heat-Induced Inhibition of Photosynthesis by Increasing Carboxylation Efficiency and Enhancing Antioxidant Systems in Lycopersicon esculentum. *J. Plant Growth Regul.* **2008**, *27*, 49–57.
139. El-Bassiony, A.M.; Ghoname, A.A.; El-Awadi, M.E.; Fawzy, Z.F.; Gruda, N. Ameliorative Effects of Brassinosteroids on Growth and Productivity of Snap Beans Grown Under High Temperature. *Gesunde Pflanz.* **2012**, *64*, 175–182.
140. Nahar, K.; Hasanuzzaman, M.; Alam, M.M.; Fujita, M. Exogenous glutathione confers high temperature stress tolerance in mung bean (Vigna radiata L.) by modulating antioxidant defense and methylglyoxal detoxification system. *Environ. Exp. Bot.* **2015**, *112*, 44–54.
141. Yang, J.D.; Yun, J.Y.; Zhang, T.H.; Zhao, H.L. Presoaking with nitric oxide donor SNP alleviates heat shock damages in mung bean leaf discs. *Bot. Stud.* **2006**, *47*, 129–136.
142. Kumar, S.; Kaur, R.; Kaur, N.; Bhandhari, K.; Kaushal, N.; Gupta, K.; Bains, T.S.; Nayyar, H. Heat-stress induced inhibition in growth and chlorosis in mungbean (Phaseolus aureus Roxb.) is partly mitigated by ascorbic acid application and is related to reduction in oxidative stress. *Acta Physiol. Plant.* **2011**, *33*, 2091.
143. Kaushal, N.; Gupta, K.; Bhandhari, K.; Kumar, S.; Thakur, P.; Nayyar, H. Proline induces heat tolerance in chickpea (Cicer arietinum L.) plants by protecting vital enzymes of carbon and antioxidative metabolism. *Physiol. Mol. Biol. Plants* **2011**, *17*, 203–213.
144. Kumar, S.; Kaushal, N.; Nayyar, H.; Gaur, P. Abscisic acid induces heat tolerance in chickpea (Cicer arietinum L.) seedlings by facilitated accumulation of osmoprotectants. *Acta Physiol. Plant.* **2012**, *34*, 1651–1658.

145. Viégas, R.A.; Silveira, J.A.G. da; Lima Junior, A.R. de; Queiroz, J.E.; Fausto, M.J.M. Effects of NaCl-salinity on growth and inorganic solute accumulation in young cashew plants. *Rev. Bras. Eng. Agrícola e Ambient.* **2006**, *5*, 216–222.

146. Colla, G.; Rouphael, Y.; Leonardi, C.; Bie, Z. Role of grafting in vegetable crops grown under saline conditions. *Sci. Hortic. (Amsterdam).* **2010**, *127*, 147–155.

147. Miceli, A.; Moncada, A.; D'Anna, F. Effect of salt stress in lettuce cultivation. *Acta Hortic.* **2003**, *609*, 371–375.

148. Kaya, C.; Higgs, D.; Sakar, E. Response of two leafy vegetables grown at high salinity to supplementary potassium and phosphorus during different growth stages. *J. Plant Nutr.* **2002**, *25*, 2663–2676.

149. Taïbi, K.; Taïbi, F.; Ait Abderrahim, L.; Ennajah, A.; Belkhodja, M.; Mulet, J.M. Effect of salt stress on growth, chlorophyll content, lipid peroxidation and antioxidant defence systems in Phaseolus vulgaris L. *South African J. Bot.* **2016**, *105*, 306–312.

150. Yarsi, G.; Sivaci, A.; Dasgan, H.Y.; Altuntas, O.; Binzet, R.; Akhoundnejad, Y. Effects of salinity stress on chlorophyll and carotenoid contents and stomata size of grafted and ungrafted galia C8 melon cultivar. *Pakistan J. Bot.* **2017**, *49*, 421–426.

151. Munns, R.; Termaat, A. Whole-plant responses to salinity. *Aust. J. Plant Physiol.* **1986**, *13*, 143–160.

152. Blaylock, A.D. Soil salinity, salt tolerance, and growth potential of horticultural and landscape plants. *Univ. Wyoming Coop. Ext. Serv. B-988* **1994**.

153. Maas, E. V.; R., G.S. Crop yields as affected by salinity. *Agronomy* **1999**, *38*, 55–110.

154. Shaheen, S.; Naseer, S.; Ashraf, M.; Akram, N.A. Salt stress affects water relations, photosynthesis, and oxidative defense mechanisms in Solanum melongena L. *J. Plant Interact.* **2013**, *8*, 85–96.

155. Xu, C.; Mou, B. Responses of Spinach to Salinity and Nutrient Deficiency in Growth, Physiology, and Nutritional Value. *J. Am. Soc. Hortic. Sci.* **2016**, *141*, 12–21.

156. Bano, S.; Ashraf, M.; Akram, N.A.; Al-Qurainy, F. Regulation in some vital physiological attributes and antioxidative defense system in carrot (Daucus carota L.) under saline stress. *J. Appl. Bot. food Qual. = Angew. Bot. /* **2012**, *85*.

157. Agastian, P.; Kingsley, S.J.; Vivekanandan, M. Effect of salinity on photosynthesis and biochemical characteristics in mulberry genotypes. *Photosynthetica* **2000**, *38*, 287–290.

158. Sayyad-Amin, P.; Jahansooz, M.R.; Borzouei, A.; Ajili, F. Changes in photosynthetic pigments and chlorophyll-a fluorescence attributes of sweet-forage and grain sorghum cultivars under

salt stress. *J. Biol. Phys.* **2016**, *42*, 601–620.

159. Moud, A.M.; Maghsoudi, K. Salt Stress Effects on Respiration and Growth of Germinated Seeds of Different Wheat (Triticum aestivum L.) Cultivars. *World J. Agric. Sci.* **2008**, *4*, 351–358.
160. Flores, P.; Botella, M.Á.; Cerdá, A.; Martínez, V. Influence of nitrate level on nitrate assimilation in tomato (Lycopersicon esculentum) plants under saline stress . *Can. J. Bot.* **2004**, *82*, 207–213.
161. Breusegem, F. Van Reactive oxygen species in plant cell death. *Plant Physiol.* **2006**, *141*, 384–390.
162. Bulgari, R.; Trivellini, A.; Ferrante, A. Effects of Two Doses of Organic Extract-Based Biostimulant on Greenhouse Lettuce Grown Under Increasing NaCl Concentrations. *Front. Plant Sci.* **2019**, *9*, 1–14.
163. Barassi, C.A.; Ayrault, G.; Creus, C.M.; Sueldo, R.J.; Sobrero, M.T. Seed inoculation with Azospirillum mitigates NaCl effects on lettuce. *Sci. Hortic. (Amsterdam).* **2006**, *109*, 8–14.
164. Fasciglione, G.; Casanovas, E.M.; Quillehauquy, V.; Yommi, A.K.; Goñi, M.G.; Roura, S.I.; Barassi, C.A. Azospirillum inoculation effects on growth, product quality and storage life of lettuce plants grown under salt stress. *Sci. Hortic. (Amsterdam).* **2015**, *195*, 154–162.
165. del Amor, F.M.; Cuadra-Crespo, P. Plant growth-promoting bacteria as a tool to improve salinity tolerance in sweet pepper. *Funct. Plant Biol.* **2012**, *39*, 82–90.
166. Hamaoui, B.; Abbadi, J.M.; Burdman, S.; Rashid, A.; Sarig, S.; Okon, Y. Effects of inoculation with Azospirillum brasilense on chickpeas (Cicer arietinum) and faba beans (Vicia faba) under different growth conditions . *Agronomie* **2003**, *21*, 553–560.
167. Del Pilar Cordovilla, M.; Berrido, S.I.; Ligero, F.; Lluch, C. Rhizobium strain effects on the growth and nitrogen assimilation in Pisum sativum and Vicia faba plant growth under salt stress. *J. Plant Physiol.* **1999**, *154*, 127–131.
168. Mayak, S.; Tirosh, T.; Glick, B.R. Plant growth-promoting bacteria that confer resistance to water stress in tomatoes and peppers. *Plant Sci.* **2004**, *166*, 525–530.
169. Yildirim, E.; Taylor, A.G.; Spittler, T.D. Ameliorative effects of biological treatments on growth of squash plants under salt stress. *Sci. Hortic. (Amsterdam).* **2006**, *111*, 1–6.
170. Mesut Çimrin, K.; Türkmen, Ö.; Turan, M.; Tuncer, B. Phosphorus and humic acid application alleviate salinity stress of pepper seedling. *African J. Biotechnol.* **2010**, *9*, 5845–5851.
171. Adil Aydin Humic acid application alleviate salinity stress of bean (Phaseolus vulgaris L.) plants decreasing membrane leakage. *African J. Agric. Reseearch* **2012**, *7*.

172. DEMIR, K.; GÜNES, A.; INAL, A.; ALPASLAN, M. Effects of Humic Acids on the Yield and Mineral Nutrition of Cucumber (Cucumis Sativus L.) Grown With Different Salinity Levels. *Acta Hortic.* **1999**, 95–104.

173. Guinan, K.J.; Sujeeth, N.; Copeland, R.B.; Jones, P.W.; O'Brien, N.M.; Sharma, H.S.S.; Prouteau, P.F.J.; O'Sullivan, J.T. Discrete Roles for Extracts of Ascophyllum Nodosum in Enhancing Plant Growth and Tolerance To Abiotic and Biotic Stresses. *Acta Hortic.* **2013**, 127–135.

174. Ross, R.; Holden, D. Commercial extracts of the brown seaweed Ascophyllum nodosum enhance growth and yield of strawberries. *HortScience* **2010**, *45*, s141–s141.

175. EL Arroussi, H.; Benhima, R.; Elbaouchi, A.; Sijilmassi, B.; EL Mernissi, N.; Aafsar, A.; Meftah-Kadmiri, I.; Bendaou, N.; Smouni, A. Dunaliella salina exopolysaccharides: a promising biostimulant for salt stress tolerance in tomato (Solanum lycopersicum). *J. Appl. Phycol.* **2018**, *30*, 2929–2941.

176. Abdel Latef, A.A.H.; Srivastava, A.K.; Saber, H.; Alwaleed, E.A.; Tran, L.S.P. Sargassum muticum and Jania rubens regulate amino acid metabolism to improve growth and alleviate salinity in chickpea. *Sci. Rep.* **2017**, *7*, 1–12.

177. Rady, M.M.; Varma C., B.; Howladar, S.M. Common bean (Phaseolus vulgaris L.) seedlings overcome NaCl stress as a result of presoaking in Moringa oleifera leaf extract. *Sci. Hortic. (Amsterdam).* **2013**, *162*, 63–70.

178. Semida, W.M.; Rady, M.M. Presoaking application of propolis and maize grain extracts alleviates salinity stress in common bean (Phaseolus vulgaris L.). *Sci. Hortic. (Amsterdam).* **2014**, *168*, 210–217.

179. Rady, M.M.; Mohamed, G.F. Modulation of salt stress effects on the growth, physio-chemical attributes and yields of Phaseolus vulgaris L. plants by the combined application of salicylic acid and Moringa oleifera leaf extract. *Sci. Hortic. (Amsterdam).* **2015**, *193*, 105–113.

180. Howladar, S.M. A novel Moringa oleifera leaf extract can mitigate the stress effects of salinity and cadmium in bean (Phaseolus vulgaris L.) plants. *Ecotoxicol. Environ. Saf.* **2014**, *100*, 69–75.

181. Rady, M.M.; Desoky, E.S.M.; Elrys, A.S.; Boghdady, M.S. Can licorice root extract be used as an effective natural biostimulant for salt-stressed common bean plants? *South African J. Bot.* **2019**, *121*, 294–305.

182. Semida, W.M.; Abd El-Mageed, T.A.; Hemida, K.; Rady, M.M. Natural bee-honey based

biostimulants confer salt tolerance in onion via modulation of the antioxidant defence system. *J. Hortic. Sci. Biotechnol.* **2019**, *0*, 1–11.

183. Elliott, J.; Deryng, D.; Müller, C.; Frieler, K.; Konzmann, M.; Gerten, D.; Glotter, M.; Flörke, M.; Wada, Y.; Best, N.; et al. Constraints and potentials of future irrigation water availability on agricultural production under climate change. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 3239–44.
184. Calvo, P.; Nelson, L.; Kloepper, J.W. Agricultural uses of plant biostimulants. *Plant Soil* **2014**, *383*, 3–41.
185. Kałuzewicz, A.; Krzesiński, W.; Spizewski, T.; Zaworska, A. Effect of biostimulants on several physiological characteristics and chlorophyll content in broccoli under drought stress and re-watering. *Not. Bot. Horti Agrobot. Cluj-Napoca* **2017**, *45*, 197–202.
186. Xu, C.; Leskovar, D.I. Effects of A. nodosum seaweed extracts on spinach growth, physiology and nutrition value under drought stress. *Sci. Hortic. (Amsterdam).* **2015**, *183*, 39–47.
187. Goñi, O.; Quille, P.; O'Connell, S. Ascophyllum nodosum extract biostimulants and their role in enhancing tolerance to drought stress in tomato plants. *Plant Physiol. Biochem.* **2018**, *126*, 63–73.
188. Petrozza, A.; Santaniello, A.; Summerer, S.; Di Tommaso, G.; Di Tommaso, D.; Paparelli, E.; Piaggesi, A.; Perata, P.; Cellini, F. Physiological responses to Megafol® treatments in tomato plants under drought stress: A phenomic and molecular approach. *Sci. Hortic. (Amsterdam).* **2014**, *174*, 185–192.
189. Romero, A.M.; Vega, D.; Correa, O.S. Azospirillum brasilense mitigates water stress imposed by a vascular disease by increasing xylem vessel area and stem hydraulic conductivity in tomato. *Appl. Soil Ecol.* **2014**, *82*, 38–43.
190. Vurukonda, S.S.K.P.; Vardharajula, S.; Shrivastava, M.; SkZ, A. Enhancement of drought stress tolerance in crops by plant growth promoting rhizobacteria. *Microbiol. Res.* **2016**, *184*, 13–24.
191. Kumar, A.; Verma, J.P. Does plant—Microbe interaction confer stress tolerance in plants: A review? *Microbiol. Res.* **2018**, *207*, 41–52.
192. ARSHAD, M.; SHAHAROONA, B.; MAHMOOD, T. Inoculation with Pseudomonas spp. Containing ACC-Deaminase Partially Eliminates the Effects of Drought Stress on Growth, Yield, and Ripening of Pea (Pisum sativum L.). *Pedosphere* **2008**, *18*, 611–620.
193. Heidari, M.; Golpayegani, A. Effects of water stress and inoculation with plant growth promoting rhizobacteria (PGPR) on antioxidant status and photosynthetic pigments in basil

(Ocimum basilicum L.). *J. Saudi Soc. Agric. Sci.* **2012**, *11*, 57–61.

194. Halpern, M.; Bar-Tal, A.; Ofek, M.; Minz, D.; Muller, T.; Yermiyahu, U. The Use of Biostimulants for Enhancing Nutrient Uptake. In *Advances in Agronomy*; 2015; Vol. 130, pp. 141–174 ISBN 9780128021378.
195. Toscano, S.; Romano, D.; Massa, D.; Bulgari, R.; Franzoni, G.; Ferrante, A. Biostimulant applications in low input horticultural cultivation systems. *Italus Hortus* **2019**, *25*, 27–36.
196. Koleška, I.; Hasanagić, D.; Todorović, V.; Murtić, S.; Klokić, I.; Paradiković, N.; Kukavica, B. Biostimulant prevents yield loss and reduces oxidative damage in tomato plants grown on reduced NPK nutrition. *J. Plant Interact.* **2017**, *12*, 209–218.
197. Anjum, K.; Ahmed, M.; Baber, J.K.; Alizai, M.A.; Ahmed, N.; Tareen, M.H. Response of Garlic Bulb Yield To Bio-Stimulant (Bio-Cozyme) Under Calcareous Soil. *Life Sci. Int. J.* **2014**, *8*, 3058–3062.
198. Papenfus, H.B.; Kulkarni, M.G.; Stirk, W.A.; Finnie, J.F.; Van Staden, J. Effect of a commercial seaweed extract (Kelpak®) and polyamines on nutrient-deprived (N, P and K) okra seedlings. *Sci. Hortic. (Amsterdam).* **2013**, *151*, 142–146.
199. Spinelli, F.; Fiori, G.; Noferini, M.; Sprocatti, M.; Costa, G. A novel type of seaweed extract as a natural alternative to the use of iron chelates in strawberry production. *Sci. Hortic. (Amsterdam).* **2010**, *125*, 263–269.
200. Cerdán, M.; Sánchez-Sánchez, A.; Jordá, J.D.; Juárez, M.; Sánchez-Andreu, J. Effect of commercial amino acids on iron nutrition of tomato plants grown under lime-induced iron deficiency. *J. Plant Nutr. Soil Sci.* **2013**, *176*, 859–866.
201. Miflin, B.J.; Lea, P.J. The pathway of nitrogen assimilation in plants. *Phytochemistry.* **1976**, *15*, 873–885.
202. Porra, R.J. Invited Review Recent Progress in Porphyrin and Chlorophyll Biosynthesis. *Regulation* **1997**, *65*, 492–516.
203. Paradiković, N.; Vinković, T.; Vinković Vrček, I.; Tkalec, M. Natural biostimulants reduce the incidence of BER in sweet yellow pepper plants (Capsicum annuum L.). *Agric. Food Sci.* **2013**, *22*, 307–317.
204. Mayak, S.; Tirosh, T.; Glick, B.R. Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. *Plant Physiol. Biochem.* **2004**, *42*, 565–572.

Published as: Bulgari, R., Franzoni, G., & Ferrante, A. (2019). Biostimulants application in horticultural crops under abiotic stress conditions. Agronomy, 9(6), 306.

AIM

The purpose of the Ph. D. research project was to evaluate the potential as biostimulants of different products applied on leafy vegetable – rocket and lettuce – in response to abiotic stresses conditions.
Plants responses to stressful conditions, treatments and to the combination of the two factors have been evaluated both from a biochemical and a molecular point of view, with the aim to deepen the knowledge on the mechanism of action of these potential biostimulant.

The first part of the work was about the evaluation of the effect of aqueous extracts prepared from flowers and leaves of Borago officinalis L.. The study of borage extracts in our laboratory started few years before my Ph.D. and borage was tested as raw material for the production of extract to apply on plants as biostimulants. This plant was chosen since it is particularly rich in bioactive compounds already exploited in numerous fields. Borage extracts were prepared and tested on lettuce and rocket salad under non-stressful conditions and results obtained showed a biostimulant effect. In particular, flavonoids, phenols, proteins and photosynthetic pigments increased especially in response to flower extract. At the same time, a reduction of nitrates concentration due to the enhanced activity of NR was observed in rocket leaves. Based on these results, the first activity was the preparation of new borage extracts with the aim to evaluate the effect of different maceration times on their efficacy.
Afterwards, the efficacy of a borage extract was indagated on plants grown under stressful conditions. In particular, it was applied on rocket salad subjected to a period of high salinity. Plants responses induced by borage treatment and salt stress were evaluated through the gene expression analysis of different transcription factors involved in stress responses in rocket.

The second part of this work involved the collaboration with a private company to study the effectiveness of a biostimulant prototype on lettuce salad grown under different stressful conditions. In order to better understand the effect of the product we compared its effect with the effect of a glutamic acid solution, the most abundant ingredient. This approach aimed to see if the efficacy of the biostimulant prototype were only due to the presence of this amino acid.

The last part of the work was carried out at Cardiff University and was aimed to study of the effect of glutamic acid treatment on VOCs profile of rocket salad subjected to a period of high salinity.

CHAPTER 1

Effects of borage extracts prepared with different maceration times on rocket and lettuce.

INTRODUCTION

1. Plant extracts

Plants have been used since ancient time not only for food but also as a source of biologically active agents, typically for the treatment of diseases in traditional medicine of several cultures. They were applied on an empirical basis, without any knowledge on their activity or components. Botanical active substances are defined as one or more components found in plants and they are obtained by subjecting plants or parts of plants to different processes of extraction.

Even if a decline in interest for their application occurred in past years, plants offer a unique resource of botanical active substances due to their structural and biological diversity. Secondary metabolites plant composition might vary in the qualitative and quantitative traits because it is influenced by the plant growing environmental conditions. Nowadays, plant extracts are gaining much importance due to their potential and because of the change in consumers behaviour. Indeed, besides their medical or pharmaceutical use, plant extracts request is increasing as food and beverage additive and cosmetics. Moreover, their market includes also the application in food preservation industry and in agricultural sector as raw materials for agrochemical or biostimulant products.

One of the most investigated group of natural products is represented by secondary metabolites. They are defined as natural products that are not essential for vegetative growth, but they have an adaptive role in plants. For example, they could enable plants to resist pathogens, deter insect or other animal attack due to their toxic nature. They could be involved in signaling processes as defence mechanism regulator molecules, or in different type of communication to attract pollinators or in interplant. Generally, they exert an important function in ecological interaction between plant and the surrounding environment. Secondary metabolites can be divided into three distinct groups: Terpenes, Phenolics and N and S containing compounds [1–3]. Terpenes are generally insoluble in water and they are responsible of the odours of plants. They are classified as monoterpenes, sesquiterpenes, diterpene, triterpenes and polyterpenes, on the basis of their chemical structure. Phenolic compounds are one of the most studied class of secondary metabolites due to their wide range of biological functions. They are responsible of the colour of plants and they are involved in several physiological mechanism

during plant growth and reproduction. Based on their chemical formula, they can be divided into phenols, coumarins, lignins, lignans, tannins, phenolic acids and flavonoids. Nitrogen and sulphur containing secondary metabolites are mostly involved in plant defences mechanisms. The first class is represented by alkaloids, the second one includes GSH, GSL, phytoalexins, thionins, defensins and allinin.

1.1. Extraction methods

A wide range of extraction methods and technologies are available for the separation of these compounds from the inactive or inert components (Table 2) [4–6]. Most of these techniques are based on the extracting power of different solvents and the application of heat, pressure and/or mixing. Infusion, maceration, digestion, decoction, percolation, and Soxhlet extraction are the conventional extraction techniques. These methods use organic solvents (such as hexane, acetone, methanol, ethanol, etc.) or water and are generally carried out under atmospheric pressure. Alternative approaches include: Accelerated Solvent Extraction (ASE), Supercritical Fluid Extraction (SFE), Microwave Assisted Extraction (MAE), Ultrasound-Assisted Extraction (UAE), Subcritical Water Extraction (SWE), Pulsed-Electric Field extraction (PEF), Enzyme-Assisted Extraction (EAE), and Rapid Solid-Liquid Dynamic Extraction (RSLDE). These methods have emerged in order to mitigate limitations of the traditional ones, reducing the extraction time and the amount of organic solvents used. They also improve the yield and the bioactivities of the extracts.

Table 2. Techniques used for extraction of bioactive compounds from plant materials and main aspects related to them.

Extraction technique	**Main aspects**
Infusion	Fresh infusion is prepared by macerating the crude material for a short period of time with cold or boiling water [4].
Maceration	Maceration is a widely used technique. Plant materials is placed in a stoppered container with a solvent and allowed to stand at room temperature for a period of minimum 3 days with frequent agitation. The procedure lead to soften and break the plant's cell wall in order to release the soluble phytochemicals. After 3 days, the mixture is pressed and filtrated [4].
Digestion	It is a form of maceration technique in which heat is applied during the process of extraction. It is used when the solvent efficacy increases with the temperature without affecting the stability of the substances [4].
Decoction	The raw material is boiled in a specified volume of water for a defined time; then, the concentrated extract is cooled and strained or filtered. This procedure is suitable for extracting water-soluble, heat-stable constituents [4].
Percolation	This procedure is commonly used to extract active ingredients in the preparation of tinctures. After few hours of moisturizing in an appropriate menstruum, the raw material is packed in a percolator Additional menstruum is added and the mixture is let macerating for 24 hours. At the

	end of this period the solvent is percolated. The mixed liquid is filtrated or decanted [4].
Hot Continuous Extraction (Soxhlet)	This technique is used when the desired compound has a limited solubility in a solvent. The material is placed in a porous bag while the extracting solvent is placed in a different flask. The solvent is heated, its condensed vapours drips into the bag containing the raw material and the extraction of the compounds is by contact [4].
Aqueous Alcoholic Extraction by Fermentation	The crude material is soaked for a specified period of time, during which it undergoes fermentation and generates alcohol in situ; this facilitates the extraction of the active constituents contained in the plant material [4].
Counter-current extraction (CCE)	The wet raw material is and is moved within a cylindrical extractor where it is in contact with the extraction solvent. The process is highly efficient, and it require little time and posing no risk from high temperature [7].
Accelerated solvent extraction (ASE)	This technique also called pressurized liquid extraction or pressurized fluid extraction, because of the use of elevated temperature and pressure that allows the extraction process to be completed within a short time and with a small quantity of solvent. The process is similar to the hot continuous extraction [8].
Microwave assisted extraction (MAE)	MAE utilizes microwave energy with frequencies range of approximately 300 MHz to 1000 GHz Microwaves can cause temperature rise of an entire material, enhancing the migration of dissolved ions and promotes solvent penetration into the matrix. It is considered as selective methods that favour polar molecules and solvents with high dielectric constant [9].
Ultrasound Extraction (Sonication)	The procedure involves the use of ultrasound with frequencies ranging from 20 kHz to 2000 kHz; this increases the permeability of cell walls and produces cavitation. The mechanical effects of ultrasound provide a greater solvent penetration into cellular matrices and improve the transfer of mass because of micro-streaming process. It is an efficient extraction technique that drastically reduces process times, increases yields and often the quality of the extract It is able to disrupt the biological cell walls which release the cell contents Sometimes it can produce free radicals [10].
Supercritical Fluid Extraction (SFE)	The pure matter has a critical point corresponding to a given pressure and temperature. When it is subjected to a pressure and a temperature superior to those of its critical point, it is in a phase called "supercritical" (SC) or supercritical fluid (SF). The SF has an intermediate behaviour between the liquid state and the gaseous state. Absolutely, it has high density such as that of liquids, a coefficient of diffusivity between that of liquids and gases, and a low viscosity (as the one of gases) [11].
Phytonics Process	This process involves the use of a new non-toxic solvent based on hydrofluorocarbon-134a, having a boiling point of 25 °C and a vapour pressure of 5.6 bar at ambient temperature [4].
Subcritical water extraction (SWE)	Water is used with temperatures of between 100 °C and 374.1 °C (critical point of water) and with a certain pressure, which varies according to the temperature, and which maintains the water in its liquid form. SWE allows the extraction of medium-polar to non- polar molecules without the use of organic solvents. SWE can provide higher extraction yields in shorter extraction times than Soxhlet and SFE methods. However, the efficiency of extraction is influenced by factors such as temperature, pH, and pressure, among others [12].
Pulsed-Electric Field extraction (PEF)	The raw material is placed between two electrodes where high voltage pulse (20 – 80 kV/cm) are applied. The electric field affects the permeability of cell membrane, causes an increase of porosity and enhances the extraction of the intercellular substances [13].

Enzyme-Assisted Extraction (EAE)	This technique is based on the ability of enzymes to catalyse specific reactions. They are able to break cell walls and membranes, favouring the extraction of bioactive substances. EAE is eco-friendly technology that allows to reduce the amount of solvent [14].
Rapid Solid-Liquid Dynamic Extraction (RSLDE)	This method is based on the generation of a negative gradient pressure between the inside and the outside of the solid matrix. First a pressure of about 8 -10 atm is applied on the solid in order to let the liquid penetrate the solid. Then the pressure is removed and the liquid inside moves outside transporting the desired substances. It can be conducted at room temperature or sub room temperature [15].

The choice of the method, the solvent and the temperature, depends on different factors such as the nature of the targeted compounds (polarity or thermo-sensitivity), the organ of plants used, and the economic feasibility of the process to the particular situation. Moreover, also the time on the extraction affects both the yield and the composition of the extract. Thus, a standardized extraction method which take into consideration all these parameters is required in order to obtain a stable and high-quality extract.

However, even if the newest extraction techniques promote the efficiency of the extraction of specific components, sometimes the high costs make them unaffordable. For example, maceration and decoction extract methods resulted more applicable, convenient, and economical for small and medium enterprises or in developing countries, compared with the modern techniques, as suggested by Vongsak et al. [16] and Sithisarn et al. [17].

1.2. Plant extract in agriculture

To protect from insects or pathogens attack plants have developed the ability to synthesize specific molecules with a toxic or repulsive effect on their enemies. These properties have been exploited over the centuries in the preparation of many pesticides products, both in small scale application and as raw material for commercial formulation [18,19]. Their use declined from 1940s when they have been almost entirely replaced by the synthetic pesticides. Nevertheless, nowadays the interest about natural based products is increasing since modern agriculture is moving toward a more sustainable use of the resources, by minimizing the input and the harmful effects of several practices. Moreover, the limitations in the application of synthetic products increased, due to the high costs and their potential negative impacts on the environment and human health. These reasons motivated the research to look for different solutions and plant-based products become an interesting alternative to replace or at least reduce the application of the conventional chemical products since plants extracts are generally cheap, environmentally friendly, and readily available. Thus, basing on their traditional

application and by studying the diverse properties of many different plant species, several trials have been done in order to indagate the effectiveness of plant extracts.

Some examples about the application of plant extract as antifungal or pesticide are reported by Gurjar et al. [20] and Naboulsi et al. [2]. Some of these products are still applied in small-scale agriculture or in organic farming due to the facility in their preparation or the prohibition to use synthetic alternatives.

Besides their use in plant protection, green approaches for promoting plant performance using natural supplementations are highly requested. Several preparations based on plants showed interesting effects stimulating plant growth, improving quality traits or enhancing tolerance to abiotic stresses.

Aqueous extract of nettle, generally called *nettle water*, has been used in horticulture, to stimulate plants growth for a long time. Peterson and Jensèn [21] indagated its effect on wheat, barley and tomato plants and they observed an increase in roots fresh weight and length in wheat and a higher shoot fresh weight both in barley and tomato. Moreover, chlorophyll content was higher in plant treated with nettle water too.

Another common example of widely exploited plants is *Moringa oleifera*. It is considered as one of the most useful plants in the world due to its high content in vitamins, minerals, and antioxidants in almost every part of the tree. Several studies reported its biostimulant effect on different plants species, both in stressful and optimal environmental conditions [22–27]. Positive effects have been observed after the application of licorice root on plant grown under salt stress [28].

Beside the application as crude extracts, plants might be considered as the raw material for the extraction of several bioactive compounds that can be used in the formulation of more complex product. For example, different biostimulant products contain protein hydrolysate, amino acids or mineral compounds obtained from plant material.

2. Borage

Borage (*Borago officinalis* L.) is an annual crop which is cultivated for several purposes; its beneficial properties are widely acknowledged, and it has been used in pharmaceutical and culinary fields since ancient times [29]. Nowadays, interest in this herb has been renewed due to the high level and quality of gamma-linolenic acid (GLA) presents in its seeds, which is used in medicine, cosmetic [30,31] or as food supplement [32]. Moreover, the antioxidant activity of borage extracts has been exploited in food preservation [33,34] and packaging industry [35–37]. Besides the applications in human or animal fields, a recent study showed a biostimulant effect of borage extracts on lettuce plants [38].

2.1. Taxonomical classification

Kingdom*: Plantae*

Subkingdom*: Tracheobionta*

Superdivision*: Spermatophyta*

Division*: Magnoliophyta*

Class*: Magnoliopsida*

Subclass*: Asteridae*

Order*: Lamiales*

Family*: Boraginaceae*

Genus*: Borago L.*

Species*: Borago officinalis L.*

2.2. Botanical characteristics

Borago officinalis is an annual plant belonging to the family of Boraginaceae, with an indeterminate vegetative growth habit. Normally, one primary stem grows from basal rosette of leaves, although sporadically multiple stems appear equally dominant. From the main stem primary, secondary, tertiary and, sometimes quaternary axillary stems develop [39]. Stems are hollow, succulent, cylindrical and, occasionally susceptible to lodging. Borage is generally an erect plant which height ranges from 70 to 100 cm, but low plant densities affect its habit leading to a spreading growth.

Leaves are simple, alternate with an obtuse apex and an entire crenate margin. The shape may be obovate, ovate or oblong and range from 5 to 15 cm long and from 3 to 8 cm wide. Basal leaves are stalked, arranged in a rosette, and the petiole is decurrent while upper leaves are sessile or have a short petiole. Leaf colour is green, but the shade is darker on the upper surface and lighter on the lower page. White, tough and unicellular trichomes cover both leaves, stems and calyces. Borage flowers are normally bright blue or blue-violet, but sometimes pink or white coloured flowers may appear. Five ovate-lanceolate petals are united to form a star-shaped corolla which is approximately 2 cm in diameter. Five short white scales with pink-violet tips are present at the base of the corolla where petals fuse together. Each petal is about 1 cm long and 0.5 cm wide. The single, short, gynobasic style with a capitate stigma is surrounded by 5 black stamens attached near the base of the

corolla and the introse anthers form a kind of conical structure. Each flower is supported by a long pedicel ending with a deeply clefy calyx composed of 5 green and conical sepals. The ovary is situated above the flower parts (superior) and it changes to a fruit with 3 to 4 green nutlets when immature, turning brownish-black at maturity. Since flowers are produced continuously and mature over an extended period, seeds of different developmental stage are present on the plant at the same time [40,41].

The rooting system is a taproot with a single dominant large structure from which a network of smaller and long roots emerges. For this reason, borage does not well tolerate transplants.

2.3. Origin and cultivation

Most of the studies reports that borage is native to Mediterranean areas, probably to North Africa and then was transferred in to Spain and other regions of Europe and Asia Minor. Indeed, unlike other species from *Borago* genus restricted to north-west Africa or limited in some Italian regions, *Borago officinalis* is a widespread species distributed in several county of and beyond the boundaries of the Mediterranean basin. Here it is present as wild weed, cultivated as garden plant or as crop vegetable [42,43]. Otherwise, some authors say that the plant is native to India and Iran where its leaves have been consumed as tea for years [44].

Nowadays it is cultivated all around the world, like Canada, Australia, America or New Zealand for the production of its seed oil rich in GLA. However, information about cultivation and best management practices are still limited and not-well defined. Borage is a very adaptable plant; it can grow in different type of soil and in a pH scale range from 4.5 to 8.2. It requires low to moderate moisture; it survive in drought conditions but a wet and well drained medium with a pH around 6.6 is preferred. Borage plants are sensitive to salt stress affecting their growth in terms of leaf area, dry weight, stem length and diameter [45–47]. Also a decrease in plant fecundity, resulting from abnormalities during pollen developmental process, has been observed in plants grown in saline environment [48]. Nevertheless, due to its ability to withstand saline condition and to uptake sodium and chlorine, the possibility to use it in bioremediation has been taken into consideration [49].

Borage plants grow better under moderate exposure to sun because intense radiations change their status to rosette leaves [29]. They have high resistance to cold and the suitable period of cultivation is spring or autumn. Temperature is a critical but essential point in plant cultivation, it influences seeds germination, the duration of the growing cycle and the quality of the production. For these reasons, several trials have been performed in order to find the cardinal temperatures for borage cultivation and define the better sowing and harvesting dates. Ghaderi et al. [50] reported that the minimum, optimum and maximum germination temperatures for Iranian accessions of borage are 5, 30 and 40 °C respectively. A further experiment conducted with a thermogradient table determined minimum, optimum and maximum temperatures for borage germination of

9, 23 and 30 °C, respectively [51]. A recent study reported that borage growth was stimulated at 24 °C compared to 21 °C, but impaired at 27 °C caused an impaired growth and a decrease in flower buds number [52].Indeed, even if borage plants are able to grow below 6 °C they flower only when temperature is higher, affecting also the production of the seeds [53]. Most processing companies require a minimum 22% GLA which is not easy to obtain at latitudes lower than 38° [54]. Therefore, borage is usually grown at higher latitudes. GLA accumulate in later stages of development and the maximum GLA content is not reached until physiological maturity. Its accumulation in borage seeds is affected by temperature and, in agreement with other oilseed crops, GLA increase as temperature decrease during seed filling [55]. It means that a non-uniform mixture of seed at several maturity levels and different fatty acids content are harvested. Moreover, once seeds reach the physiological maturity, they are often lost due to shattering. All these aspects strongly affect seed yield and composition and make harvest management decisions very difficult. Several studies aim to define the best harvest time [55–57] and method [58] and, also, to identify non-shattering borage mutants [59,60]. On the contrary, flowering twigs are collected before the start of seed formation when borage is cultivated for medical purpose.

Plant density is another important point in borage cultivation; to avoid a spreading growth habit a value of 100 000 shrubs per hectare, sowing about 5-7 kg is recommended. Researchers report also 30 cm distance between rows and 10 cm distance between plants on row as the best attendance [29]. In Berti et al. [53] study the maximum yield was obtained with a planting density between 172 222 and 205 000 plant per hectare, at 60 and 40 cm between raw, respectively and with a seeding rate of 7 kg per hectare. Others experiments indicated that a sowing rate of 16 kg per hectare may be positive if seed germinability is poor, but generally 8 kg is enough [61]. Fertilization is not always adopted in borage cultivation because the amount of nutrient in soil is usually enough. However, several trials have been performed to evaluate the effects of different fertilization strategies on borage plants. The majority of them reported a positive effect of nitrogen and potassium fertilization on plants growth, number of branches, dry matter and grain yield, number of flower, mucilage percentage and essential oil yield [54,62–64]; another study showed an opposite effect on GLA concentration in borage seeds, according to the source of nitrogen (urea or ammonium nitrate) added [65]. In some cases the N P K fertilization did not significantly affect plant growth and biomass, probably due to the high fertility of the soil [54,55]. Furthermore, a positive response to sulphur application was observed in seed yield [54].

Irrigation as well as fertilization, is not generally applied in borage cultivation, even if it has been observed that water stress negatively affect flower development, nectar sugar content, pollen viability and grain yield [52,66,67].

2.4. Chemical composition

Over the years, many researches have been conducted to study the chemical composition of *Borago officinalis*. The phytochemical analyses were carried out to identify different kinds of components from stems, leaves, flowers and seeds, and several extraction techniques have been used. Since its importance in pharmacological, medical and nutritional application, the majority of the studies focused on the isolation of the most active compounds, in particular GLA from seeds, and little work has been reported about minor and trace elemental composition of leaves and flowers. Several researches described the fatty acid composition of borage and even with some differences in the obtained results, almost all of these confirmed that borage leaves are rich in polyunsaturated fatty acids (PUFA) especially α-linolenic, stearidonic, linoleic and γ-linolenic acids. Besides PUFA, some monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) were identified. MUFA were represented by oleic, hexadecenoic and palmitoleic acids; SFA by palmitic, arachidic and stearic acids [68–71]. In addition, others fatty acids such as meristic, lauric, elcosenoic, behenic, lignoceric and nevronic acids have been detected in green parts of borage plants by del Rio-Celestino et al. [72]. Furthermore, others lipid classes like phospholipid, glycolipid and neutral lipids were investigate [73,74]. Fatty acid composition of borage flowers and leaves was similar; few differences in the percentage of the components were observed and flowers were characterized by high content of linoleic, palmitic and γ-linolenic acids [69].

Fatty acids are precursors for a large number of volatile compounds responsible for the fresh, green and fruity odor of fruits and vegetables. Different compounds belonging to several classes have been identified in borage leaves and flowers [68,75]. Two aliphatic hydrocarbons represented by nonadecane and tetracosane were the most abundant, followed by an alcohol ((Z)-3-hexenol), a keton (camphor), a phenol (carvacrol), several aldhydes and monoterpenic hydrocarbon classes in small amount. Salem et al. [76,77] identified (E)-(E),2-4 decadienal as the main compound of the essential oils from borage stem and flower while Zribi et al. [78] identified benzenacetaldehyde, octanal and nonanal as major compounds in essential oil, flowers and leaves, respectively.

In spite of the high levels of fatty acids, few researches paid particular attention to the phenolic profile of borage and its antioxidant activity [57,79–82]. Tannins and anthocyanins were present in low amount in leaves extracts compared with total flavonoids (quercetin, isoquercetin, catechin-7-O-glucoside, naringenin O-hexoside, luteolin 7-O-glucoside, vitexin and isovitexin, luteolin 7,30,40-trimethyl ether, kaempferol 3,7,40-trimethyl ether, naringenin O-hexoside) and total flavonols. As the main class of flavonoids, anthocyanins are present also in borage flowers as pigments. They are responsible for change their colour from pink to blue and their concentration increase during flower development. The major compound is represented by petunidin 3,5 diglucoside, followed by delphinidin 3,5 diglucoside [76]. Karimi et al. [83] confirmed the presence of phenolic

and flavonoid in borage flowers and among them the salicylic acid and myricetin were the most abundant compounds.

Others antioxidant compounds such as rosmarinic, syringic and sinapic acids were found both in borage leaves [84,85] and seeds [86]. A few sterols (β- and ɤ- sitosterol, campesterol, stigmasterol, cholesterol, ergosta-5,24-dien-3-ol) and a secoiridoid (oleuropein) with antioxidant properties were identified in leaves extracts too [80,81]. Moreover, a new lignan glucoside (officinalioside), together with three megastigmane glucosides (actinidioionoside, roseoside, crotalionoside C) and one flavonoid galactoside (kaempferol 3-O-β-D-galactopyranoside) was isolated from the aerial parts of borage [87]. A derivative of aspidospermine (1-acetyl-19,21-epoxy-15,16-dimethoxyaspidospermidine-17-ol) exerting an anti-amyloid activity by assisting the proper folding of the protein has recently found in borage leaves extract [44].

In addition to the antioxidant compounds, a few amount of toxic pyrrolizidine alkaloids (lycopsamine and supinidine viridiflorate) were identified in leaves, seeds and flowers of borage [88–91]. Concerning the mineral composition, borage showed a great amount of potassium, followed by sodium, calcium magnesium and iron. Manganese, zinc and copper were present in minor amounts [92–94]. Moreover, a variable percentage of mucilage, resin and gum was found in leaves, stems and flowers.

The quality and the ratio of borage tissues components change upon growing stages, as reported by several authors [75,76,79,95–98]. For example, nonadecane and tetracosane were predominant during growth period, then a decrease of hydrocarbons and aldehydes and a progressive increase in alcohols was observed. Moreover, the total fatty acids showed an initial increase followed by a decrease during senescence, due to loss of membrane lipids. The evolution of the chemical composition was related to the stem elongation. Indeed, a progressive increase in fiber and lignin fraction and a decrease in raw protein was described [92]. Moreover, since the level of micronutrients, mineral and trace elements as well as the chemical composition of plant tissues are affected by the chemical and physical properties of soil, and by the environmental condition [78], some differences were found from one study to another.

Current interest in borage cultivation is for its seeds which contain a high percentage of GLA in the oil. In mature seeds it ranges between 170 and 280 g kg^{-1}. Palmitic, stearic, oleic, linoleic, linolenic and erucic acids were also identified. As well as chemical composition in borage leaves or flowers, the fatty acid composition in borage seeds changes during time and growing conditions [46,99]. This phenomenon is particularly important in the definition of the best harvest time. A general increase in GLA concentration has reported [51,53,74,100].

Several phenolic acids have been found in borage seeds; their role is particularly important preventing the oxidation of the oil and as a source of antioxidant in food or pharmaceutical formulation [57,101]. In addition to rosmarinic, syringic and sinapic acids content in defatted seeds, as reported above [86], Zadernowski et al. [102] observed that ferulic acid represent the highest portion of total free phenolic compounds, followed by 2-

hydroxy-4-methoxybenzoic, gallic, p-coumaric, and cinnamic acids. Small quantities of caffeic acid was also identified. Similar results have been obtained by Mhamdi et al. [75] who also reported that borage seed oil is a good source of β-caryophyllene.

2.5. Borage uses

Borage has been used since ancient times for culinary and medical purpose in different countries. Its leaves are used in salads, soups or in different dishes and beverages [72,80,103]. Flowers sometimes can be served fresh or candied as edible decoration of cocktails and confectionery [69]. Since its antioxidant properties were demonstrated, several studies have focused on its application to food preservation such as in the preparation of gelatine films from fish [35,104], preventing oxidation in sausages [34] and oil [84], or extending lamb shelf life [36,37]. Besides its consumption as edible plant, many health effects have been attributed to the borage plant. They have been discussed by a great number of authors and published in scientific papers [29,105–107]. It is indicated to alleviate and heal colds, bronchitis, and respiratory infections in general for its anti-inflammatory and balsamic properties. In naturopathy borage is used for the regulation of metabolism and the hormonal system and is considered as a good remedy for PMS and menopause. Borage is used as natural medicament to improve the intellective processes, activating and improving memory records [106].

Borage uses are mostly related to the high content of GLA and his properties. GLA is a precursor of a prostaglandin in the human body, which is vital in many functions. Human organism is not capable to synthesize it; therefore, its supplementation could be a value for preventing and/or treating various degenerative pathologies. GLA is generally prescribed as anti-inflammatory but it is also used for the treatment of multiple sclerosis, diabetes, arthritis and dermatitis [105,108,109]. Based on the traditional uses of borage, studies investigated its efficacy in gastrointestinal (colic, cramps, diarrhoea), respiratory (asthma, bronchitis), cardiovascular (cardiotonic, antihypertensive and blood pressure), and urinary (diuretic and kidney/bladder disorders) disorders [110]. Moreover, Rezk et al. [111] reported its beneficial effect in reducing hepatotoxicity and protecting the cells from the oxidative stress induced by radiation exposure. Recently, borage treatments showed a beneficial anxiolytic effect in rats and in patients with OCD [112,113].

The juice and fresh herb are used in cosmetics as rejuvenating or nutrients masks. The juice is responsible for inflammation alleviation and promotion of the regeneration of the skin. However, the ointment from the fresh herb is effective in treatment of eczema and wounds. The most common use of oil in cosmetology are capsules; their regular intake positively affects the structure of hair and condition of nails. Due to its numerous properties, borage use as animal feed has been proposed [97].

As mentioned before, a recent study proposed the potential use of borage as an affordable source of plant biostimulants [10]. Indeed, results obtained from this work showed that extracts from borage leaves and flowers affected the primary and secondary metabolism of lettuce crop by increasing leaf pigments and photosynthetic activity both at harvest and during storage.

Besides its uses for human/animal health and food, Al-Moubaraki [114] observed the inhibitive effect of aqueous borage extracts against the corrosion of mild steel, proposing its application in mechanical field.

AIM

The purpose of this experiment was the evaluation of the effect of different maceration times on the efficacy of the of the aqueous extracts obtained from borage leaves and flowers. All treatments have been tested on two different leafy vegetables – *Diplotaxis tenuifolia*, L. and *Lactuca sativa* L. - grown under non-stressful conditions.

MATERIALS AND METHODS

1. Plant material, growth conditions and experimental design

1.1. Preparation of borage extracts

For extracts preparation Borage plants were cultivated in a greenhouse at the Faculty of Agricultural and Food Sciences of Milan, under controlled conditions. Borage seeds were obtained 7 g of NPK fertilizer one time during the growing cycle. Sampling was carried out starting from flowering stage (April-May) and leaves (LE) and flowers (FE) were collected separately, weighed and stored at -20 °C until use. Borage extracts were prepared following the method used by Bulgari et al. [38] with a slight change on the period of maceration. Plant material was roughly minced, transferred into clean glass jars (3 L) with lid and soaked in water. The volume of the water was added in proportion to the weight of plant material in a ratio of 1:2 (w/v). The jars were kept in a dark place, at room temperature (~ 25 °C) and stirred once in a while. Four different maceration times were assayed: 1 (T1), 3 (T2), 7 (T3) and 14 (T4) days. At the end of each period the solid residue was separated from the liquid and discarded. The aqueous phase was filtered using a syringe filter with a 0.45 µm pore size and then diluted with water (10 mL/L) to be used as plant treatments. Final extracts were stored at -20 °C until use.

1.2. Rocket cultivation

The trial was carried out two times at the Faculty of Agricultural and Food Science of Milan in 2017/2018. Rocket plants (*Diplotaxis tenuifolia,* L.; ISI Sementi S.p.A., Italy) were grown hydroponically into plastic tank (35 x 25 x 20 cm) with 10 L of a standard Hoagland's solution (Table 3). Seeds of rocket were manually sown into polystyrene trays with an agri-perlite substrate. Plants were grown in an experimental greenhouse under controlled conditions (natural ambient light condition).

Table 3. Composition of the Hoagland nutrient solution used for rocket plants cultivation

Compounds	Concentration [mM]
$Ca(NO_3)_2$	2.19
KNO_3	4.55
NH_4NO_3	3.87
K_2HPO_4	1.38
$MgSO_4$	0.83
K_2SO_4	1.09
Oligo green*	0.02 (g L^{-1})
H_2SO_4	Up to pH 5.5 – 6.5

*Oligogreen: is a mineral water-soluble powder fertilizer that provides the plant with micronutrients, essential for the most important bio-chemical reactions. Green Has Italia.

Plants were treated with 20 mL of water (control) and 20 mL of each borage extract. Treatments were applied as foliar spray onto leaves until run-off. The application was performed as foliar spray twice during the growing periods: the first one, 23 days after sowing (DAS) and the second one 28 DAS, one day before the harvest.

1.3. Lettuce cultivation

The trial was carried out at the Faculty of Agricultural and Food Science of Milan in 2018. Two-week-old plantlets of lettuce (*Lactuca sativa*, L. var *longifolia*; ISI Sementi S.p.A., Italy) were transplanted into 20 cm diameter plastic pot on a peaty substrate and grown in an experimental greenhouse under controlled conditions (natural ambient light condition). Plants were treated with 20 mL of water (control) and 20 mL of each borage extract. Treatments were applied as foliar spray onto leaves until run-off. The application was performed as foliar spray twice during the growing periods: the first one, 15 days after transplant (DAT) and the second one 30 DAT. Lettuce plants were harvested at commercial maturity stage at 46 DAT.

2. Non-destructive analyses

2.1. Chlorophyll

Leaves chlorophyll content was estimated *in vivo* using a chlorophyll content meter (CL-01 Chlorophyll Content Meter, Hansatech Instruments, UK). Measurement were performed at the end of each trials. The results were express as chlorophyll index (relative units).

2.2. Chlorophyll a fluorescence

Chlorophyll *a* fluorescence was measured *in vivo* using two different instruments: a hand-portable fluorometer (Handy-PEA, Hansatech Instruments, UK) and a field portable pulse modulated chlorophyll fluorometer (FMS2, Hansatech Instruments, UK). Before all measurement with Handy-PEA, leaves were dark-adapted with the leaf clips for 30-40 minutes. Then were exposed to a saturating light (3000 µmol m^{-2} s^{-1}) provided by an array of three high-intensity light-emitting diodes for 1 second. Chlorophyll *a* fluorescence was measured and then analysed with the JIP-test. Information about the structural and functional status of photosynthetic apparatus was

provided by the parameters measured, such as the maximum quantum of photosystem II (Fv/Fm) or the performance index (PI).

Modulated chlorophyll fluorescence under ambient light regime was measured using the FMS2, fluorimeter (Hansatech Instruments, UK). In order to calculate the electron transport rate (ETR) PAR value is recorded by a light sensor on the leaf-clip. The steady-state fluorescence (Fs) was measured with the measuring radiation. After that, a pulse of saturating light was imposed to obtain the maximum fluorescence level in light adapted leaves (Fm'). The effective PSII quantum efficiency (ϕPSII) and the electron transport rate (ETR) were calculated by the FMS software. Measurements were carried out at the end of each trial.

3. Destructive analyses

3.1. Total fresh biomass and dry weight

Fresh above ground biomass was measured for each tank/pot at the end of the experiment by cutting plants at collar. The leaf dry weight was determined by oven-drying samples in 105 °C until constant weight was reached.

3.2. Chlorophylls and carotenoids

Chlorophylls and carotenoids were determined in rocket leaves at harvest. Pigments were extracted using 5 mL of 99.9% (v/v) methanol. Leaf disc samples (30 mg), obtained with a 5 mm diameter cork borer, were kept in dark room for 24 h at 4 °C. After that absorbance reading were measured at 665.2 and 652.4 nm for chlorophylls and 470 nm for total carotenoids. Pigments levels were calculated by Lichtenthaler's formula and expressed on a fresh weight basis [115].

3.3. Phenols and anthocyanin

Total phenols and anthocyanin were extracted in 3 mL of methanol acidified with hydrochloric acid. Leaf disc samples (30 mg), obtained with a 5 mm diameter cork borer, were kept in dark room for 24 h at 4 °C. After that absorbance reading were measured at 320 nm for total phenols, and at 535 nm for anthocyanin. Phenolic index was expressed as Abs320 nm g^{-1} FW. Anthocyanins concentration was expressed in cyanidin-3-glucoside equivalents using a molar extinction coefficient (ε) of 29,600 L M^{-1} cm^{-1}.

3.4. Nitrate

Nitrate concentration was determined by the method of Cataldo et al. [116]. Fresh leaf tissue was homogenized in distilled water (1 g fresh tissue per 4 mL distilled water). The homogenate was centrifuged at 4000 rpm for 15 min at RT (ALC centrifuge-model PK130R) and the recovered supernatant was used for the colorimetric analysis. Twenty microliters of the extract were added to 80 mL of 5% (w/v) salicylic acid in concentrated H_2SO_4 (SA-H_2SO_4). Afterward 3 mL of 1.5 N NaOH were added. The samples were cooled to RT and absorbance at 410 nm was measured with a spectrophotometer. Nitrate content was calculated referring to a KNO_3 standard calibration curve. Nitrate concentration was expressed as mg of KNO_3per kg of fresh weight.

3.5. Sucrose and total sugars

Sucrose content was measured using the resorcinol method. Approximately 1 g of leaf tissue was homogenized in a mortar with 3 mL of water. The mixture was centrifuged at 4000 rpm for 15 min at RT. Sucrose assay was performed by mixing 0.2 mL of supernatant with 0.2 mL of 2 N NaOH and incubated in a water bath at 100 °C for 10 min, then 1.5 mL of resorcinol buffer (containing 30% hydrochloric acid, 1.2 mM resorcinol, 4.1 mM thiourea 1.5 M acetic acid) was added to samples and incubated in a water bath at 80 °C for 10 min. After cooling at room temperature, the optical density was determined spectrophotometrically at 500 nm and the sucrose concentration was calculated using a standard curve.

The total sugars were determined on the same extract using the anthrone method with slight modifications [117]. The anthrone reagent (10.3 mM) was prepared dissolving anthrone in 95% H_2SO_4. The reagent was left to stand for 30-40 min before use, 0.5 mL extract was placed on top of 2.5 mL of anthrone reagent incubated in ice for 5 min and then vortexed vigorously. The tubes were heated to 95 °C for 10 min and left to cool in ice. Readings were performed at 620 nm. Calibration curve was carried out using a glucose standard solution.

4. Statistical analysis

Statistical analysis was performed with GraphPad Prism 6 and data were subjected to one-way ANOVA. Differences among means were determined by Tuckey post-test ($P < 0.05$). If normality assumption for ANOVA was violated the Kruskal-Wallis test was applied.

RESULTS

1 Rocket (1st growing cycle)

1.1 Total fresh biomass and dry weight

Fresh biomass was measured at harvest for each tank and yield was calculated as grams of fresh weight per square meter. Highest and lowest values were reached by the plants treated with the FE (T2) and with the LE (T3). The values were 2888 g m^{-2} and 2166 g m^{-2}, respectively. Since each tank was a singular experimental unit ANOVA cannot be used and data about fresh and dry weight are not shown.

1.2 Chlorophyll

Chlorophyll content non-destructively measured and expressed as relative units and by the destructive method are reported below (Figure 1 A, B). The results obtained from the two techniques showed a similar trend mostly in response to FE treatments. Indeed, in FE-treated plants an initial increase between the (T1) and (T2) is followed by a decrease from (T2) and (T4). Instead, in LE-treated plants all values were similar regardless of period of maceration. Nevertheless, statistical analysis did not show any significant difference among treatments.

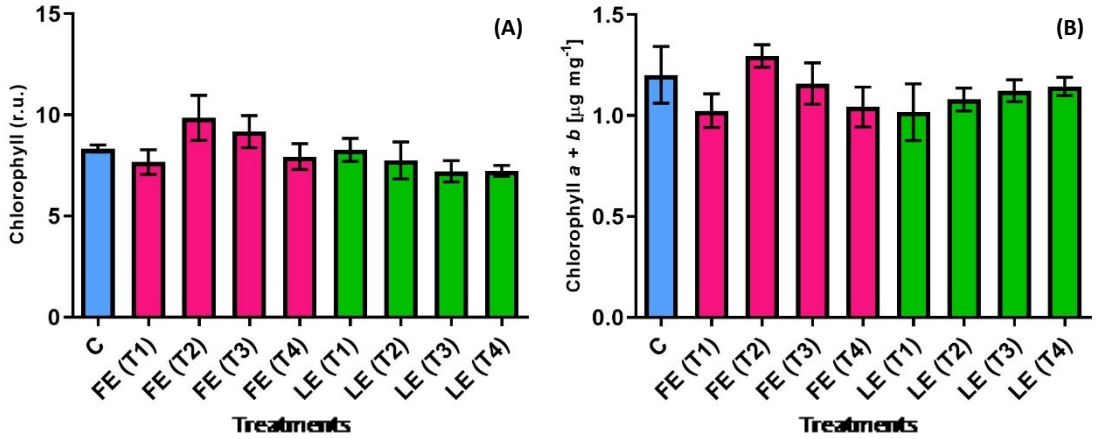


Figure 1. Chlorophyll content determined *in vivo* **(A)** and chlorophyll *a+b* concentration **(B)** in rocket leaves treated with water (control = C) and with borage extracts (flowers extract = FE or leaves extract = LE) obtained with different maceration times: 1 day (T1), 3 days (T2), 7 days (T3) and 14 days (T4). Values are means ± SE (n = 5) (A); (n = 3) (B). Data were subjected to one-way ANOVA. Different letters, where present, represent significant differences among treatments.

1.3 Carotenoids

The content of carotenoids (Figure 2) in rocket leaves showed the same trend of chlorophyll content. The highest (0.22 µg mg^{-1} FW) and the lowest (0.12 µg mg^{-1} FW) average values were observed in plants treated with FE (T2) and LE (T1) respectively. A significant difference ($P < 0.05$) resulted between these two treatments. Borage extracts generally induced a decrease of this parameter, even if the observed differences among treatments and control were not statistically significant.

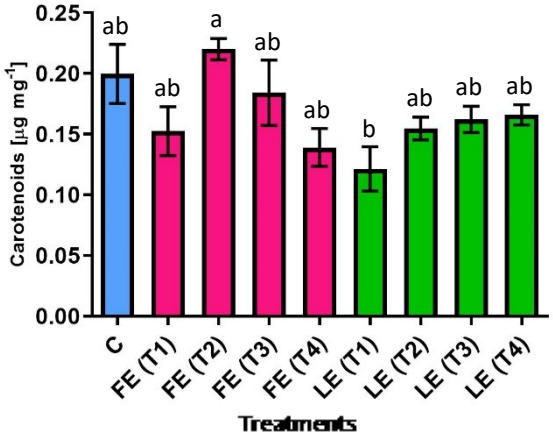


Figure 2. Carotenoids content in rocket leaves treated with water (control = C) and with borage extracts (flowers extract = FE or leaves extract = LE) obtained with different maceration times: 1 day (T1), 3 days (T2), 7 days (T3) and 14 days (T4). Values are means ± SE (n = 3) Data were subjected to one-way ANOVA. Different letters, where present, represent significant differences among treatments.

1.4 Phenols and anthocyanin

Phenols and anthocyanin contents are listed in Figure 3 A and B. The phenol index resulted significantly ($P < 0.05$) higher in plant treated with FE (T2) compared with most of the others borage extract treatments. In particular, values recorded in response to FE (T3), LE (T1) and LE (T2) treatments were decreased of -24.4%, -23.5% and -24.8%, respectively. Moreover, the concentration of anthocyanin in rocket leaves showed a similar trend. The highest value (20.85 Cyanidin eq. mg 100 g^{-1}) was recorded in leaf tissue of plants treated with FE (T2). The treatments with FE (T3), FE (T4), LE (T1) and LE (T2) caused a decrease of anthocyanin concentration about -28.6%, -26.5%, -28.6% and -27.6%, respectively. No significant ($P < 0.05$) difference was observed among borage extracts treatments and control.

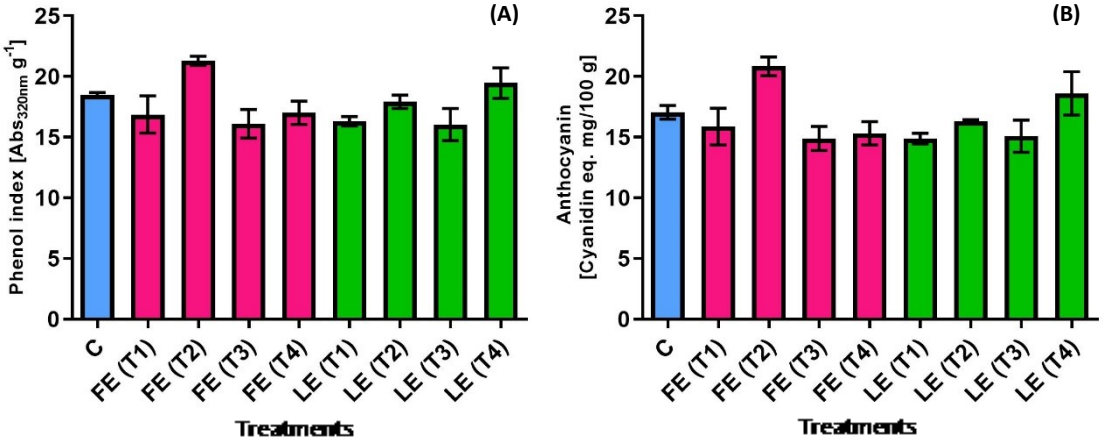


Figure 3. Phenol index **(A)** and anthocyanin content **(B)** in rocket leaves treated with water (control = C) and with borage extracts (flowers extract = FE or leaves extract = LE) obtained with different maceration times: 1 day (T1), 3 days (T2), 7 days (T3) and 14 days (T4). Values are means ± SE (n = 3). Data were subjected to one-way ANOVA. Different letters, where present, represent significant differences among treatments.

1.5 Chlorophyll a fluorescence

The maximum quantum efficiency of PSII (*Fv/Fm*) (Figure 4 A) did not show any significant ($P < 0.05$) difference in response to treatments. All samples were much higher than 0.83, commonly referred to as the threshold value between stressed and unstressed leaf tissue, with an average of 0.86. The vitality index of PSII generally called performance index (*PI*) did not show any significant ($P < 0.05$) change after treatment with borage extracts and all values were similar to the control (Figure 4 B). A similar pattern based on maceration time was observed in response to FE and LE treatments. The time needed to reach the maximum fluorescence intensity (*TFm*) is a good indicator of the energy transfer from reaction centres to plastoquinones (Figure 4 C). *TFm* average value in leaves tissue of control plants were 267.5 millisecond and all treatments with FE induced a slightly decrease, albeit not significantly, regardless of maceration time. The highest value (500 ms) was observed in plants treated with LE (T3) and statistical analysis revealed significant ($P < 0.05$) differences between this treatment and control and with most of the other borage extract treatments. A related parameter is the *Area* (data not show). It is defined as the total complementary area between fluorescence induction curve and represent a useful tool to probe electron transport chain capacity and redox state and it is proportional to the pool size of the electron acceptors Q_A on the reducing side of PSII. If we normalize the *Area* on the maximum variable fluorescence (*Fm*) to obtain the *Sm* parameter in order to quantitatively compare different samples, significant differences were revealed (Figure 4 D). In particular, results obtained showed that the *Sm* value in FE (T3) treated plants was significantly ($P < 0.05$) lower than plant treated with LE (T1) and (T3) of about -28% for both.

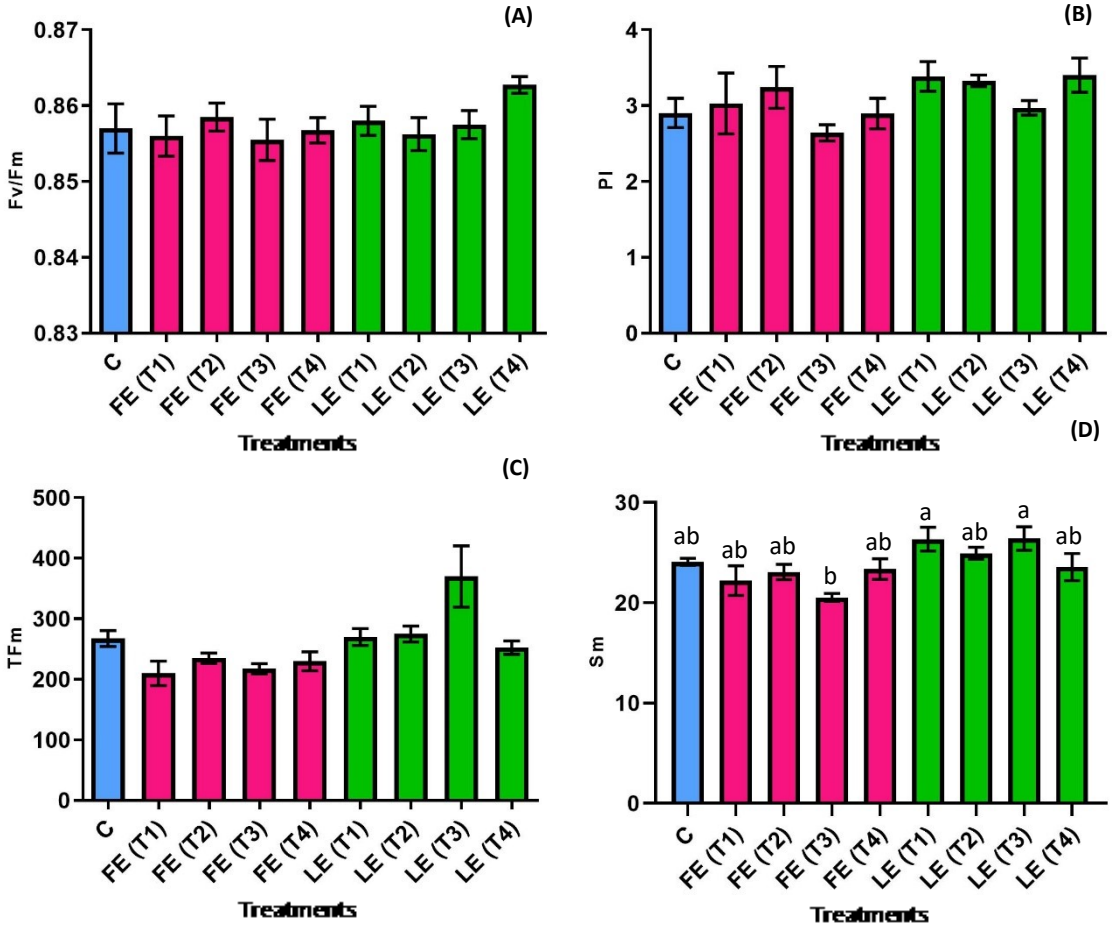


Figure 4. Maximum quantum efficiency of PSII (Fv/Fm) **(A)**, performance index (PI) **(B)**, the time needed to reach the maximum fluorescence intensity (TFm) **(C)** and the total complementary area between fluorescence induction curve normalized per Fm (Sm) **(D)** in rocket leaves treated with water (control = C) and with borage extracts (flowers extract = FE or leaves extract = LE) obtained with different maceration times: 1 day (T1), 3 days (T2), 7 days (T3) and 14 days (T4). Values are means ± SE (n = 4). Data were subjected to one-way ANOVA. Different letters, where present, represent significant differences among treatments.

The electron transport rate (ETR) in rocket leaves tissues (Figure 5 A) were not significantly affected by any treatment with borage extracts even if a similar pattern based on maceration time was observed in FE and LE. Instead, the effective quantum yield of photosystem II in the light (ϕPSII) showed a decrease in rocket leaves treated with FE (T2) (Figure 5 B). Since the ϕPSII data group violated the normality assumption for ANOVA a non-parametric test (Kruskal-Wallis) was applied and no significant differences resulted.

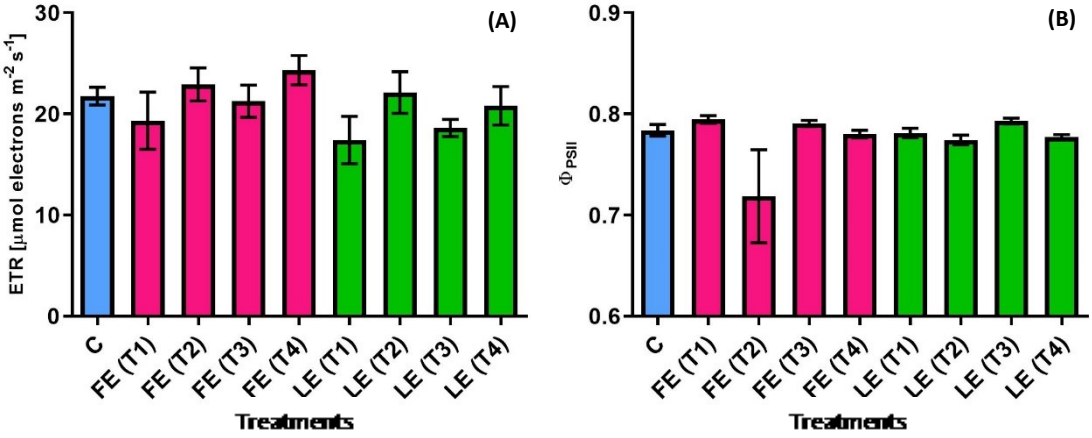


Figure 5. The electron transport rate (ETR) **(A)** and the effective quantum yield of photosystem II in the light (ϕ_{PSII}) **(B)** in rocket leaves treated with water (control = C) and with borage extracts (flowers extract = FE or leaves extract = LE) obtained with different maceration times: 1 day (T1), 3 days (T2), 7 days (T3) and 14 days (T4). Values are means ± SE (n = 5). Data were subjected to one-way ANOVA or Kruskal-Wallis test. Different letters, where present, represent significant differences among treatments.

1.6 Nitrate

Borage extracts did not significantly affect the concentration of nitrate in rocket leaves and results obtained are reported in **Error! Reference source not found.**. The average values ranged from 5238.65 mg kg^{-1} in control plants to 6112.36 mg kg^{-1} in FE (T2) treated plants. All values were below the threshold imposed by EU regulation N. 1258/2011.

1.7 Sucrose and total sugars

The concentration of sucrose and total sugars (Table 4) in rocket leaves were not significantly affected by any of the borage extract treatments. However, a slight increase of sucrose content associated with the duration of maceration resulted in samples treated with LE. In particular, values grew from 344.6 mg kg^{-1} at (T1) to 480.7 mg kg^{-1} at (T4). The same pattern was not observed in total sugar concentration. On the contrary, plants treated with FE showed the same trend in both sucrose and total sugars concentration.

Table 4. Nitrate and sugars concentrations of rocket leaves treated with water (control = C) and with borage extracts (flowers extract = FE or leaves extract = LE) obtained with different maceration times: 1 day (T1), 3 days (T2), 7 days (T3) and 14 days (T4). Values are means ± SE (n = 3). Data were subjected to one-way ANOVA. Different letters, where present, represent significant differences among treatments.

Treatment	Nitrate [mg kg^{-1} FW]	Sucrose [mg kg^{-1} FW]	Total sugars [mg kg^{-1} FW]
Control	5238.65 ± 200.18	428.52 ± 52.21	1941.80 ± 254.09
FE (T1)	5616.66 ± 775.76	367.15 ± 13.88	1807.91 ± 302.19
FE (T2)	6112.36 ± 219.70	333.82 ± 38.03	1675.99 ± 157.45
FE (T3)	5418.63 ± 64.31	423.28 ± 54.51	2100.35 ± 359.78
FE (T4)	5663.41 ± 286.51	387.04 ± 40.68	1724.28 ± 156.09
LE (T1)	5878.62 ± 312.58	344.64 ± 53.14	1545.68 ± 46.04
LE (T2)	5278.36 ± 161.41	368.60 ± 20.43	1762.19 ± 141.47
LE (T3)	5519.42 ± 121.03	402.54 ± 8.42	1696.64 ± 27.05
LE (T4)	5301.84 ± 336.62	480.76 ± 47.46	1682.31 ± 55.49

2 Rocket (2nd growing cycle)

2.1 Total fresh biomass and dry matter

Fresh biomass was measure at harvest for each tank and yield was calculated as grams of fresh weight per square meter. Values ranged from Highest and lowest values were reached by the plants treated with the LE (T1) and with the FE (T4), respectively. The values were 3022 g m^{-2} and 1945 g m^{-2}, respectively. Since each tank was a singular experimental unit ANOVA cannot be used and data about fresh and dry weight are not shown.

2.2 Chlorophyll

Figure 6 A and B reported the chlorophyll content estimated *in vivo* and the chlorophyll *a+b* concentration measured by the destructive method. Unlike the first growing cycle, results obtained from the two techniques did not show a similar trend. Indeed, in LE-and FE-treated plants all chlorophyll values expressed as relative units were similar regardless of period of maceration. Instead, the destructive analysis highlighted two different patterns between samples treated with FE and LE. Indeed, FE treatments led to an initial decrease from (T1) to (T3), followed by a final increase at (T4). Opposite trend resulted in plants treated with LE. Nevertheless, statistical analysis did not show any significant difference.

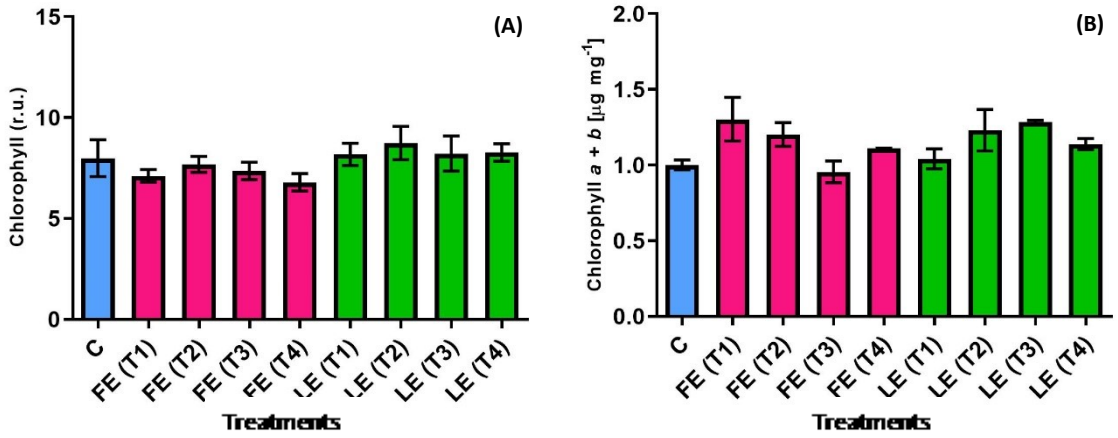


Figure 6. Chlorophyll content determined *in vivo* **(A)** and chlorophyll *a+b* concentration **(B)** in rocket leaves treated with water (control = C) and with borage extracts (flowers extract = FE or leaves extract = LE) obtained with different maceration times: 1 day (T1), 3 days (T2), 7 days (T3) and 14 days (T4). Values are means ± SE (n = 5) (A); (n = 3) (B). Data were subjected to one-way ANOVA. Different letters, where present, represent significant differences among treatments.

2.3 Carotenoids

Like in the first growing cycle, the concentration of carotenoids (Figure 7) in rocket leaves followed the trend of chlorophyll *a+b* content. Different patterns ware observed comparing the two trials. The highest (0.256 µg mg^{-1} FW) and the lowest (0.181 µg mg^{-1} FW) average values were observed in plants treated with FE (T1) and FE (T2) respectively but no significant difference resulted between these two treatments.

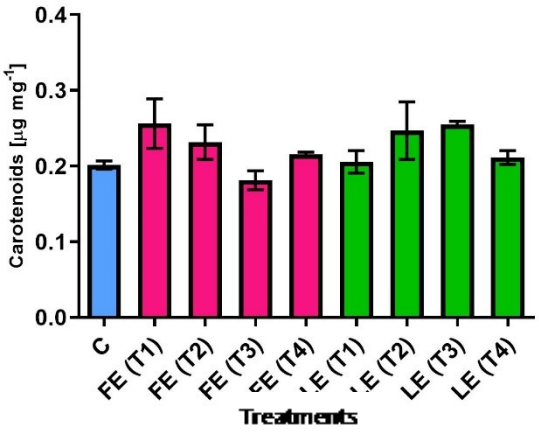


Figure 7. Carotenoids content in rocket leaves treated with water (control = C) and with borage extracts (flowers extract = FE or leaves extract = LE) obtained with different maceration times: 1 day (T1), 3 days (T2), 7 days (T3) and 14 days (T4). Values are means ± SE (n = 3) Data were subjected to one-way ANOVA. Different letters, where present, represent significant differences among treatments.

2.4 Phenols and anthocyanin

Table 5 listed the phenols and anthocyanin contents measured in leaf tissues. Phenols index (expressed as Abs_{320nm} g^{-1}) in control plant was around 19.68 and all borage extracts generally induced an increase in this parameter, mostly at (T1) and (T2) maceration time. The highest average was observed in sample treated with FE (T2) with a value +29.2% higher that control. The concentration of anthocyanin in rocket leaves showed a similar trend. Moreover, the pattern is similar to the results obtained in the first growing cycle. However, statistical analysis did not reveal any significant difference among treatments.

Table 5. Phenol index and anthocyanin content in rocket leaves treated with water (control = C) and with borage extracts (flowers extract = FE or leaves extract = LE) obtained with different maceration times: 1 day (T1), 3 days (T2), 7 days (T3) and 14 days (T4). Values are means ± SE (n = 3). Data were subjected to one-way ANOVA. Different letters, where present, represent significant differences among treatments.

Treatment	Phenol index [Abs_{320nm} g^{-1}]	Anthocyanin [Cyanidin eq. mg/100 g]
Control	19.68 ± 0.66	16.57 ± 0.35
FE (T1)	23.96 ± 0.83	20.30 ± 0.77
FE (T2)	25.43 ± 1.47	21.19 ± 0.53
FE (T3)	20.21 ± 2.24	18.15 ± 1.18
FE (T4)	22.91 ± 1.20	19.74 ± 0.67
LE (T1)	22.61 ± 1.57	19.99 ± 1.25
LE (T2)	25.30 ± 2.75	21.29 ± 2.86
LE (T3)	21.60 ± 0.07	18.70 ± 0.16
LE (T4)	22.47 ± 0.86	19.52 ± 0.35

2.5 Chlorophyll a fluorescence

Borage treatments did not significantly affect the maximum quantum efficiency of PSII (*Fv/Fm*) (Figure 8 A) even if (T1) and (T2) extracts slightly enhanced this parameter, regardless the raw material (Borage flowers or leaves). All samples were higher than 0.83, usually considered as optimal value of healthy plant. At the same way, the performance index (*PI*) values (Figure 8 B) of plants treated with borage extracts were always higher than control plants but differences were not significant. *TFm* represent the time needed to reach the maximum fluorescence intensity is reported in Figure 8 C. The average value of control leaves was around 230 milliseconds, a little lower if compared with the first growing cycle. FE treatments induced a slight decrease from (T1) to (T3) and a final pick at (T4). In particular (T1) and (T4) time points were higher than control of +13% and +19.6%, respectively. The highest value (275 ms) was observed in plants treated with FE (T4) while the lowest value (215 ms) resulted in plants treated with LE (T3), in contrast to first trial results. However, statistical analysis did not reveal any significant difference among treatments. Similar result was obtained from the analysis of *Sm* parameter (Figure 8 D) and the significant changes observed in the first growing cycle were not revealed in the second one.

All borage extracts induced a general increase in electron transport rate (ETR) levels (Figure 9 A) of rocket leaves if compared with the control. The lowest (23.2 µmol m^{-2} s^{-1}) and the highest (37.6 µmol m^{-2} s^{-1}) values were reached in control and in plants treated with FE (T1). ETR value of control plants was significantly ($P < 0.05$) lower than FE (T1, T2, T3) and LE (T1, T2, T4) treatments of -38.3%, -29.1%, -25.4%, -27%, -24.9% and -33%, respectively. Moreover, plants treated with FE (T1) were significantly ($P < 0.05$) higher than plants treated with

FE (T3), FE (T4), LE (T2) and LE (T4). FE-treated plants showed a decrease in ETR levels over the maceration time. Different effect was observed in the effective quantum yield of photosystem II in the light (ϕ_{PSII}) (Figure 9 B). Most of the plants treated with borage extracts showed similar values with the control (0.78) while a significant ($P < 0.05$) decrease resulted after the treatments with FE (T1), FE (T2) and LE (T4).

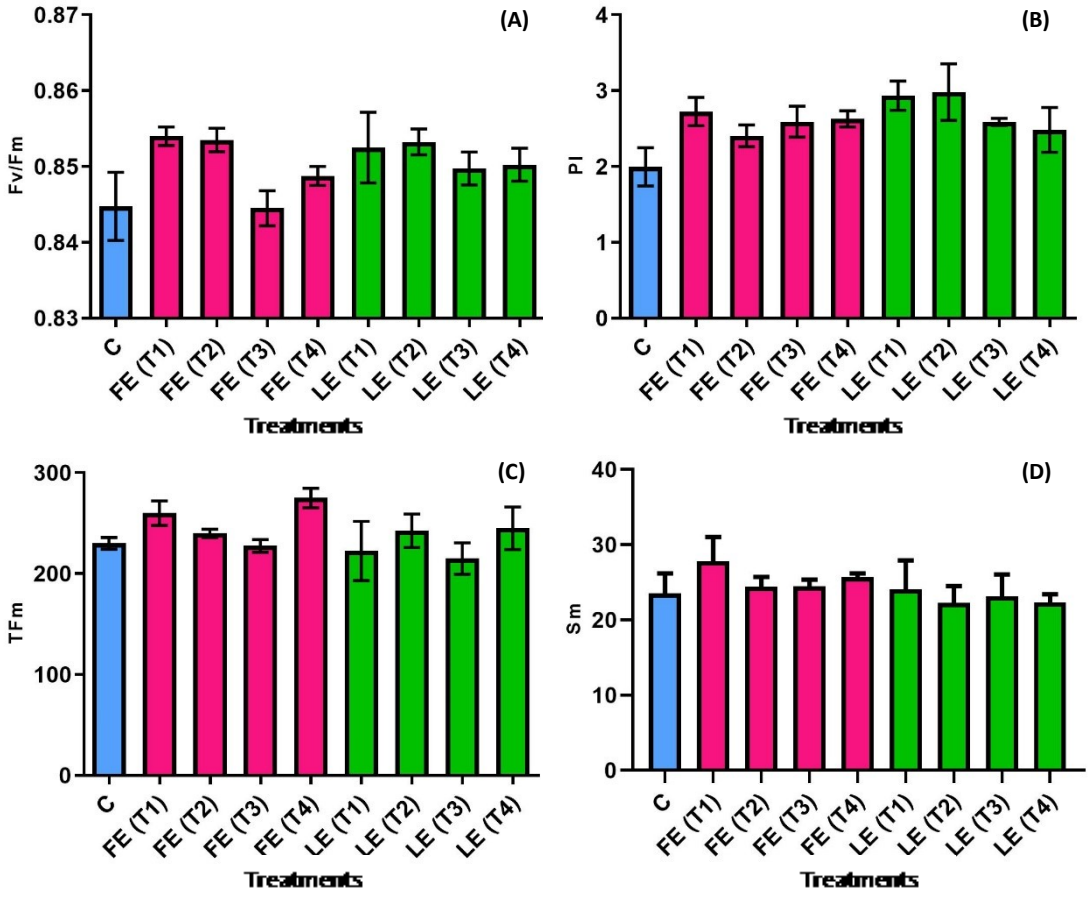


Figure 8. Maximum quantum efficiency of PSII (Fv/Fm) **(A)**, performance index (PI) **(B)**, the time needed to reach the maximum fluorescence intensity (TFm) **(C)** and the total complementary area between fluorescence induction curve normalized per Fm (Sm) **(D)** in rocket leaves treated with water (control = C) and with borage extracts (flowers extract = FE or leaves extract = LE) obtained with different maceration times: 1 day (T1), 3 days (T2), 7 days (T3) and 14 days (T4). Values are means ± SE (n = 4). Data were subjected to one-way ANOVA. Different letters, where present, represent significant differences among treatments.

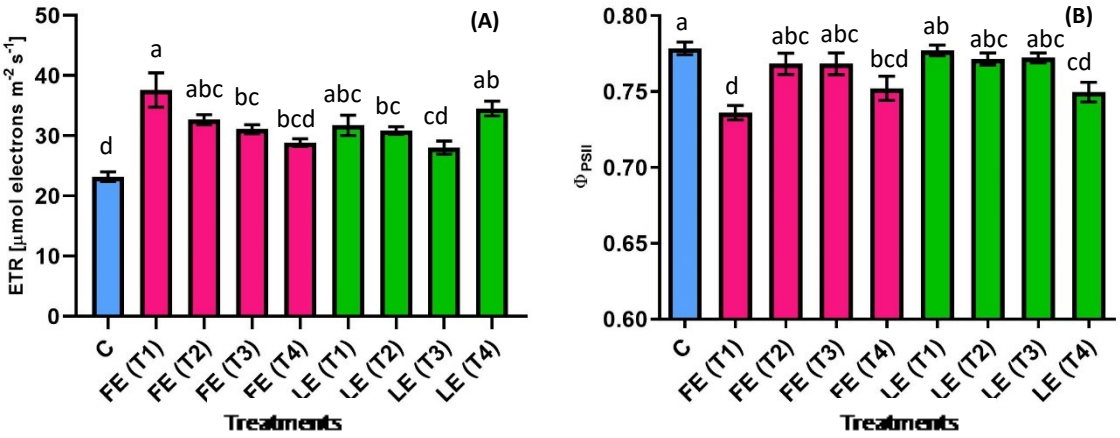


Figure 9. The electron transport rate (ETR) **(A)** and the effective quantum yield of photosystem II in the light (ϕ_{PSII}) **(B)** in rocket leaves treated with water (control = C) and with borage extracts (flowers extract = FE or leaves extract = LE) obtained with different maceration times: 1 day (T1), 3 days (T2), 7 days (T3) and 14 days (T4). Values are means ± SE (n = 5). Data were subjected to one-way ANOVA. Different letters, where present, represent significant differences among treatments.

2.6 Nitrate

Table 6 lists the nitrate concentration measured in rocket leaves. In general, plants treated with borage extracts showed a slight but not significative decrease of nitrate levels compared with the control (6056.3 mg kg^{-1}). Only FE (T4) treatment induce an opposite effect and the average value of nitrate content was 7550.1 mg kg^{-1} FW exceeding the maximum level of nitrate concentration in rocket leaves imposed by EU regulation N. 1258/2011. The statistical analysis revealed significative ($P < 0.05$) differences among this treatment and FE (T1, T3) and LE (T1, T2, T3) treatments.

2.7 Sucrose and total sugars

The levels of sucrose (Table 6) in rocket plants treated with borage extracts were generally lower than the control (414.35 mg kg^{-1} FW). In particular, values recorded in response to FE (T1, T2, T4) and LE (T1, T2) treatments were significantly ($P < 0.05$) decreased of-26.6%, -21%, -23%, -19.8% and -22.5%, respectively. The lowest sucrose concentration was observed in samples treated with FE (T1) and the value was around 303.49 mg kg^{-1} FW. Borage extracts treatments induced a general decrease also in the concentration of total sugars (Table 6). The highest value was around 2901.366 mg kg^{-1} FW and was found in leaves of control plants. Samples treated with FE showed an increase of the concentration of total sugars in relation with maceration time. A

similar trend was observed also in LE-treated plants, after an initial decrease from (T1) to (T2). Nonetheless, no significant ($P < 0.05$) differences among treatments resulted from the statistical analysis.

Table 6. Nitrate and sugars concentrations of rocket leaves treated with water (control = C) and with borage extracts (flowers extract = FE or leaves extract = LE) obtained with different maceration times: 1 day (T1), 3 days (T2), 7 days (T3) and 14 days (T4). Values are means ± SE (n = 3). Data were subjected to one-way ANOVA. Different letters, where present, represent significant differences among treatments.

Treatment	Nitrate [mg kg^{-1} FW]	Sucrose [mg kg^{-1} FW]	Total sugars [mg kg^{-1} FW]
Control	6056.34 ± 673.67 ab	414.35 ± 35.43 a	2901.366 ± 199.14
FE (T1)	4509.88 ± 246.38 b	303.49 ± 9.60 bcd	1227.857 ± 345.42
FE (T2)	4842.85 ± 407.35 ab	326.55 ± 14.38 bcd	1787.41 ± 460.18
FE (T3)	4577.91 ± 207.07 b	354.31 ± 13.73 ad	1891.63 ± 485.37
FE (T4)	7550.83 ± 728.49 a	318.84 ± 1.26 bcd	2641.086 ± 288.75
LE (T1)	2764.00 ± 1335.32 b	332.46 ± 4.68 bcd	2151.289 ± 255.18
LE (T2)	4316.18 ± 159.04 b	321.27 ± 3.15 bcd	1372.175 ± 261.03
LE (T3)	4150.97 ± 976.44 b	364.52 ± 11.22 ab	1642.149 ± 114.20
LE (T4)	5236.57 ± 800.75 ab	358.66 ± 5.31 ac	2749.499 ± 703.27

3 Lettuce

3.1 Total fresh biomass and dry matter

Total fresh biomass and dry matter of lettuce plants are reported in Table 7. The average fresh weight of control plants at harvest was 57.8 g. Borage treatments did not affect plant growth and all values were similar to the control. A slight increase (+26%) was observed in response of LE (T2) treatment, but it was not significative ($P < 0.05$). No significative differences ($P < 0.05$) were observed also in the amount of dry matter.

Table 7. Fresh biomass and dry matter in lettuce leaves treated with water (control = C) and with borage extracts (flowers extract = FE or leaves extract = LE) obtained with different maceration times: 1 day (T1), 3 days (T2), 7 days (T3) and 14 days (T4). Values are means ± SE (n = 4). Data were subjected to one-way ANOVA. Different letters, where present, represent significant differences among treatments.

Treatment	Fresh biomass [g/plant]	Dry matter [%]
Control	57.8 ± 1.82	7.57 ± 0.52
FE (T1)	58.9 ± 2.53	7.76 ± 0.22
FE (T2)	62.9 ± 5.05	7.56 ± 0.47
FE (T3)	60.2 ± 5.05	7.24 ± 0.33
FE (T4)	60.2 ± 5.03	7.72 ± 0.34
LE (T1)	65.6 ± 5.11	7.48 ± 0.22
LE (T2)	72.7 ± 9.79	7.00 ± 0.26
LE (T3)	61.7 ± 4.57	7.42 ± 0.13
LE (T4)	58.4 ± 1.11	7.42 ± 0.28

3.2 Chlorophyll and carotenoids

The levels of chlorophyll estimated and measured with two different techniques are reported in Figure 10 A and B, respectively. The estimation of chlorophyll content *in vivo* showed a slight increase of samples treated with borage extracts but only the difference (+35.7%) between control and LE (T4) treated plants was significative ($P < 0.05$). The average value of chlorophyll *a+b* concentration in rocket leaves was around 0.68 µg mg^{-1} FW but no significant ($P < 0.05$) changes were observed after the application of borage extracts. The content of carotenoids (data not shown) in lettuce plants was not affected by any of the borage extracts treatments and all values averaged from 0.13 µg mg^{-1} FW to 0.18 µg mg^{-1} FW.

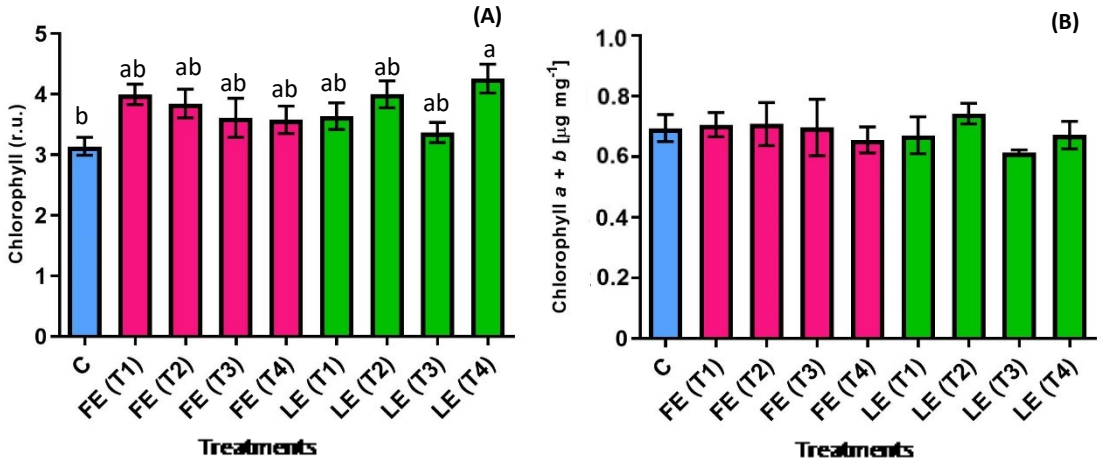


Figure 10. Chlorophyll content determined *in vivo* **(A)** and chlorophyll *a+b* concentration **(B)** in lettuce leaves treated with water (control = C) and with borage extracts (flowers extract = FE or leaves extract = LE) obtained with different maceration times: 1 day (T1), 3 days (T2), 7 days (T3) and 14 days (T4). Values are means ± SE (n = 12) (A); (n = 4) (B). Data were subjected to one-way ANOVA. Different letters, where present, represent significant differences among treatments.

3.3 Phenols and anthocyanin

The concentrations of phenols and anthocyanin are reported in Figure 11 A and B, respectively. Phenol index values averaged from 28.2 in LE (T2) treated plants to 39.6 in FE (T2) treated plants. Two different trends resulted by the application of FE or LE in relation with the maceration time.

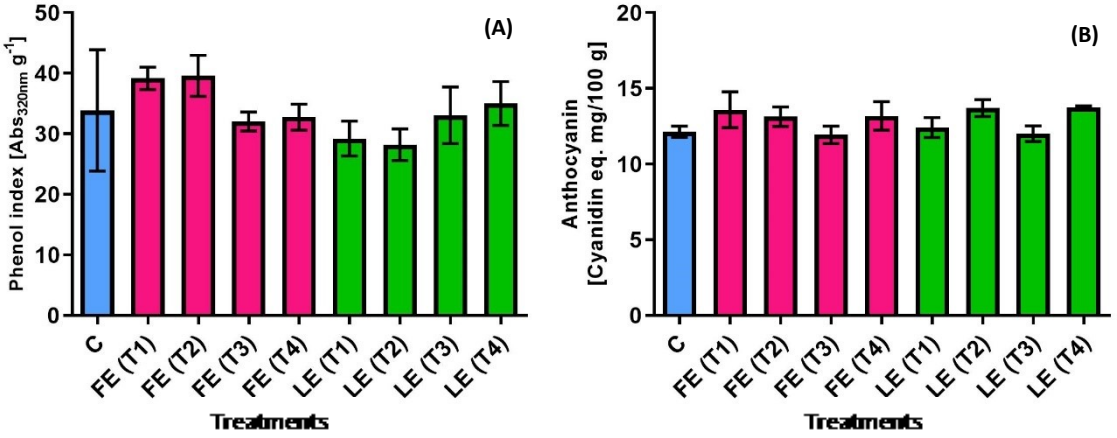


Figure 11. Phenol index **(A)** and anthocyanin content **(B)** in lettuce leaves treated with water (control = C) and with borage extracts (flowers extract = FE or leaves extract = LE) obtained with different maceration times: 1 day (T1), 3 days (T2), 7 days (T3) and 14 days (T4). Values are means ± SE (n = 4). Data were subjected to one-way ANOVA. Different letters, where present, represent significant differences among treatments.

Indeed, (T1) and (T2) were higher than (T3) and (T4) in plants treated with FE, whereas the opposite effect resulted by LE application. However, no significant ($P < 0.05$) differences were observed in phenols index, probably due to the high variability observed in control plants. Anthocyanin content was around 12 mg of cyanidin eq. per 100 g FW both in control and in samples treated with borage extracts.

3.4 Chlorophyll fluorescence

The maximum quantum efficiency of PSII (*Fv/Fm*) (Table 8) did not show any significant ($P < 0.05$) change in response to borage treatments. All values were higher than 0.83, commonly referred to as the threshold value between stressed and unstressed leaf tissue. At the same time, no significant differences were detected in PI, ETR or ϕPSII parameters (Table 8).

Table 8. Maximum quantum efficiency of PSII (Fv/Fm), performance index (PI), electron transport rate (ETR) and the effective quantum yield of photosystem II in the light (ϕ_{PSII}) in lettuce leaves treated with water (control = C) and with borage extracts (flowers extract = FE or leaves extract = LE) obtained with different maceration times: 1 day (T1), 3 days (T2), 7 days (T3) and 14 days (T4). Values are means ± SE (n = 4). Data were subjected to one-way ANOVA. Different letters, where present, represent significant differences among treatments.

Treatment	Fv/Fm	PI	ETR	ϕPSII
Control	0.853 ± 0.002	1.37 ± 0.038	43.96 ± 9.12	0.671 ± 0.02
FE (T1)	0.852 ± 0.003	1.45 ± 0.075	45.17 ± 5.31	0.694 ± 0.02
FE (T2)	0.849 ± 0.002	1.31 ± 0.023	47.11 ± 8.62	0.680 ± 0.01
FE (T3)	0.842 ± 0.005	1.22 ± 0.133	53.85 ± 5.26	0.696 ± 0.01
FE (T4)	0.854 ± 0.003	1.50 ± 0.100	52.52 ± 0.54	0.681 ± 0.02
LE (T1)	0.854 ± 0.006	1.56 ± 0.195	51.27 ± 5.35	0.683 ± 0.01
LE (T2)	0.842 ± 0.007	1.42 ± 0.034	46.80 ± 9.03	0.702 ± 0.02
LE (T3)	0.848 ± 0.002	1.51 ± 0.060	40.29 ± 3.04	0.682 ± 0.01
LE (T4)	0.85 ± 0.002	1.46 ± 0.066	44.43 ± 1.60	0.700 ± 0.01

DISCUSSION AND CONCLUSION

The interest on plant extracts with biostimulants properties is increasing as they have been shown to improve plant growth and quality [26,27,118,119]. Moreover, the application of plant extracts as a potential alternative or additional products to the currently used agrochemicals fit the need of a more sustainable agriculture [120–122]. Thus, several companies and research groups are focused on the study and development of innovative products based on plants. Plants are a rich source of diverse bioactive compounds, however, the high variability in their composition, the difficulties in the choice of the best extraction method, and the stability of the final product are just some of the problems in the formulation of new plant-based products [2,5,123]. Hence, several experiments need to be carried out to assess their effects. Based on the results obtained from previous experiments on *Borago officinalis* [38], the aim of this study was to investigate the effect of the maceration time on the properties of the extracts and their effectiveness on two vegetable crops, rocket and lettuce. First of all, we decided to prepare our extracts from plants grown under controlled conditions instead of collecting the raw material from wild plants since most of the bioactive compounds found in plants are secondary metabolites and they are closely related to the environmental conditions [1]. The purpose was to avoid any unexpected change in the external conditions that might affect the composition of the plants.

To screen the effect of borage extracts, several biochemical analyses were performed on leaves at harvest. In general, no biostimulant effect has been observed, neither in rocker nor in lettuce salad. Indeed, almost no significant difference resulted in any of the analysed parameters and plants treated with different extracts showed a similar response to the control treated with water. Moreover, a certain degree of variability in the results appeared between two growing cycles in rocket trials. The different responses might have been related to the growing seasons or to some change occurred in the composition of the extracts during storage.

One of the characteristic responses of biostimulant products is an increase in chlorophyll content in treated plants. This effect has been observed in a wide range of crop species, environmental conditions and in response of different biostimulant formulations [26,124–128]. Chlorophylls content is an important parameter giving some indication of the physiological status of the plant. Indeed, it is closely related to photosynthetic potential and plant productivity; it can be used as index to have an idea of the nutrient status of the plant since most of the nitrogen is incorporated in chlorophyll molecules, and it is also responsible for the visual appearance of the leaves. This aspect is particularly important in vegetables as quality trait and affecting consumer preferences [129]. The increase in chlorophyll level is basically due to an increase in the biosynthesis of chloroplasts, a reduction in chlorophyll degradation and a delay in senescence. In this study the level of chlorophyll in plants treated with borage extracts did not significantly changed. In particular, in rocket trials, different trends emerged even if the statistical analysis did not prove any significant differences. In particular, different pattern appeared

according to the portion of the borage used to prepare the extract and to the maceration time. The content of chlorophyll *a+b* slightly decreased in plants treated with flowers extracts as the time of extraction increases while, an opposite behaviour has been observed in plants treated with leaves extracts. The same result emerged in lettuce experiment where the chlorophyll content measured as relative units was significantly higher in plants treated with LE (T4) compared with the control, in agreement with those observed by Bulgari et al. [38] in borage extracts.

Analogously, carotenoids content exhibited a similar trend of chlorophylls in all our experiments. This result was expected since these molecules serve as accessory pigments in light harvesting and have an important role in the protection of chlorophylls from photooxidation [130,131]. Moreover, they increase the nutritional value of the product and his health benefit for humans by providing precursors for the synthesis of vitamin A and antioxidants involved in the reduction of various diseases [132]. Nevertheless, the differences observed in chlorophylls content were more emphasized in the analysis of total carotenoids. Their content decreased in plants treated with borage extracts, especially in the first growing cycle of rocket. Besides their importance in photosynthetic tissues, carotenoid also serve as precursors for two phytohormones (abscisic acid and stigolactones) and several apocarotenoids [130,131] and some of them exhibit also strong aroma properties. The accumulation of carotenoids and their stability in plant tissues are affected by several factors such as light, temperature, and water stress [133]. Moreover, it is also known that epoxidation and de-epoxidation reactions in xanthophyll cycle are influenced by the alteration in pH gradients [134,135]. Nevertheless, all the mechanisms underlying their accumulation are not well understood yet.

Even if we observed a decrease of carotenoids concentration after borage treatments, no damage in photosynthetic apparatus resulted from the analysis of chlorophyll fluorescence indexes. Indeed, the performance index (PI) and the quantum maximum efficiency of PSII (Fv/Fm) were similar to control plants and Fv/Fm values was higher than 0.83, commonly considered as a threshold between stressed and non-stressed leaves. Thus, borage treatments might not have caused any stress in photosynthetic tissues of rocket leaves and a different cause was responsible of the reduction in carotenoids content. This effect was not observed neither in the second growing cycle of rocket nor in lettuce plants. Moreover, all borage treatments stimulated the photosynthetic activity by enhancing the electron transport rate (ETR) of rocket plants during the second growing cycle. The increase in the value of ETR has been observed by several authors after the application of different biostimulant products, both in stressful and non-stressful conditions [136,137]. However, at the same time, rocket plants treated with some of the borage extracts showed a decrease in the effective quantum yield of photosystem II in the light (ϕPSII).

Vegetables and in particular leafy vegetables such as rocket and lettuce are considered a high source of nitrate in human diet. Nitrate is found in nature as part of the nitrogen cycle, and play an important role in plant growth,

nutrition, and development [94,138,139]. Its accumulation in plant tissues is a complex trait and is influenced by many internal and external factors such as the plant species/cultivar, the amount of fertilizer used, the light intensity, and temperature. Nitrate is relatively non-toxic by itself, but once transformed in nitrite by human body it become dangerous because if it reacts with amine or amides it can produce N-nitroso compounds increasing the risk of several diseases [140]. For this reason, the European Union imposed limits in nitrate concentration for the commercialization of several leafy products, including rocket and lettuce salad [141]. Thus, the determination of nitrate content has received increased attention and much research has been conducted to minimize the accumulation of nitrate in vegetables. Recently, it was proven that different biostimulant products reduce the nitrate levels in several plant species [124,125,142–145]. Among them, previous experiments showed that also borage extracts are able to decrease the nitrate accumulation in rocket by incrementing the activity of nitrate reductase. In the present study only a slight but not significative decrease of nitrate levels was observed in plants treated with almost all borage extracts, except for FE (T4) during the second growing cycle. This treatment induced an opposite effect and nitrate content was 7550.1 mg kg^{-1} FW, exceedingly also the maximum level of nitrate concentration imposed by EU regulation N. 1258/2011.

At the same time, a decrease in sucrose levels was observed in plants treated with borage extracts, while the total sugars amount was not significantly affected. These results are in contrast to previous experiments on borage extracts where sucrose level increased in in rocket leaves after flower extract treatments [38]. Sucrose is the metabolic link between photosynthetic carbon production and carbon utilisation. It is the end product of photosynthesis and it function as carbon skeleton for the production of several molecules. In plants, sucrose is transported from source organs to sink organs, where it is stored or metabolised. It is also considered as a signal molecule, involved in the regulation of different metabolic pathways. In present study, the lower amount of sucrose observed in rocket leaves might be attributed to a faster metabolization or transport and not to a decrease in its biosynthesis. Indeed, as confirmed by the levels of photosynthetic pigment or by the analyses of chlorophyll fluorescence indexes, borage extracts did not negatively affect the photosynthetic apparatus.

Overall, based on the current results, even if different trends resulted from the application of borage extracts, neither the time of maceration nor the part of the plants used showed a clear effect on the efficacy of borage treatments. Moreover, the high variability observed, did not help us to understand the effective properties and the mechanisms of action of these extracts. At the same time did not allow us to discriminate the different borage treatment. The high variability and the ineffectiveness of the treatments are probably related to the borage cultivation system. Indeed, it is known that environmental stress such as drought, salinity, soil strength, and nutrient status, have a great influence on the accumulation of the active compounds in plants. Moreover, the longer time of maceration chosen in the previous experiment might have led the extraction of different active molecules.

REFERENCES

1. Mazid, M.; Khan, T.; Mohammad, F. Role of Secondary Metabolites in Chemical Defence Mechanisms in Plants. *Front. Life Sci.* **2011**, *3*, 232–249.
2. Naboulsi, I.; Aboulmouhajir, A.; Kouisni, L.; Bekkaoui, F.; Yasri, A. Plants extracts and secondary metabolites , their extraction methods and use in agriculture for controlling crop stresses and improving productivity : A review. *Acad. J. Med. Plants* **2018**, *6*, 223–240.
3. Starmans, D.A.J.; Nijhuis, H.H. Extraction of secondary metabolites from plant material: A review. *Trends Food Sci. Technol.* **1996**, *7*, 191–197.
4. *Extraction Technologies for Medicinal and Aromatic Plants*; Handa, S.S., Khanuja, S.P.S., Longo, G., Rakesh, D.D., Eds.; Trieste, 2008; Vol. 6;.
5. Azmir, J.; Zaidul, I.S.M.; Rahman, M.M.; Sharif, K.M.; Mohamed, A.; Sahena, F.; Jahurul, M.H.A.; Ghafoor, K.; Norulaini, N.A.N.; Omar, A.K.M. Techniques for extraction of bioactive compounds from plant materials: A review. *J. Food Eng.* **2013**, *117*, 426–436.
6. Azwanida, N. A Review on the Extraction Methods Use in Medicinal Plants, Principle, Strength and Limitation. *Med. Aromat. Plants* **2015**, *04*, 3–8.
7. Raynie, D.E. EXTRACTION. In *Encyclopedia of Separation Science*; Elsevier, 2000; pp. 118–128.
8. Mustafa, A.; Turner, C. Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review. *Anal. Chim. Acta* **2011**, *703*, 8–18.
9. Chan, C.-H.; Yusoff, R.; Ngoh, G.-C.; Kung, F.W.-L. Microwave-assisted extractions of active ingredients from plants. *J. Chromatogr. A* **2011**, *1218*, 6213–6225.
10. Vinatoru, M.; Toma, M.; Radu, O.; Filip, P.I.; Lazurca, D.; Mason, T.J. The use of ultrasound for the extraction of bioactive principles from plant materials. *Ultrason. Sonochem.* **1997**, *4*, 135–139.
11. Lang, Q. Supercritical fluid extraction in herbal and natural product studies - a practical review. *Talanta* **2001**, *53*, 771–782.
12. Gbashi, S.; Adebo, O.A.; Piater, L.; Madala, N.E.; Njobeh, P.B. Subcritical Water Extraction of Biological Materials. *Sep. Purif. Rev.* **2017**, *46*, 21–34.
13. *Electrotechnologies for Extraction from Food Plants and Biomaterials*; Lebovka, N., Vorobiev, E., Eds.; Food Engineering Series; Springer New York: New York, NY, 2009; ISBN 978-0-387-79373-3.
14. Puri, M.; Sharma, D.; Barrow, C.J. Enzyme-assisted extraction of bioactives from plants. *Trends Biotechnol.* **2012**, *30*, 37–44.

15. Naviglio, D. Naviglio's principle and presentation of an innovative solid-liquid extraction technology: Extractor Naviglio®. *Anal. Lett.* **2003**, *36*, 1647–1659.
16. Vongsak, B.; Sithisarn, P.; Mangmool, S.; Thongpraditchote, S.; Wongkrajang, Y.; Gritsanapan, W. Maximizing total phenolics, total flavonoids contents and antioxidant activity of Moringa oleifera leaf extract by the appropriate extraction method. *Ind. Crops Prod.* **2013**, *44*, 566–571.
17. Sithisarn, P.; Supabphol, R.; Gritsanapan, W. Comparison of free radical scavenging activity of Siamese neem tree (Azadirachta indica A. Juss var. siamensis Valeton) leaf extracts prepared by different methods of extraction. *Med. Princ. Pract.* **2006**, *15*, 219–222.
18. Siegwart, M.; Graillot, B.; Blachere Lopez, C.; Besse, S.; Bardin, M.; Nicot, P.C.; Lopez-Ferber, M. Resistance to bio-insecticides or how to enhance their sustainability: a review. *Front. Plant Sci.* **2015**, *6*, 1–19.
19. Benner, J.P. Pesticidal compounds from higher plants. *Pestic. Sci.* **1993**, *39*, 95–102.
20. Gurjar, M.S.; Ali, S.; Akhtar, M.; Singh, K.S. Efficacy of plant extracts in plant disease management. *Agric. Sci.* **2012**, *03*, 425–433.
21. Peterson, R.; Jensén, P. Effects of nettle water on growth and mineral nutrition of plants. II. pot-and water-culture experiments. *Biol. Agric. Hortic.* **1986**, *4*, 7–18.
22. Abdel-Rahman, S.S.A.; Abdel-Kader, A.A.S. Response of Fennel (Foeniculum vulgare, Mill) plants to foliar application of moringa leaf extract and benzyladenine (BA). *South African J. Bot.* **2019**, 1–10.
23. Abou-Sreea, A.I..; Matter, F.M.. . USING MORINGA LEAF EXTRACT AS BIOSTIMULANT AND GIBERRELLIC ACID FOR ENHANCING FENNEL (Foeniculum vulgare var . azoricum Mill .) GROWTH AND OIL YIELD. *Acta Sci. Intellectus* **2016**, *2*.
24. Abd El-Mageed, T.A.; Semida, W.M.; Rady, M.M. Moringa leaf extract as biostimulant improves water use efficiency, physio-biochemical attributes of squash plants under deficit irrigation. *Agric. Water Manag.* **2017**, *193*, 46–54.
25. Howladar, S.M. A novel Moringa oleifera leaf extract can mitigate the stress effects of salinity and cadmium in bean (Phaseolus vulgaris L.) plants. *Ecotoxicol. Environ. Saf.* **2014**, *100*, 69–75.
26. Abdalla, M.M. The potential of Moringa oleifera extract as a biostimulant in enhancing the growth, biochemical and hormonal contents in rocket (Eruca vesicaria subsp. sativa) plants. *Int. J. Plant Physiol. Biochem.* **2013**, *5*, 42–49.
27. Culver, M.; Fanuel, T.; Chiteka, A.Z. Effect of Moringa Extract on Growth and Yield of Tomato.

Greener J. Agric. Sci. **2012**, *2*, 207–211.

28. Rady, M.M.; Desoky, E.S.M.; Elrys, A.S.; Boghdady, M.S. Can licorice root extract be used as an effective natural biostimulant for salt-stressed common bean plants? *South African J. Bot.* **2019**, *121*, 294–305.
29. Asadi-Samani, M.; Bahmani, M.; Rafieian-Kopaei, M. The chemical composition, botanical characteristic and biological activities of Borago officinalis: A review. *Asian Pac. J. Trop. Med.* **2014**, *7*, S22–S28.
30. Hebard, A.B.; Coupland, K.; Boughton, D.N.; Surette, M. PLANT DERIVED LIPID USEFUL FOR NUTRACEUTICAL AND COSEMECEUTICAL APPLICATIONS 2008.
31. Flider, F.J. *INFORM - International News on Fats, Oils and Related Materials*. 2005, p. 279.
32. Fan, Y.-Y.; Chapkin, R.S. Importance of Dietary γ-Linolenic Acid in Human Health and Nutrition. *J. Nutr.* **1998**, *128*, 1411–1414.
33. Aliakbarlu, J.; Tajik, H. Antioxidant and antibacterial activities of various extracts of borago officinalis flowers. *J. Food Process. Preserv.* **2012**, *36*, 539–544.
34. Ciriano, M.G.I. de; García-Herreros, C.; Larequi, E.; Valencia, I.; Ansorena, D.; Astiasarán, I. Use of natural antioxidants from lyo1. Kalhor, H.R.; Ashrafian, H. Identification of an aspidospermine derivative from borage extract as an anti-amyloid compound: A possible link between protein aggregation and antimalarial drugs. Phytochemistry 2017, 140. *Meat Sci.* **2009**, *83*, 271–277.
35. Gómez-Estaca, J.; Giménez, B.; Montero, P.; Gómez-Guillén, M.C. Incorporation of antioxidant borage extract into edible films based on sole skin gelatin or a commercial fish gelatin. *J. Food Eng.* **2009**, *92*, 78–85.
36. Bellés, M.; Alonso, V.; Roncalés, P.; Beltrán, J.A. Display stability of fresh and thawed lamb supplemented with vitamin E or sprayed with an antioxidant borage seed extract. *J. Sci. Food Agric.* **2018**, *98*, 2871–2879.
37. Bellés, M.; Alonso, V.; Roncalés, P.; Beltrán, J.A. Effect of borage and green tea aqueous extracts on the quality of lamb leg chops displayed under retail conditions. *Meat Sci.* **2017**, *129*, 153–160.
38. Bulgari, R.; Morgutti, S.; Cocetta, G.; Negrini, N.; Farris, S.; Calcante, A.; Spinardi, A.; Ferrari, E.; Mignani, I.; Oberti, R.; et al. Evaluation of Borage Extracts As Potential Biostimulant Using a Phenomic, Agronomic, Physiological, and Biochemical Approach. *Front. Plant Sci.* **2017**, *8*, 1–16.
39. Janick, J.; Simon, J.E.; Quinn, J.; Beaubaire, N. Borage: a source of gamma linolenic acid. In

Herbs, Spices, and Medicinal Plants: Recent Advances in Botany, Horticulture, and Pharmacology; 1990; pp. 145–168.

40. Beaubaire, N.A.; Simon, J.E. PRODUCTION POTENTIAL OF BORAGO OFFICINALIS L. *Acta Hortic.* **1987**, 101–114.
41. SIMPSON, M.J.A. A description and code of development of borage Borago officinalis. *Ann. Appl. Biol.* **1993**, *123*, 187–192.
42. Mansion, G.; Selvi, F.; Guggisberg, A.; Conti, E. Origin of Mediterranean insular endemics in the Boraginales: Integrative evidence from molecular dating and ancestral area reconstruction. *J. Biogeogr.* **2009**, *36*, 1282–1296.
43. SELVI, F.; COPPI, A.; BIGAZZI, M. Karyotype Variation, Evolution and Phylogeny in Borago (Boraginaceae), with Emphasis on Subgenus Buglossites in the Corso-Sardinian System. *Ann. Bot.* **2006**, *98*, 857–868.
44. Kalhor, H.R.; Ashrafian, H. Identification of an aspidospermine derivative from borage extract as an anti-amyloid compound: A possible link between protein aggregation and antimalarial drugs. *Phytochemistry* **2017**, *140*, 134–140.
45. Jaffel, K.; Sai, S.; Bouraoui, N.K.; Ammar, R.B.; Legendre, L.; Lachâal, M.; Marzouk, B. Influence of salt stress on growth, lipid peroxidation and antioxidative enzyme activity in borage (Borago officinalis L.). *Plant Biosyst. - An Int. J. Deal. with all Asp. Plant Biol.* **2011**, *145*, 362–369.
46. Jaffel-Hamza, K.; Sai-Kachout, S.; Harrathi, J.; Lachaâl, M.; Marzouk, B. Growth and Fatty Acid Composition of Borage (Borago officinalis L.) Leaves and Seeds Cultivated in Saline Medium. *J. Plant Growth Regul.* **2013**, *32*, 200–207.
47. Chakovari, S.Z.; Enteshari, S.; Qasimov, N.; Chakovari, Z.; Enteshari, S.; Qasimov, N. Effect of salinity stress on biochemical parameters and growth of borage (Borago officinalis L.). *Iran. J. Plant Physiol.* **2016**, *6*, 1673–1685.
48. TORABI, F.; MAJD, A.; ENTESHARI, S.; IRIAN, S.; NABIUNI, M. Effects of Salinity on the Development of Hydroponically Grown Borage (Borago officinalis L.) Male Gametophyte. *Not. Bot. Horti Agrobot. Cluj-Napoca* **2013**, *41*, 65.
49. Badi, H.N.; Sorooshzadeh, A. Evaluating potential of Borage (Borago officinalis L.) in bioremediation of saline soil saffron production in vertical system View project Effects of auxin and copper on growth of saffron View project. *Artic. AFRICAN J. Biotechnol.* **2011**, *10*, 146–153.
50. Ghaderi, F.A.; Solatani, A.; Sadeghipour, H.R. Cardinal Temperatures of Germination in

Medicinal Pumpkin (Cucurbita pepo convar. pepo var. styriaca), Borago (Borago officinalis L.) and Black Cumin (Nigella sativa L.). *Asian J. plant Sci.* **2008**, *7*, 574–578.

51. Gilbertson, P.K.; Berti, M.T.; Johnson, B.L. Borage cardinal germination temperatures and seed development. *Ind. Crops Prod.* **2014**, *59*, 202–209.
52. Descamps, C.; Quinet, M.; Baijot, A.; Jacquemart, A.L. Temperature and water stress affect plant–pollinator interactions in Borago officinalis (Boraginaceae). *Ecol. Evol.* **2018**, *8*, 3443–3456.
53. Berti, M.; Wilckens, R.; Fischer, S.; Araos, R. Borage: A new crop for Southern Chile. *Trends new Crop. new uses* **2002**, 501–505.
54. Berti, M.; Fischer, S.; Wilckens, R.; Hevia, F.; Johnson, B.L. BORAGE (Borago officinalis L.) RESPONSE TO N, P, K, AND S FERTILIZATION IN SOUTH CENTRAL CHILE. *Chil. J. Agric. Res.* **2010**, *70*, 228–236.
55. El Hafid, R.; Blade, S.F.; Hoyano, Y. Seeding date and nitrogen fertilization effects on the performance of borage (Borago officinalis L.). *Ind. Crops Prod.* **2002**, *16*, 193–199.
56. SIMPSON, M.J.A. Comparison of swathing and desiccation of borage Borago officinalis and estimation of optimum harvest stage. *Ann. Appl. Biol.* **1993**, *123*, 105–108.
57. Mhamdi, B.; Aidi Wannes, W.; Sriti, J.; Jellali, I.; Ksouri, R.; Marzouk, B. Effect of harvesting time on phenolic compounds and antiradical scavenging activity of Borago officinalis seed extracts. *Ind. Crops Prod.* **2010**, *31*, 2004–2007.
58. Galambosi, B.; Domokos, J.; Sairanen, J. Experiences with different methods of harvesting borage (Borago officinalis L.). *Zeitschrift fur Arznei- und Gewurzpflanzen* **2014**, *19*, 61–66.
59. De Haro-Bailón, A.; Del Rio, M. Isolation of chemically induced mutants in Borage (Borago officinalis L.). *JAOCS, J. Am. Oil Chem. Soc.* **1998**, *75*, 281–283.
60. De Haro, A.; Del Río, M.; Alcaide, B.; Rapoport, H.; Cabrera, A. Characterisation and evaluation of species of the Boraginaceae family as source of gamma-linolenic acid for mediterranean conditions. *Acta Hortic.* **2004**, *629*, 231–237.
61. Laurence, R. *Borage production for oil and gamma linolenic acid*; 2004;
62. Hendawy, S.F.; El-Gengaihi, S. Comparative responses of borago officinalis and echium vulgare to different nitrogen and phosphorus sources. *J. Herbs, Spices Med. Plants* **2010**, *16*, 12–23.
63. Ebrahimi, A.; Moaveni, P.; Farahani, H.A. Effects of planting dates and compost on mucilage variations in borage (Borago officinalis L .) under different chemical fertilization systems. **2010**, *1*, 58–61.

64. Mahmoodi, P.; Yarnia, M.; Rashidi, V.; Amirnia, R.; Tarinejhad, A. Effects of nano and chemical fertilizers on physiological efficiency and essential oil yield of borago officinalis L. *Appl. Ecol. Environ. Res.* **2018**, *16*, 4773–4788.
65. El-Gengaihi, S.; Wahba, H.E.; Reffaat, A.M. The response of borage plants (Borago officinalis L.) to different nitrogen source and rates. *Egypt. J. Hortic.* **2000**, *27*, 409–423.
66. Ghassemi-golezani, K.; Dastborhan, S.; Zehtab-salmasi, S. Seed Priming and Field Performance of Borage (*Borago officinalis* L.) under Different Irrigation Treatments. *Int. J. Agron. Plant Prod.* **2013**, *4*, 82–87.
67. Tafazolli, E.; Zeraatkish, Y.; Moosavi, S.N. The Impacts Economic Analysis of Deficit Irrigation and Integrated Application of Biological Fertilizers on Cultivation of Borage in Iran. **2016**, 59–65.
68. Aidi Wannes, W.; Mhamdi, B.; Saidani Tounsi, M.; Marzouk, B. Lipid and volatile composition of borage (Borago officinalis L.) leaf Wissem. *Trends Phytochem. Res.* **2017**, *1*, 183–198.
69. Borowy, A.; Chwil, M.; Kapłan, M. Biologically active compounds and antioxidant activity of borage (Borago officinalis L.) flowers and leaves. *Acta Sci. Pol. Hortorum Cultus* **2017**, *16*, 169–180.
70. Griffiths, G.; Brechany, E.Y.; Jackson, F.M.; Christie, W.W.; Stymne, S.; Keith Stobart, A. Distribution and biosynthesis of stearidonic acid in leaves of Borago officinalis. *Phytochemistry* **1996**, *43*, 381–386.
71. Mhamdi, B.; Aidi Wannes, W.; Marzouk, B. Biochemical evaluation of borage (Borago officinalis) rosette leaves through their essential oil and fatty acid composition. *Ital. J. Biochem.* **2007**, *56*, 176–9.
72. Río-Celestino, M. del; Font, R.; de Haro-Bailón, A. Distribution of fatty acids in edible organs and seed fractions of borage (Borago officinalis L.). *J. Sci. Food Agric.* **2008**, *88*, 248–255.
73. Aidi Wannes, W.; Mhamdi, B.; Saidani Tounsi, M.; Marzouk, B. Lipid and volatile composition of borage (Borago officinalis L.) leaf. *Trends Phytochem. Res.* **2017**, *1*, 143–148.
74. Stähler, K.; Quek, S.Y.; Miller, M.R. Investigation of γ-linolenic acid and stearidonic acid biosynthesis during a life cycle of Borago officinalis L. *JAOCS, J. Am. Oil Chem. Soc.* **2011**, *88*, 1715–1725.
75. Mhamdi, B.; Wannes; Aidi, W.; Bourgou, S.; Marzouk, B. Biochemical characterization of borage (Borago officinalis L.) seeds. **2007**, *33*, 331–341.
76. Salem, N.; Msaada, K.; Hammami, M.; Limam, F.; Vasapollo, G.; Marzouk, B. Variation in anthocyanin and essential oil composition and their antioxidant potentialities during flower

development of Borage (Borago officinalis L.). *Plant Biosyst.* **2014**, *148*, 444–459.

77. Salem, N.; Msaada, K.; Hammami, M.; Jday, A.; Salem, S.; Limam, F.; Marzouk, B. Variations in Tunisian borage essential oil profiles and their antioxidant activities during flowering. *Nat. Prod. Res.* **2014**, *28*, 1919–1922.
78. Zribi, I.; Bleton, J.; Moussa, F.; Abderrabba, M. GC-MS analysis of the volatile profile and the essential oil compositions of Tunisian Borago Officinalis L.: Regional locality and organ dependency. *Ind. Crops Prod.* **2019**, *129*, 290–298.
79. Mhamdi, B.; Biotechnologie, C. De; Wannes, W.A.; Biotechnologie, C. De Phenolic compound and antiradical scavenging activity changes during Borago officinalis stalk leaf development. **2010**.
80. Zemmouri, H.; Ammar, S.; Boumendjel, A.; Messarah, M.; El Feki, A.; Bouaziz, M. Chemical composition and antioxidant activity of Borago officinalis L. leaf extract growing in Algeria. *Arab. J. Chem.* **2014**.
81. Conforti, F.; Sosa, S.; Marrelli, M.; Menichini, F.; Statti, G.A.; Uzunov, D.; Tubaro, A.; Menichini, F.; Loggia, R. Della In vivo anti-inflammatory and in vitro antioxidant activities of Mediterranean dietary plants. *J. Ethnopharmacol.* **2008**, *116*, 144–151.
82. Segovia, F.J.; Luengo, E.; Corral-Pérez, J.J.; Raso, J.; Almajano, M.P. Improvements in the aqueous extraction of polyphenols from borage (Borago officinalis L.) leaves by pulsed electric fields: Pulsed electric fields (PEF) applications. *Ind. Crops Prod.* **2015**, *65*, 390–396.
83. Karimi, E.; Oskoueian, E.; Karimi, A.; Noura, R.; Ebrahimi, M. Borago officinalis L. flower: a comprehensive study on bioactive compounds and its health-promoting properties. *J. Food Meas. Charact.* **2018**, *12*, 826–838.
84. Bandoniene, D.; Murkovic, M. The detection of radical scavenging compounds in crude extract of borage (Borago officinalis L.) by using an on-line HPLC-DPPH method. *J. Biochem. Biophys. Methods* **2002**, *53*, 45–49.
85. Bandoniene, D.; Murkovic, M.; Venskutonis, P.R. Determination of rosmarinic acid in sage and borage leaves by high-performance liquid chromatography with different detection methods. *J. Chromatogr. Sci.* **2005**, *43*, 372–376.
86. Wettasinghe, M.; Shahidi, F.; Amarowicz, R.; Abou-Zaid, M.M. Phenolic acids in defatted seeds of borage (Borago officinalis L.). *Food Chem.* **2001**, *75*, 49–56.
87. Samy, M.N.; Hamed, A.N.E.S.; Sugimoto, S.; Otsuka, H.; Kamel, M.S.; Matsunami, K. Officinalioside, a new lignan glucoside from Borago officinalis L. *Nat. Prod. Res.* **2015**, *30*, 967–972.

88. Larson, K.M.; Roby, M.R.; Stermitz, F.R. Unsaturated pyrrolizidines from borage (borago officinalis) a common garden herb. **1983**, *47*, 747–748.

89. Dodson, C.D.; Stermitz, F.R. Pyrrolizidine Alkaloids from Borage (Borago Officinalis) Seeds and Flowers. *J. Nat. Prod.* **1986**, *49*, 727–728.

90. Herrmann, M.; Joppe, H.; Schmaus, G. Thesinine-4'-O-β-D-glucoside the first glycosylated plant pyrrolizidine alkaloid from Borago officinalis. *Phytochemistry* **2002**, *60*, 399–402.

91. Vacillotto, G.; Favretto, D.; Seraglia, R.; Pagiotti, R.; Traldi, P.; Mattoli, L. A rapid and highly specific method to evaluate the presence of pyrrolizidine alkaloids in Borago officinalis seed oil. *J. Mass Spectrom.* **2013**, *48*, 1078–1082.

92. Medrano, A.; Masoud, T.A.; Martinez, M.C. Mineral and proximate composition of borage. *J. Food Compos. Anal.* **1992**, *5*, 313–318.

93. Volpe, M.G.; Nazzaro, M.; Di Stasio, M.; Siano, F.; Coppola, R.; De Marco, A. Content of micronutrients, mineral and trace elements in some Mediterranean spontaneous edible herbs. *Chem. Cent. J.* **2015**, *9*, 17–19.

94. Bianco, V.V.; Santamaria, P.; Elia, A. NUTRITIONAL VALUE AND NITRATE CONTENT IN EDIBLE WILD SPECIES USED IN SOUTHERN ITALY. *Acta Hortic.* **1998**, 71–90.

95. Aghofack-Nguemezi, J.; Tatchago, V. Changes in Glycerolipid and Sterol Contents in Leaves of Borago officinalis L. Plants as Related to Growth and Development. *Pakistan J. Biol. Sci.* **2006**, *9*, 686–691.

96. Sayanova, O.; Shewry, P.R.; Napier, J.A. Characterization and expression of a fatty acid desaturase from Borago officinalis. *J. Exp. Bot.* **1999**, *50*, 411–412.

97. Peiretti, P.G.; Palmegiano, G.B.; Salamano, G. Quality and fatty acid content of borage (Borago officinalis L.) during the growth cycle. *Ital. J. Food Sci.* **2004**, *16*, 177–184.

98. Jamieson, G.R.; Reid, E.H. The leaf lipids of some members of the boraginaceae family. *Phytochemistry* **1969**, *8*, 1489–1494.

99. Peiretti, P.G.; Tassone, S.; Masoero, G. Lipid maturity trend in crops as characterized by α -linolenic acid decay and by NIRS study. **2015**, 4–16.

100. Galwey, N.W.; Shirlin, A.J. Selection of borage (Borago officinalis) as a seed crop for pharmaceutical uses. *Heredity (Edinb).* **1990**, *65*, 249–257.

101. Mohajer, S.; Taha, R.M.; Ramli, R. Bin; Mohajer, M. Phytochemical constituents and radical scavenging properties of Borago officinalis and Malva sylvestris. *Ind. Crops Prod.* **2016**, *94*, 673–681.

102. Zadernowski, R.; Naczk, M.; Nowak-Polakowska, H. Phenolic acids of borage (Borago

officinalis L.) and evening primrose (Oenothera biennis L.). *JAOCS, J. Am. Oil Chem. Soc.* **2002**, *79*, 335–338.

103. Biscotti, N.; Pieroni, A.; Luczaj, L. The hidden Mediterranean diet: Wild vegetables traditionally gathered and consumed in the Gargano area, Apulia, SE Italy. *Acta Soc. Bot. Pol.* **2015**, *84*, 327–338.
104. Giménez, B.; Gómez-Guillén, M.C.; Pérez-Mateos, M.; Montero, P.; Márquez-Ruiz, G. Evaluation of lipid oxidation in horse mackerel patties covered with borage-containing film during frozen storage. *Food Chem.* **2011**, *124*, 1393–1403.
105. Gupta, M.; Singh, S.; Linn, B.O. BORAGO OFFICINALIS LINN . AN IMPORTANT MEDICINAL PLANT OF MEDITERRANEAN REGION : A REVIEW. *Int. J. Pharm. Sci. Res.* **2010**, *5*, 27–34.
106. Pieszaki, M.; MIKOŁAJCZAK, P.; MANIKOWSKA, K. Borage (Borago officinalis L.) – a valuable medicinal plant used in herbal medicine. **2012**, *58*, 95–103.
107. Tewari, D.; Bawari, S.; Patni, P.; Sah, A.N. *Borage (Borago officinalis L.)*; Elsevier Inc., 2019; ISBN 9780128124918.
108. Tollesson, A.; Frithz, A. Borage oil, an effective new treatment for infantile seborrhoeic dermatitis. *Br. J. Dermatol.* **1993**, *129*, 95–95.
109. Leventhal, L.J. Treatment of Rheumatoid Arthritis with Gammalinolenic Acid. *Ann. Intern. Med.* **1993**, *119*, 867.
110. Gilani, A.H.; Bashir, S.; Khan, A. ullah Pharmacological basis for the use of Borago officinalis in gastrointestinal, respiratory and cardiovascular disorders. *J. Ethnopharmacol.* **2007**, *114*, 393–399.
111. Rezk, M.M.; Sarhan, K.A.; Ammar, A.A.A. Ameliorative Effect of Borage Seeds Oil against Radiation- Induced Hepatotoxicity in Rats Ameliorative Effect of Borage Seeds Oil against Radiation- Induced Hepatotoxicity in Rats. **2018**.
112. Komaki, A.; Rasouli, B.; Shahidi, S. Anxiolytic Effect of Borago officinalis (Boraginaceae) Extract in Male Rats. *Avicenna J. Neuro Psych Physiol.* **2015**, *2*, 31–33.
113. Sarris, J.; Camfield, D.; Berk, M. Complementary medicine, self-help, and lifestyle interventions for Obsessive Compulsive Disorder (OCD) and the OCD spectrum: A systematic review. *J. Affect. Disord.* **2012**, *138*, 213–221.
114. Al-Moubaraki, A.H. Potential of borage flowers aqueous extract, Borago officinalis L., against the corrosion of mild steel in phosphoric acid. *Anti-Corrosion Methods Mater.* **2018**, *65*, 53–65.
115. Lichtenthaler, H.K. Chlorophylls and carotenoids: Pigments of photosynthetic

biomembranes. In; 1987; pp. 350–382.

116. Cataldo, D.A.; Maroon, M.; Schrader, L.E.; Youngs, V.L. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun. Soil Sci. Plant Anal.* **1975**, *6*, 71–80.
117. Yemm, E.W.; Willis, A.J. The estimation of carbohydrates in plant extracts by anthrone. *Biochem. J.* **1954**, *57*, 508.
118. Pardo-García, A.I.; Martínez-Gil, A.M.; Cadahía, E.; Pardo, F.; Alonso, G.L.; Salinas, M.R. Oak extract application to grapevines as a plant biostimulant to increase wine polyphenols. *Food Res. Int.* **2014**, *55*, 150–160.
119. Szparaga, A.; Piskier, T. THE EFFECT OF TREATING THE SEEDS OF Brassica oleracea L . WITH AQUEOUS EXTRACTS ON THE GERMINATION CAPACITY AND SEED. **2017**.
120. Toscano, S.; Romano, D.; Massa, D.; Bulgari, R.; Franzoni, G.; Ferrante, A. Biostimulant applications in low input horticultural cultivation systems. *Italus Hortus* **2019**, *25*, 27–36.
121. Xu, L.; Geelen, D. Developing Biostimulants From Agro-Food and Industrial By-Products. *Front. Plant Sci.* **2018**, *9*, 1–13.
122. Chojnacka, K. Innovative bio-products for agriculture. *Open Chem.* **2015**, *13*, 932–937.
123. Lapornik, B.; Prošek, M.; Wondra, A.G. Comparison of extracts prepared from plant by-products using different solvents and extraction time. *J. Food Eng.* **2005**, *71*, 214–222.
124. Vernieri, P.; Borghesi, E.; Tognoni, F.; Serra, G.; Ferrante, A.; Piaggesi, A. Use of biostimulants for reducing nutrient solution concentration in floating system. *Acta Hortic.* **2006**, *718*, 477–484.
125. Vernieri, P.; Borghesi, E.; Ferrante, A. Application of Biostimulants in Floating System for Improving Rocket Quality Bioactive Compounds Biosynthesis and Metabolism in Fruit and Vegetables View project Plant responses to biostimulants application View project. **2005**, 1–4.
126. Bulgari, R.; Podetta, N.; Cocetta, G.; Piaggesi, A.; Ferrante, A. The effect of a complete fertilizer for leafy vegetables production in family and urban gardens. *Bulg. J. Agric. Sci.* **2014**, *20*, 1361–1367.
127. Abbas, S.M.; Akladious, S.A. Application of carrot root extract induced salinity tolerance in cowpea (Vigna sinensis L.) seedlings. *Pakistan J. Bot.* **2013**, *45*, 795–806.
128. Peterson, R.; Jensén, P. Effects of Nettle Water on Growth and Mineral Nutrition of Plants. I. Composition and Properties of Nettle Water. *Biol. Agric. Hortic.* **1985**, *2*, 303–314.
129. Ferrante, A.; Incrocci, L.; Maggini, R.; Serra, G.; Tognoni, F. Colour changes of fresh-cut leafy

vegetables during storage. *J. Food, Agric. Environ. J. Food Agric. Environ.* **2004**, *22*, 40–44.

130. Nisar, N.; Li, L.; Lu, S.; Khin, N.C.; Pogson, B.J. Carotenoid metabolism in plants. *Mol. Plant* **2015**, *8*, 68–82.
131. Yuan, H.; Zhang, J.; Nageswaran, D.; Li, L. Carotenoid metabolism and regulation in horticultural crops. *Hortic. Res.* **2015**, *2*, 15036.
132. RAO, A.; RAO, L. Carotenoids and human health. *Pharmacol. Res.* **2007**, *55*, 207–216.
133. Othman, R.; Hassan, N.M.; Hatta, F.A.M. Influence of Environmental Stress toward Carotenogenesis Regulatory Mechanism through In Vitro Model System. In *Carotenoids*; InTech, 2017; Vol. i, p. 13.
134. Morosinotto, T.; Caffarri, S.; Dall'Osto, L.; Bassi, R. Mechanistic aspects of the xanthophyll dynamics in higher plant thylakoids. *Physiol. Plant.* **2003**, *119*, 347–354.
135. Rockholm, D.C.; Yamamoto, H.Y. Violaxanthin De-Epoxidase *'*. **1996**, 697–703.
136. Kocira, S.; Sujak, A.; Oniszczuk, T.; Szparaga, A.; Szymanek, M.; Karakuła-Juchnowicz, H.; Krawczuk, A.; Kupryaniuk, K. Improvement of the photosynthetic activity of Moldavian dragonhead (Dracocephalum moldavica L.) through foliar application of a nitrophenolate–based biostimulant . *BIO Web Conf.* **2018**, *10*, 01009.
137. Bulgari, R.; Trivellini, A.; Ferrante, A. Effects of Two Doses of Organic Extract-Based Biostimulant on Greenhouse Lettuce Grown Under Increasing NaCl Concentrations. *Front. Plant Sci.* **2019**, *9*, 1–14.
138. Qiu, W.; Wang, Z.; Huang, C.; Chen, B.; Yang, R. Nitrate accumulation in leafy vegetables and its relationship with water. *J. soil Sci. plant Nutr.* **2014**, 0–0.
139. Bosnir, D.B.J.; Bevardi, M.; Boskovic, A.G.; Lasic, S.M.D.; Krivohlavek, A.; Racs, A.; Mojosovic-Cuic, A.; Trstenjak, N.U. Nitrate in Leafy Green Vegetables and Estimated Intake. *African J. Tradit. Complement. Altern. Med.* **2017**, *14*, 31–41.
140. Chowdhury, A.; Das, A. Nitrate Accumulation and Vegetable Quality. *Int. J. Sci. Res.* **2016**, *4*, 1668–1672.
141. Cavaiuolo, M.; Ferrante, A. Nitrates and glucosinolates as strong determinants of the nutritional quality in rocket leafy salads. *Nutrients* **2014**, *6*, 1519–1538.
142. Dudaš, S.; Šola, I.; Sladonja, B.; Erhatić, R.; Ban, D.; Poljuha, D. The Effect of Biostimulant and Fertilizer on "Low Input" Lettuce Production. *Acta Bot. Croat.* **2016**, *75*, 253–259.
143. Kunicki, E.; Grabowska, A.; Sękara, A.; Wojciechowska, R. The effect of cultivar type, time of cultivation, and biostimulant treatment on the yield of spinach (Spinacia oleracea L.). *Folia Hortic.* **2010**, *22*, 9–13.

144. Tarantino, E.; Disciglio, G.; Frabboni, L.; Libutti, A.; Gatta, G.; Gagliaridi, A.; Tarantino, A. Effects of Biostimulant Application on Quali- Quantitative Characteristics of Cauliflower , Pepper and Fennel Crops under Organic and Conventional Fertilization. *Waset.Org* **2015**, *9*, 712–716.

145. Amanda, A.; Ferrante, A.; Valagussa, M.; Piaggesi, A. Effect of biostimulants on quality of baby leaf lettuce grown under plastic tunnel. *Acta Hortic.* **2009**, *807*, 407–412.

CHAPTER 2

Effects of borage extract on rocket salad under salt stress

INTRODUCTION

Salinity stress is a major abiotic stress affecting plant growth, quality and productivity. More than 30% of the irrigated land of the world is affected by salinization and this number was projected to increase considering the climate change scenario and the environmental pollution [1,2]. Salinity can be due to salts dissolved in the irrigation water or by marine aerosol in the coastal areas. Therefore, the study of the physiological and molecular mechanisms of tolerance to salt stress is central to obtain crops that are able to survive and produce under stressful conditions.

It is known that salt stress impairs plants for different reasons: it causes hyperosmotic stress, ion imbalance, and as consequence oxidative damage. In the short term (after minutes, hours and up the first day of stress), salt stress is perceived by roots as osmotic stress, caused by the reduced ability of the plants to take up water. In the long term (days, weeks or years), plant growth is limited by the ion toxicity and nutrient imbalance occurring in the cytoplasm due to the accumulation of salt and the competitive uptake mechanisms with other ions. The first phase, when the salt is not penetrated in plant tissues, is also known as the water-deficit effect of salinity and the cellular and metabolic processes involved are in common with drought stress. The second phase is the salt-specific effect and it is due to the excess of ions inside the plant [3].

Several metabolic processes such as photosynthesis [4,5], respiration [6], phytohormone regulation [7], and protein biosynthesis are altered by salinity. The effects of salinity on plants vary depending on different factors, such as the level of salt concentration, the duration of the exposure, the plant phenological stage, the interaction with the environmental conditions, and the ability of a species or cultivar to grow in saline condition.

According to this ability, plants are generally divided into glycophytes and halophytes. Glycophytes growth is inhibited by concentrations of NaCl around 100-200 mM, whereas halophytes can survive at higher concentrations of NaCl, typically around 300-500 mM [8]. The threshold value used as criteria to define if a plant belongs to glycophytes or halophytes changed during time and according to different authors. For instance, Flowers et al. [9] initially proposed a value of 300 mM and then 200 mM, whereas other authors referred to a lower threshold about 85 mM [10]. This situation has led to a confusion in the number of the species belonging to one or the other category. For example, wild rocket (*Diplotaxis tenuifolia* L.) is a perennial plant originating from the Mediterranean area presents in coastal areas. Recently, wild rocket has been classified as salt tolerant with potential as vegetable crop for saline agriculture. De Vos et al. [11] investigated

the responses of this specie under the effects of increasing salinity. They observed that no growth reduction resulted up to 100 mM NaCl and a decrease about 20% occurred at 200 mM, mostly due to the modifications in leaf morphology than salt toxicity. Moreover, rocket plants were able to survive at concentration about 300 mM.

Tolerant plants are able to implement different adaptation strategies including morphological, physiological, biochemical, and molecular changes in order to grow and complete their growing cycle on saline substrate. Although salt stress is primarily perceived by the root apparatus, leaf growth is more sensitive to salinity and two of the main strategies for salt tolerance are the minimisation of the uptake of salt by the roots and its distribution in all tissues in order to avoid the accumulation in leaves [12]. During the osmotic-stress phase, plant growth in saline condition seems to be regulated by hormonal signal, more than water relations. ABA is the main candidate in this mechanism, since it is present in xylem sap and it increases after drought and salt stress. In the second phase, specific mechanisms controlling Na^+ and Cl^- concentrations occur, such as the exclusion of salt from the phloem, its accumulation in older leaves, in the leaf base or stems, and its compartmentalization in the vacuoles.

Shavrukov [13] pointed out the differences between salt stress and salt shock. Salt shock is considered as an extreme form of salt stress and it is defined as the sudden exposition of plants to high levels of NaCl. This situation rarely occurs in natural or agricultural systems where the NaCl increases gradually but it is still applied in the study of salt stress on plants. The osmotic and the ionic component can be distinguished also in salt shock, albeit with some differences. Indeed, when plants are exposed to salt shock, they have to face a large difference in osmotic pressure, causing plasmolysis and the leakage of the nutrient solution in the apoplast. Genes involved in osmotic responses are rapidly activated in roots cells. During this phase plants are not able to control the movement of the solutes and salt is quickly transported from the roots to the leaves. Thus, the ionic phase occurs earlier if compared to salt stress. This effect is common to all plant species regardless their level of salt tolerance. Differences among species are present in terms of cell damages and in the efficiency of recovery.

Plants response to abiotic stresses is a complex regulatory network involving different pathways and interaction among signalling molecules, defence proteins and stress-responsive genes [14]. Tolerant plants may have some peculiar stress-responsive genes which are not present in susceptible plants and nowadays most of the researches are focused on identifying these genes and their role in the molecular mechanisms of tolerance. These genes encode various salt stress responsive proteins and transcription factors involved in stress responses. The first ones directly act against salt stress by protecting the plants from dehydration, regulating the biosynthesis of osmo-protectants, while the latter are involved in the regulation of the gene expression and signal transduction.

Transcription factors have different domains, transcriptional domains and a DNA binding domain. TFs bind to specific *cis* regulating DNA sequence modulating the expression of salt-responsive genes. Several TFs share the same binding domain and on the basis of this sequence they are classified in families, like NAM-ATAF1.2-CUC (NAC), APETALA2/Ethylene Responsive Factor (AP2/ERF), Basic Leucine Zipper Domain (bZIP), MYB, and WRKY [15]. Cavaiuolo et al. [16] performed a transcriptomic analysis of short-term acclimation in *Diplotaxis tenuifolia* after salt shock exposition and identified 20879 active genes and 12995 silent genes in response stressful conditions. Among these, 29 TFs were upregulated and 44 downregulated under salinity, being NAC, AP2/ERF, and bZIP families the most represented.

NAC (NAM, ATAF1,2, CUC2) transcription factors are plant-specific proteins that are present in a wide range of species. NAC proteins are involved in numerous processes, including plant development, senescence and responses to abiotic stresses [17]. Several studies showed that most of the NAC genes are induced by stressful condition and take part in stress tolerance mechanisms and transgenic plants overexpressing NAC TFs resulted more tolerant to various environmental stress [18–21]. NAC family members are also involved in a complex crosstalk with other TFs and between different pathways [22]. For example, *NAC019*, *ANAC055*, and *ANAC072* are induced by salinity and their overexpression has been shown to upregulate several genes related with stress responses, such as *RD29A* through the interaction with *ABF3*, an ABA-responsive element binding factor [18,23]. At the same time, their expression is controlled by other TFs of MYB family [24]. *NAC019* and *NAC055* are associated with jasmonic acid and ethylene signaling pathways, whereas *NAC072* is involved in ABA-dependent stress response [23]. *ANAC069* is a protein involved in auxin and salt stress signals during seeds germination [25], and it has been reported to be regulated by *ATDOF5.8* transcription factor [26]. *ANAC092*, also known as *ORE1*, is a positive regulator of salt stress response and is directly upregulated by a phytochrome interacting factor 4 (PIF4), both during leaves senescence and under salt stress [27]. *ANAC029*, on the contrary, plays a role as negative regulator in salt stress responses, by repressing *AREB1* [28].

The MYB TFs family is not plant-specific but is present in all eukaryote organisms. This family includes a large number of proteins related to the regulation of plant development, metabolisms and the responses to biotic and abiotic stresses. In plants, most of the genes associated with the responses to environmental stress belongs to R2R3-MYB group [29]. *MYB96* and *MYB30* are involved in the biosynthesis and transport of cuticular wax and lipids [30,31]. Their upregulation under salt stress condition might be linked to the lipids role in the mitigation of stress responses or in the stress signalling [32]. Moreover, MYB30 has been reported to play an important role in root elongation through ROS-dependent processes [33], in the ABA signalling pathways [34] and in the expression of several brassinosteroids target genes [35,36].

Basic helix-loop-helix (bHLH) as well as MYB family, is presents both in plants and animals, and is one of the largest family in *Arabidopsis*. Members of bHLH family are divided in two big groups and take part in diverse physiological and developmental processes, such as the light signal transduction, roots air development, metal homeostasis, stomatal differentiation, flavonoid biosynthesis, and fruit dehiscence [37]. *bHLH122* play a role in the regulation of osmotic stress probably through ABA dependent mechanism. Moreover, *bHLH122* overexpressing plants were more tolerant to drought, salt and osmotic stress [38]. *BEE2*, *HBI1* and *IBH1* belong to bHLH family and are involved in the early response for brassinosteroids signaling pathway [39,40].

The AP2/ERF is a plant-specific transcription factor family divided in four subfamilies the AP2, DREB, ERF and RAV. Transcription factors of DREB and ERF subfamilies have been reported to be the main factors involved in abiotic stress responses [41]. DREB members bind to the *cis* acting dehydration responsive element (DRE), whereas ERF TFs bind to the ethylene responsive element (ERE). In Arabidopsis, *DREB2* genes are highly induced by drought, salinity and heat stress [42–44]. In particular, *DREB2A* resulted to play an important role by regulating the gene expression under osmotic stress conditions through ABA-independent pathway [45]. *ERF107* and *ERF39* are two members of ERF subfamily. *ERF107*, also known as *DEWAX* acts as negative regulator of wax biosynthesis [46] and has been reported to play and important role in salt stress responses [47]. *ERF39* acts as positive regulator of genes involved in primary cell wall biosynthesis, by modulating the production on cellulose in response to environmental stimuli [48]. ERF003 has been reported to ameliorate wheat adaptation to salt and water stress by activating several stress related genes [49].

WRKY is one of the most studies TFs family in plants and is well known to participate both in biotic and abiotic stress responses. Recently, it has been reported that WRKY proteins can act as repressors or activators of important plant processes. *WRKY54* is involved in brassinosteroids-plant growth and co-operate with *WRKY70* in the regulation of osmotic stress [50,51]. Moreover, soybean plants overexpressing *WRKY54* gene showed a better tolerance to drought and salt stress [52].

The zinc-finger motifs are present in several transcription factors regulate important biological processes. Indeed, modifications of mutations in genes encoding for a zinc-finger protein resulted in developmental anomalies and a decrease in the responses to abiotic stresses.
C3H49 is involved in seedling growth rate, plant size, leaf and flower morphology and senescence [53,54], and take part in hormone signalling, salt and drought stress responses [55]. Plant overexpressing C3H49 are more sensitive to ABA and are more tolerant to water stress [53]. ZAT12 protein is necessary for the expression of a key enzyme involved in ROS scavenging and it has been reported to take part in high light and cold acclimation [56,57]. Furthermore, it acts as negative regulator of Fe acquisition [58] and under salt stress

condition is dependent on two ethylene insensitive transcription factors, *EIN3* and *EIL1*. In yeast, the upregulation of *ZAT12* resulted in increased salt tolerance and a reduced ROS accumulation [59].

Basic region/leucine zipper (bZIP) family includes proteins with a basic region that binds DNA and a leucine zipper region for protein dimerization. bZIP TFs play different roles in light signaling, biotic and abiotic stress responses, seeds maturation, and flower development. ABF3 TF possesses an ABA response element binding factor and belongs to group-A bZIP. Tfs of this group are involved in ABA signal in response to several abiotic stresses [60,61]

Homeodomain-leucine zipper (HDZip) is a large Transcription factors family specific to plants and its members are grouped in four classes with different functions. *ATHB7* and *ATHB12* are involved in ABA dependent signalling pathways and both are upregulate by high level of ABA and in water stress conditions [62,63].

The study of TFs role, their regulation, and the target genes represents one of the modern genetic engineering strategies to improve crop tolerance against abiotic stresses. TFs are considered a good target also because most of them are early responsive genes under stress condition and they are able to control a set of genes involved in plant stress resistance [64,65]. This approach reveals the complexity of transcriptional regulation of plant genes since an overlap in the transcription factors expression in response to multiple abiotic stresses often appears. Indeed, besides the regulation of stress responses, TFs participate to different biological and physiological processes such as plant development or senescence, regulating a cluster of downstream target genes [22,66]. Thus, linking specific TFs with a single stress response in a big network of pathways is a big challenge. Beside the study of plants stress responses mechanisms, transcriptome analysis is a strategy more frequently used to understand the effects and the mode of action of biostimulants on plants both under stressful and non-stressful conditions [67,68].

AIM

The object of this study was to evaluate the response of *Diplotaxis tenuifolia* L. to salt stress in combination with a foliar application of a borage extract. This response was analysed in terms of change in different physiological parameters, such as chlorophyll, chlorophyll *a* fluorescence, carotenoids, phenols, and anthocyanin but also from a molecular point of view. Thus, the expression of some of the transcription factors typically involved in salt stress response was studied. The borage extract used in this experiment showed positive effect both on primary and secondary metabolism of lettuce and rocket plants grown under non-stressful condition in previous experiments. Thus, we decided to evaluate its potential biostimulant effects in terms of salt stress tolerance.

MATERIALS AND METHODS

1. Plant material, stress treatment and experimental plan

The trial was carried out at the Faculty of Agricultural and Food Science of Milan in 2018. Rocket plants (*Diplotaxis tenuifolia*, L.; ISI Sementi S.p.A., Italy) were grown hydroponically into plastic tanks (35 x 25 x 20 cm) with 10 L of a modified Hoagland medium and the concentration of nutrient in the solution is reported in Table 9. Seeds of rocket were manually sown into polystyrene trays filled with an agri-perlite substrate on 20 February 2018. Cultivation took place in an experimental greenhouse under controlled conditions.

Table 9. Composition of the Hoagland nutrient solution used for rocket plants cultivation.

Compounds	Concentration [mM]
$Ca(NO3)_2$	2.19
KNO3	4.55
NH4NO3	3.87
K2HPO4	1.38
MgSO4	0.83
K2SO4	1.09
Oligo green*	0.02 (g L-1)
H2SO4	Up to pH 5.5 – 6.5

*Oligogreen: is a mineral water-soluble powder fertilizer that provides the plant with micronutrients, essential for the most important bio-chemical reactions. Green Has Italia

The experimental design was a combination of two factors: stress and treatment, each of them with two levels. Salt stress was imposed by transferring plants to a fresh nutrient solution containing 200 mM NaCl, 35 days after sowing at 08.00 h (on March the 26th 2018). The nutrient solution of non-stressed plants was also changed with a fresh one. Treatments consisted of 20 mL of water (control) and 20 mL of a borage extract. The plant extract used in this experiment had been previously prepared and tested by our research group, as described by Bulgari et al. [69]. The borage flower extract diluted 10 mL L^{-1} has been chosen as treatment since the positive results obtained in this study and in previous unpublished experiments. Treatments were applied on 25 March 2018, as foliar spray onto leaves until run-off 24 hours before the beginning of the stress.

For physiological analyses, leaf tissues were collected from four biological replicates after 1, 2, and 4 days of stress and stored at-20 °C until analyses. For gene expression analyses, leaf tissues were collected after 2, 4, 6, 9, and 24 hours of exposure to salt (10.00 h, 12.00 h, 14.00 h, 17.00 h and 8.00 h). Samples were shock frozen in liquid nitrogen before storage at −80 °C until used for RNA isolation.

2. Non-destructive analyses

2.1 Chlorophyll

Leaves chlorophyll content was estimated *in vivo* using a chlorophyll content meter (CL-01 Chlorophyll Content Meter, Hansatech Instruments). The results were express such as chlorophyll index (relative units).

2.2 Chlorophyll a fluorescence

Chlorophyll a fluorescence was measured *in vivo* using a hand-portable fluorometer (Handy-PEA, Hansatech Instruments). Before all measurement leaves were dark-adapted with the leaf clips for 30-40 minutes. Then were exposed to a saturating light (3000 µmol m^{-2} s^{-1}) provided by an array of three high-intensity light-emitting diodes for 1 s. Chlorophyll *a* fluorescence was measured and then analysed with the JIP-test. Information about the structural and functional status of photosynthetic apparatus was provided by the parameters measured, such as the maximum quantum of photosystem II (Fv/Fm) or the performance index (PI) derived from JIP test calculation.

3. Destructive analyses

3.1 Total chlorophylls and carotenoids

Chlorophylls and carotenoids were extracted from rocket leaves using 5 mL of 99.9% (v/v) methanol. Leaf disc samples (30 mg), obtained with a 5 mm diameter cork borer were kept in dark room for 24 h at 4 °C. After that absorbance reading were measured at 665.2 and 652.4 nm for chlorophylls and 470 nm for total carotenoids with a spectrophotometer. Pigments levels were calculated by Lichtenthaler's formula and expressed on a fresh weight basis [70].

3.2 Phenolic index and total anthocyanin

Phenolic index and total anthocyanin were determined from leaf disc samples (30 mg), obtained with a 5 mm diameter cork borer. Leaf samples were transferred to a tube containing 3 mL of methanol acidified with hydrochloric acid (1%) and kept in dark room for 24 h at 4 °C. Absorbance reading were measured at 320 nm for total phenols, and at 535 nm for anthocyanin with a spectrophotometer. Phenolic index was expressed as

Abs320 nm g^{-1} FW. Anthocyanins concentration was expressed in cyanidin-3-glucoside equivalents using a molar extinction coefficient (ε) of 29,600 L M^{-1} cm^{-1}.

3.3 Nitrate

Nitrate concentration was determined by the method of Cataldo et al. [71]. Fresh leaf tissue was homogenized in distilled water (1 g fresh tissue per 4 mL distilled water). The homogenate was centrifuged at 4000 rpm for 15 min at RT (ALC centrifuge-model PK130R) and the recovered supernatant was used for the colorimetric analysis. Twenty microliters of the extract were added to 80 mL of 5% (w/v) salicylic acid in concentrated H_2SO_4 (SA-H_2SO_4). Afterward 3 mL of 1.5 N NaOH were added. The samples were cooled at room temperature and absorbance at 410 nm was measured with a spectrophotometer. Nitrate content was calculated referring to a KNO_3 standard calibration curve. Nitrate content was calculated referring to a KNO_3 standard calibration curve. Nitrate concentration was expressed as mg of NO_3-N per kg of fresh weight.

3.4 Reducing and total sugars

Reducing sugars were measured using the dinitrosalicylic (DNS) acid method. This colorimetric technique consists of a redox reaction between the 3,5-dinitrosalicyclic acid and the reducing sugars present in the sample [72]. Approximately 1 gram of leaf tissue was homogenized in a mortar with 3 mL of water. The mixture was centrifuged at 4000 rpm for 15 min at RT. DNS assay was performed by mixing 0.2 mL of supernatant with 0.2 mL of DNS and incubated in a water bath at 100 °C for 5 min, then 1.5 mL of water was added to samples. After cooling at room temperature, the optical density was determined spectrophotometrically at 530 nm, using a glucose standard curve.

The total sugars were determined on the same extract using the anthrone method with slight modifications [73]. The anthrone reagent (10.3 mM) was prepared dissolving anthrone in 95% H_2SO_4. The reagent was left to stand for 30-40 min before use, 0.5 mL extract was placed on top of 2.5 mL of anthrone reagent incubated in ice for 5 min and then vortexed vigorously. The tubes were heated to 95 °C for 10 min and left to cool in ice. Readings were performed at 620 nm. Calibration curve was carried out using a glucose standard solution.

3.5 Lipid peroxidation

Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) in accordance with the method described by Heath and Parker [74]. Approximately 1 gram of leaf tissue was homogenized in a mortar with 3 mL of 0.1% (w/v) trichloroacetic acid (TCA). The mixture was centrifuged at 4500 rpm for 10 min at room temperature. TBARS assay was performed by mixing 1 mL of supernatant with 4 mL of 20% (w/v) TCA, 25 µL of 0.5% thiobarbituric acid (TBA) and incubated in a water bath at 95 °C for 30 min. After cooled on ice, the tubes were centrifugated at 4000 rpm for 10 min and the optical density was determined spectrophotometrically at 532 and 600 nm. Absorbance at 600 nm was subtracted from the absorbance at 532 nm (as an index of non-specific turbidity) and the concentration of TBARS was calculated using the Lambert-Beer law with an extinction coefficient εM= 155 mM-1 cm-1 and expressed as malondialdehyde (MDA) equivalents (nmol g-1) according to Du and Bramlage [75].

3.6 Osmolytes

Fresh leaf tissue was homogenized in distilled water (1 fresh tissue per 4 mL distilled water). The homogenate was centrifuged at 4000 rpm for 15 min at RT and the recovered supernatant was analysed. Its osmolarity was determined using an automatic freezing point depression osmometer (Digital Osmometer, Roebling, Berlin, Germany) calibrated with sodium chloride solutions.

3.7 Abscisic acid

Approximately 1 g of leaf tissue was homogenized in a mortar with 3 mL of water, the mixture was centrifuged at 4000 rpm for 15 min at RT and the supernatant was collected and then stored at -80 °C until analysis. The abscisic acid (ABA) concentration was determined by an indirect enzyme linked immuno-sorbent assay based on the use of DBPA1 monoclonal antibody, raised against S(+)-ABA using the Plant Growth Regulator Immunoassay Detection Kits (Sigma-Aldrich) according to manufacturer instructions.

4. Total RNA isolation and analysis of gene expression

Starting from the *Diplotaxis tenuifolia* L. RNAseq database (SRP study accession number SRP102718) created at the University of Milan [16], the sequences of 25 genes were identified in order to be used as molecular markers for salt stress (Table 10). The sequences were selected among those showing significant changes in

their expression (RPKM) in response to 24 hours of exposure to salt stress condition. All sequences were blasted using NCBI BLAST database and their involvement in salt stress responses mechanisms have been confirmed through the literature review. Among them, 23 TFs have been identified and 1 did not show any correspondence to known gene sequences, thus it has been reported as unknown 2. Specific primers (Table 11) for all selected sequences (24 transcription factors and *RD29A*) were designed using the program Primer-Blast available at the National Center for Biotechnology Information website (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Table 10. Transcription factors selected from the *Diplotaxis tenuifolia* RNAseq database, grouped by family and divided according to the results obtained by Cavaiuolo et al., (2017).

TF FAMILY	UPREGULATED	DOWNREGULATED
ERF / AP2	*DtERF039*	*DtDREB2A, DtERF107, DtERF003*
WRKY		*DtWRKY54*
bHLH	*DtbHLH-122,*	*DtBEE2, DtHBI1-like, DtIBH1-like*
ZINC FINGER	*DtC3H49*	*DtZAT12*
HD-ZIP	*DtHB12, DtHB7*	
b-ZIP	*DtABF3*	*DtbZIP63*
NAC	*DtNAC72, DtANAC019, DtNAC29, DtANAC069, DtNAC92-NAC59,*	
MYB	*DtMYB94,*	*DtMYB30*
-		*UNKNOWN2*

Table 11. Primers sequences and Melting temperature (Tm) for qRT-PCR analysis**.**

GENE	PRIMER PAIR	SEQUENCE (5'->3')	Tm (°C)
DtNAC72	Forward primer	TCATGCACGAGTATCGCCTC	59.97
	Reverse primer	AGAGCTCTGTTCTTCACGGC	60.04
DtHB12	Forward primer	TGGTTTCAGAACAAGAGGGCT	59.51
	Reverse primer	ATTTTCTGGTCCTGTGGTGC	58.38
DtERF039	Forward primer	TTAGGATCGGTGCTTGCTGG	60.11
	Reverse primer	CGAACTTTCGTGGGGTCAGA	59.97
DtANAC019	Forward primer	CTGGATACCCAAACCCGACC	60.11
	Reverse primer	ACTCGGGTACAGAACTCGGA	59.96
DtMYB94	Forward primer	ACTGGAGATCCGTGCCTACT	60.03
	Reverse primer	CACCTGTTGCCCAAAAGAGC	59.97
DtbHLH122	Forward primer	AACAGAGGAGACGACGGAGA	59.96
	Reverse primer	GAGCGAGATTATTCGCCGGA	60.04
DtHB7	Forward primer	AGCTGGCTCCACAATGTTCA	59.89
	Reverse primer	AAGTGTGTGAGACGGGACAC	59.90
DtABF3	Forward primer	GACTGCTGAGGAAAGCCACT	59.96
	Reverse primer	GAGGAACTCCGGTGACATCC	59.82

DtC3H49	Forward primer	GTACATGCGGAAATGGTCGC	59.97
	Reverse primer	TCAGAAGACTTCACACCGGC	59.97
DtRABC2B	Forward primer	GCTGCTCGTGAGCTGATTTG	59.90
	Reverse primer	ACACGAGCGGTCTTGCTTTA	59.97
DtNAC29	Forward primer	CTTTGTCTGTACCGGTCGCT	60.04
	Reverse primer	ACAAGTTCGACCCATGGCAA	60.18
DtANAC069	Forward primer	GACGATTTCGCCAACGACAG	59.91
	Reverse primer	CTCATTTCACACGGCGCATT	59.83
DtNAC92/ NAC59	Forward primer	CGGTCGAACCATCAAAACCG	59.83
	Reverse primer	GCAACCGAGGACAAGGGTTA	59.96
DtDREB2A	Forward primer	AGGAAAGTACCCGCGAAAGG	60.04
	Reverse primer	GTCGGAAAGGTACCAAGCCA	59.96
BEE2	Forward primer	ACTGGTAAAGCCGGTATGCT	59.09
	Reverse primer	CTACGGATCCATGCTGGTGT	59.53
DtbZIP63	Forward primer	TCGCAACTCTCCTCATCGAC	59.55
	Reverse primer	TCCACACTATGCCTCAGGTT	58.34
DtWRKY54	Forward primer	ACTTGGACCGTGGAAGCTAA	58.95
	Reverse primer	ACATCTCAGGGTCTCGCTCA	60.32
DtMYB30	Forward primer	TTCACTTGGCGAAGAAGGCT	59.89
	Reverse primer	CGAGGCATACGTGGTAGAGG	59.69
DtERF107	Forward primer	CAGTCGGGCCATGTAGTTGT	60.04
	Reverse primer	GAAACGATGTACCGGAGCCT	59.82
DtIBH1-like	Forward primer	TGTCCCCGGTGGAGAGTTTA	60.18
	Reverse primer	ATGCGGTCCTATCGACCAAC	59.90
DtERF003	Forward primer	AGGCAGCAAGGCTAATGTGT	59.96
	Reverse primer	ATTCTTGACGCCGTGAGTGT	59.97
UNKNOWN2	Forward primer	GAGCTTAGCTTCTGAGTGGTGT	60.03
	Reverse primer	ACAACCACCAGCGTAACCAA	60.11
DtHBI1-like	Forward primer	AATGGCTGCAACAGCAACAA	59.54
	Reverse primer	TCCAAAACCAGATCCCGGC	60.00
DtZAT12-like	Forward primer	ACTCCGCATAACGGACAAGG	60.11
	Reverse primer	ATTAACTCGACGGTGGAGGC	59.82
DtRD29A	Forward primer	TCCACGTGTTGCTTATCCCC	60.04
	Reverse primer	AACTCCGGGATACGGTCAGA	60.03
EF1α	Forward primer	TCTTGGTAGACGCCTTCACG	65.3
	Reverse primer	AGGAAGCGGTGTCATTGTTG	65.0

Frozen leaves of rocket plants were thoroughly ground with liquid N using cold mortar and pestle. Approximately 100 mg was transferred to a cryotube and stored at −80 °C. Total RNA was isolated using the Spectrum Plant Total RNA Kit with on-column DNase-treatment (Sigma-Aldrich, Italy) following the steps of protocol A with slight modification.

The concentration and the purity of RNA were assessed by measuring the absorbance at 230 nm, 260 nm and 280 nm using a NanoDrop N-1000 spectrophotometer (NanoDrop technologies). A ratio of absorbance at 260 and 280 ≈ 2.0 is generally accepted as pure for RNA and expected 260/230 values are commonly in the range of 2.0-2.2, usually higher than the respective 260/280 value.

Three µg of RNA were reversely transcribed to cDNA using the SuperScript III cDNA Synthesis Kit according to the manufacturer's instruction (Invitrogen, Italy).

The SYBR® Green PCR Master Mix (Applied Biosystems) was used for the quantitative RT-PCR analysis. The reaction mix was prepared by adding 10 µL of SYBR Green, 0.4 µL of forward and reverse primers, 2 µL of

cDNA diluted 1:20, and 7.2 µL of RNase free water. The total volume for each PCR reaction was 20 µL. Analysis was performed using the ABI7300 (Applied Biosystem) thermocycler and PCR program and reactions were run in triplicate from two biological replicates.

The expression levels were analyzed with the AB software program and results were calculated using the $2^{-\Delta\Delta ct}$ method described by Livak and Schmittgen [76]. According to this method, the data are presented as fold change in gene expression normalized to a housekeeping gene and relative to a calibrator. The Elongation factor 1 alpha (EF1α) was used as reference gene (housekeeping) due to the highest stability in its expression levels, whereas the non-stressed and non-treated sample after 2h was chosen as internal calibrator.

5. Statistical analyses

Data obtained from physiological analyses were subjected to a two-way ANOVA whereas, data related to gene expression analysis were subjected to a three-way ANOVA. Differences among means were determined by Tuckey post-test ($P < 0.05$). Statistics were performed using GraphPad Prism version 6 or 8 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Additional information is reported in each figure's legend.

RESULTS

1. Physiological analyses results

In Table 12 and Figure 12 the overall responses of rocket plants to salt stress growing condition, in combination with a treatment with the borage extract are summarized. In general, physiological parameters were significantly affected more by salinity than treatments, with some exceptions. For example, after 2 days the application of borage extract had a significant effect on the abscisic acid level in rocket leaves, both under stress and non-stressful conditions. Moreover, after 4 days borage treatment significantly increased the content of total sugars in plants.

In treated and untreated rocket plants, the content of abscisic acid, lipid peroxidation, osmolytes concentration, and reducing sugars considerably increased in response to salinity condition. On the contrary, nitrate concentration decreased in stressed plants, regardless the treatment, as reported by the intense colour in the heat map (Figure 12). Total carotenoids, chlorophyll *a* + *b*, and phenols slightly decreased in all conditions, if compared with the unstressed and untreated plants after one day.

Table 12. Physiological response of rocket plants treated with borage extract under salt stress condition after 1, 2 and 4 days. S means STRESS, T means TREATMENT, SxT means the INTERACTION between stress and treatment.

	1 DAS			2 DAS			4 DAS		
	SxT	**S**	**T**	**SxT**	**S**	**T**	**SxT**	**S**	**T**
chlorophyll	ns	ns	ns	**	ns	ns	ns	**	**
chlorophyll *a+b*	ns	ns	ns	ns	ns	ns	ns	*	ns
carotenoids	ns	ns	ns	ns	ns	ns	*	*	ns
phenols	ns	ns	ns	ns	ns	ns	ns	ns	ns
anthocyanin	ns	ns	ns	ns	ns	ns	ns	ns	ns
Fv/Fm	*	*	ns	ns	*	ns	ns	*	ns
PI	ns	*	ns	ns	ns	ns	ns	ns	ns
nitrate	ns	**	ns	**	**	ns	ns	**	ns
reducing sugars	ns	**	ns	ns	ns	ns	ns	ns	ns
total sugars	ns	**	ns	ns	ns	ns	ns	ns	*
lipid peroxidation	ns	**	ns	ns	**	ns	ns	**	ns
osmolytes	ns	ns	ns	ns	ns	ns	ns	ns	ns
abscisic acid	ns	**	ns	ns	ns	*	ns	**	ns

** and * indicate respectively differences at P ≤ 0.05 and P ≤ 0.01 probability level, ns indicates not significant difference.

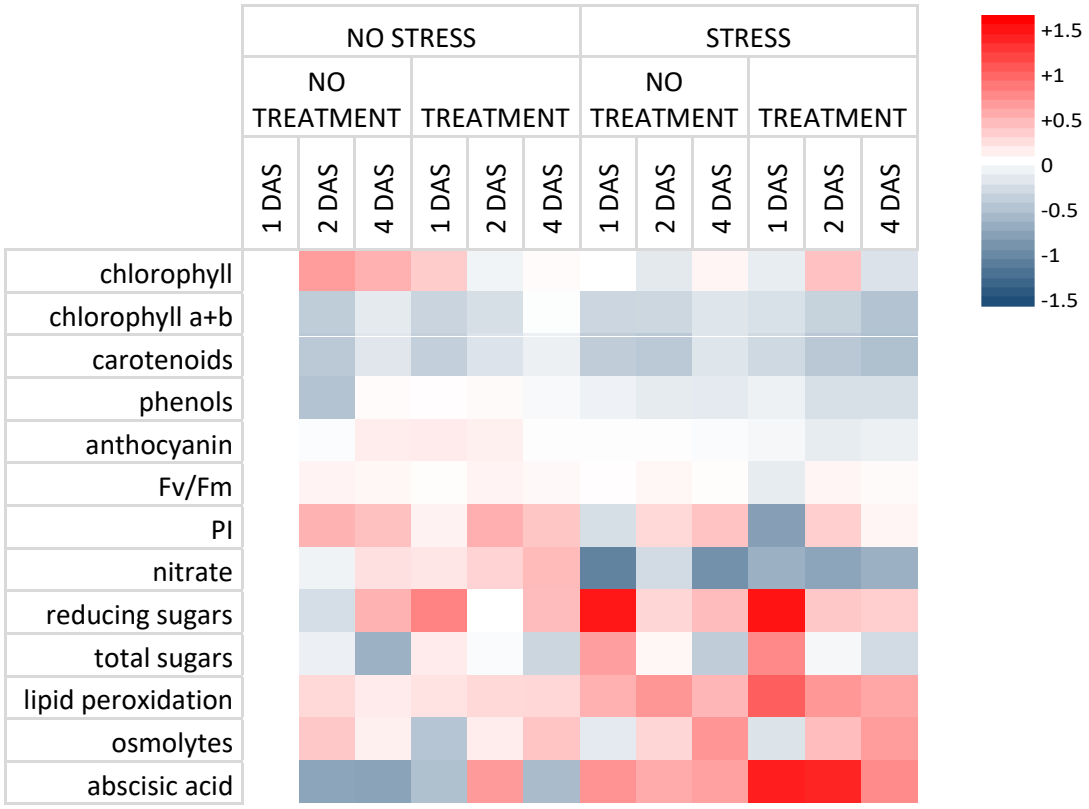


Figure 12. Heat map analysis summarizing the plant responses to salt stress and borage treatment. Results were calculated as Logarithm base 2 (Log2) of untreated and treated plants under salt stress and non-stress growing condition, after 1, 2 and 4 days of stress. Results were visualized using a false colour scale with red indicating an increase and blue a decrease of plants values compared to non-stressed and non-treated values after 1 day (1 DAS). No differences were visualized by white squares.

1.1 Chlorophyll

The chlorophyll content measured *in vivo* as relative units did not show any significant change one day after the beginning of the stress (Figure 13 A), not in response to salt, neither to borage treatment. All values were around 10 r.u.. A significant interaction between stress and treatment was observed after two days of stress (Figure 13 B). In particular, in non-stressed condition chlorophyll content of plants treated with borage extract was significantly lower than control while an opposite effect resulted under stress condition. After 4 days (Figure 13 C) chlorophyll content was similar in stressed plants (regardless of treatment) and non-stressed plants treated with borage extract, however, their values were significantly lower than control plants grown without stress.

Different trends of chlorophyll content were observed during the experimental time course: under non-stressful condition chlorophyll content increased in control plants from 10 r.u. to almost 15 r.u., while it did not change in plants treated with borage extract. Stressed plants did not show any variation during time, only a slight increase was observed after 2 days in plant treated with borage extract (Figure 13 B).

Figure 13. Chlorophyll content determined *in vivo* in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken after 1 day (A), 2 days (B) and 4 days (C) of stress. Values are means ± SE (n = 11). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

Chlorophyll content obtained with the destructive method (Figure 14) did not show the same trend of the previous analyses. In particular, no significant change appeared in chlorophyll *a+b* concentration of rocket leaves, after 1 and 2 days of stress (Figure 14 A and B) and the average values were around 1 µg mg^{-1} FW. On the contrary salt stress led to a significant decrease in the concentration of chlorophyll *a+b* in plants treated with borage extract after 4 days of stress (Figure 14 C).

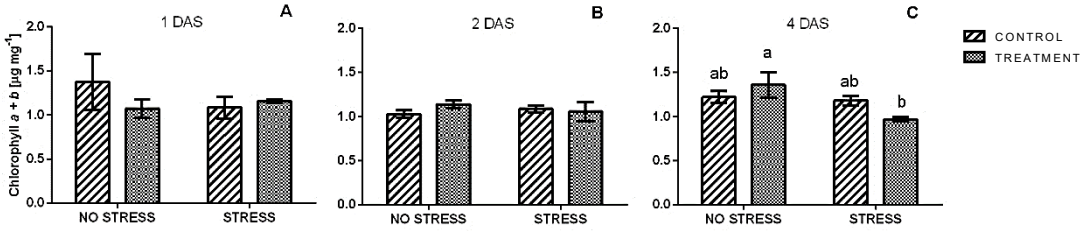


Figure 14. Chlorophyll *a + b* concentration in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken after 1 day (A), 2 days (B) and 4 days (C) of stress. Values are means ± SE (n = 4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

1.2 Total carotenoids

The content of carotenoids (Figure 15) in rocket leaves showed the same trend observed in chlorophyll *a+b* analyses, as expected. In particular, no significant change appeared after 1 and 2 days of stress and all values were similar to non-stressed control (Figure 15 A and B). After 4 days of stress, carotenoids level of plants treated with borage extract significantly decreased, while it was not affected in control plants (Figure 15 C).

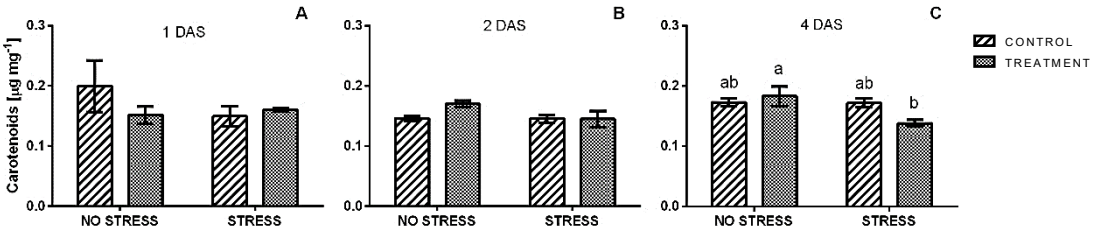


Figure 15. Carotenoids concentration in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken after 1 day (A), 2 days (B) and 4 days (C) of stress. Values are means ± SE (n = 4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

1.3 Phenol index and total anthocyanin

Phenol index (expressed as ABS_{320nm} g^{-1}) and total anthocyanin contents measured in rocket leaves are listed in Table 13. Both parameters were not affected by the salt stress, in any of the time point analysed. Moreover, statistical analysis did not detect any significant difference between samples neither in response to borage treatment.

Table 13. Phenol index and anthocyanin concentration in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken after 1 day, 2 days and 4 days of stress. Values are means ± SE (n = 4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

STRESS	TREATMENT	Phenol index [ABS_{320nm} g^{-1}]			Anthocyanin [Cyanidin eq. mg/100 g]		
		1 DAS	2 DAS	4 DAS	1 DAS	2 DAS	4 DAS
NO STRESS	CONTROL	19.67 ± 2.15	13.83 ± 3.62	20.00 ± 1.88	19.59 ± 1.54	19.31 ± 2.02	21.17 ± 1.71
NO STRESS	TREATMENT	19.87 ± 0.86	20.10 ± 0.63	19.06 ± 0.79	21.27 ± 0.58	20.91 ± 0.44	19.43 ± 0.54
STRESS	CONTROL	18.22 ± 0.94	17.58 ± 0.86	17.43 ± 1.08	19.45 ± 0.26	19.45 ± 0.63	19.22 ± 0.77
STRESS	TREATMENT	18.19 ± 1.84	16.45 ± 1.18	16.37 ± 0.80	18.74 ± 1.67	17.55 ± 1.01	18.00 ± 0.87

1.4 Chlorophyll a fluorescence

The maximum quantum efficiency of PSII (Fv/Fm) gives us an information on the plant's potential photosynthetic ability under stress conditions. Generally, an average value of 0.83 is considered the stress threshold for herbaceous plants, whereas lower values indicate stressful conditions for the plants with limitation of physiological processes. A significant interaction between stress condition and treatment resulted after one day of stress (Figure 16 A). Fv/Fm values were really close to the threshold in non-stressed plants. The same value was observed in control plants grown under salt stress. Instead, stressed plants treated with borage extract showed a lower value of about 0.73. After 2 and 4 days of stress (Figure 16 B and C) Fv/Fm ratio increased and all levels were higher than 0.83, regardless the stress condition or the treatment.

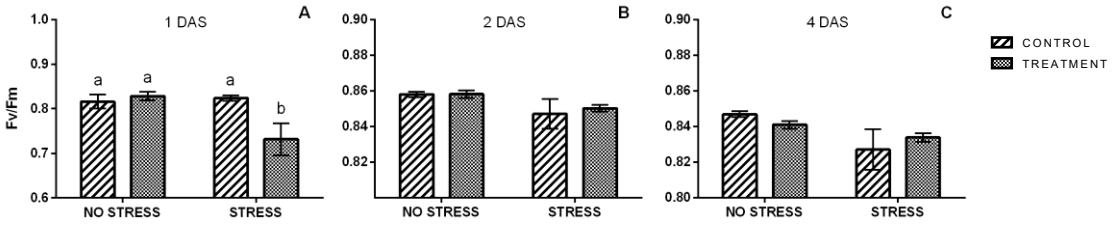


Figure 16. Maximum quantum efficiency of PSII (Fv/Fm) measured in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken 1 day (A), 2 days (B) and 4 days (C) of stress. Values are means ± SE (n = 9). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

The performance index (PI) did not show any significant change, not in response to salt stress, neither to treatment. After one day of stress (Figure 17 A) the results had a similar trend to Fv/Fm ratio and stressed plants treated with borage extract had a lower value if compared with the other conditions. Except for this, all values averaged from 2.2 to 2.8. After 2 and 4 days of stress (Figure 17 B and C) PI increased in all samples and values averaged from 2.8 to 3.7. Salt stress significatively affected the PI both time point, however no significant difference emerged among samples.

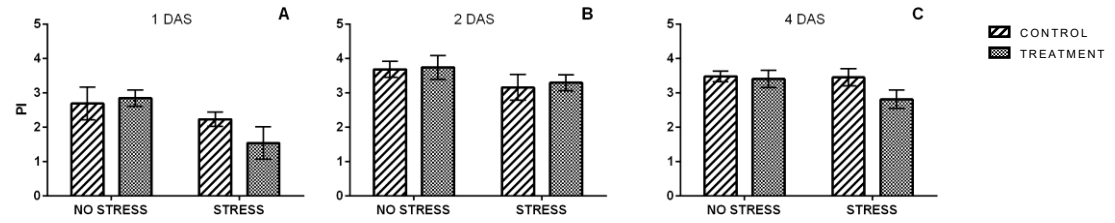


Figure 17. Performance index (PI) measured in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken after 1 day (A), 2 days (B) and 4 days (C) of stress. Values are means ± SE (n = 9). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

1.5 Nitrate

In general, salinity induced a significant decrease of the nitrate concentration in rocket leaves, regardless the treatment. After one day of stress (Figure 18 A) nitrate content of non-stressed plants was 3836 and 4264 mg kg^{-1} FW in control and after borage treatment, respectively. At the same time nitrate levels were halved under stress condition. In particular, nitrate concentrations were 1856 mg kg^{-1} FW in control plants and 2423 mg kg^{-1} FW in plants treated with borage extract. A similar effect has been observed also after 4 days, while after 2 days a significant interaction between stress and treatment was detected.

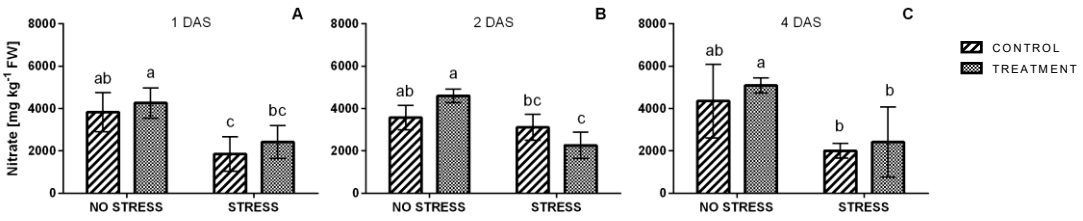


Figure 18. Nitrate concentration in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken after 1 day (A), 2 days (B) and 4 days (C) of stress. Values are means ± SE (n = 4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

1.6 Reducing and total sugars

A significant effect of salinity has been detected one day after the beginning of the stress (Figure 19 A) when the average value of reducing sugars increased from 2.9 mg g^{-1} FW in non-stress condition to 7.5 mg g^{-1} FW under salt stress. A slight but not significant increase was also observed in non-stressed conditions, in response to borage treatment. After 2 and 4 days of stress the concentration of reducing sugars decreased and no significant differences were observed among samples. In particular, all values were similar to control plants

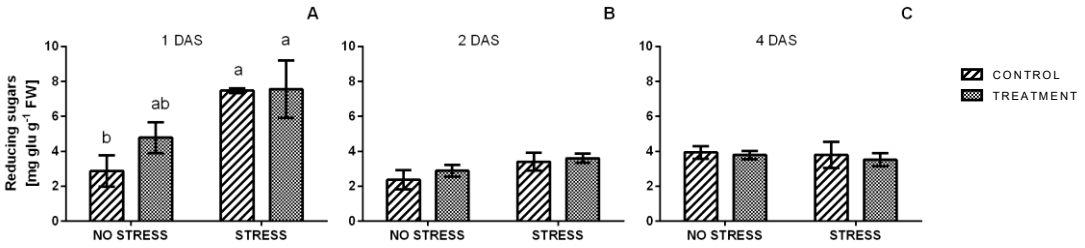


Figure 19. Reducing sugars concentration in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress for 24 h. Measures were taken after 1 day (A), 2 days (B) and 4 days (C) of stress. Values are means ± SE (n = 4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

grown in non-stressful condition and values ranged from 2.4 to 3.9 mg g^{-1} FW. A similar trend resulted in the concentration of total sugars, as reported in Figure 20.

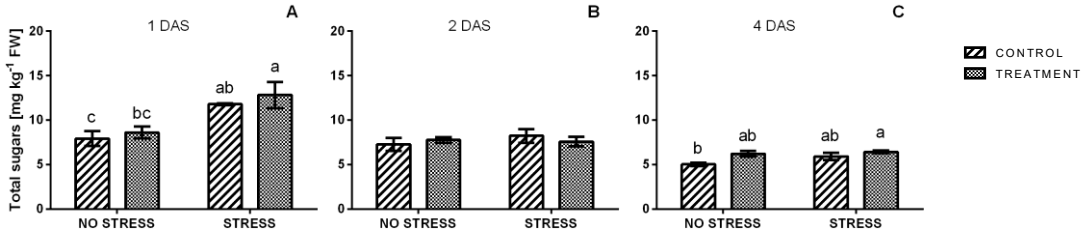


Figure 20. Total sugars concentration in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken after 1 day (A), 2 days (B) and 4 days (C) of stress. Values are means ± SE (n = 4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

1.7 Lipid peroxidation

In general, a significant increase due to salt stress resulted from the ANOVA. The level of lipid peroxidation in control plants grown under non-stress conditions was around 12 nmol g^{-1} FW in each time point, and a similar value was observed in response to borage treatment. After one day of stress (Figure 21 A) a significant difference resulted between plants treated with borage extract under stress and non-stressed condition, while only a slight increase was observed in control plants. In particular, the highest level of lipid peroxidation resulted in plants treated with borage extract and grown under salt stress (20.7 nmol g^{-1} FW). After 2 and 4 days, the level of lipid peroxidation in stressed samples slightly decreased to about 15 nmol g^{-1} FW. However, it remained significantly higher than non-stressed samples.

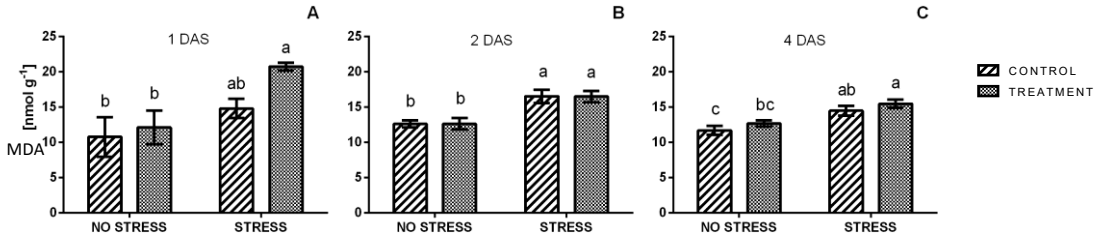


Figure 21. Concentration of malondialdehyde (MDA) in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken 1 day (A), 2 days (B) and 4 days (C) of stress. Values are means ± SE (n = 4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

1.8 Osmolytes

The levels of osmolytes in rocket leaves (Figure 22) was not affected by the stress condition or by the borage treatment, in any of the time point analysed. In particular, after one day of salt stress all values averaged from 0.12 to 0.17 Osm kg-1g-1. A slight increase appeared after 2 and 4 days (Figure 22 B and C), however no significant difference has been detected among samples.

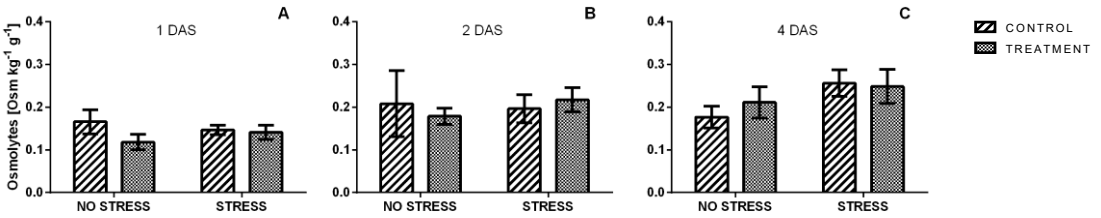


Figure 22. Osmolytes concentration in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken after 1 day (A), 2 days (B) and 4 days (C) of stress. Values are means ± SE (n = 4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

1.9 Abscisic acid

After one and four days (Figure 23 A and C) a significant effect of the stress resulted from the ANOVA. Initially, the average concentration of ABA in non-stressed samples was around 60 mg g^{-1} and no significant effect resulted after the application of the borage extract. The ABA contents in plants grown under stress condition were significantly higher than in non-stressed ones and averaged from 110.4 to 178.5 mg g^{-1}. The highest level was reached in samples treated with borage extract and it was three time higher than in non-stressed samples. After two days of stress (Figure 23 B) the concentration of ABA unchanged in almost all sample if compared with the previous time point. Moreover, the ANOVA showed a significant effect of treatment. After four days the concentration of ABA in all samples decreased, and no significant difference was detected even if stress samples were higher than non-stressed ones.

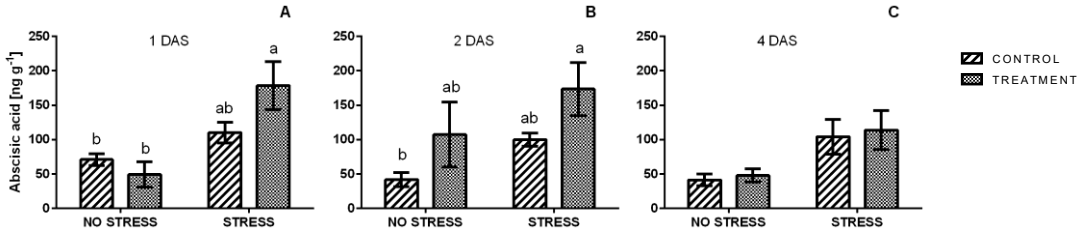


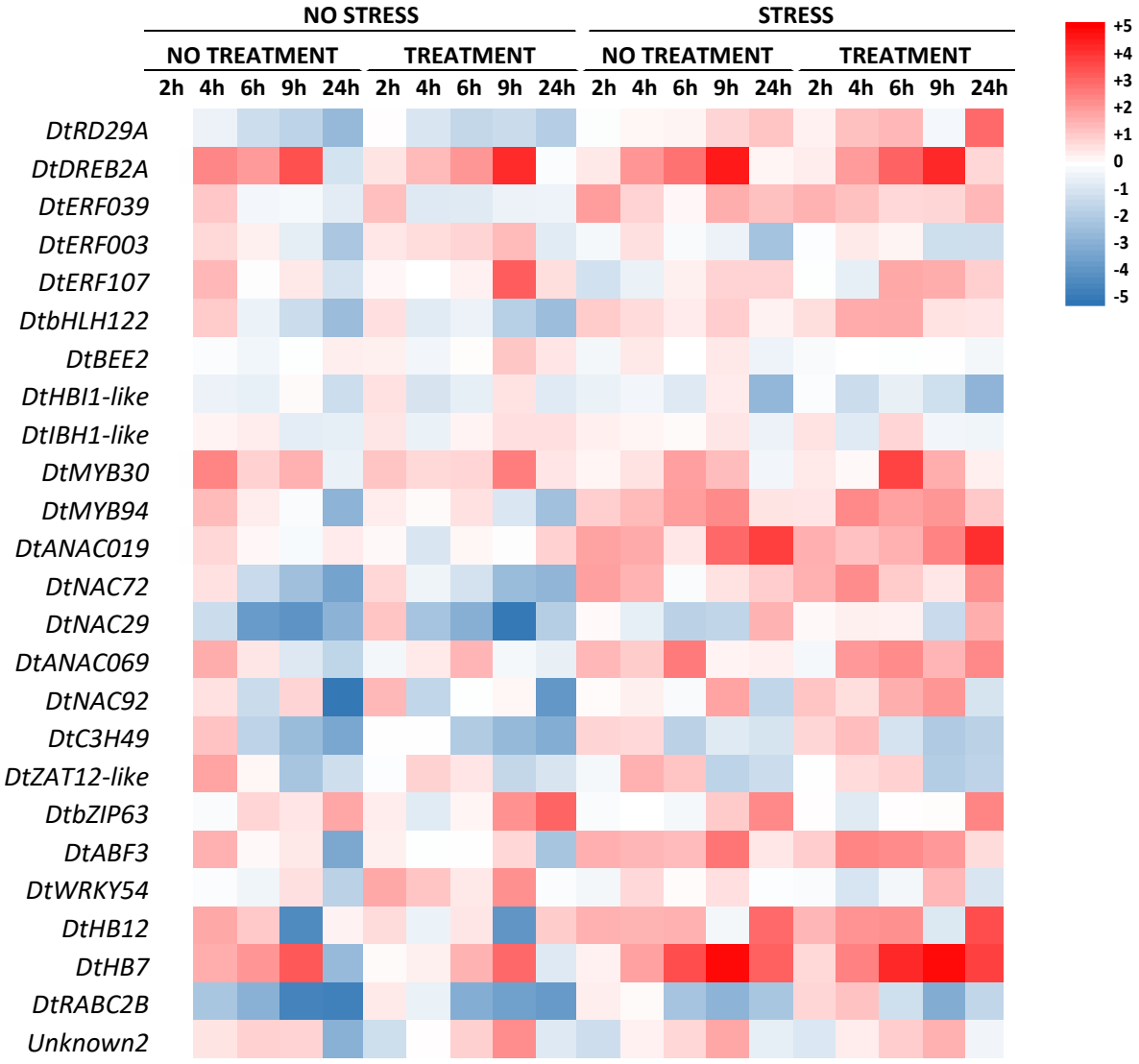
Figure 23. Abscisic acid concentration in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken 1 day (A), 2 days (B) and 4 days (C) of the stress. Values are means ± SE (n = 4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$)

2. Gene expression analysis results

The purity of RNA extracted from rocket leaves subjected to salt stress and treated with borage extract was measured using the NanoDrop. On average, the A260/280 ratio of RNA samples was 2.30 and the ratio of A260/230 was 2.20, indicating that the RNA was not contaminated by proteins or phenols.

Selected primers were tested in a qRT-PCR in order to check if they bind and amplify the RNA via melting curve analysis. Results obtained showed that all primers worked successfully.

Table 6. Heat map showing temporal expression of selected transcription factors associated with salt stress responses in rocket plants grown under salt stress condition and treated with a borage extract. Data are - ddCt calculated as - $(Ct,_{Target} - Ct,_{housekeeping})_{Time\ x}$ - $(Ct,_{Target} - Ct,_{housekeeping})_{Time\ 0}$, where time x is any time point and time 0 is the expression of the target gene normalized to the housekeeping in non-stressed and non-treated samples after 2 hours. The rows represent the transcription factors, and within each row the blue shaded areas indicate lower expression, whereas the red shaded areas indicate higher expression. No differences were visualized by white squares.



The changes in the expression of the transcription factors involved in the salt stress response have been clustered into a heatmap (Table 6). Further on, a graph representing the expression analysis of each transcription factor is presented and described. Different trends in the transcript levels resulted in response to salt stress, borage treatment and during time. Under no stress condition a similar pattern of expression was observed for *DtRD29A*, *DtbHLH122*, *DtNAC72*, *DtNAC29*, *DtC3H49*, and *DtRABC2B* with a progressive decrement within 24 h. Their trend did not change in response to borage treatment. The minimum level was observed for *DtNAC29* after 9 h and for *DtRABC2B* after 24 h. On the contrary, a constant increment of the expression levels resulted for *DtDREB2A* and *DtHB7* within 9 h. After 24 h the expression of almost all of TFs was downregulated in plants grown under non-stress condition, regardless of the treatment. In contrast, stressful growing condition induced a general increase in the expression levels of almost all transcription factors, as shown by the red colour shades in the heatmap. If we analyse the results obtain each time point, we observed that after 2 h of stress exposition the expression of almost all TFs was strongly induced. At the same time borage treatment increase the expression in plants grown under non stressful condition. After 4 h, a decrease in the expression levels appeared in response to borage treatment in unstressed plants. Non-treated plants showed similar levels of expression in both growing conditions, whereas the combination of stress and treatment slightly induced the transcript accumulation of almost all TFs.

After 6 h, a strong induction of the expression levels resulted in response to salt stress, mostly if combined with borage treatment. A similar effect appeared also after 9 h. After 24 h the expression of almost all of TFs was downregulated in plants grown under non stress condition regardless the treatment. On the contrary, salt stress maintains the expression levels high also after 24 h, mostly in combination of borage treatment.

Expression of the DtRD29A gene

The expression pattern of *DtRD29A* (Figure 24) was affected both by the stress condition, the treatment and the time. In general, a constant decrease in the expression levels resulted in non-stressed leaves within 24 h, both in treated and control plants. After 24 h the values of $2^{-\Delta\Delta Ct}$ were 0.18 and 0.3 in control and treated plants, respectively. An opposite trend was detected in rocket grown under salt stress condition: a gathering but slight increase was observed in control plants, whereas the expression of *DtRD29A* rose faster in plants treated with borage extract. In particular, a constant increase resulted within 6 h, followed by a decrease at 9 h and a final growth after 24 h. At this time point the $2^{-\Delta\Delta Ct}$ value of treated plants was almost 8 times larger than the initial point (non-stressed and non-treated at 2 h) while the value of control plants was around 2.3.

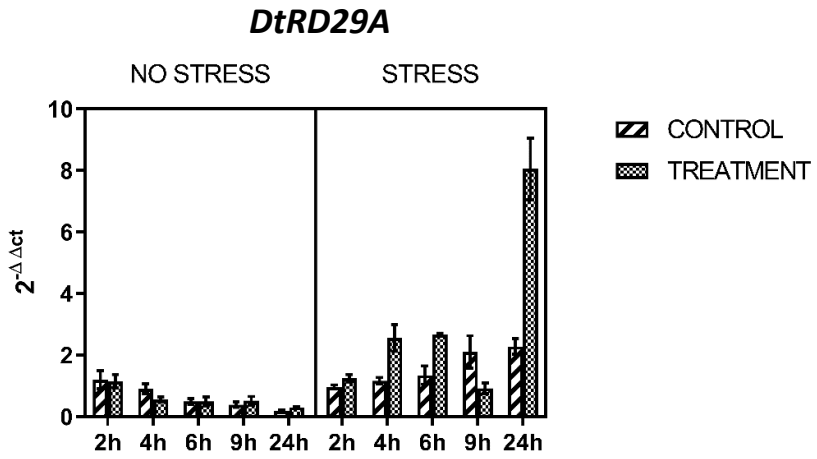


Figure 24. Changes in the expression of *DtRD29A* in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken 2, 4, 6, 9, 24 hours after the initial exposure to salt stress. Values are means ± SE (n = 6). Data were subjected to three-way ANOVA.

Expression of DtDREB2A, DtERF107, DtERF003, DtERF039 – (AP2/ERF transcription factors family)

The analysis of the selected transcription factors belonging to AP2/ERF family showed different expression patterns in response to the experimental conditions, as reported in Figure 25. Changes in the expression of *DtDREB2A* are reported in Figure 25 A. Under non-stress condition the expression levels in control plants increased up to 11 within 9 h and decreased to 0.6 at 24 h. This trend did not change after the application of the borage treatment and by the stress condition. Only few differences appeared in some time points. In particular, at 9 h under non-stress condition the expression of *DtDREB2A* in plants treated with borage extract was higher (+72%) if compared with the control ones at the same time point. On the contrary, under salt stress condition the highest level was reached in control plants, and the expression resulted almost 24-fold higher than the calibrator (non-stressed and non-treated at 2 h). Expression of *DtERF107* (Figure 25 B) in unstressed and untreated plants was generally low. A slight increase appeared at 4 h whereas no differences were observed in other time points. After borage treatment the expression did not change, except after 9 h where the $2^{-\Delta\Delta Ct}$ value increased more than 12 times. Under salt stress condition no changed resulted in control plants, whereas borage treatment induced a slight increase after 6 and 9 h of stress. The *DtERF039* expression profile (Figure 25 C) in control plants under non stress condition was low and similar to that of the *DtERF107* (Figure 25 B). Borage extract induced a slight increase by almost 3 times at 2 h, whereas the $2^{-\Delta\Delta Ct}$.values in all the other time points were around 0.6. Salt stress generally led to the accumulation of *DtERF039* mRNA in all samples. In control plants the expression was induced more than 4 times after 2 h of stress, then decreased

within 6 h and increased again at 9 and 24 h. In treated plants the expression was not affected after 2 h of salt stress and the level was similar to those treated and unstressed. However, all values were significantly higher than those observed in non-stress condition. *DtERF003* expression levels (Figure 25 D) were very low in control plants both under non stress and stress conditions. After 24 h of salt stress expression decreased up to 0.2. Two different trends appeared in plants treated with the borage extract, depending on the stress condition. In particular, under optimal condition borage treatment induced the expression by 3 times within 9 h, whereas salt stress generally lowered all values and the trend observed in treated plant was similar to that of control.

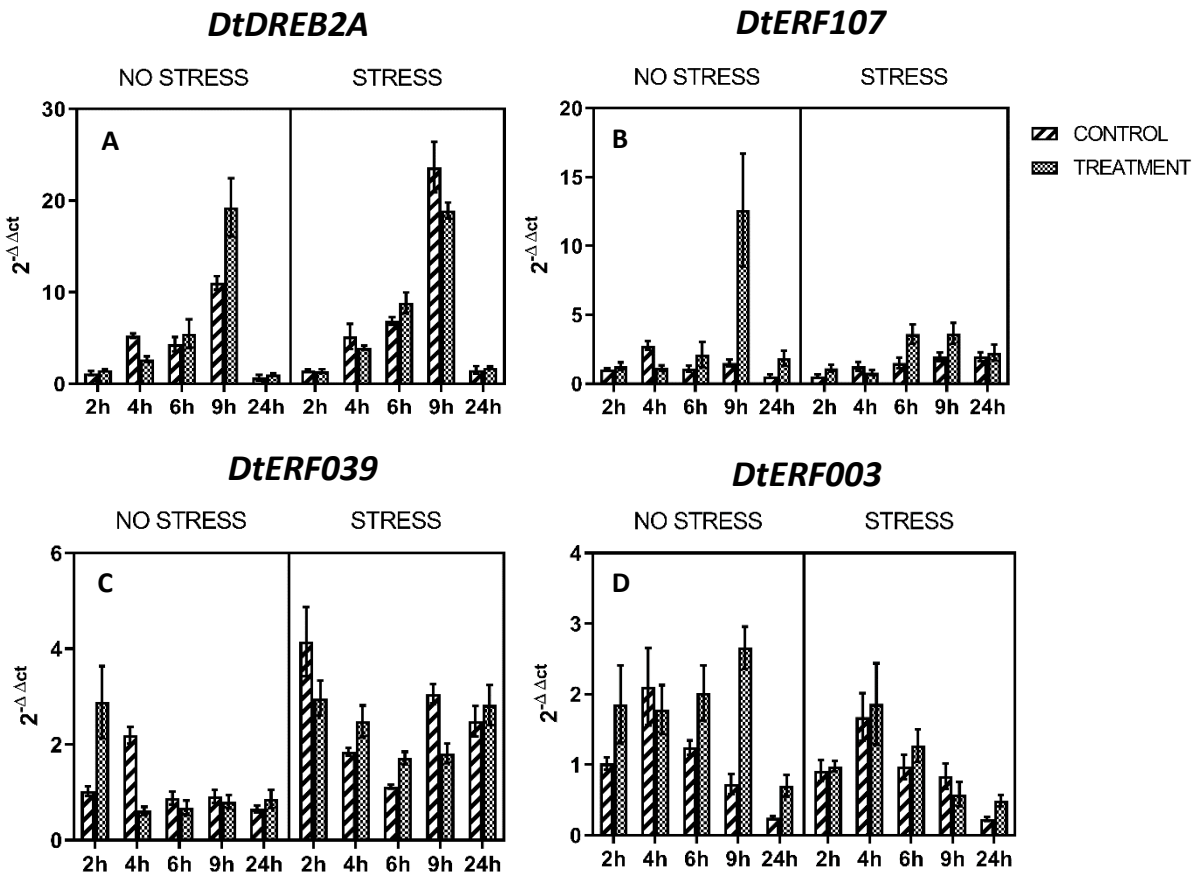


Figure 25. Changes in the expression of *DtDREB2A* (A), *DtERF107* (B), *DtERF003* (C), *DtERF039* (D) in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken 2, 4, 6, 9, 24 hours after the initial exposure to salt stress. Values are means ± SE (n = 6). Data were subjected to three-way ANOVA.

Expression of DtbHLH122, DtBEE2, DtHBI1-like, DtIBH1-like – (bHLH transcription factors family)

The expression patterns of selected transcription factors belonging to bHLH family are reported in Figure 26. In general, their expression was low and only few differences appeared in response to stress or treatment. The expression of *DtbHLH122* (Figure 26 A) in control plants grown under non stressful condition was double at 4 h, then constantly decreased to 0.2 after 24 h. Borage treatment affected the trend and anticipated the peak at 2 h and not 4 h. Salt stress generally induced the expression of this transcription factor, mostly after 4 and 6 h in plants treated with borage extract. The expression of *DtBEE2* transcription factor (Figure 26 B) in control plants under non-stress condition did not change within 9 h but it rapidly increased more than 5 times after 24 h. In plants treated with borage extract only a slight increase appeared after 9 h. No change resulted under salt stress and all values were similar to the unstressed control at 2 h. Different pattern resulted in the expression of *DtHBI1-like* transcription factor (Figure 26 C). In particular, under non-stress condition all values fell between 0.5 and 2, regardless the treatment. A constant decrease to 0.2 was observed within 24 h in response to salt stress both in control and in treated plants. No clear effect related to salt stress, time or treatment resulted in the expression of *DtIBH1-like* (Figure 26 D). Indeed, control plants showed a slight increase within 6 h in non-stress condition and within 9 h under salt stress, but the values were always lower than 1.5. Borage treatment induced different responses at different time points. In particular, under non-stress condition it the expression increased at 2, 9 and 24 h, whereas it decreased at 4 and 6 h.

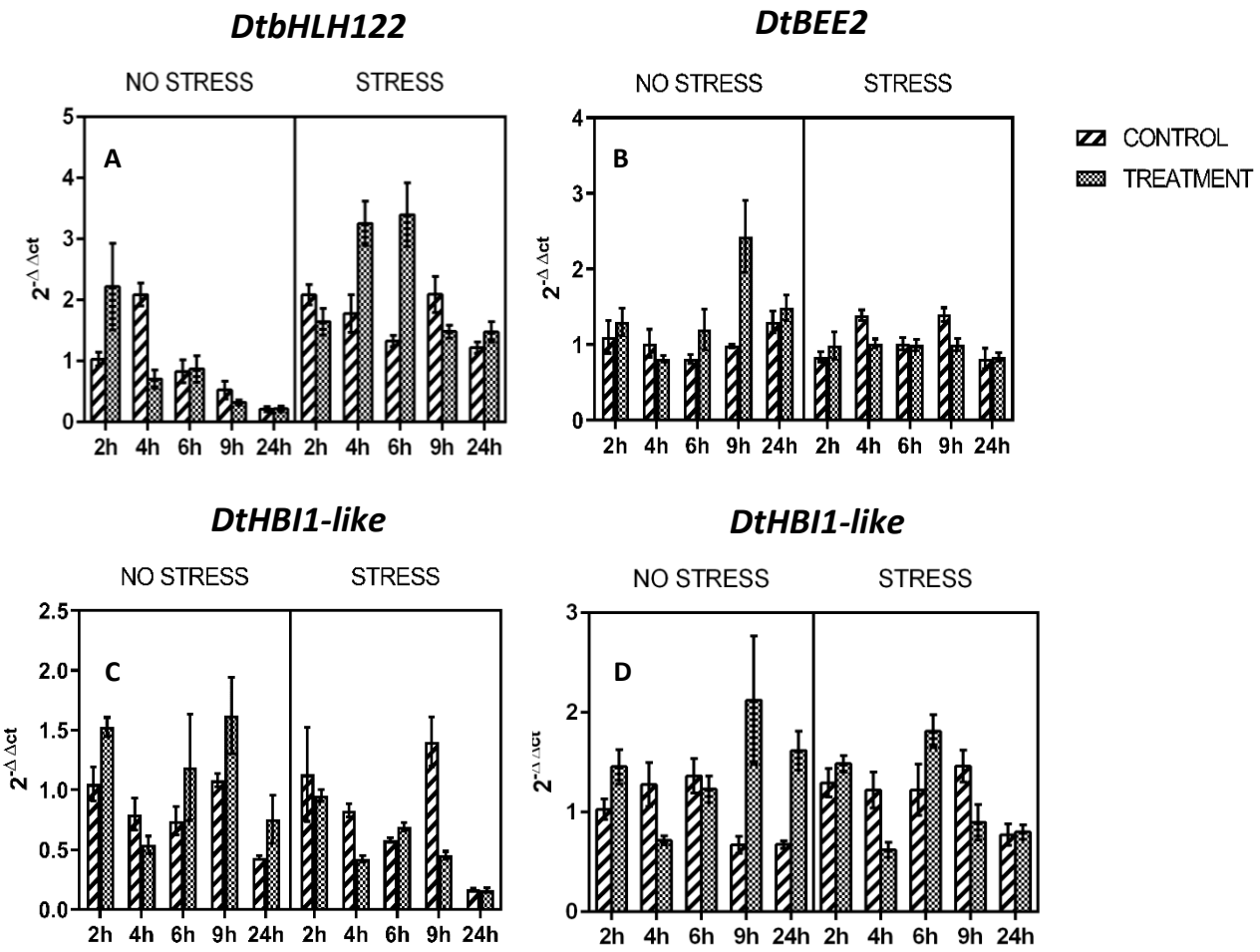


Figure 26. Changes in the expression of *DtbHLH122* (A), *DtBEE2* (B), *DtHBI1-like* (C) and *DtIBH1-like* (D) in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken 2, 4, 6, 9, 24 hours after the initial exposure to salt stress. Values are means ± SE (n = 6). Data were subjected to three-way ANOVA

Expression of DtMYB30 and DtMYB94 – (MYB transcription factors family)

Under non-stressful condition the expression of *DtMYB30* (Figure 27 A) in control plants was generally low, only a peak appeared at 4 h. In plants treated with borage extract the expression increased within 9 h reaching the $2^{-\Delta\Delta Ct}$.value of 7.4, then decreased after 24 h. Salt stress lowered the expression in non-treated plants, whereas induced the expression of *DtMYB30* in treated plants after 6 h. In particular, transcript accumulation was 16 times higher than the unstressed control at 2 h. The expression levels of *DtMYB94* are reported in Figure 27 B. Control plants showed a similar pattern of those observed in the expression of *DtMYB30* (Figure 27 A) with an increase at 4 h followed by a constant decrease up to 0.2 at 24 h. Similar trend resulted also in plants treated with borage extract. The expression levels were induced under salt stress mostly after 4, 6 and 9 h, both in control and treated samples. In particular, in control plants the expression showed

a constant growth up to 5 at 9 h and then decreased after 24 h, whereas treated plants reached the same peak after 4 h.

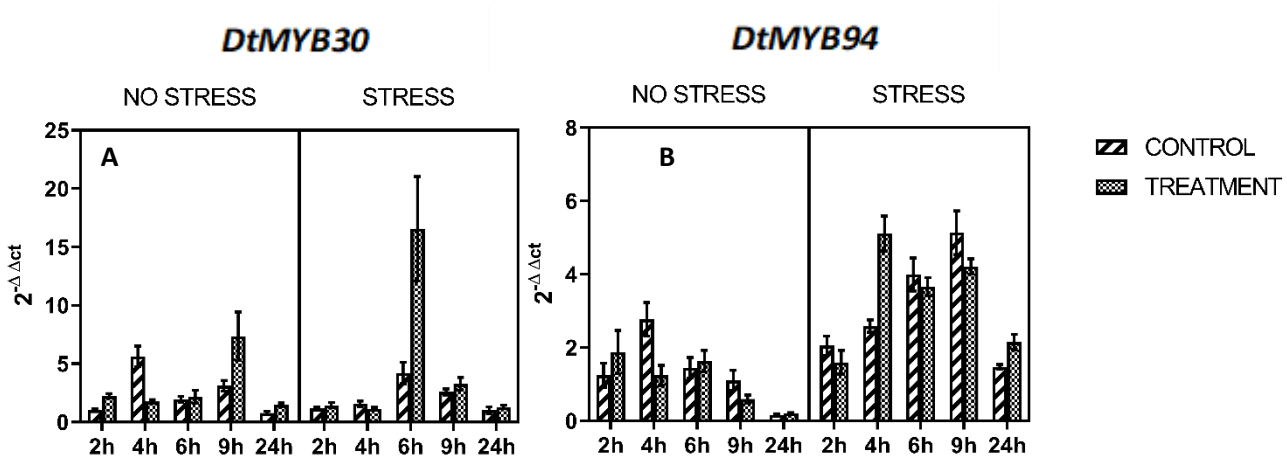


Figure 27. Changes in the expression of *DtMYB30* (A) and *DtMYB94* (B) in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken 2, 4, 6, 9, 24 hours after the initial exposure to salt stress. Values are means ± SE (n = 6). Data were subjected to three-way ANOVA

Expression of DtNAC29, DtNAC72, DtNAC69, DtNAC92, DtANAC019 – (NAC transcription factors family)

Different expression patterns resulted from the analysis of the selected transcription factors belonging to the NAC family in response to the experimental condition, as reported in Figure 28. Under non-stress condition the expression of *DtNAC29* and *DtNAC72* was very similar (Figure 28 A and B). In particular, a constant decrease was observed within 24 h, regardless the treatment. Borage treatment affected the accumulation of the *DtNAC29* and *DtNAC72* only at 2 h. Under salt stress the expression of both transcription factors showed two different trends. In particular, within 9 h salt stress did not affect the expression of *DtNAC29* in control plants while treated plants showed a slight increase. After 24 h the expression levels in both treated and non-treated samples increased by 4 and 3 times, respectively. Differently, the expression of *DtNAC72* was induced by 4 times after 2 h of stress in control plants. Afterwards, the amount of transcript decreased within 6 h and slightly increased at 24 h. Treated plants showed a different pattern and the expression of *DtNAC72* resulted 5 times higher after 4 and 24 hours of stress. The expression of *DtANAC69* (Figure 28 C) in untreated and unstressed plants increased more than 3 times at 4 h, then progressively decreased and reached the value of 0.3 after 24 h.

The accumulation of the transcripts was not affected by the borage treatment, except after 6 h, when a peak more than 5 times higher appeared. Salt stress generally induced the expression of this transcription factor. In particular, control plants showed a constant increased after 2, 4 and 6 hours and then decreased at 9 and 24 hours. Borage treatment in combination with salt stress induced the expression of *DtANAC69* after 4 h. Values were high until 24 h. The expression levels of *DtNAC92* are reported in Figure 28 D. An increase more than 2 times was observed in control plants under non-stressed condition within 9 h but after 24 h the expression level decreased and reached the $2^{-\Delta\Delta Ct}$ value of 0.1. Salt stress induced the expression in control plants only after 9 h, whereas it induced an overall increase in plants treated with borage extract already after 2 hours.

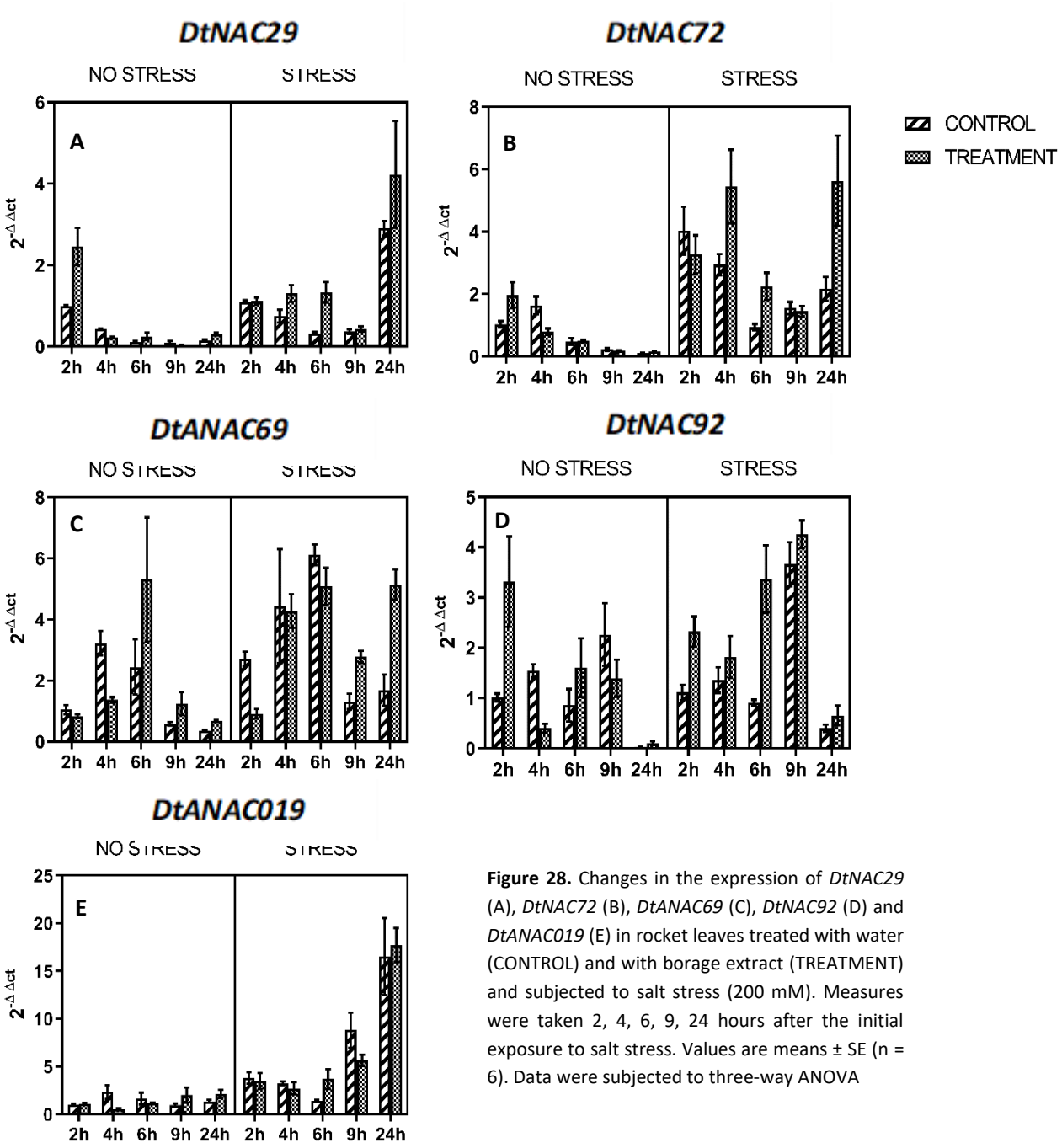


Figure 28. Changes in the expression of *DtNAC29* (A), *DtNAC72* (B), *DtANAC69* (C), *DtNAC92* (D) and *DtANAC019* (E) in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken 2, 4, 6, 9, 24 hours after the initial exposure to salt stress. Values are means ± SE (n = 6). Data were subjected to three-way ANOVA

After 24 h *DtNAC92* was downregulated in both conditions. The expression levels of *DtANAC019* (Figure 28) was similar to those observed in *DtRD29A* (Figure 24). Indeed, under non-stressed condition the expression did not change during time or in response to borage treatment. On the contrary, salt stress induced a constant increased in all samples, regardless the treatment. In particular, values grown from a $2^{-\Delta\Delta Ct}$ value of 4 to almost 18 after 24 hours of exposure to salt stress.

Expression of DtC3H49 and DtZAT12-like – (ZINC FINGER transcription factors family)

A similar pattern of expression was observed for *DtC3H49* and *DtZAT12-like* (Figure 29). In particular, *DtC3H49* was strongly downregulated after 6 hours, regardless the treatment or the growing condition. After 2 and 4 hours of salt stress the expression slightly increased in control and treated plants, if compared with the non-stress condition. In a similar way, *DtZAT12-like* expression was downregulated in all samples, both under stress and non-stress condition after 9 and 24 h. Moreover, at 4 h control plants showed a peak in the expression, under both growing conditions.

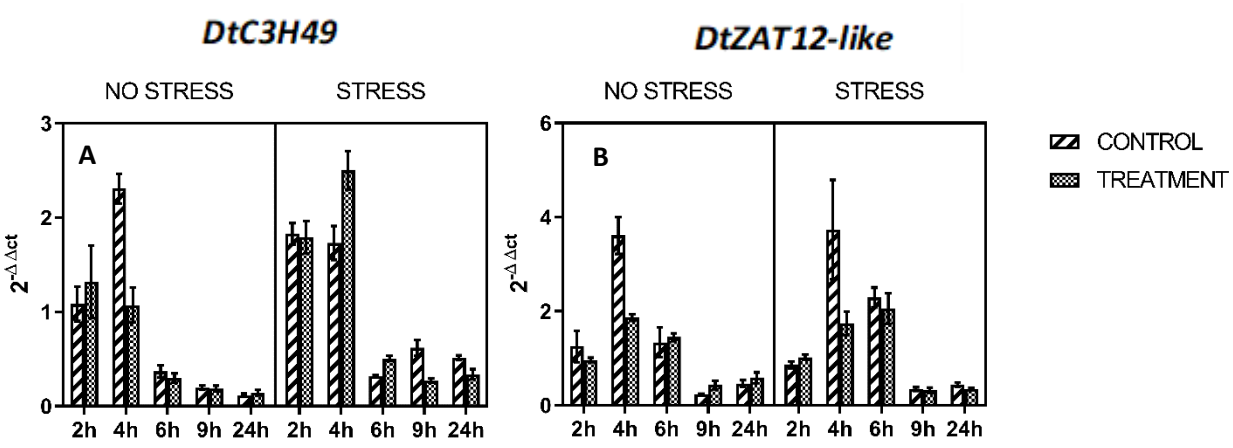


Figure 29. Changes in the expression of *DtC3H49* (A) and *DtZAT12-like* (B) in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken 2, 4, 6, 9, 24 hours after the initial exposure to salt stress. Values are means ± SE (n = 6). Data were subjected to three-way ANOVA.

Expression of DtABF3 and DtbZIP63 – (b-ZIP transcription factors family)

Under non-stressful condition the expression of *DtABF3* (Figure 30 A) was generally low and all values ranged from 1 to 3 within 9 h. After 24 h the expression was downregulated, both in control and in treated plants. Salt stress induced the expression of *DtABF3* in all samples and time points. Control plants showed a decrease from a value of 3 after 2 h to 1.4 after 24 h of stress. A peak of about 6.7 appeared after 9 h of stress. In plants treated with borage extract the expression increased after 4 h of stress, reaching a value of 6 and then constantly decreased within 24 h reaching a minimum of 1.6. The expression analysis of *DtbZIP63* transcription factor is reported in Figure 30 B. In general, no changes have been observed within 6 h, neither in control nor in treated plants and regardless the growing condition. Under non-stress condition, the expression of *DtbZIP63* was 3 times higher at 24 h in control plants, whereas it was strongly upregulated after 9 and 24 h and values were 5 and more than 8 times higher in treated plants. A pick around 5 resulted also under salt stress condition after 24 h both in control and in treated plants.

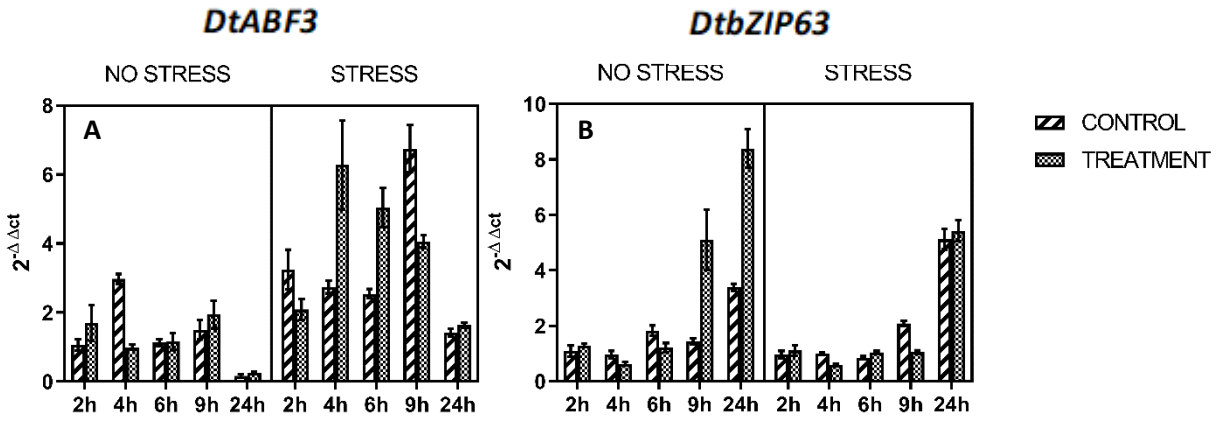


Figure 30. Changes in the expression of *DtABF3* (A) and *DtbZIP63* (B) in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken 2, 4, 6, 9, 24 hours after the initial exposure to salt stress. Values are means ± SE (n = 6). Data were subjected to three-way ANOVA.

Expression of DtWRKY54 – (WRKY transcription factors family)

The expression levels of *DtWRKY54* (Figure 31) in control plants were generally low within 24 h, both in non-stressed and in stressed conditions. On the contrary, the expression was induced by borage treatment, mostly under non-stress condition. After 2 h the $2^{-\Delta\Delta Ct}$ value was 3 times higher than control. It constantly decreased during time, apart from a peak after 9 h. Under salt stress the expression levels were reduced and the highest value, around 3 resulted in treated plants after 9 hours of exposure to salt.

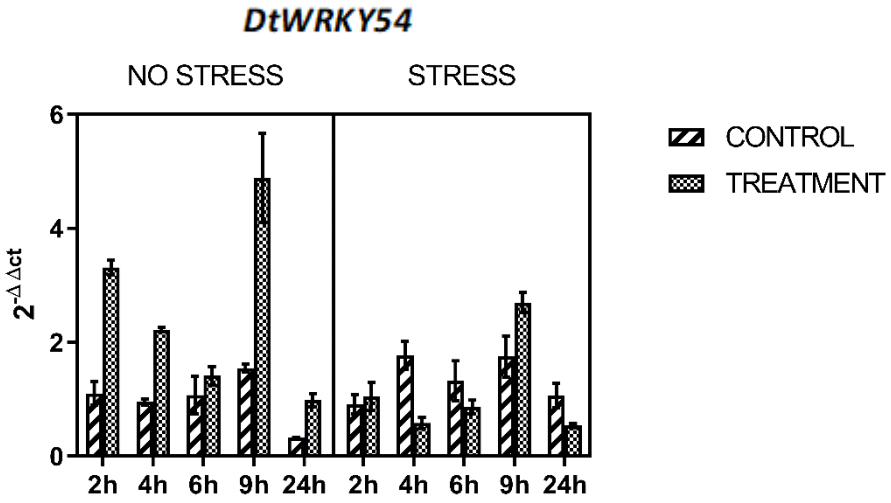


Figure 31. Changes in the expression of *DtWRKY54* in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken 2, 4, 6, 9, 24 hours after the initial exposure to salt stress. Values are means ± SE (n = 6). Data were subjected to three-way ANOVA.

Expression of DtHB12 and DtHB7 – (HD-ZIP transcription factors family)

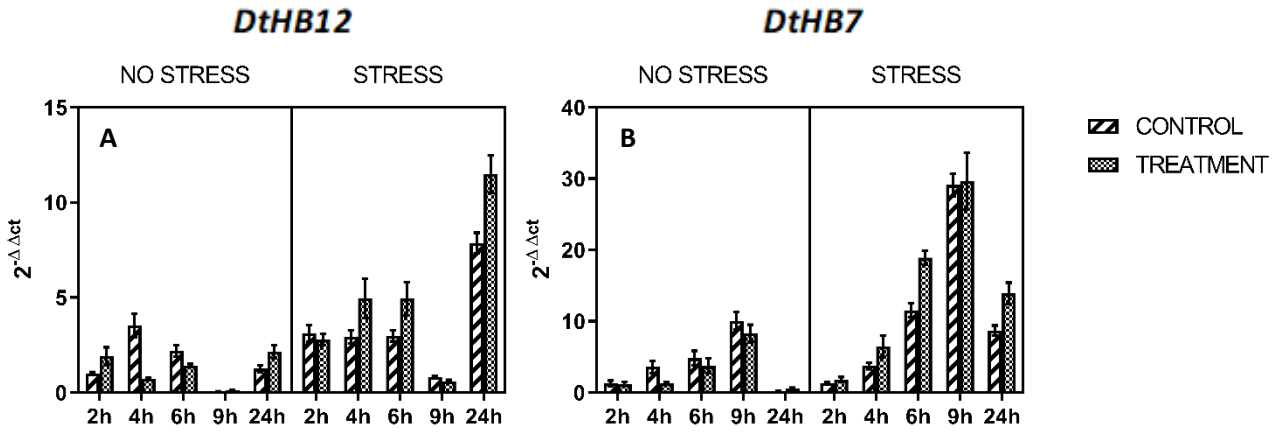


Figure 32. Changes in the expression of *DtHB12* (A) and *DtHB7* (B) in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken 2, 4, 6, 9, 24 hours after the initial exposure to salt stress. Values are means ± SE (n = 6). Data were subjected to three-way ANOVA.

The expression patterns of the selected transcription factors belonging to HD-ZIP are reported in Figure 32. Both *DtHB12* and *DtHB7* expression increased in response to salt stress. In particular, the expression of *DtHB12* (Figure 32 A) in plants grown under non-stress condition did not changed much during time and the maximum level was reached in control plants after 4 h. After 9 h the transcription factor was downregulated, both in treated and control plants. The same effect resulted also in plants grown under salinity. Salt stress

induced the expression levels in all samples and a constant increase resulted during time. The maximum levels were reached after 24 h of exposure both in control and in treated plants; values were 8 and 12 time higher than internal target, respectively. The expression pattern of *DtHB7* was very similar in control and treated plants during time. Moreover, expression levels increased in response to salt stress but the trend unchanged. In particular, the expression rose until 9 h, then it decreased at 24 h in both growing conditions. The maximum levels were reached after 9 h of salt exposure and the expression was almost 30 times higher than internal calibrator, both in control and in treated plants.

Expression of RABC2B

The expression levels of *DtRABC2B* in rocket leaves (Figure 33) revealed a global down-regulation within 24 h. In particular, under non stress condition the $2^{-\Delta\Delta Ct}$ value of control plants dropped to 0.2 after 4 h and reached the minimum point (0.04) after 24 h. A similar trend resulted in plants grown under salt stress, even if the decrease started 2 hours late. Plants treated with borage extract showed higher value, mostly in combination with salt stress and after 2 and 4 hours of exposure to salt.

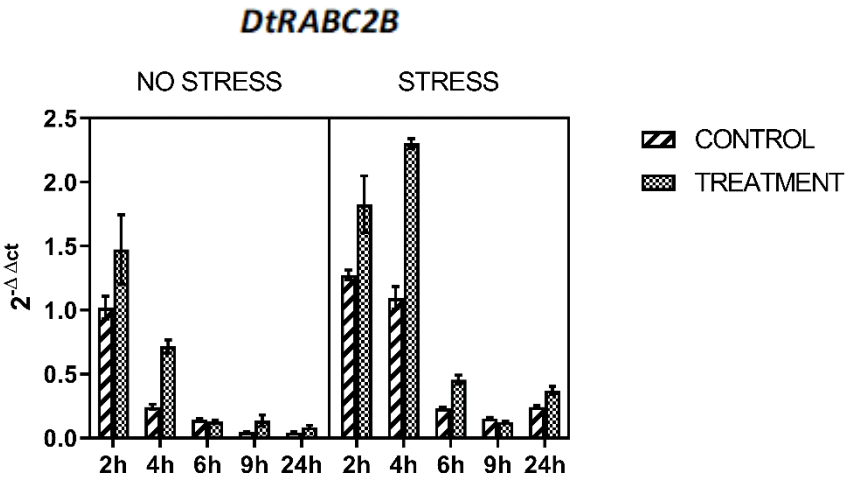


Figure 33. Changes in the expression of *DtRABC2B* in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken 2, 4, 6, 9, 24 hours after the initial exposure to salt stress. Values are means ± SE (n = 6). Data were subjected to three-way ANOVA.

Expression of Unknown 2

Changes in the expression of an unknown transcription factors named *Unknown2* is reported in Figure 34 A. In control plants grown under non stress condition, the expression grew until 6 h by almost 3 times and then decreased to 0.6 at 24 h. Borage treatment slightly decreased the expression level at 2 h whereas a peak almost 7 times higher resulted after 9 h. Under salt stress, both control and treated plants had the same trend excluding after 24 h. In particular, the expression gradually increased during time from a $2^{-\Delta\Delta Ct}$ value of 0.5 to almost 3. After 24 h of stress all values dropped around 1.

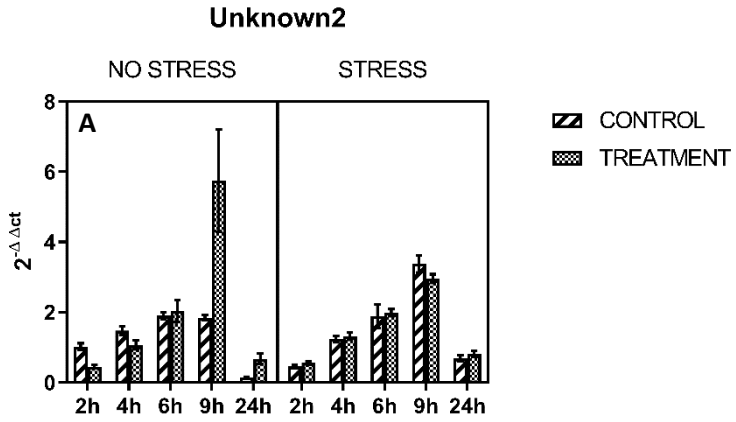


Figure 34. Changes in the expression of an Unknown transcription factors named Unknown2 (A) in rocket leaves treated with water (CONTROL) and with borage extract (TREATMENT) and subjected to salt stress (200 mM). Measures were taken 2, 4, 6, 9, 24 hours after the initial exposure to salt stress. Values are means ± SE (n = 6). Data were subjected to three-way ANOVA.

DISCUSSION AND CONCLUSION

The extreme concentration of sodium chloride in growing media causes different damages to vegetables crops depending on the crop tolerance threshold. It reduces plant productivity, limits the photosynthesis, decreases the chlorophyll biosynthesis, and impairs the uptake of water and nutrients. All these physiological, anatomical, and metabolic changes lead to a reduction of the product quality and marketability [77,78]. However, the response of vegetables to salt stress and the amount of the damage accused, essentially change depending on several interacting parameters, such as the cultivar, the concentration of the salt, the time of exposure, and the plant phenological stage affected by the stress [14]. The application of biostimulant products has been proven to be a useful strategy for increasing plant tolerance to salinity [79].

Diplotaxis tenuifolia can be considered as salt tolerant species since its ability to keep considerable growth rates under increasing salinity without any relevant variation to physiological parameters [11,80]. In the present study, rocket plants have been exposed to 200 mM NaCl for four days, just before the harvest, in order to evaluate the plant responses to salt shock with no acclimatisation period. To better understand the nature of the different processes activated to cope the stress, a transcriptome analysis and the evaluation of different physiological and biochemical mechanisms have been performed.

The content of chlorophyll and the chlorophyll fluorescence-related parameters did not significatively change in response to salt stress. These results are consistent with those reported in other studies, in which salt stress did not cause significant alteration in photosynthetic apparatus after few days of exposition to stressful conditions [81–83]. It is known that these parameters are considered biochemical markers of salt tolerance since chlorophyll levels and PSII efficiency usually decrease quickly in sensitive plants [84]. The content of chlorophyll in green leafy vegetable is also important because it defines the visual appearance of the product and influences the consumer choice [85]. The application of biostimulant products has been shown to increase the biosynthesis of chlorophyll in different vegetables [86,87].

This experiment confirms that *D. tenuifolia* is a salt tolerant crop [11] since its ability to counteract a sudden exposition to a high level of salinity, at least under our experimental conditions. At the same time, the short treatment time with the borage extract did not increase the chlorophyll content as well as the PSII efficiency in rocket plants grown under salt and non-salt conditions. On the contrary, after one day of exposition to salt stress plants treated with borage extract showed a significant decrease of Fv/Fm ratio. Since this value unchanged in untreated plants in response to salt treatment and in plant treated with the borage extract grown in non-stressful condition, it may indicate that treatment induced the plant temporarily susceptible to salinity. The same effect observed in chlorophyll concentration resulted also in carotenoids level and it makes sense because of their role as accessory light-harvesting pigments and protecting chlorophyll molecules. These results were compatible with those obtained by Bulgari et al. [69] after two applications of the same

borage extract on rocket and lettuce plants grown under non-stressful conditions. No significant modification of chlorophyll and carotenoids content were also detected in *Eruca sativa* Mill. grown under salt stress, as reported by Barbieri et al. [88]. A moderate salinity has a positive effect increasing the concentration of phytochemicals, such as flavonoids, phenolic acids, and tannins [89–92]. These polyphenolic compounds play an important role in plant protection against reactive oxygen species (ROS), which are produced in plant tissues when physiological metabolism is impaired by various environmental stresses. Besides their roles in plants, phenolic compounds are beneficial for human health [93]. They exert important function, such as reduction of cardiovascular disease, diabetes, cancer, stroke, inhibition of pathogens, anti-inflammation and anti-allergic effects, scavenging activity against free radicals. All these activities are related not only to their antioxidant capacity, but also to their selective action on different signaling pathways [94]. At the same time, several biostimulant products were reported to enhance the secondary metabolism and increase the synthesis of phytochemicals [95]. Borage extracts have shown to be effective in improving total phenols, flavonoids and antioxidant capacity in lettuce plants [69]. This was not the case in present study, as no significant changes were recorded, not in response to salt, not to treatment. A high variability in total phenols concentration was observed also by Hamilton et al. [96] within species and between trials. The different response obtained in our experiment compared to those reported by Bulgari et al. [69] by applying the same extract could be related to the number of treatments or the different species. A significant increase of total sugars appeared in leaf tissues after 1 day of salt exposition, both in treated and non-treated plants. Afterwards, values declined to non-stress levels and remained stable until harvest (4 days of salt stress). This trend may be related to the increase observed in reducing sugars concentration. The accumulation of sugars has been reported in different plants species exposed to salinity [97,98]. Carbohydrates are products of photosynthesis and they are the building blocks and source of energy to plant growth. Besides these roles, sugars are involved in several processes related to plant stress responses, acting as signalling molecules, osmoprotectants or antioxidants [99,100]. As reported by Shavrukov [13], when plants are suddenly exposed to a high level of salinity, they suffer osmotic shock and plasmolysis, regardless their level of tolerance. Indeed, the only difference between a sensitive and a tolerant plant is the degree of damage and the time needed to restore the physiological function. In accordance to this, a significant increase in lipid peroxidation resulted in all stressed samples. The MDA content in unstressed plants treated and non-treated, was similar to those observed by Ozdener et al. [101] in *Eruca sativa* Mill. It means that the application of borage extract did not cause any damage to cell membranes.

The accumulation of osmolytes is a typical response of plant exposed to salt stress condition in order to maintain the cell turgor pressure and stabilize proteins and other cell components against denaturing effects. Plants with an improved osmolyte biosynthesis generally showed enhanced stress tolerance [102–104]. In this experiment no significant change in osmolytes accumulation was observed, and this result was

unexpected. Abscisic acid is a plant hormone involved in different roles in plant growth and development either in physiological processes as well as in response to abiotic stresses. It has been suggested that some responses to osmotic stress could be modulated by ABA [105]. Frike et al. [106] reported that ABA accumulates in leaf tissues more than 6-times within 10 minutes in response to an environmental stress and within the first hour following the stress event, ABA has a growth-promoting function. A slight but non-significant increase in ABA levels resulted in plants grown under salt stress. This is in line to those observed by He and Cramer [107]. They compared the ABA accumulation in two *Brassica* species and found that ABA concentration increases more in salt-sensitive species than in tolerant ones. Results obtained in present experiment showed a slight increase of ABA content in treated plants, mostly in combination with the salinity. It may suggest that borage extract stimulates a little the production of ABA.

Rocket is a nitrate-accumulating vegetable and it is known that nitrate content its leaves changes depending on the cultivation systems as well as the environmental conditions, such as light intensity, photoperiod, temperature and abiotic stresses [108,109]. After the ingestion nitrates undergo different reactions that may lead to the formation of cancerogenic compounds like nitrosamines [110]. For this reason, the European Commission Regulation established limitation for the commercialization of several leafy vegetable (EU No 1258/2011). The maximum levels of nitrate for rocket salad are 7000 mg NO_3^- kg^{-1} if harvested from October to March, and 6000 mg NO_3^- kg^{-1} if harvested from April to September. In this experiment, reduced nitrate content was observed in plants exposed to salinity for 1, 2 and 4 days. These findings are consistent with those reported by Barbieri et al. [88] and Urrestarazu et al. [111] studies, in which a reduction of nitrate levels in rocket and lettuce salad resulted after high salinity treatment. Strategies to decrease the concentration of nitrate are important for the production of fresh vegetables and different biostimulant products showed a positive effect reducing the nitrate levels in several crop species [87,112,113]. However, nitrate contents were not affected by borage treatment, not under control or stress condition. This result was unexpected since, on the contrary, Bulgari [114] observed an increment of the NR activity *in vivo* and a substantial reduction of nitrate concentration in rocket plants treated with the same borage extract. This variability might be due to differences in the experimental plans (period of cultivation, number of treatments, timing of the application) or to hypothetical changes in the composition of the extract during storage.

At molecular level the transcriptional regulation depends on the interaction between transcription factors and a broad range of target genes involved in different stress responses pathways. TFs are involved in the primary stress responses, when plants induce a set of genes during the first few hours of stress exposition. For these reasons, we chose 24 transcription factors among different families and we analysed their expression within 24 hours of salt stress. Moreover, since their expression is dynamic and often transient, the measure at one single time point is not enough to understand their activity, thus we evaluated their expression over time after

2, 4, 6, 9 and 24 hours. TFs have been identified and selected from a *Diplotaxis tenuifolia* RNAseq database among those showing significant up or downregulation in response to 24 h of salt exposition.

RD29A is extremely sensitive to various abiotic stressors, it is considered as a stress-response marker gene and is used for different abiotic stress treatments. In this experiment the *DtRD29A* expression constantly decreased under control condition, both in untreated and treated plants. These values are consistent with those reported in another paper, in which the gene is not expressed at significant levels under what is considered as normal plant growing conditions [115]. As expected *DtRD29A* transcripts increased under salt stress condition, whereas its expression resulted particularly affected by the combination of NaCl and borage treatment. This result might suggest that rocket plants treated with borage extract were more sensitive to salt stress if compared with the non-treated ones. The presence of one ABRE (Abscisic Acid-Responsive Element) motif in the promoter region of the gene and the increase in ABA level in the same tissues may suggest that *DtRD29A* expression was affected by the stress and the treatment through ABA-dependent signal transduction pathway. Interestingly, Lee et al.[116] observed that *RD29A* expression pattern changes when induced by single or combined salt and ABA treatments and the combination of the inputs leads to unique dynamic behaviour that cannot be explained by the sum of the single responses. Li et al. [23] proposed an interaction between an ABRE binding protein (*ABF3*) and another transcription factor (*NAC72*) in the regulation of the expression of *RD29A*.

In our experiment we observed a rapid increased in *DtNAC72* expression induced by salt exposition similar to that reported by Tran et al. [117] in *Arabidopsis thaliana* exposed to 250 mM NaCl for 24 h. Fujita et al. [118] observed a slower increase of the expression during time, probably due to the lower concentration of NaCl used in the experiment. Moreover, the same authors reported the existence of two different pathways for the *NAC72* expression, one ABA-dependent in non-stressful condition, and the other ABA-independent under salt stress. It might explain why borage treatment induced the expression of *DtNAC72* under salinity and not under normal growing conditions. On the contrary, a gradual increase appeared in the expression of *DtNAC019* in response to salt stress but in this case borage extract did not show any effect. Even if *NAC019* and *NAC72* are homologs, their response to salt and treatment is quite different. Results obtained in the present study confirmed that both TFs are involved in salinity response but probably in two different pathways. Moreover, the different response to the borage treatment suggests that they are induced through different ways. *DtABF3* expression as well as *DtNAC72* did not show a gradual increase but it was rapidly affected by salt stress. ABF3 plays an important role in ABA signaling both in normal and stressful condition and it is induced by ABA and osmotic stress. Several papers confirmed its importance in the regulation of stress responses genes and an improved resistance to abiotic stresses was observed in plants overexpressing that gene [54 and references therein].

Besides the ABRE motif, two DRE (dehydration-responsive element) motifs are present in *RD29A* promoter region, where DRE-binding proteins are able to bind and affect the expression of the gene through ABA-independent way. Among DRE-binding proteins, in this experiment we evaluated the expression of *DtDREB2A* gene belonging to AP2/ERF transcription factor family. The trend of expression observed for this TF seemed not to be affected by the stress or by the treatment and its pattern of expression suggests that it might have a circadian rhythm. These results was confirmed also by Dubois et al. [120] in *Arabidopsis*, in response to mild drought condition. The authors observed that *DREB2A* was induced by drought during the day and repressed by drought during the night. Since the expression of the gene is controlled by a growth regulating factor, they supposed that during the day *DREB2A* is up-regulated and is involved in stress defence responses, whereas during the night the growing process is favoured.

MYB is a large family of transcription factors well-known to be involved in drought responses [121]. In this experiment we examined the expression of two TFs belonging to this family, *DtMYB94* and *DtMYB30*. The expression of *DtMYB94* constantly increased in response to salinity and was similar with those reported by Lee and Suh [31] in *Arabidopsis*. At the same time, *DtMYB30* expression showed a different pattern in response to salt and borage treatment. In general, its expression was lower in plants grown under stress. Recent studies reported that both *MYB30* and *MYB94* are involved in the activation of cuticular wax biosynthesis [30,31]. Moreover, *MYB30* acts as a positive regulator in ABA signaling response [34], in the accumulation pattern of very-long-chain fatty acids such as waxes, phospholipids and complex sphingolipids [122], and promoting the expression of a subset of brassinosteroids (BRs) target genes [35,36]. As discussed above, an increase in lipid peroxidation resulted in plant grown under salt stress and treated with borage extract. These finding may suggest that rocket plants reacted to salt stress through the accumulation of cuticular waxes and the production of new lipids molecules.

WRKY54, as well as *MYB30* is involved in BRs signaling pathway [51]. *DtWRKY54* transcription factor was low expressed in control and untreated plants and was slightly induced by salt stress. A similar results was observed in *Arabidopsis* by Zhou et al. [52] who suggested a link between *WRKY54* and *DREB2A* since the existence of a binding site (w-box) for *WRKY54* in the promoter region of *DREB2A*. The same authors observed that plants modified to over-express *WRKY54* were more tolerant to salt stress. In our experiment borage treatment induced the expression of Dt*WRKY54* under normal growing condition whereas it caused a general down regulation in plants exposed to salinity. Moreover, *WRKY54* co-operate with *WRKY70* as negative regulators of the plant response to osmotic stress [50], so the lower expression levels observed in plants grown under salinity but previously treated with borage extract could suggest a better tolerance to osmotic stress.

C3H49 and *ZAT12-like* transcription factors belong to the zinc finger family and they are important component of the oxidative stress response [123]. C3H49, also known as OZF2 or ZTF2 was reported to act as signaling protein also in ABA and salt stress responses through the ABI2-mediated signaling pathway [55]. It is also

involved in seedling growth rate, plant size, leaf and flower morphology and senescence [53,54]. ZAT12 protein is required for the expression of cytosolic Ascorbate peroxidase 1, a key enzyme involved in H_2O_2 scavenging [56,57]. Generally, a higher expression of genes involved in the oxidative stress response is associated to enhanced stress tolerance [124]. In our experiment, both transcription factors seemed to be regulated by the circadian clock, however no information about it have been found in literature. Salt stress slightly induce *DtC3H49* but not *DtZAT12-like* expression, whereas borage treatment did not show any clear effect.

Rocket plants grown under salt stress showed high levels in the expression of *DtHB7* and *DtHB12*. Both these transcription factors belong to HD ZIP family and they negative regulators of ABA signaling by acting as positive regulators of a protein phosphatase (PP2C) genes [63]. *DtHB7* resulted more upregulated than *DtHB12*, confirming what observed by Zimmermann et al. [125]. Moreover, it was reported that plants overexpressing *AtHB7* showed a higher chlorophyll content [62], it might be linked to the high chlorophyll levels observed in rocket plants grown under salt stress.

ERF003, *ERF107* and *ERF39* are member of the ERF transcription factor subfamily and they are involved in abiotic stress responses through binding the ethylene-responsive element (ERE) [41]. Their expression have been reported to increase in response to high concentration of salt and to contribute to salt and drought tolerance [47,49,126]. In our experiment, we observed that *DtERF107* and *DtERF39* expression was induced in plants exposed to salt stress, in accordance with reported above. On the contrary, the expression of *DtERF003* rapidly increased in response to borage treatment but no changes appeared in plants exposed to salinity, suggesting that in rocket *DtERF003* is not involved in salt stress response.

An overall upregulation of *DtbHLH122* expression resulted in rocket plants exposed to salt stress. *BHLH122* has been reported to be a positive regulator of drought and osmotic stress signaling resistance and its expression is at least partly independent from ABA signaling [38]. The same authors reported that *Arabidopsis* plants that overexpress *bHLH122* showed an increased resistance to water, salt and osmotic stresses. Moreover, bHLH122 was able to bind the promotor of a gene involved in ABA catabolism, repress its expression and lead to an accumulation of ABA. These results might explain those observed in our experiment. Indeed, rocket plant grown under salt stress and treated with borage extract showed an up-regulation of *DtbHLH122* transcript as well as an increased concentration of ABA content.

Besides *bHLH122*, we also analysed the expression of other three TFs of bHLH family, *BEE2*, *HBI1* and *IBH1* all involved in brassinosteroids signaling pathway. *BEE2* and *HBI1-like* are induced by BRs and repressed by ABA while *IBH1* is reported to inhibit both *BEE2* and *HBI1*. Moreover, plants overexpressing *IBH1* showed a decreased stress response [127]. All TFs resulted generally low expressed in rocket plants regardless the growing condition. These results, together with the low expressions levels of *DtMYB30* and *DtWRKY54* and the up regulation of *DtNAC72*, a negative regulator in the BRs signaling pathway, might suggest that stress

responses in rocket plants are mediated more through ABA signaling pathways than brassinosteroids signaling.

This is further confirmed by the increased concentration of ABA in the leaf tissues of plant grown under salt stress. It is also known that an antagonistic interaction between ABA and BRs exist.

bZIP63 is a transcription factor involved in the processes regulating the circadian phase through the regulation of low-energy response. It is a target of a sugar-sensing kinase (*SnRK1*), a conservative gene usually activated during starvation [128,129]. In our experiment we observed that *DtbZIP63* is low expressed in control plants and only a peak is observed after 24 h. This time point is corresponding to 8:00 am, and the accumulation of *DtbZIP63* transcripts might be linked to the low concentration of sugars during night. Indeed, it has been reported that *bZIP63* expression is usually repressed by sugars and ABA [130]. Interestingly, borage extract induced the expression of the gene under non stressful condition even if the level of sugars and ABA in rocket leaves didn't change. Moreover, *bZIP63* is reported to function as a negative regulator of osmotic stress tolerance in *Arabidopsis* seeds germination [131], thus the low expression detected in stressed plants might also confirm the tolerance of rocket plants.

RABC2B is a protein involved in signal transduction and intracellular transport. In rocket plants *DtRABC2B* was generally low expressed in both growing condition, in contrast to those reported by Liu et al. [132], who observed an induction in response to salt stress in *Arabidopsis*. Borage treatment induced *DtRABC2B* expression, mostly after 2 and 4 hours of stress exposition. However, to date there are only few information about the biological role and the regulation of these proteins in plants.

NAC92, *NAC29* and *NAC69* are involved in multiple abiotic stress responses. Their expression levels were generally low in leaves under non stress condition, as reported also by Xue et al. [133] and were induced by salt stress at different time points. NAC69 shares high similarity in the NAC domain with NAC72 and NAC19, and this is confirmed by the early induction of *DtNAC69* in response to salt stress. On the contrary, *DtNAC29* and *DtNAC92* expression was induced only after 24 and 9 h respectively.

The trend of expression of the unknown transcription factor was similar to those observed in *DtHB7* and *DtDREB2A*. Moreover, as well as *DtBEE2*, *DtWRKY54* and *DtERF107*, a peak of transcript appeared after 9 h.

An overall upregulation of the gene expression appeared in response to salt stress condition. In general, the results obtained in the present experiment confirmed those reported in the transcriptome for most of the TFs analysed. Novel findings emerged from the *bZIP63*, *ERF107*, *DREB2A* and *NAC92* expression. Moreover, the analysis of the gene expression over time allowed us to see the different trends in the TFs. For example, the expression levels of C3H49 and RABC2B were higher in plant exposed to salt stress if compared with the control after 24 h as reported by Cavaiuolo et al.[16] . However, the trend of their expression shows a constant decrease during time.

REFERENCES

1. Ivushkin, K.; Bartholomeus, H.; Bregt, A.K.; Pulatov, A.; Kempen, B.; de Sousa, L. Global mapping of soil salinity change. *Remote Sens. Environ.* **2019**, *231*, 111260.
2. Shrivastava, P.; Kumar, R. Soil salinity: A serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. *Saudi J. Biol. Sci.* **2015**, *22*, 123–131.
3. Munns, R. Genes and salt tolerance: bringing them together. *New Phytol.* **2005**, *167*, 645–663.
4. Hniličková, H.; Hnilička, F.; Martinková, J.; Kraus, K. Effects of salt stress on water status, photosynthesis and chlorophyll fluorescence of rocket. *Plant, Soil Environ.* **2017**, *63*, 362–367.
5. Najar, R.; Aydi, S.; Sassi-Aydi, S.; Zarai, A.; Abdelly, C. Effect of salt stress on photosynthesis and chlorophyll fluorescence in Medicago truncatula. *Plant Biosyst. - An Int. J. Deal. with all Asp. Plant Biol.* **2019**, *153*, 88–97.
6. Jacoby, R.P.; Taylor, N.L.; Millar, A.H. The role of mitochondrial respiration in salinity tolerance. *Trends Plant Sci.* **2011**, *16*, 614–623.
7. Peleg, Z.; Blumwald, E. Hormone balance and abiotic stress tolerance in crop plants. *Curr. Opin. Plant Biol.* **2011**, *14*, 290–295.
8. Cheeseman, J.M. The evolution of halophytes, glycophytes and crops, and its implications for food security under saline conditions. *New Phytol.* **2015**, *206*, 557–570.
9. Flowers, T.J.; Troke, P.F.; Yeo, A.R. The Mechanism of Salt Tolerance in Halophytes. *Annu. Rev. Plant Physiol.* **1977**, *28*, 89–121.
10. Glenn, E.P.; Brown, J.J.; Blumwald, E. *Salt tolerance and crop potential of halophytes*; 1999; Vol. 18; ISBN 0735268999130.
11. de Vos, A.C.; Broekman, R.; de Almeida Guerra, C.C.; van Rijsselberghe, M.; Rozema, J. Developing and testing new halophyte crops: A case study of salt tolerance of two species of the Brassicaceae, Diplotaxis tenuifolia and Cochlearia officinalis. *Environ. Exp. Bot.* **2013**, *92*, 154–164.
12. Munns, R.; Termaat, A. Whole-plant responses to salinity. *Aust. J. Plant Physiol.* **1986**, *13*, 143–160.
13. Shavrukov, Y. Salt stress or salt shock: which genes are we studying? *J. Exp. Bot.* **2013**, *64*, 119–127.
14. Parida, A.K.; Das, A.B. Salt tolerance and salinity effects on plants: a review. *Ecotoxicol.*

Environ. Saf. **2005**, *60*, 324–349.

15. Ciarmiello, L.F.; Woodrow, P.; Piccirillo, P.; De Luca, A.; Carillo, P. *Transcription Factors and Environmental Stresses in Plants*; 2014; Vol. 1; ISBN 9780128010884.
16. Cavaiuolo, M.; Cocetta, G.; Spadafora, N.D.; Müller, C.T.; Rogers, H.J.; Ferrante, A. Gene expression analysis of rocket salad under pre-harvest and postharvest stresses: A transcriptomic resource for Diplotaxis tenuifolia. *PLoS One* **2017**, *12*, e0178119.
17. Olsen, A.N.; Ernst, H.A.; Leggio, L. Lo; Skriver, K. NAC transcription factors: structurally distinct, functionally diverse. *Trends Plant Sci.* **2005**, *10*, 79–87.
18. LIU, X.; HONG, L.; LI, X.-Y.; YAO, Y.; HU, B.; LI, L. Improved Drought and Salt Tolerance in Transgenic Arabidopsis Overexpressing a NAC Transcriptional Factor from Arachis hypogaea. *Biosci. Biotechnol. Biochem.* **2011**, *75*, 443–450.
19. Nakashima, K.; Takasaki, H.; Mizoi, J.; Shinozaki, K.; Yamaguchi-Shinozaki, K. NAC transcription factors in plant abiotic stress responses. *Biochim. Biophys. Acta - Gene Regul. Mech.* **2012**, *1819*, 97–103.
20. Hu, H.; Dai, M.; Yao, J.; Xiao, B.; Li, X.; Zhang, Q.; Xiong, L. Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. *Proc. Natl. Acad. Sci.* **2006**, *103*, 12987–12992.
21. Zheng, X.; Chen, B.; Lu, G.; Han, B. Overexpression of a NAC transcription factor enhances rice drought and salt tolerance. *Biochem. Biophys. Res. Commun.* **2009**, *379*, 985–989.
22. Kumar, J.; Singh, S.; Singh, M.; Srivastava, P.K.; Mishra, R.K.; Singh, V.P.; Prasad, S.M. Transcriptional regulation of salinity stress in plants: A short review. *Plant Gene* **2017**, *11*, 160–169.
23. Li, X.; Li, X.; Li, M.; Yan, Y.; Liu, X.; Li, L. Dual Function of NAC072 in ABF3-Mediated ABA-Responsive Gene Regulation in Arabidopsis. *Front. Plant Sci.* **2016**, *7*, 1–9.
24. Hickman, R.; Hill, C.; Penfold, C.A.; Breeze, E.; Bowden, L.; Moore, J.D.; Zhang, P.; Jackson, A.; Cooke, E.; Bewicke-Copley, F.; et al. A local regulatory network around three NAC transcription factors in stress responses and senescence in Arabidopsis leaves. *Plant J.* **2013**, *75*, 26–39.
25. Jung, J.-H.; Park, C.-M. Auxin modulation of salt stress signaling in Arabidopsis seed germination. *Plant Signal. Behav.* **2011**, *6*, 1198–1200.
26. He, L.; Su, C.; Wang, Y.; Wei, Z. ATDOF5.8 protein is the upstream regulator of ANAC069 and is responsive to abiotic stress. *Biochimie* **2015**, *110*, 17–24.
27. Sakuraba, Y.; Bülbül, S.; Piao, W.; Choi, G.; Paek, N.-C. Arabidopsis EARLY FLOWERING3 increases salt tolerance by suppressing salt stress response pathways. *Plant J.* **2017**, *92*,

1106–1120.

28. Seok, H.-Y.; Woo, D.-H.; Nguyen, L.V.; Tran, H.T.; Tarte, V.N.; Mehdi, S.M.M.; Lee, S.-Y.; Moon, Y.-H. Arabidopsis AtNAP functions as a negative regulator via repression of AREB1 in salt stress response. *Planta* **2017**, *245*, 329–341.
29. Xiong, H.; Li, J.; Liu, P.; Duan, J.; Zhao, Y.; Guo, X.; Li, Y.; Zhang, H.; Ali, J.; Li, Z. Overexpression of OsMYB48-1, a novel MYB-related transcription factor, enhances drought and salinity tolerance in rice. *PLoS One* **2014**, *9*, 1–13.
30. Lee, S.B.; Kim, H.U.; Suh, M.C. MYB94 and MYB96 Additively Activate Cuticular Wax Biosynthesis in Arabidopsis. *Plant Cell Physiol.* **2016**, *57*, 2300–2311.
31. Lee, S.B.; Suh, M.C. Cuticular Wax Biosynthesis is Up-Regulated by the MYB94 Transcription Factor in Arabidopsis. *Plant Cell Physiol.* **2015**, *56*, 48–60.
32. Okazaki, Y.; Saito, K. Roles of lipids as signaling molecules and mitigators during stress response in plants. *Plant J.* **2014**, *79*, 584–596.
33. Mabuchi, K.; Maki, H.; Itaya, T.; Suzuki, T.; Nomoto, M.; Sakaoka, S.; Morikami, A.; Higashiyama, T.; Tada, Y.; Busch, W.; et al. MYB30 links ROS signaling, root cell elongation, and plant immune responses. *Proc. Natl. Acad. Sci.* **2018**, *115*, E4710–E4719.
34. Zheng, Y.; Schumaker, K.S.; Guo, Y. Sumoylation of transcription factor MYB30 by the small ubiquitin-like modifier E3 ligase SIZ1 mediates abscisic acid response in Arabidopsis thaliana. *Proc. Natl. Acad. Sci.* **2012**, *109*, 12822–12827.
35. Li, L.; Yu, X.; Thompson, A.; Guo, M.; Yoshida, S.; Asami, T.; Chory, J.; Yin, Y. Arabidopsis MYB30 is a direct target of BES1 and cooperates with BES1 to regulate brassinosteroid-induced gene expression. *Plant J.* **2009**, *58*, 275–286.
36. Ashraf, M.; Akram, N.A.; Arteca, R.N.; Foolad, M.R. The Physiological, Biochemical and Molecular Roles of Brassinosteroids and Salicylic Acid in Plant Processes and Salt Tolerance. *CRC. Crit. Rev. Plant Sci.* **2010**, *29*, 162–190.
37. Feller, A.; Machemer, K.; Braun, E.L.; Grotewold, E. Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. *Plant J.* **2011**, *66*, 94–116.
38. Liu, W.; Tai, H.; Li, S.; Gao, W.; Zhao, M.; Xie, C.; Li, W.X. bHLH122 is important for drought and osmotic stress resistance in Arabidopsis and in the repression of ABA catabolism. *New Phytol.* **2014**, *201*, 1192–1204.
39. Moreno, J.E.; Moreno-Piovano, G.; Chan, R.L. The antagonistic basic helix-loop-helix partners BEE and IBH1 contribute to control plant tolerance to abiotic stress. *Plant Sci.* **2018**, *271*, 143–150.
40. Malinovsky, F.G.; Batoux, M.; Schwessinger, B.; Youn, J.H.; Stransfeld, L.; Win, J.; Kim, S.-

K.; Zipfel, C. Antagonistic Regulation of Growth and Immunity by the Arabidopsis Basic Helix-Loop-Helix Transcription Factor HOMOLOG OF BRASSINOSTEROID ENHANCED EXPRESSION2 INTERACTING WITH INCREASED LEAF INCLINATION1 BINDING bHLH1. *Plant Physiol.* **2014**, *164*, 1443–1455.

41. Mizoi, J.; Shinozaki, K.; Yamaguchi-Shinozaki, K. AP2/ERF family transcription factors in plant abiotic stress responses. *Biochim. Biophys. Acta - Gene Regul. Mech.* **2012**, *1819*, 86–96.
42. Huang, B.L.; Zhang, X.K.; Li, Y.Y.; Li, D.Y.; Ma, M.Y.; Cai, D.T.; Wu, W.H.; Huang, B.Q. Cloning and characterization of the dehydration-responsive element-binding protein 2A gene in Eruca vesicaria subsp sativa. *Genet. Mol. Res.* **2016**, *15*, 1–10.
43. Lata, C.; Prasad, M. Role of DREBs in regulation of abiotic stress responses in plants. *J. Exp. Bot.* **2011**, *62*, 4731–4748.
44. Kazuo Nakashima, Zabta K. Shinwari, Yoh Sakuma, Motoaki Seki, Setsuko Miura, K.; Shinozaki, and K.Y.-S. Organization and expression of two Arabidopsis DREB2 genes encoding DRE-binding proteins involved in deh .... **2000**, 657–665.
45. Yoshida, T.; Mogami, J.; Yamaguchi-Shinozaki, K. ABA-dependent and ABA-independent signaling in response to osmotic stress in plants. *Curr. Opin. Plant Biol.* **2014**, *21*, 133–139.
46. Go, Y.S.; Kim, H.; Kim, H.J.; Suh, M.C. Arabidopsis Cuticular Wax Biosynthesis Is Negatively Regulated by the DEWAX Gene Encoding an AP2/ERF-Type Transcription Factor. *Plant Cell* **2014**, *26*, 1666–1680.
47. Sun, Z.-M.; Zhou, M.-L.; Dan-Wang; Tang, Y.-X.; Lin, M.; Wu, Y.-M. Overexpression of the Lotus corniculatus Soloist Gene LcAP2/ERF107 Enhances Tolerance to Salt Stress. **2016**, *23*, 442–449.
48. Saelim, L.; Akiyoshi, N.; Tan, T.T.; Ihara, A.; Yamaguchi, M.; Hirano, K.; Matsuoka, M.; Demura, T.; Ohtani, M. Arabidopsis Group IIId ERF proteins positively regulate primary cell wall-type CESA genes. *J. Plant Res.* **2019**, *132*, 117–129.
49. Rong, W.; Qi, L.; Wang, A.; Ye, X.; Du, L.; Liang, H.; Xin, Z.; Zhang, Z. The ERF transcription factor TaERF3 promotes tolerance to salt and drought stresses in wheat. *Plant Biotechnol. J.* **2014**, *12*, 468–479.
50. Li, J.; Besseau, S.; Törönen, P.; Sipari, N.; Kollist, H.; Holm, L.; Palva, E.T. Defense-related transcription factors WRKY70 and WRKY54 modulate osmotic stress tolerance by regulating stomatal aperture in Arabidopsis. *New Phytol.* **2013**, *200*, 457–472.
51. Chen, J.; Nolan, T.; Ye, H.; Zhang, M.; Tong, H.; Xin, P.; Chu, J.; Chu, C.; Li, Z.; Yin, Y. Arabidopsis WRKY46, WRKY54 and WRKY70 Transcription Factors Are Involved in

Brassinosteroid-Regulated Plant Growth and Drought Response. *Plant Cell* **2017**, *29*, tpc.00364.2017.

52. Zhou, Q.-Y.; Tian, A.-G.; Zou, H.-F.; Xie, Z.-M.; Lei, G.; Huang, J.; Wang, C.-M.; Wang, H.-W.; Zhang, J.-S.; Chen, S.-Y. Soybean WRKY-type transcription factor genes, GmWRKY13, GmWRKY21, and GmWRKY54, confer differential tolerance to abiotic stresses in transgenic Arabidopsis plants. *Plant Biotechnol. J.* **2008**, *6*, 486–503.
53. Lee, S.J.; Jung, H.J.; Kang, H.; Kim, S.Y. Arabidopsis zinc finger proteins AtC3H49/AtTZF3 and AtC3H20/AtTZF2 are involved in ABA and JA responses. *Plant Cell Physiol.* **2012**, *53*, 673–686.
54. Bogamuwa, S.P.; Jang, J.C. Tandem CCCH zinc finger proteins in plant growth, development and stress response. *Plant Cell Physiol.* **2014**, *55*, 1367–1375.
55. Huang, P.; Ju, H.-W.; Min, J.-H.; Zhang, X.; Chung, J.-S.; Cheong, H.-S.; Kim, C.S. Molecular and Physiological Characterization of the Arabidopsis thaliana Oxidation-Related Zinc Finger 2, a Plasma Membrane Protein Involved in ABA and Salt Stress Response Through the ABI2-Mediated Signaling Pathway. *Plant Cell Physiol.* **2012**, *53*, 193–203.
56. Rizhsky, L.; Davletova, S.; Liang, H.; Mittler, R. The Zinc Finger Protein Zat12 Is Required for Cytosolic Ascorbate Peroxidase 1 Expression during Oxidative Stress in Arabidopsis. *J. Biol. Chem.* **2004**, *279*, 11736–11743.
57. Davletova, S.; Schlauch, K.; Coutu, J.; Mittler, R. The Zinc-Finger Protein Zat12 Plays a Central Role in Reactive Oxygen and Abiotic Stress Signaling in Arabidopsis. **2005**, *139*, 847–856.
58. Le, C.T.T.; Brumbarova, T.; Ivanov, R.; Stoof, C.; Weber, E.; Mohrbacher, J.; Fink-Straube, C.; Bauer, P. ZINC FINGER OF ARABIDOPSIS THALIANA12 (ZAT12) Interacts with FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT) Linking Iron Deficiency and Oxidative Stress Responses. *Plant Physiol.* **2016**, *170*, 540–557.
59. Ben Daniel, B.-H.; Cattan, E.; Wachtel, C.; Avrahami, D.; Glick, Y.; Malichy, A.; Gerber, D.; Miller, G. Identification of novel transcriptional regulators of Zat12 using comprehensive yeast one-hybrid screens. *Physiol. Plant.* **2016**, *157*, 422–441.
60. Nguyen, N.H.; Jung, C.; Cheong, J.-J. Chromatin remodeling for the transcription of type 2C protein phosphatase genes in response to salt stress. *Plant Physiol. Biochem.* **2019**, *141*, 325–331.
61. Fernando, V.C.D.; Al Khateeb, W.; Belmonte, M.F.; Schroeder, D.F. Role of Arabidopsis ABF1/3/4 during det1 germination in salt and osmotic stress conditions. *Plant Mol. Biol.* **2018**, *97*, 149–163.

62. Ré, D.A.; Capella, M.; Bonaventure, G.; Chan, R.L. Arabidopsis AtHB7 and AtHB12 evolved divergently to fine tune processes associated with growth and responses to water stress. *BMC Plant Biol.* **2014**, *14*, 150.

63. Valdés, A.E.; Övernäs, E.; Johansson, H.; Rada-Iglesias, A.; Engström, P. The homeodomain-leucine zipper (HD-Zip) class I transcription factors ATHB7 and ATHB12 modulate abscisic acid signalling by regulating protein phosphatase 2C and abscisic acid receptor gene activities. *Plant Mol. Biol.* **2012**, *80*, 405–418.

64. Hoang, X.L.T.; Nhi, D.N.H.; Thu, N.B.A.; Thao, N.P.; Tran, L.-S.P. Transcription Factors and Their Roles in Signal Transduction in Plants under Abiotic Stresses. *Curr. Genomics* **2017**, *18*.

65. Khan, S.-A.; Li, M.-Z.; Wang, S.-M.; Yin, H.-J. Revisiting the Role of Plant Transcription Factors in the Battle against Abiotic Stress. *Int. J. Mol. Sci.* **2018**, *19*, 1634.

66. Singh, K. Transcription factors in plant defense and stress responses. *Curr. Opin. Plant Biol.* **2002**, *5*, 430–436.

67. Kimotho, R.N.; Baillo, E.H.; Zhang, Z. Transcription factors involved in abiotic stress responses in Maize (Zea mays L.) and their roles in enhanced productivity in the post genomics era. *PeerJ* **2019**, *7*, e7211.

68. Trevisan, S.; Manoli, A.; Ravazzolo, L.; Franceschi, C.; Quaggiotti, S. mRNA-Sequencing Analysis Reveals Transcriptional Changes in Root of Maize Seedlings Treated with Two Increasing Concentrations of a New Biostimulant. *J. Agric. Food Chem.* **2017**, *65*, 9956–9969.

69. Bulgari, R.; Morgutti, S.; Cocetta, G.; Negrini, N.; Farris, S.; Calcante, A.; Spinardi, A.; Ferrari, E.; Mignani, I.; Oberti, R.; et al. Evaluation of Borage Extracts As Potential Biostimulant Using a Phenomic, Agronomic, Physiological, and Biochemical Approach. *Front. Plant Sci.* **2017**, *8*, 1–16.

70. Lichtenthaler, H.K. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. In; 1987; pp. 350–382.

71. Cataldo, D.A.; Maroon, M.; Schrader, L.E.; Youngs, V.L. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun. Soil Sci. Plant Anal.* **1975**, *6*, 71–80.

72. Miller, G.L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* **1959**, *31*, 426–428.

73. Yemm, E.W.; Willis, A.J. The estimation of carbohydrates in plant extracts by anthrone. *Biochem. J.* **1954**, *57*, 508.

74. Heath, R.L.; Packer, L. Photoperoxidation in isolated chloroplasts. *Arch. Biochem. Biophys.* **1968**, *125*, 189–198.
75. Du, Z.; Bramlage, W.J. Modified thiobarbituric acid assay for measuring lipid oxidation in sugar-rich plant tissue extracts. *J. Agric. Food Chem.* **1992**, *40*, 1566–1570.
76. Livak, K.J.; Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods* **2001**, *25*, 402–408.
77. Machado, R.; Serralheiro, R. Soil Salinity: Effect on Vegetable Crop Growth. Management Practices to Prevent and Mitigate Soil Salinization. *Horticulturae* **2017**, *3*, 30.
78. Maksimovic, I.; Ili, A. Effects of Salinity on Vegetable Growth and Nutrients Uptake. In *Irrigation Systems and Practices in Challenging Environments*; InTech, 2012.
79. Bulgari, R.; Franzoni, G.; Ferrante, A. Biostimulants Application in Horticultural Crops under Abiotic Stress Conditions. *Agronomy* **2019**, *9*, 306.
80. Caruso, G.; Parrella, G.; Giorgini, M.; Nicoletti, R. Crop Systems, Quality and Protection of Diplotaxis tenuifolia. *Agriculture* **2018**, *8*, 55.
81. Stępień, P.; Kłbus, G. Water relations and photosynthesis in Cucumis sativus L. leaves under salt stress. *Biol. Plant.* **2006**, *50*, 610–616.
82. Cocetta, G.; Mishra, S.; Raffaelli, A.; Ferrante, A. Effect of heat root stress and high salinity on glucosinolates metabolism in wild rocket. *J. Plant Physiol.* **2018**, *231*, 261–270.
83. Urlić, B.; Dumičić, G.; Romić, M.; Ban, S.G. The effect of N and NaCl on growth, yield, and nitrate content of salad rocket (Eruca sativa Mill.). *J. Plant Nutr.* **2017**, *40*, 2611–2618.
84. Ashraf, M.; Harris, P.J.C. Photosynthesis under stressful environments: An overview. *Photosynthetica* **2013**, *51*, 163–190.
85. Grossman, R.; Wisenbilt, J. What we know about consumers' color choices. *J. Mark. Pract. Appl. Mark. Sci.* **1999**, *5*, 78-88.
86. Bulgari, R.; Cocetta, G.; Trivellini, A.; Vernieri, P.; Ferrante, A. Biostimulants and crop responses: A review. *Biol. Agric. Hortic.* **2015**, *31*, 1–17.
87. Vernieri, P.; Borghesi, E.; Tognoni, F.; Serra, G.; Ferrante, A.; Piaggesi, A. Use of biostimulants for reducing nutrient solution concentration in floating system. *Acta Hortic.* **2006**, *718*, 477–484.
88. Barbieri, G.; Bottino, A.; Di Stasio, E.; Vallone, S.; Maggio, A. Proline and light as quality enhancers of rocket (Eruca sativa Miller) grown under saline conditions. *Sci. Hortic. (Amsterdam).* **2011**, *128*, 393–400.
89. De Pascale, S.; Maggio, A.; Fogliano, V.; Ambrosino, P.; Ritieni, A. Irrigation with saline water improves carotenoids content and antioxidant activity of tomato. *J. Hortic. Sci.*

Biotechnol. **2001**, *76*, 447–453.

90. Falcinelli, B.; Sileoni, V.; Marconi, O.; Perretti, G.; Quinet, M.; Lutts, S.; Benincasa, P. Germination under moderate salinity increases phenolic content and antioxidant activity in rapeseed (Brassica napus var oleifera Del.) sprouts. *Molecules* **2017**, *22*.
91. Valifard, M.; Mohsenzadeh, S.; Kholdebarin, B.; Rowshan, V. Effects of salt stress on volatile compounds, total phenolic content and antioxidant activities of Salvia mirzayanii. *South African J. Bot.* **2014**, *93*, 92–97.
92. Zhou, Y.; Tang, N.; Huang, L.; Zhao, Y.; Tang, X.; Wang, K. Effects of salt stress on plant growth, antioxidant capacity, glandular trichome density, and volatile exudates of schizonepeta tenuifolia briq. *Int. J. Mol. Sci.* **2018**, *19*.
93. Vermerris, W.; Nicholson, R. Phenolic Compounds and their Effects on Human Health. In *Phenolic Compound Biochemistry*; Springer Netherlands: Dordrecht; pp. 235–255.
94. Ozcan, T.; Akpinar-Bayizit, A.; Yilmaz-Ersan, L.; Delikanli, B. Phenolics in Human Health. *Int. J. Chem. Eng. Appl.* **2014**, *5*, 393–396.
95. Yakhin, O.I.; Lubyanov, A.A.; Yakhin, I.A.; Brown, P.H. Biostimulants in Plant Science: A Global Perspective. *Front. Plant Sci.* **2017**, *7*.
96. Hamilton, J.M.; Fonseca, J.M. Effect of saline irrigation water on antioxidants in three hydroponically grown leafy vegetables: Diplotaxis tenuifolia, eruca sativa, and lepidium sativum. *HortScience* **2010**, *45*, 546–552.
97. Amirjani, M.R. Effect of Salinity Stress on Growth, Sugar Content, Pigments and Enzyme Activity of Rice. *Int. J. Bot.* **2011**, *7*, 73–81.
98. Ashraf, M. Organic substances responsible for salt tolerance in Eruca sativa. *Biol. Plant.* **1994**, *36*, 255–259.
99. Bolouri-Moghaddam, M.R.; Le Roy, K.; Xiang, L.; Rolland, F.; Van Den Ende, W. Sugar signalling and antioxidant network connections in plant cells. *FEBS J.* **2010**, *277*, 2022–2037.
100. Sami, F.; Yusuf, M.; Faizan, M.; Faraz, A.; Hayat, S. Role of sugars under abiotic stress. *Plant Physiol. Biochem.* **2016**, *109*, 54–61.
101. Ozdener, Y.; Aydin, B.K. The effect of zinc on the growth and physiological and biochemical parameters in seedlings of Eruca sativa (L.) (Rocket). *Acta Physiol. Plant.* **2010**, *32*, 469–476.
102. Iqbal, N.; Nazar, R. *Osmolytes and Plants Acclimation to Changing Environment: Emerging Omics Technologies*; Iqbal, N., Nazar, R., A. Khan, N., Eds.; Springer India: New Delhi, 2016; ISBN 978-81-322-2615-4.

103. Slama, I.; Abdelly, C.; Bouchereau, A.; Flowers, T.; Savouré, A. Diversity, distribution and roles of osmoprotective compounds accumulated in halophytes under abiotic stress. *Ann. Bot.* **2015**, *115*, 433–447.

104. Zhou, Q.; Yu, B.J. Accumulation of inorganic and organic osmolytes and their role in osmotic adjustment in NaCl-stressed vetiver grass seedlings. *Russ. J. Plant Physiol.* **2009**, *56*, 678–685.

105. Sah, S.K.; Reddy, K.R.; Li, J. Abscisic Acid and Abiotic Stress Tolerance in Crop Plants. *Front. Plant Sci.* **2016**, *7*, 1–26.

106. Fricke, W.; Akhiyarova, G.; Veselov, D.; Kudoyarova, G. Rapid and tissue-specific changes in ABA and in growth rate in response to salinity in barley leaves. *J. Exp. Bot.* **2004**, *55*, 1115–1123.

107. He, T.; Cramer, G.R. Abscisic acid concentrations are correlated with leaf area reductions in two salt-stressed rapid-cycling Brassica species. *Plant Soil* **1996**, *179*, 25–33.

108. Santamaria, P.; Gonnella, M.; Elia, A.; Parente, A.; Serio, F. Ways of reducing rocket salad nitrate content. *Acta Hortic.* **2001**, *548*, 529–536.

109. Cavaiuolo, M.; Ferrante, A. Nitrates and glucosinolates as strong determinants of the nutritional quality in rocket leafy salads. *Nutrients* **2014**, *6*, 1519–1538.

110. Habermeyer, M.; Roth, A.; Guth, S.; Diel, P.; Engel, K.-H.; Epe, B.; Fürst, P.; Heinz, V.; Humpf, H.-U.; Joost, H.-G.; et al. Nitrate and nitrite in the diet: How to assess their benefit and risk for human health. *Mol. Nutr. Food Res.* **2015**, *59*, 106–128.

111. Urrestarazu, M.; Postigo, A.; Salas, M.; Sánchez, A.; Carrasco, G. Nitrate accumulation reduction using chloride in the nutrient solution on lettuce growing by NFT in semiarid climate conditions. *J. Plant Nutr.* **1998**, *21*, 1705–1714.

112. Amanda, A.; Ferrante, A.; Valagussa, M.; Piaggesi, A. Effect of biostimulants on quality of baby leaf lettuce grown under plastic tunnel. *Acta Hortic.* **2009**, *807*, 407–412.

113. Disciglio G., Frabboni L., T.A. and T.E. Applying Natural Fertiliers to Herbaceous Crops. *J. life Sci.* **2014**, *8*, 504–510.

114. Bulgari, R. STUDIES OF QUALITY AND NUTRIENT USE EFFICIENCY IN VEGETABLE CROPS GROWN UNDER DIFFERENT SUSTAINABLE CROPPING SYSTEM, 2018.

115. Msanne, J.; Lin, J.; Stone, J.M.; Awada, T. Characterization of abiotic stress-responsive Arabidopsis thaliana RD29A and RD29B genes and evaluation of transgenes. *Planta* **2011**, *234*, 97–107.

116. Lee, S.Y.; Boon, N.J.; Webb, A.A.R.; Tanaka, R.J. Synergistic activation of RD29A via integration of salinity stress and abscisic acid in arabidopsis thaliana. *Plant Cell Physiol.*

2016, *57*, 2147–2160.

117. Tran, L.-S.P.; Nakashima, K.; Sakuma, Y.; Simpson, S.D.; Fujita, Y.; Maruyama, K.; Fujita, M.; Seki, M.; Shinozaki, K.; Yamaguchi-Shinozaki, K. Isolation and Functional Analysis of Arabidopsis Stress-Inducible NAC Transcription Factors That Bind to a Drought-Responsive cis -Element in the early responsive to dehydration stress 1 Promoter. *Plant Cell* **2004**, *16*, 2481–2498.
118. Fujita, M.; Fujita, Y.; Maruyama, K.; Seki, M.; Hiratsu, K.; Ohme-Takagi, M.; Tran, L.S.P.; Yamaguchi-Shinozaki, K.; Shinozaki, K. A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. *Plant J.* **2004**, *39*, 863–876.
119. Wang, Z.; Su, G.; Li, M.; Ke, Q.; Kim, S.Y.; Li, H.; Huang, J.; Xu, B.; Deng, X.P.; Kwak, S.S. Overexpressing Arabidopsis ABF3 increases tolerance to multiple abiotic stresses and reduces leaf size in alfalfa. *Plant Physiol. Biochem.* **2016**, *109*, 199–208.
120. Dubois, M.; Claeys, H.; Van den Broeck, L.; Inzé, D. Time of day determines Arabidopsis transcriptome and growth dynamics under mild drought. *Plant Cell Environ.* **2017**, *40*, 180–189.
121. Benny, J.; Pisciotta, A.; Caruso, T.; Martinelli, F. Identification of key genes and its chromosome regions linked to drought responses in leaves across different crops through meta-analysis of RNA-Seq data. *BMC Plant Biol.* **2019**, *19*, 194.
122. Raffaele, S.; Vailleau, F.; Léger, A.; Joubès, J.; Miersch, O.; Huard, C.; Blée, E.; Mongrand, S.; Domergue, F.; Roby, D. A MYB Transcription Factor Regulates Very-Long-Chain Fatty Acid Biosynthesis for Activation of the Hypersensitive Cell Death Response in Arabidopsis. *Plant Cell* **2008**, *20*, 752–767.
123. Kawa, D.; Testerink, C. Regulation of mRNA decay in plant responses to salt and osmotic stress. *Cell. Mol. Life Sci.* **2017**, *74*, 1165–1176.
124. Miller, G.; Suzuki, N.; Rizhsky, L.; Hegie, A.; Koussevitzky, S.; Mittler, R. Double Mutants Deficient in Cytosolic and Thylakoid Ascorbate Peroxidase Reveal a Complex Mode of Interaction between Reactive Oxygen Species, Plant Development, and Response to Abiotic Stresses. *Plant Physiol.* **2007**, *144*, 1777–1785.
125. Zimmermann, P.; Hirsch-hoffmann, M.; Hennig, L.; Gruissem, W. GENEVESTIGATOR . Arabidopsis Microarray Database and Analysis Toolboxl [wI. **2014**, *136*, 2621–2632.
126. Song, C.P.; Galbraith, D.W. AtSAP18, an orthologue of human SAP18, is involved in the regulation of salt stress and mediates transcriptional repression in Arabidopsis. *Plant Mol. Biol.* **2006**, *60*, 241–257.
127. Zhang, C.; Bai, M. yi; Chong, K. Brassinosteroid-mediated regulation of agronomic traits in

rice. *Plant Cell Rep.* **2014**, *33*, 683–696.

128. Mair, A.; Pedrotti, L.; Wurzinger, B.; Anrather, D.; Simeunovic, A.; Weiste, C.; Valerio, C.; Dietrich, K.; Kirchler, T.; Nägele, T.; et al. SnRK1-triggered switch of bZIP63 dimerization mediates the low-energy response in plants. *Elife* **2015**, *4*, 1–33.
129. Frank, A.; Matiolli, C.C.; Viana, A.J.C.; Hearn, T.J.; Kusakina, J.; Belbin, F.E.; Wells Newman, D.; Yochikawa, A.; Cano-Ramirez, D.L.; Chembath, A.; et al. Circadian Entrainment in Arabidopsis by the Sugar-Responsive Transcription Factor bZIP63. *Curr. Biol.* **2018**, *28*, 2597-2606.e6.
130. Kunz, S.; Pesquet, E.; Kleczkowski, L.A. Functional dissection of sugar signals affecting gene expression in Arabidopsis thaliana. *PLoS One* **2014**, *9*, 1–10.
131. Veerabagu, M.; Kirchler, T.; Elgass, K.; Stadelhofer, B.; Stahl, M.; Harter, K.; Mira-Rodado, V.; Chaban, C. The Interaction of the Arabidopsis Response Regulator ARR18 with bZIP63 Mediates the Regulation of PROLINE DEHYDROGENASE Expression. *Mol. Plant* **2014**, *7*, 1560–1577.
132. Liu, J.-X.; Srivastava, R.; Che, P.; Howell, S.H. Salt stress responses in Arabidopsis utilize a signal transduction pathway related to endoplasmic reticulum stress signaling. *Plant J.* **2007**, *51*, 897–909.
133. Xue, G.-P.; Way, H.M.; Richardson, T.; Drenth, J.; Joyce, P.A.; McIntyre, C.L. Overexpression of TaNAC69 Leads to Enhanced Transcript Levels of Stress Up-Regulated Genes and Dehydration Tolerance in Bread Wheat. *Mol. Plant* **2011**, *4*, 697–712.

CHAPTER 3

Evaluation of a biostimulant prototype formulation on leafy vegetable grown under different stressful conditions.

INTRODUCTION

Fruit and vegetables intake is essential in human diet, because they are rich in many nutrients, fibre, minerals, carbohydrates, and phytochemicals involved in the reduction of chronic diseases. Therefore, growing high-quality vegetables becomes one of the most important goals of current agriculture, in order to meet the needs of the growing population, the increasing demand for healthy food, and because of their high economical value.

Among horticultural species, lettuce is one of the most popular vegetable worldwide, cultivated either in open field or in protected environment. Second to rocket, lettuce is cultivated as ready to eat salad, also known as baby-leaf vegetables for the fresh-cut industry. These vegetables are cultivated with very high density and harvested at young stage when their height ranges from 50 to 120 mm and they are mainly eaten as fresh leaves. Since almost all steps in baby-leaf cultivation are mechanized, the uniformity of the product at harvest is an essential point. This can be reached through the correct application of water and nutrients during the growing cycle to maintain a constant soil moisture [1]. Irrigation is crucial in terms of yield and quality of the product especially in leafy vegetables where the percentage of water is very high (90-95%). Moreover, around 99% of transpired water is involved in cooling plants, the remaining part works as nutrient transport.

Since there is a linear relationship between yield and crop water consumption, irrigation often exceed the real requirements of the culture [2]. It is a common practice in baby-leaf during the week before the harvest and especially the evening before, ensure the water supply in order to maximize the yield, increase the turgidity of the leaves and minimize postharvest losses. The amount of water in lettuce leaves is not important only from a nutritional point of view, but also to keep the textural characteristics of the leaves, associated to the turgor pressure. Moreover, a couple of day before the harvest nutrient solution is generally replaced by water with the aim of reducing the nitrate concentration.

As mentioned above, vegetables are often overirrigated by the farmers and, consequently, the water use efficiency (WUE) is reduced. At leaf-plant level, WUE can be expressed as the ratio between net CO2 assimilated through the photosynthesis and the water lost with transpiration expressed as transpiration rate (E) or stomatal conductance (gs).

It is well known that water is a critical resource of agriculture and its correct management is important both to supply the right amount of water to the culture avoiding stress symptoms and also to reduce resource waste.

When water availability is not enough to meet the crop demand, plants spend part of their energy to uptake water from the substrate, thus plant growth and productivity are affected. The amount of water present in the substrate can be easily monitored by tensiometers or TDR probes, and measurement can be used in water management to adjust the time and the volume of irrigation.

To enhance WUE it is important to reduce water input and promote plant growth through several strategies, such as the correct choice of the cultivars, the application of irrigation techniques with high efficiency, the reduction of water loss from the soil, the increase of soil nutrient status and other factors. Among these, regulated deficit irrigation (RDI) is a common practice based on the reduction of water supply with the aim to reduce the agricultural water use, increase water productivity (WUE), product quality, and also farmers' profit [3–8]. Water use efficiency increases under deficit irrigation, but it decreases if the uniformity of irrigation is low. This technique needs a good knowledge of plants water requirements and tolerance in order to avoid water stress conditions and increase the risk of high salinity. Indeed, is not possible to entirely eliminate water loss because the movement of water in the soil is necessary for salt leaching. Plants response to deficit irrigation is complex and depends on several factors such as the environmental condition, location, growing period, and phenological phase.

Moreover, it has been observed that WUE can increase if water stressed plants are re-watered. Indeed, leaf photosynthetic rates quickly recover after the re-watering period and the levels can exceed those of unstressed plants, increasing also the yield [9,10].

Nevertheless, the ability of plants to fully recover after a period of drought depends on the crop species and on the severity of the stress. For instance, Huang et al. [11] evaluated different scenario of water stress and re-watering on wetland plants. Results obtained show that a moderate water stress increase the WUE whereas if the soil moisture decrease below a threshold the recovery could be difficult even after re-watering.

In order to avoid yield losses due to a not accurate water management, the application of biostimulant products might be beneficial for plants growth and quality of the production. These products, containing bioactive molecules, have a beneficial effect on plants and improve their capability to face adverse environmental conditions, acting on primary or secondary metabolism. Furthermore, since one of their main effects is to improve water use efficiency, their application could be a possible strategy to reduce the amount of water added to crops. Moreover, studies reported that plants treated with different biostimulant products quickly restored after the end of water stress [12–14].

A similar effect of drought is induced by salt stress. Indeed, in the first phase of plant response to high salinity, plants reduce the water uptake in order to avoid salt loading in the cells. This is usually called osmotic or water-deficit effect of salt stress [15].

Lettuce has been considered to be a moderately salt sensitive crop and it is one of the most important leafy vegetable cultivated in the Mediterranean area where its production is jeopardised by the high salinity of the water frequently used for irrigation. [16–18].

Furthermore, both water and salt stress lead to the production of reactive oxygen species (ROS). They are partially reduced or excited forms of atmospheric oxygen characterized by high reactivity. ROS, through oxidative stress, mainly cause damage to DNA, RNA, proteins and lipids [19,20]. On the other hand, the maintenance of a basal level of ROS in cells is important since they act as molecular signal to regulate and maintain physiological functions (redox biology) interacting mostly with protein cysteine residues [21]. They also play a key role in signal transduction [22].Plants have developed some mechanisms to avoid damages caused by these molecules and the presence of ROS is highly balanced both by enzymes and non-enzymatic metabolites [23]. The balance between the level of ROS necessary for normal physiological functions and the toxic level is highly regulated by a group of genes that controls their production, perception and elimination [24]. Indeed, oxidative stress can have different impacts on the organism and lead to significant damage to cells and, if the system is unable to regain control, this can lead to cell death. SOD, CAT, APX, MDAR, DHAR and GR are some enzymes involved in the plant's antioxidant response. The word "antioxidant" is used to describe any compound capable of interacting with ROS without turning into a radical. The superoxide dismutase protein (SOD, EC 1.15.1.1) is the first step of defence against active oxygen species: it removes the hydroxyl radical (OH-.) by catalysing its dismutation by reducing one molecule to hydrogen peroxide (H_2O_2) and another to O_2 [25]. Since H_2O_2 is a strong oxidant, it cannot accumulate in organelles such as chloroplasts; Catalases (CAT, EC 1.11.1.6) and peroxidases are the two main systems for the enzymatic removal of H_2O_2 in plants[26]. CAT displaces H_2O_2 into H_2O and O_2, while ascorbate peroxidase (APX, EC 1.11.1.11) converts H_2O_2 to water with the help of a reducing substrate such as ascorbate. APX uses two molecules of ascorbic acid to reduce H_2O_2 to H_2O, generating two molecules of monodehydroascorbate (MDHA). Monodehydroascorbate is a radical that is rapidly converted by monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) into ascorbate and dehydroascorbate [27]. DHA is reduced by ascorbic acid through the action of dehydroascorbate reductase (DHAR, EC 1.8.5.1) using reduced glutathione (GSH) as reducing substrate. The removal of H_2O_2 through these reactions is known as the ascorbate-glutathione cycle. Ascorbic acid and glutathione are not consumed in this way, but participate in the cyclic transfer of reducing equivalents, involving four enzymes that allow the reduction of H_2O_2 to H_2O using the electron derived from NAD(P)H [28].

Among plant biostimulants categories, plant-derived protein hydrolysates are largely used to increase plant production and quality traits both in optimal and under stressful conditions. Protein-hydrolysate biostimulant can be applied both as soil drench and foliar spray. The first method is used in long term treatments whereas the second one is usually applied when a relatively rapid responses is required. Plant-derived protein hydrolysates are produced though chemical, enzymatic or thermal hydrolysis and the row materials consist in agroindustry by products.

They contain peptides, free amino acids and other bioactive compounds with hormone-like activities. Recent studies suggested that protein hydrolysates might act modifying the composition and activity of plant microbiome and facilitating the assimilation of nutrients.

Amino acids play numerous roles in plant, as organic nitrogenous compounds they are the building blocks in the biosynthesis of protein, pigments, vitamins, alkaloids, enzymes, terpenoids, coenzymes, they are involved in processes such as cell growth and signalling. Several examples of positive effect of amino acids application are reported in literature review [29–31]. Moreover, amino acids take part in plant stress responses acting as osmolytes, regulating the ion transport, the stomatal opening and in detoxification mechanisms [32]. The exogenous application of amino acids might regulate carbon and nitrogen metabolisms and promoting the assimilation of nitrogen [33]. Since amino acids are involved in many physiological processes, their mode of action as biostimulants is often unclear and difficult to be identified [34].

Glutamic acid is one of the most important amino acids in plants playing a role in the synthesis of other amino acids and nitrogen compounds. Amino acids are able to promote both primary and secondary metabolisms. Several studies have pointed out the positive effect of glutamic acid application on photosynthesis and chlorophyll fluorescence [35–38]. This is probably due to the link between photosynthetic capacity and leaf nitrogen concentration. Moreover, glutamic acid and glycine are essential metabolites in the biosynthesis of chlorophyll by being incorporated into the aminolevulinic acid [39]. Cao et al., [40] reported that exogenous application of glutamic acid improved the quality of Chinese chive and reduced the nitrate accumulation. Similar effect was observed also in lettuce plants cultivated in hydroponic system [41]. Under stressful condition glutamic acid application had a positive effect reducing physiological damage by enhancing the activity of antioxidant enzymes [42].

In addition to glutamic acid, another important amino acid involved in abiotic stress responses for proline and chlorophyll biosynthesis. This molecule is essential for primary metabolism as free amino acid and as component of proteins. It is well known that proline concentration in plants increase under several abiotic and biotic stress conditions such as drought, salt stress, UV radiations, heavy metals, oxidative stress and so on. Its accumulation is particularly important for plant tolerance to stressful conditions not only because of its role as compatible osmolyte, but also due to its activity in the protection of protein integrity, as signaling molecule and as metal chelator [43]. Several reports indicate that the effects of exogenous proline depend on its

concentration. At low concentration, it has positive effect increasing stress tolerance [44–46], on the contrary, at high concentration it results toxic [47].

AIM

This work involved the collaboration with a private company in order to test the efficacy of a product prototype as potential biostimulant. The product contains different amino acids of vegetal origin, in particular glutamic acid. As mentioned before, the effect of a biostimulant is a result of the interaction of its constituents, and may not be explained only by the sum of them. For this reason, we chose an experimental approach comparing the effect of the biostimulant with a glutamic acid treatment at the same concentration contained in the prototype. The product evaluation process involved the following activities:

The aim of the first activity was the evaluation of the product in plant subjected to a short period of water reduction close to the harvest.

The aim of the second activity was the evaluation of the efficacy of the product in plants subjected to water stress and re-watering.

The aim of the third activity was the evaluation of the product in plants subjected to a period of salt stress.

MATERIALS AND METHODS

1. Non-destructive analyses

1.1. Chlorophyll

Leaves chlorophyll content was colorimetrically estimated *in vivo* using a chlorophyll content meter (CL-01 Chlorophyll Content Meter, Hansatech Instruments, UK). The results were express such as chlorophyll index (relative units).

1.2. Chlorophyll a fluorescence

Chlorophyll *a* fluorescence was measured *in vivo* using two different instruments: a hand-portable fluorometer (Handy-PEA, Hansatech Instruments) and a field portable pulse modulated chlorophyll fluorometer (FMS2, Hansatech Instruments). Before all measurement with Handy-PEA, leaves were dark-adapted with the leaf clips for 30-40 minutes. Then were exposed to a saturating light (3000 µmol m^{-2} s^{-1}) provided by an array of three high-intensity light-emitting diodes for 1 second. Information about the structural and functional status of photosynthetic apparatus was provided by the parameters measured, such as the maximum quantum of photosystem II (Fv/Fm), the performance index (PI), the dissipation energy per active reaction center (DIo/RC) and the density of reaction centres (RC/CSm).
Modulated chlorophyll *a* fluorescence under ambient light regime was measured using the FMS-2. In order to calculate the electron transport rate (ETR) PAR value is recorded by a light sensor on the leaf-clip. The steady-state fluorescence (Fs) was measured with the measuring radiation. After that, a pulse of saturating light was imposed to obtain the maximum fluorescence level in light adapted leaves (Fm'). The effective PSII quantum efficiency (ϕ_{PSII}) and the electron transport rate (ETR) were calculated by the FMS software.

1.3. Gas exchange and water use efficiency

Leaves gas exchange was determined in the greenhouse using a portable open gas exchange system (CIRAS2, Portable Photosynthesis System, U.S.A.). This instrument lets to set some parameters inside the cuvette, such as CO_2 concentration, vapor pressure, temperature and light intensity. Net photosynthetic rate (Pn) and transpiration rate (E) were directly measured by the instrument from the CO_2 and water vapour concentration in and out the cuvette. Otherwise leaf conductance (gs) and sub-stomatal CO_2 concentration (Ci) was calculated by the equations comprising Pn and E. Photosynthetic water use efficiency (pWUE) was calculated as the ratio of Pn to E, whereas intrinsic water use efficiency (iWUE) as the ratio of Pn to gs.

2. Destructive measurements

2.1. Yield and dry matter

Fresh vegetable yield was measured for each tank at the end of the experiment cutting lettuce plants at soil level. The leaf dry matter was calculated from the dry weight obtained by oven-drying samples in 105 °C until constant weight was reached.

2.2. Total chlorophylls and carotenoids

Chlorophylls and carotenoids pigments were extracted using 99.9% (v/v) methanol. Leaf disc samples (30 mg), obtained with a 5 mm diameter cork borer, were kept in dark room for 24 h at 4 °C. After that absorbance reading were measured at 665.2 and 652.4 nm for chlorophylls and 470 nm for total carotenoids. Pigments levels were calculated by Lichtenthaler's formula [48].

2.3. Phenols and anthocyanin

Total phenols and anthocyanin were extracted using methanol acidified with hydrochloric acid. Leaf disc samples (30 mg), obtained with a 5 mm diameter cork borer, were kept in dark room for 24 h at 4 °C. After that absorbance reading were measured at 320 nm for total phenols, and at 535 nm for anthocyanin. Phenolic index was expressed as Abs320 nm g^{-1} FW. Anthocyanins concentration was expressed in cyanidin-3-glucoside equivalents using a molar extinction coefficient (ε) of 29,600 L M^{-1} cm^{-1}.

2.4. Leaf colour

Colorimetric analysis was performed on detached leaves at the end of the trial. Lettuce leaves colour was measured using a CR-400 Chroma meter (Konica Minolta, New York, NY) in CIELAB and L*C*h colour spaces.

2.5. Abscisic acid

The abscisic acid (ABA) concentration was determined by an indirect enzyme linked immuno-sorbent assay (ELISA). Approximately 1 g of leaf tissue was homogenized in a mortar with 3 mL of water, the mixture was centrifuged at 4000 rpm for 15 min at RT and the supernatant was collected and analysed using the Plant Growth Regulator Immunoassay Detection Kits (Sigma-Aldrich) according to manufacturer instructions.

2.6. Nitrate

Nitrate concentration was determined by the method of Cataldo et al. [49]. Fresh leaf tissue was homogenized in distilled water (1 g fresh tissue per 3 mL water). The homogenate was centrifuged at 4000 rpm for 15 min at RT (ALC centrifuge-model PK130R) and the recovered supernatant was used for the colorimetric analysis. Twenty microliters of the extract were added to 80 mL of 5% (w/v) salicylic acid in concentrated H_2SO_4 (SA-H_2SO_4). Afterward 3 mL of 1.5 N NaOH were added. The samples were cooled to RT and absorbance at 410 nm was measured in a spectrophotometer. Nitrate content was calculated referring to a KNO_3 standard calibration curve. Nitrate concentration was expressed as mg KNO_3 per kg FW.

2.7. Osmolytes

Fresh leaf tissue was homogenized in distilled water (1 g fresh tissue per 3 mL distilled water). The homogenate was centrifuged at 4000 rpm for 15 min at RT and the recovered supernatant was analysed. Its osmolarity was determined using an automatic freezing point depression osmometer (Digital Osmometer, Roebling, Berlin, Germany) calibrated with sodium chloride solutions.

2.8. Proline

Proline concentration in leaf tissue was determined by the ninhydrin-based colorimetric assay improved by Bates et al. [50]. Approximately 1 g of leaf tissue was grinded with 10 mL of 3% sulfosalicylic acid. Samples were centrifugated at 4000 rpm for 5 min at RT and 100 µL of supernatant was added to a reaction mixture prepared with 3% sulfosalicylic acid, glacial acetic acid and acidic ninhydrin. The tubes were mixed, each lid was punctured with a needle to avoid high pressure and the tubes were incubated at 96 °C for 60 min and then the reaction was terminated putting the tubes on ice. The extraction was made adding 1 mL toluene to the reaction mixture. The tubes were vortexed and leaved on the bench for 5 min to allow the separation of the organic and water phases. The chromophore phase containing toluene was used to read the absorbance at 520 nm using toluene as reference. Proline concentration was calculated referring a standard calibration curve and expressed as µg per g FW.

2.9. Lipid peroxidation

Lipid peroxidation was estimated by using the TBARS assay. About 1 g of leaf tissue was homogenized in 3mL of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 4500 rpm for 10 min. 1 mL of the supernatant was added to 4 mL of 20% (w/v) TCA, 25 µL of 0.5% thiobarbituric acid (TBA). The mixture was shaken on a vortex

and incubated at 95 °C for 30 min in a water bath and cooled on ice. Absorbance at 600 nm was subtracted from the absorbance at 532 nm (as an index of non-specific turbidity) and the concentration of TBARS was calculated using the Lambert-Beer law with an extinction coefficient εM= 155 mM^{-1} cm^{-1} and expressed as malondialdehyde (MDA) equivalents (nmol g^{-1}) according to Du and Bramlage [51].

2.10. Sucrose and total sugars

Sucrose content was measured using the resorcinol method. Approximately 1 g of leaf tissue was homogenized in a mortar with 3 mL of water. The mixture was centrifuged at 4000 rpm for 15 min at RT. Sucrose assay was performed by mixing 0.2 mL of supernatant with 0.2 mL of 2 N NaOH and incubated in a water bath at 100°C for 10 min, then 1.5 mL of resorcinol buffer (containing 30% hydrochloric acid, 1.2 mM resorcinol, 4.1 mM thiourea 1.5 M acetic acid) was added to samples and incubated in a water bath at 80 °C for 10 min. After cooling at room temperature, the optical density was determined spectrophotometrically at 500 nm, using a sucrose standard curve.

The total sugars were determined on the same extract using the anthrone method [52] with slight modifications. The anthrone reagent (10.3 mM) was prepared dissolving anthrone in 95% H_2SO_4. The reagent was left to stand for 30-40 min before use, 0.5 mL extract was placed on top of 2.5 mL of anthrone reagent incubated in ice for 5 min and then vortexed vigorously. The tubes were heated to 95 °C for 10 min and left to cool in ice. Readings were performed at 620 nm. Calibration curve was carried out using a glucose standard solution.

3. Total RNA isolation and analysis of gene expression

Frozen leaves of lettuce were thoroughly ground with liquid N using cold mortar and pestle. Approximately 100 mg was transferred to a cryotube and stored at −80 °C. Total RNA was isolated using the Spectrum Plant Total RNA Kit with on-column DNase-treatment (Sigma-Aldrich, Italy) following the steps of protocol A with slight modification.

The concentration and the purity of RNA were assessed by measuring the absorbance at 230 nm, 260 nm and 280 nm using a NanoDrop N-1000 spectrophotometer (NanoDrop technologies). A ratio of absorbance at 260 and 280 ≈ 2.0 is generally accepted as pure for RNA and expected 260/230 values are commonly in the range of 2.0-2.2, usually higher than the respective 260/280 value.

Three µg of RNA were reversely transcribed to cDNA using the SuperScript IV cDNA Synthesis Kit according to the manufacturer's instruction (Invitrogen, Italy).

The SYBR® Green PCR Master Mix (Applied Biosystems) was used for the quantitative RT-PCR analysis. The reaction mix was prepared by adding 10 µL of SYBR Green, 0.4 µL of forward and reverse primers, 2 µL of cDNA diluted 1:20, and 7.2 µL of RNase free water. The total volume for each PCR reaction was 20 µL. Analysis was performed using the ABI7300 (Applied Biosystem) thermocycler and PCR program and reactions were run in triplicate from two biological replicates. Gene expression analyses were performed using gene-specific primers for: superoxide dismutase [Fe] 3, chloroplastic (*SOD*), catalase (*CAT*), L-ascorbate peroxidase 6, chloroplastic/mitochondrial (*APX*), monodehydroascorbate reductase, chloroplastic/mitochondrial (*MDAR*), dehydroascorbate reductase (*DHAR*), glutathione reductase, chloroplastic (*GR*). Primers were designed using the program Primer-Blast available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

Table 14. Primers sequences and Melting temperature (Tm) for qRT-PCR analysis

GENE	PRIMER PAIR	SEQUENCE (5'->3')	Tm (°C)
LsAPX	Forward primer	ACTCATCCGGAAAGAGAGAGC	59.25
	Reverse primer	CTGAAGAGGCTTATCCGGGC	60.25
LsMDAR	Forward primer	GCTTCTGTTGAGGAAGCCCT	59.96
	Reverse primer	GCACATCCTGACCTCTCTCG	59.90
LsGR	Forward primer	GCAATAGAGTGGAGTCAGGTGG	60.42
	Reverse primer	AACGTCTCCAACTGCCCAAA	60.11
LsSOD	Forward primer	ATCCATGCAACCAGGAGGTG	60.03
	Reverse primer	AAAACAAGCCAAACCCAGCC	59.82
LsCAT	Forward primer	AGCTTCCTGCAAATGCTCCT	59.96
	Reverse primer	GAGCAGGGTCGTGTCTTGAA	59.97
LsDHAR	Forward primer	CTGGATGGGCACCAAAGGTT	
	Reverse primer	GACCCAATAGCATCACAAACCA	
EF1α	Forward primer	TCTTGGTAGACGCCTTCACG	65.3
	Reverse primer	AGGAAGCGGTGTCATTGTTG	65.0

The expression levels were analyzed with the AB software program and results were calculated using the 2-ddct method described by Livak and Schmittgen [76]. According to this method, the data are presented as fold change in gene expression normalized to a housekeeping gene and relative to a calibrator. The Elongation

factor 1 alpha (EF1α) was used as reference gene (housekeeping) due to the highest stability in its expression levels, whereas the non-stressed and non-treated sample after 3 hours was chosen as internal calibrator.

4. Statistical analyses

Data were subjected to a two-way ANOVA and differences among means were determined by Tuckey post-test ($P < 0.05$). Statistics were performed using GraphPad Prism version 6 and 8 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Additional information is reported in each figure's legend.

1st ACTIVITY

1. Plant material, growth conditions, and experimental design

The trial was carried out two times in 2017 at the Faculty of Agricultural and Food Science of Milan. Experiment timesteps are reported in **Error! Reference source not found.**. Seeds of lettuce (*Lactuca sativa* var. 'chiara'; ISI Sementi S.p.A., Italy) were manually sown into 50 L plastic tanks filled with a thin layer of expanded clay and covered with commercial soil. Plants were grown in an experimental greenhouse under controlled conditions. The experimental design was a combination of two factors: two water regimes (well irrigated and water reduction) and five treatments (see **Error! Reference source not found.**2). In addition to glutamic acid treatment, since the private company was evaluating the possibility to add proline in the formulation, a treatment with proline alone and in combination with the product were added to the experimental plan.
Each experimental unit consisted of two tanks. Treatments were applied as foliar spray twice during the growing period: the first one, before the water reduction and the second one, at the beginning of water stress. Plants were treated with 20 mL of product.

Figure 35. Timelines of the experiments, with indication of treatment applications and sampling.

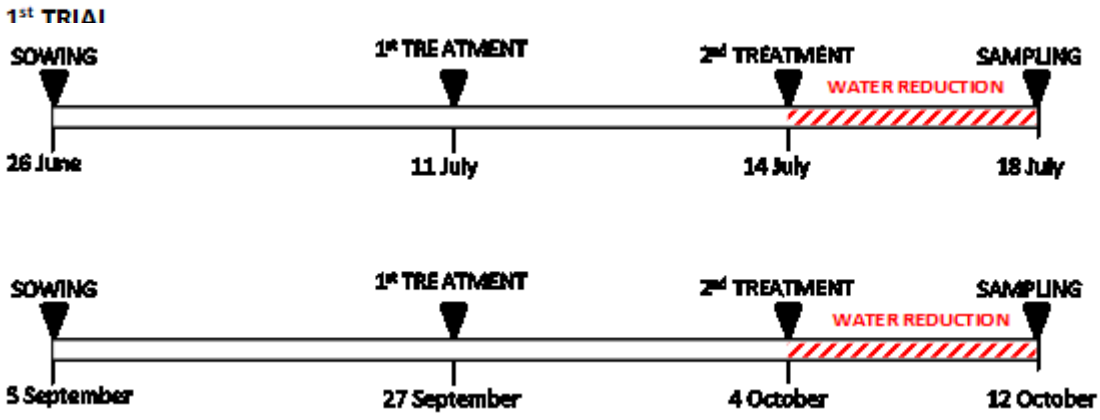


Table 15. list of treatments and concentration applied.

TREATMENT	DOSE
Control (water)	-
GHI_16_VHL	4.8 mL/L
GHI_16_VHL+ Proline	4.8 mL/L + 0.23 g/L
Proline [1 mM]	0.23 g/L
Glutamic acid [2,45 mM]	0.36 g/L

With the exception of chlorophyll *a* fluorescence, that was measured three time during each trial (one day after the first treatment, during the water reduction and at harvest), the following analyses and measurement were performed at the end of each growing cycle:

- chlorophyll;
- gas exchange;
- yield;
- total chlorophylls and carotenoids;
- phenols and anthocyanin;
- leaf colour;
- abscisic acid;
- nitrate;
- osmolytes;
- proline;
- lipid peroxidation;
- sucrose and total sugars.

2. Water management

The controlled water reduction was imposed by withholding irrigation until the soil water availability was around 30% lower than the well-watered control tanks. This condition was kept until harvest. Here "100" is referred to well-watered plants and "70" is referred to the plants which received less water amount.

Irrigation was controlled by measuring soil water content with tensiometers at 10 cm depth and TDR probes (SM100 soil, Waterscout, Spectrum Technologies, Inc.) during the growing season.

Tensiometer values were kept between -40 and -50 kPa in well-watered tanks and less than -110 kPa in stressed ones during the stress period.

2nd ACTIVITY

1. Plant material, growth conditions, experimental design, and water management

The trial was carried out in 2018 at the Faculty of Agricultural and Food Science of Milan. Experiment timesteps are reported in Figure 36. Lettuce plants (*Lactuca sativa* var. 'longifolia') were transplanted into 2,5 L plastic pot filled with a commercial soil mixed with perlite. Plants were grown in an experimental greenhouse under controlled conditions

The experimental design was a combination of two factors: two water regimes (well irrigated and water deprivation + re-watering) and three treatments (see Table 16). Each experimental unit consisted of six pots. The water stress was imposed by withholding irrigation until the plants started showing the first symptoms of stress such as the loss of turgor and by monitoring the leaf water content with the measure of chlorophyll *a* fluorescence. This condition was kept for 24 hours and then water supply was restored at the same level of non-stressed plants. Treatments were applied as foliar spray four times during the growing period and each plant was treated with 10 mL of product. Treatments were applied two times before the water deprivation, one time during the water stress and the last one during the re-watering, one day before the harvest. (Figure 36).

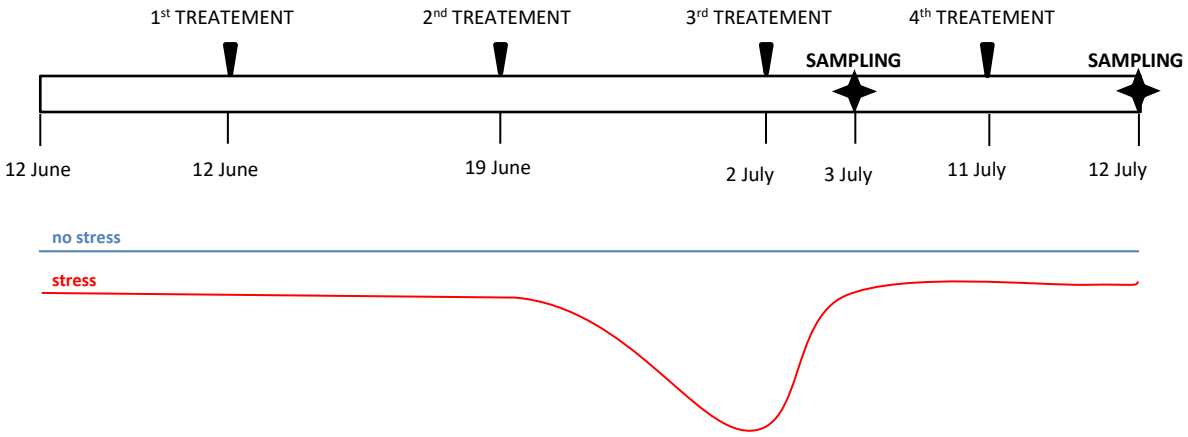


Figure 36. Timelines of the experiment, with indication of treatment applications and sampling.

Table 16. List of treatments and concentration applied.

TREATMENT	DOSE
Control (water)	-
GHI_18_VHLglu	2 mL L^{-1}
Glutamic acid	0.275 g L^{-1}

The following analyses and measurement were performed 24 hours after the third treatment (3 July), before the re-watering:

- chlorophyll;
- chlorophyll *a* fluorescence;
- nitrate;
- osmolytes;
- proline.

The following analyses and measurement were performed at the end of the growing cycle (12 July):
yield;

- dry weight and dry matter;
- chlorophyll;
- chlorophyll *a* fluorescence;
- gas exchange.

Samples for the gene expression analysis were collected 3 and 6 hours after the third treatment and stored at -80 °C until use.

3rd ACTIVITY

1. Plant material, growth conditions, experimental design and water management

The trial was carried out in 2018 at the Faculty of Agricultural and Food Science of Milan. Experiment timesteps are reported in Figure 37. Lettuce plants (*Lactuca sativa* var. 'longifolia') were transplanted into 2.5 L plastic pot filled with a commercial soil mixed with perlite Plants were grown in an experimental greenhouse under controlled conditions
The experimental design was a combination of two factors: salt stress and treatments as reported in Table 17. Each experimental unit consisted of six pots. The salt stress was imposed by irrigating the plants with a 100 mM NaCl solution. Treatments were applied as foliar spray every ten days for a total of four applications and each plant was treated with 10 mL of product. Treatments were applied one time before the salt stress, and three times during the stress.

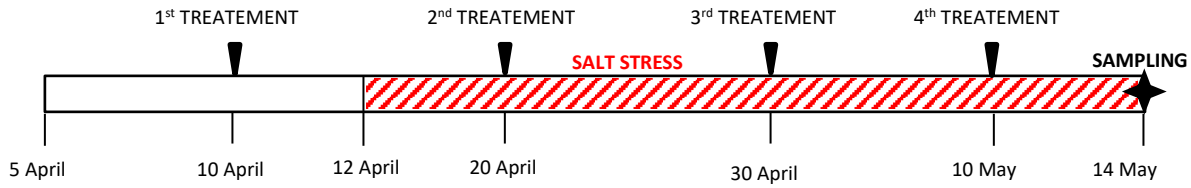


Figure 37. Timelines of the experiment, with indication of treatment applications and sampling.

Table 17. List of treatments and concentration applied.

TREATMENT	DOSE
Control (water)	-
GHI_18_VHLglu	2 mL L^{-1}
Glutamic acid	0.275 g L^{-1}

The following analyses and measurement were performed at the end of the growing cycle (14 May):

- yield;
- chlorophyll;
- chlorophyll *a* fluorescence;
- gas exchange;
- nitrate;
- abscisic acid;
- osmolytes;
- proline.

Samples for the gene expression analysis were collected 3 and 6 hours after the last treatment and stored at -80 °C until use.

RESULTS

1st ACTIVITY

1.1. Yield

Table 18 reports the yield of lettuce plants of both experiments. Yield ranged from 1323 to 2205 g m^{-2} and from 1500 to 3088 g m^{-2} in the first and second growing cycle, respectively. No significant differences have been observed among treatments or in response to the different irrigation levels. Indeed, non-treated plants grown with a lower water amount showed values very similar to non-stressed control both in the first and second growing cycle. Moreover, yields obtained in the second experiment were generally higher to the first one, with the exception of plants treated with glutamic acid and subjected to water reduction.

Table 18. Yield of lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken at the end of each growing cycle (**A**: 1st trial **B**: 2nd trial). Values are means ± SE (n =4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences (P < 0.05).

TRETMENT	WATER	YIELD [g m^{-2}]	
		1st TRIAL	2nd TRIAL
CONTROL	70	1617.647 ± 558.824	2397.059 ± 720.5882
	100	2117.647 ± 558.824	2485.294 ± 1044.118
GLUTAMIC ACID	70	2044.118 ± 367.647	1500.000 ± 352.9412
	100	2205.882 ± 205.882	2941.176 ± 147.0588
PROLINE	70	1323.529 ± 764.706	1588.235 ± 29.41176
	100	1617.647 ± 323.529	2441.176 ± 764.7059
GHI_16_VHL	70	1676.471 ± 558.824	2397.059 ± 426.4706
	100	1714.706 ± 1008.824	2264.706 ± 1117.647
GHI_16_VHL + PROLINE	70	1500.000 ± 617.647	1838.235 ± 397.0588
	100	1676.471 ± 264.706	3088.235 ± 117.6471

1.2. Chlorophylls

In the first trial chlorophyll levels estimated with the non-destructive method resulted significantly (P < 0.05) affected by the interaction between water amount and treatment. Moreover, water reduction produced a significant increase (P < 0.05) of chlorophyll index in control plants (Figure 4 A). The same effect, but not statistically significant has been observed in plants treated with the different products, except for plants treated with GHI_16_VHL that showed an opposite behaviour in response to water reduction. The lowest values (1.534 r.u.) was reached in well-watered plants treated with proline whereas maximum average was obtained in control plants grown with less water (2.153 r.u.).

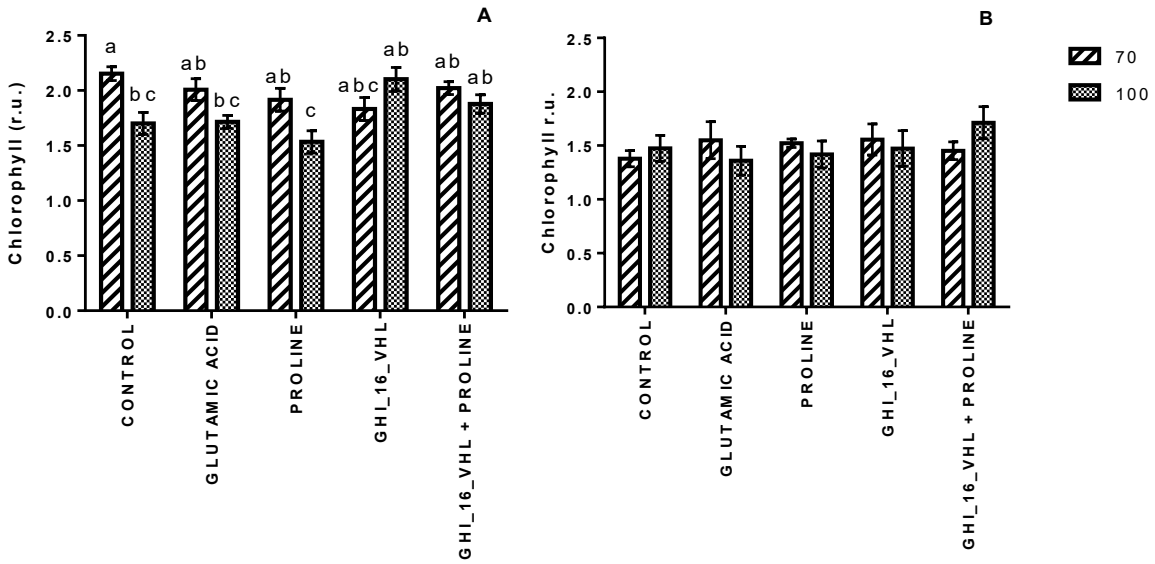


Figure 4. Chlorophyll content determined *in vivo* in lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken at the end of each growing cycle (**A**: 1st trial **B**: 2nd trial). Values are means ± SE (n = 10). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

Under well-water condition treatments with proline treatment or GHI_16_VHL + proline did not significantly affect the chlorophyll index if compared with control plants but a significant difference ($P < 0.05$) resulted between plants treated with proline alone or in combination with the biostimulant prototype. In plants grown with a lower amount of water no significant differences appeared in response to different treatments. In the second growing cycle chlorophyll levels did not show any statistical difference (Figure 4 B). All values were generally lower than first cycle and the overall average was around 1.5 r.u., regardless the water amount or the treatment.

Analyses of chlorophyll *a+b* content showed a different trend if compared with the non-destructive method, in both first and second trial (Figure 5 A, B). In particular, in the first growing cycle statistical analysis showed a significative interaction ($P < 0.05$) between the two factors but unlike the previous analysis treatments affected the chlorophyll content in plants grown with less water and not in well-watered ones. Chlorophyll *a+b* level was significantly higher ($P < 0.05$) only in plants that received less water and in combination with glutamic acid treatment. In all other cases, differences observed were not significant. The lowest value has been reached in plants treated with GHI_16_VHL + proline and grown in reduced water condition (0.279 µg mg^{-1}). Moreover, it was significantly lower than well-watered control. In the second trial all values were slightly lower than the first one, as resulted from the non-destructive analysis and the interaction between water and treatment was significant ($P < 0.05$) (Figure 5 B). Plants treated with the biostimulant prototype showed a significant ($P < 0.05$) decrease in chlorophyll *a+b* content in combination with the less water amount.

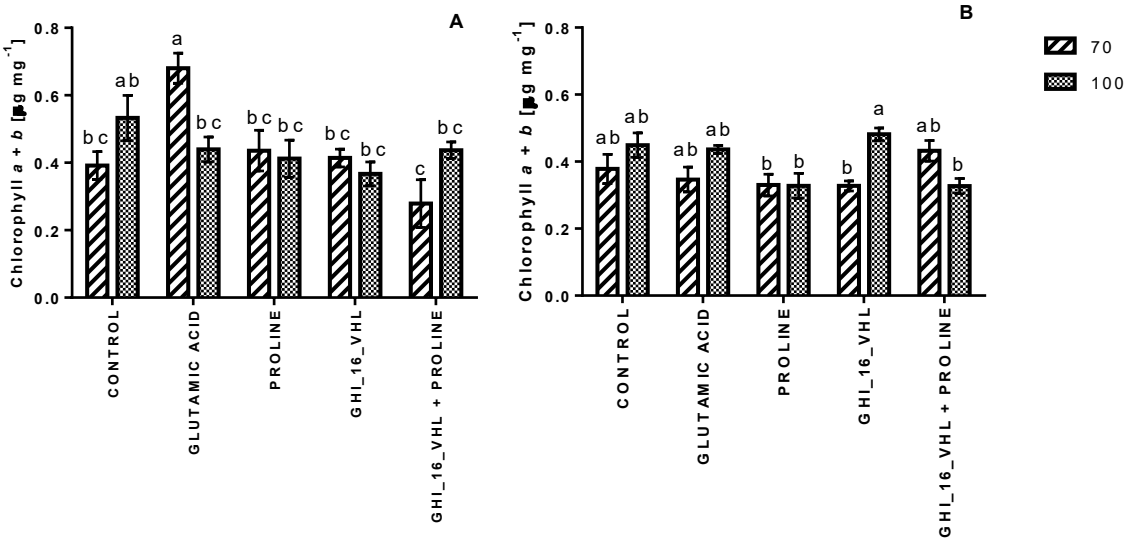


Figure 38. Chlorophyll *a+b* content in lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken at the end of each growing cycle (**A**: 1st trial **B**: 2nd trial). Values are means ± SE (n =4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences (P < 0.05).

1.3. Carotenoids

The content of carotenoids (Figure 6) in lettuce leaves showed a similar trend observed in chlorophyll *a+b* analyses. In particular, in the first trial (Figure 6 A) the interaction between water and treatment was significant, but unlike the chlorophyll content the high level of carotenoids observed in plants treated with

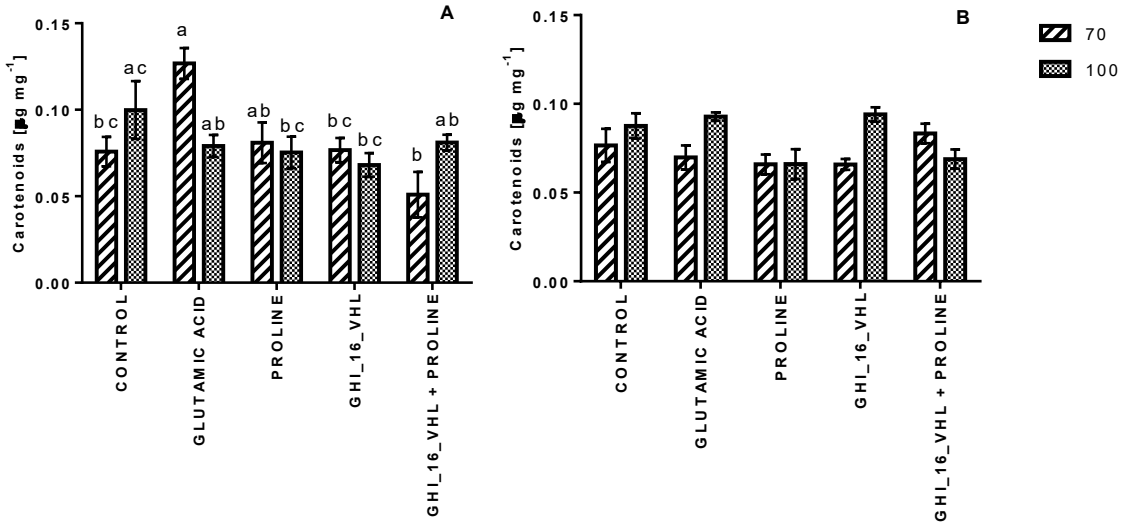


Figure 39. Carotenoids content in lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken at the end of each growing cycle (**A**: 1st trial **B**: 2nd trial). Values are means ± SE (n =4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences (P < 0.05).

glutamic acid and grown with less water did not result statistically significant if compared with the plants treated with the same product and grown in well-watered condition. Similarly, in the second growing cycle even if the interaction between factor was significant, no significant differences appeared between samples (Figure 6 B).

1.4. Phenols and anthocyanins

Phenols content expressed as phenol index had the same trend both in the first and in the second growing cycle. In particular, in the first growing cycle (Figure 7 A), the interaction between water and treatment was statistically significant ($P < 0.05$) and a significant difference resulted between well-watered plants treated with proline (10.633 ABS_{320nm} g^{-1}) and glutamic acid (5.925 ABS_{320nm} g^{-1}). However, both values were not significantly different from the control. In the second trial (Figure 7Figure 40 B), the level of phenols was generally higher compared with the first one. Treatment factor resulted statistically significant ($P < 0.05$) and phenol index of control plants grown with less water (17.355 ABS_{320nm} g^{-1}) was significantly lower than those measure in well-watered plants treated with proline (41.857 ABS_{320nm} g^{-1}) and plants treated with GHI_16_VHL + proline that received less water (33.188 ABS_{320nm} g^{-1}).

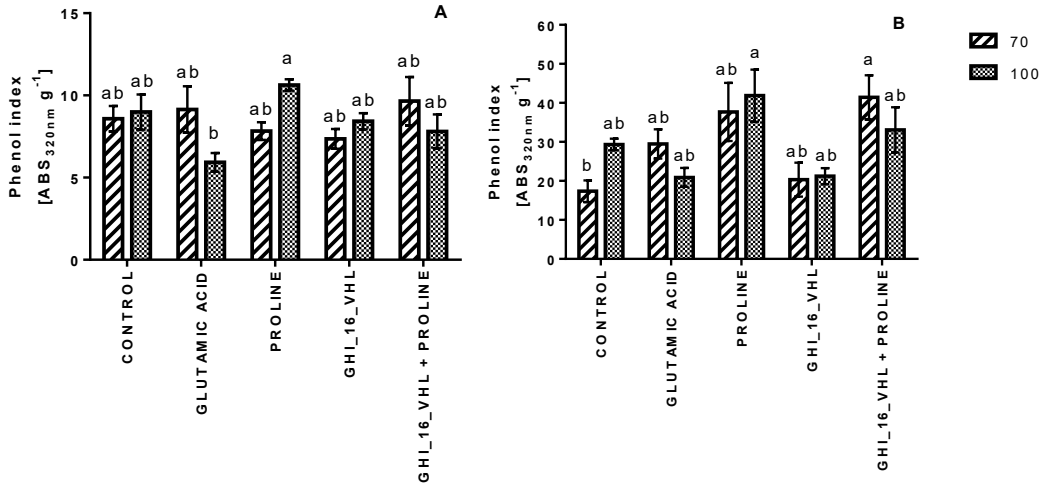


Figure 40. Phenols content expressed as phenol index in lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken at the end of each growing cycle (**A**: 1st trial **B**: 2nd trial). Values are means ± SE (n =4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

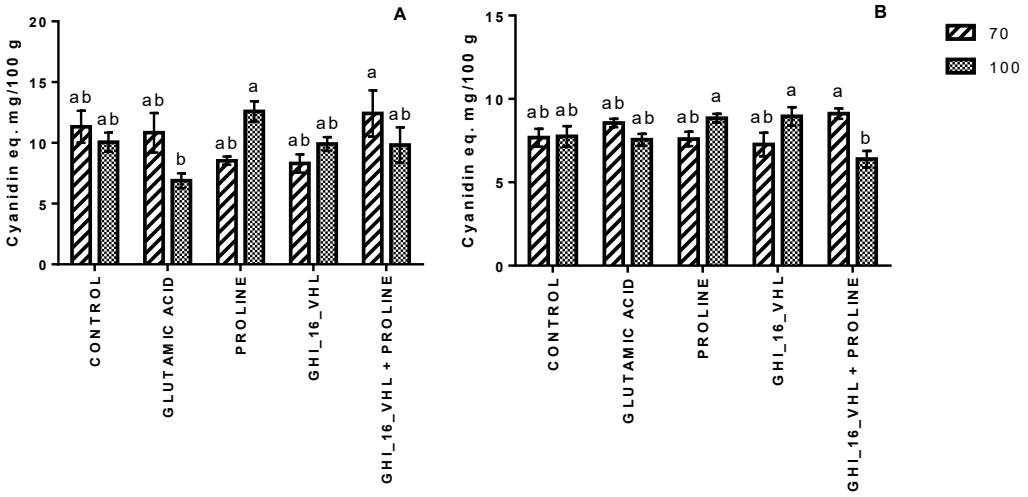


Figure 41. Anthocyanin content expressed as mg of Cyanidin equivalents per 100 grams of fresh weight in lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken at the end of each growing cycle (**A**: 1st trial **B**: 2nd trial). Values are means ± SE (n =4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

Unlike phenol index, the content of anthocyanin expressed as milligrams of Cyanidin equivalent per 100 grams of fresh weight, was higher in the first trial (Figure 8 A) than in the second one (Figure 8 B) but the interaction between water amount and treatment was statistically significant ($P < 0.05$) in both growing cycles. In the first one anthocyanin content showed the same trend of phenols index whereas in the second one a different trend resulted. In particular, in treated plants with GHI_16_VHL + proline, water reduction lead to a significant increase of anthocyanin content, from 6.389 to 9.995 (Cyanidin-3 glucoside eq. mg/100 g^{-1}).

1.5. Leaf colour

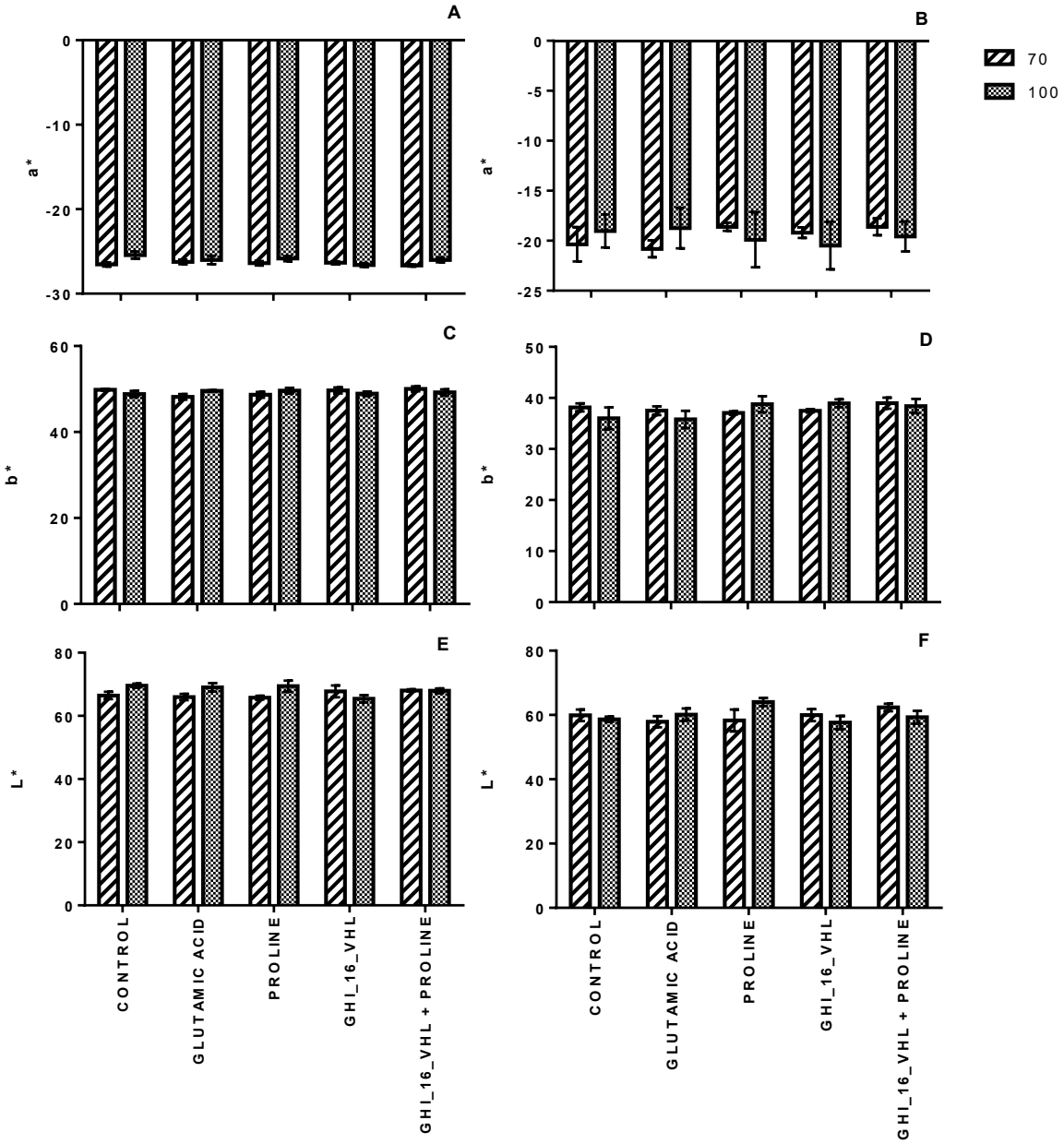


Figure 42. Colour expressed as the measured values of CIELAB a*, b* and L* of lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken at the end of each growing cycle (**A**, **C**, **E**: 1st trial **B**, **D**, **F**: 2nd trial). Values are means ± SE (n =4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

CIELAB parameters a* (ranges from green (-) to red (+)), b*(range from blue (-) to yellow (+)) and L* (lightness from black (0) to white (100)) describing the colour of lettuce leaves are represented in Figure 9Figure 42. During the first growing cycle the a* parameter (Figure 9Figure 42 A) was significantly affected by the amount of water, whereas b* (Figure 9 C) and L* (Figure 9 E) did not change, not in response to water, or in response

to treatment. In the second trial no significant difference resulted in any parameters measured (Figure 9 B, D, F). Moreover, a* values were generally higher in the first growing cycle than in the second one, where an opposite behaviour resulted for b* and L*.

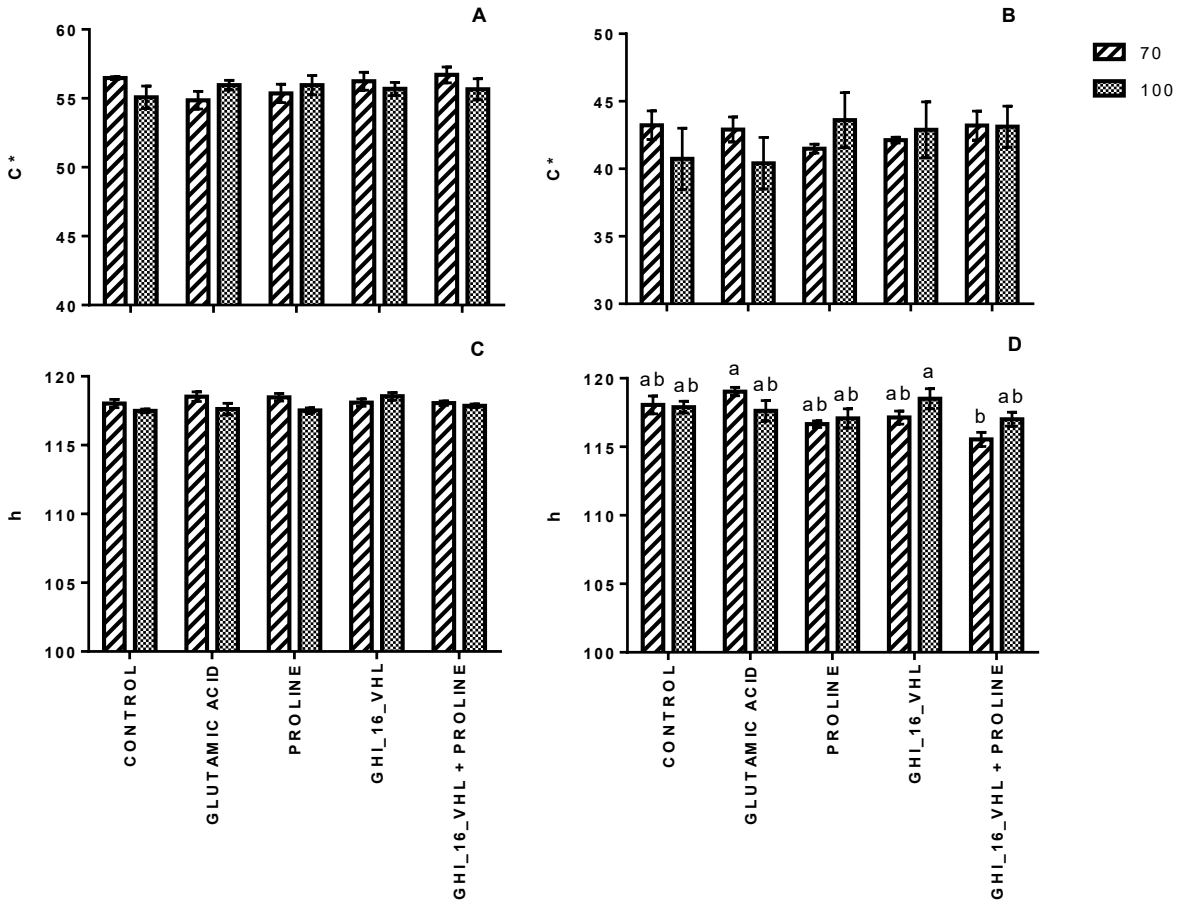


Figure 43. Colour expressed as the values C* (chroma) and h (hue) of lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken at the end of each growing cycle (**A**, **C**: 1st trial **B**, **D**: 2nd trial). Values are means ± SE (n =4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

C* and h values are calculated from a* and b* parameters and represent the chroma and the hue, respectively. C* values did not result significantly affected by water and treatment, not in the first or in the second growing cycle (Figure 10Figure 43 A, B). Interestingly hue angle was significantly affected by the quantity of water in the first trial (Figure 10Figure 43 C) and by the treatment in the second one (Figure 10Figure 43 D).

1.6. Chlorophyll a fluorescence

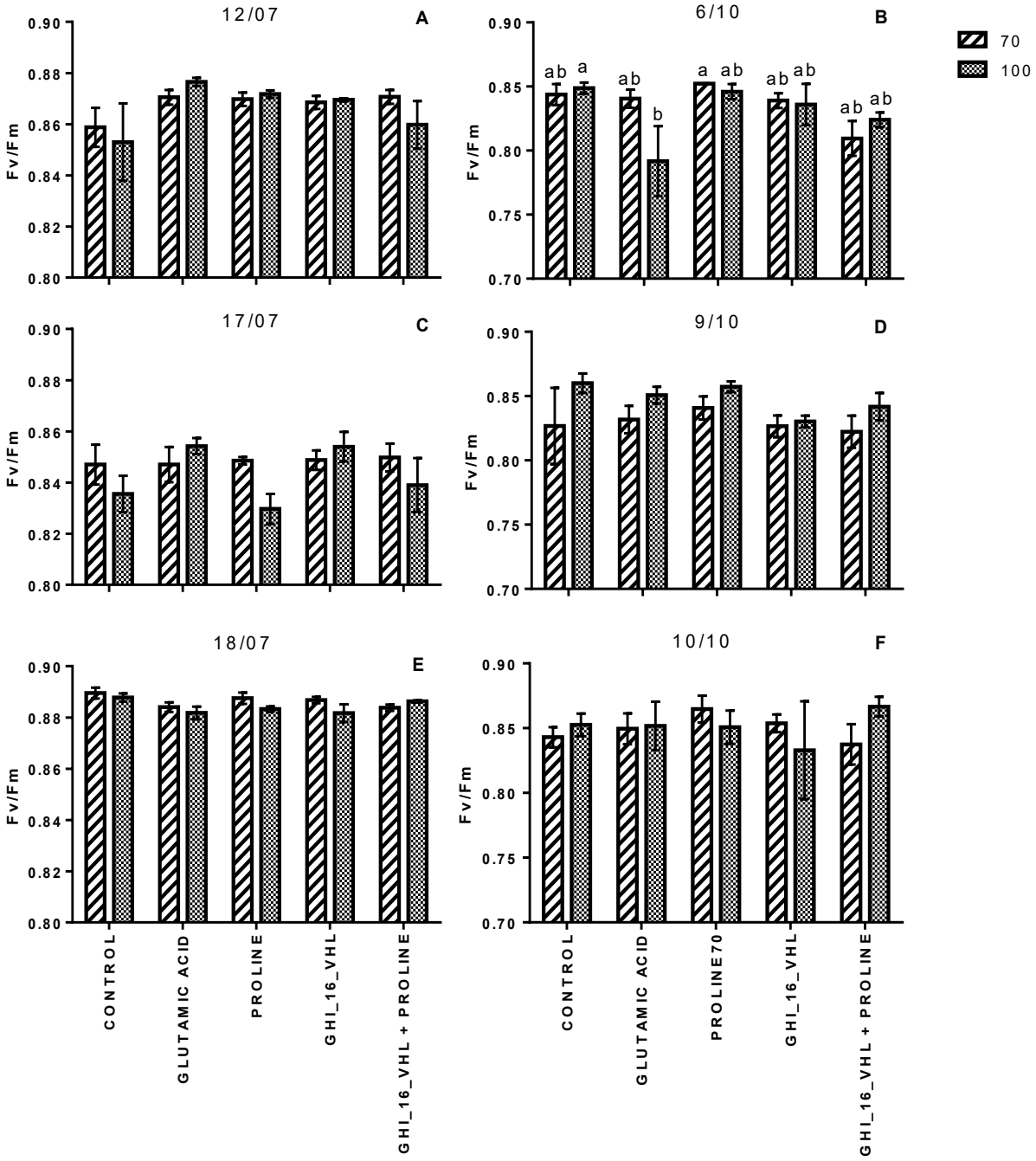


Figure 44. Maximum quantum efficiency of PSII (Fv/Fm) measured in lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken one day after the first treatment (12/07 -6/10) (**A**, **B**), during the water reduction (17/07-9/10) (**C**, **D**) and at the end of each trial (18/07-10/10) (**E**, **F**). (**A**, **C**, **E**: 1st trial **B**, **D**, **F**: 2nd trial). Values are means ± SE (n =4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

The analysis of chlorophyll *a* fluorescence provides useful information about the status of the photosynthetic apparatus and the efficiency of photosynthesis. Fv/Fm is the maximum quantum yield of PSII and is normally

used as indicator of the health status of plants. Optimal values are around 0.83 – 0.84, while lower value usually means that plants are exposed to stress causing photoinhibition. In the first growing cycle Fv/Fm ratio were around 0.87 after the first treatment (Figure 11 A). All values generally decreased during the water reduction, mostly in well-watered plants treated with water and proline (Figure 11 C), however, at the end of the trial Fv/Fm ratio increased in all samples to an average value of 0.89 (Figure 11 E). No significant effect resulted from the statistical analysis in any of the time points. In the second growing cycle Fv/Fm values were generally slightly lower than first one. A significant effect of the treatment resulted one day after the first application (Figure 11 B) and Fv/Fm average was around 0.84 except for plants treated with GHI_16_VHL + proline and well-watered plants treated with glutamic acid, that showed lower values. During the water reduction the amount of water resulted statistically significant (Figure 11 D) and a decrease in Fv/Fm ratio appeared in plants watered with less water. No significant difference resulted at the end of the cycle and all values was around 0.85 (Figure 11 F).

A similar trend has been observed in the performance index (PI) (Figure 12Figure 45) and in the number of reaction centres per cross section (RC/CSm) (Figure 13). PI is a very sensitive parameter that correlate with stress and gives us information about the status of both photosystem I and II is the performance index (PI). A slight decrease appeared during the water reduction in both growing cycles, and lettuce plants of the second growing cycle (around 1.0) showed lower values if compare with the first one (around 1.5). No significant differences resulted, not in response to treatment application or water amount.

During the first growing cycle the number of RC/CSm was significantly affected by the interaction between water and treatment one day after the first treatment (Figure 13 A) and at the end of the experiment (Figure 46 E). RC/CSm as well as PI and Fv/Fm, decreased during the water reduction from an average value of 923 to 732 and then increased again at the end of the trial. During the second growing cycle, no significant effect was detected in any time point examined.

The dissipation of energy per reaction centre (DI_0/RC) was significantly affected by the interaction of water and treatment one day after the first treatment (Figure 47 A). In particular, under well-watered condition, glutamic acid treatment induced a significant decrease in DI_0/RC value, if compared with control plants. The same trend was not visible in the following time points. During the second trial all values were generally higher than those observed in the first one and an opposite response appeared after the first treatment when well-watered plants treated with glutamic acid had a higher value than control (Figure 47 B).

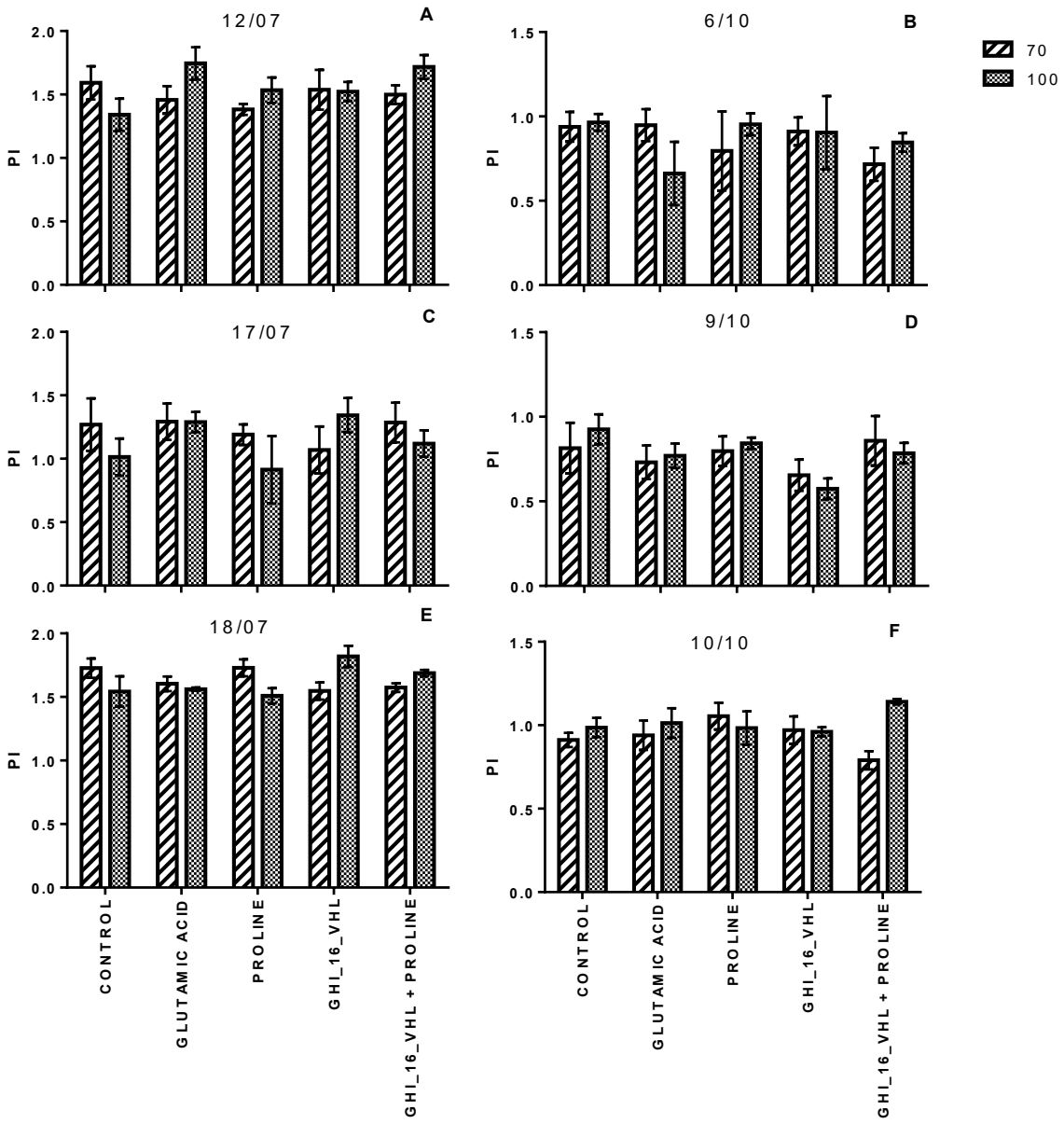


Figure 45. Performance index (PI) measured in lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken one day after the first treatment (12/07 -6/10) (**A**, **B**), during the water reduction (17/07-9/10) (**C**, **D**) and at the end of each trial (18/07-10/10) (**E**, **F**). (**A**, **C**, **E**: 1st trial **B**, **D**, **F**: 2nd trial). Values are means ± SE (n =4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

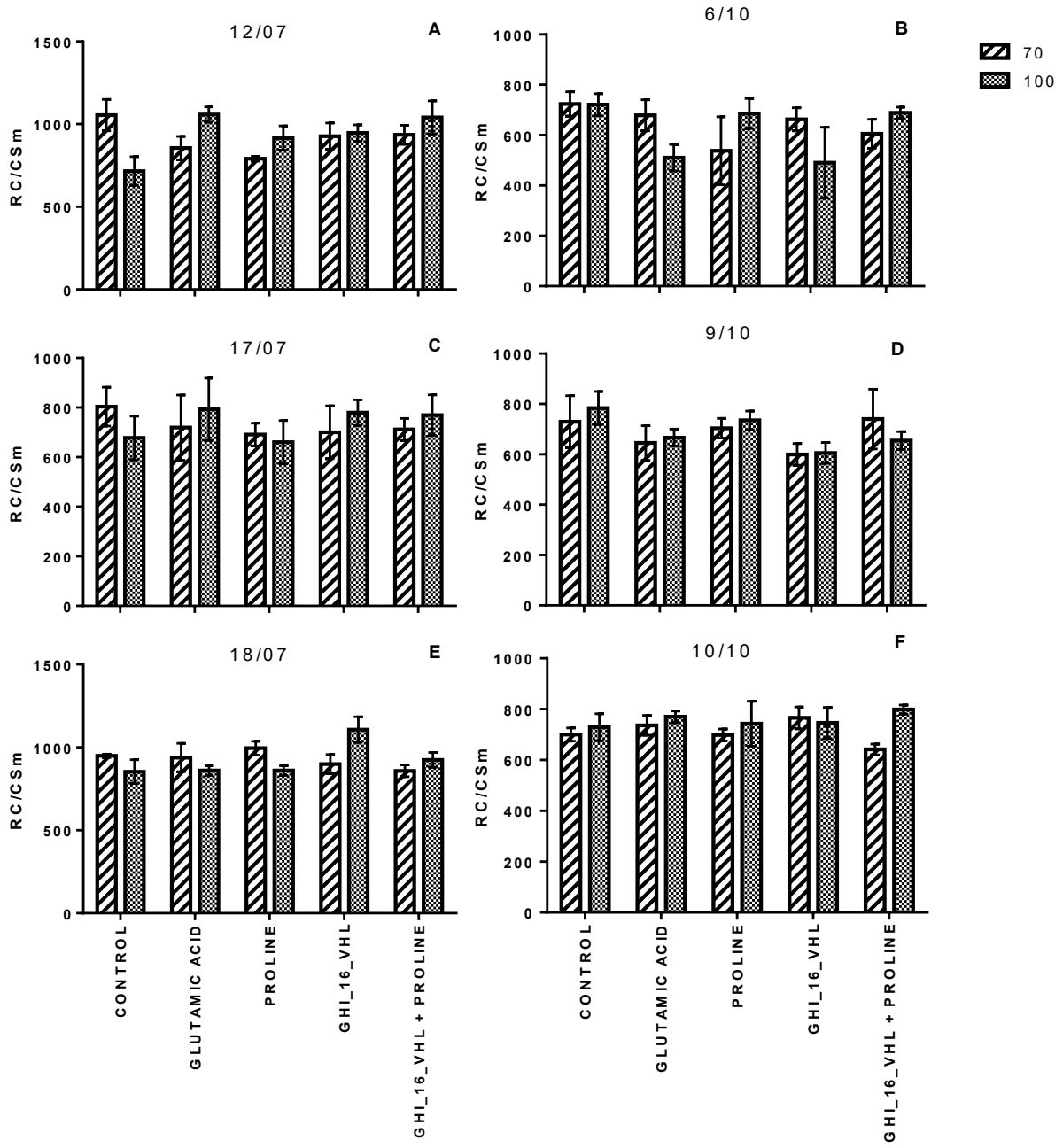


Figure 46. Number of reactions centres for cross section (RC/CSm) measured in lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken one day after the first treatment (12/07 -6/10) (**A**, **B**), during the water reduction (17/07-9/10) (**C**, **D**) and at the end of each trial (18/07-10/10) (**E**, **F**). (**A**, **C**, **E**: 1st trial **B**, **D**, **F**: 2nd trial). Values are means ± SE (n =4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

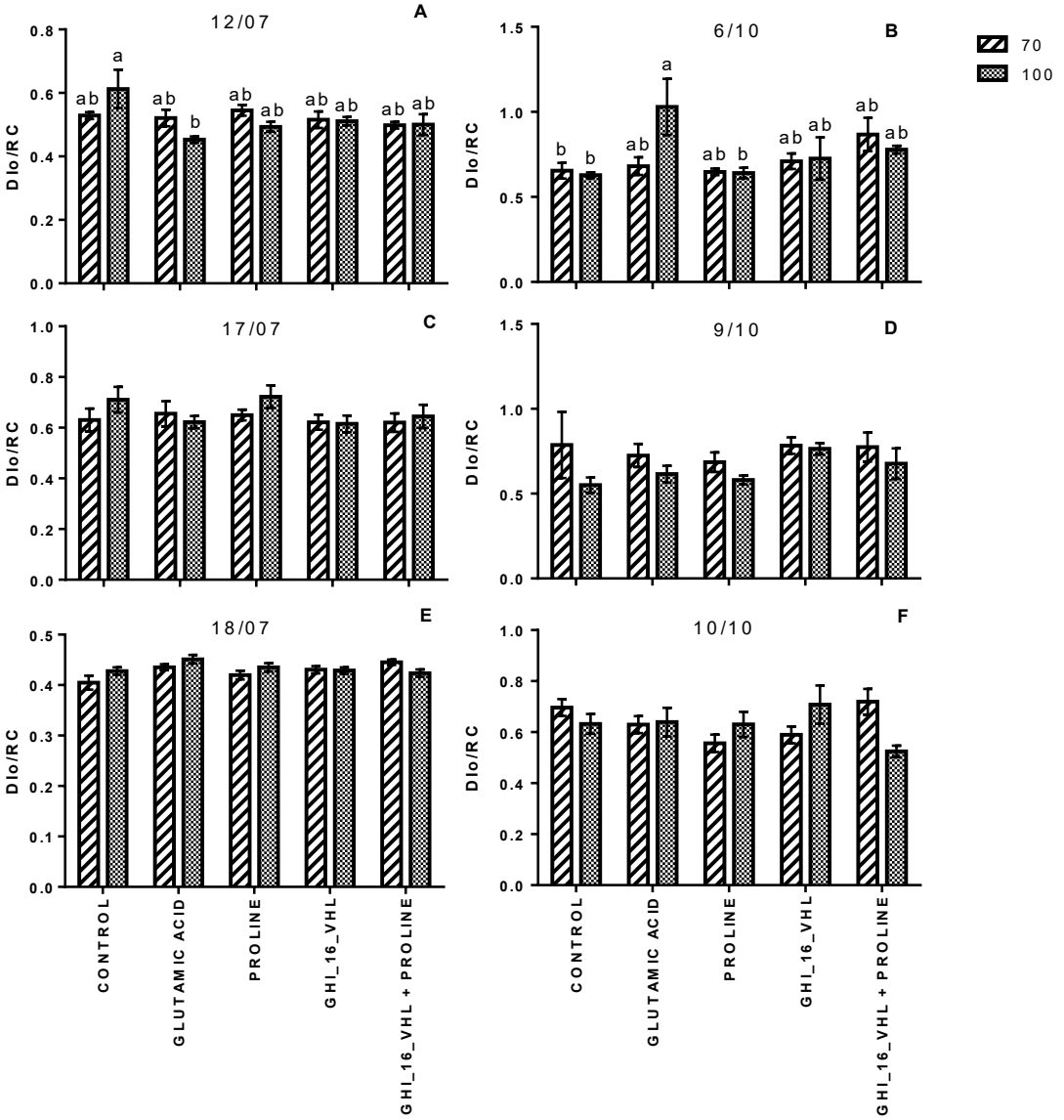


Figure 47. Dissipation of energy per reaction centre (DI_0/RC) measured in lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water deprivation: 70). Measures were taken one day after the first treatment (12/07 -6/10) (**A**, **B**), during the water deprivation (17/07-9/10) (**C**, **D**) and at the end of each trial (18/07-10/10) (**E**, **F**). (**A**, **C**, **E**: 1st trial **B**, **D**, **F**: 2nd trial). Values are means ± SE (n =4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

Fluorescence variables measured in light adapted leaves are reported in Table 19. In the first growing cycle no significant difference resulted in the effective PSII quantum efficiency of illuminated sample (ϕ_{PSII}), and average value was around 0.806. In the same experiment the electron transport rate (ETR) has been significantly ($P < 0.05$) affected by the treatments and ETR value of plants treated with glutamic acid and grown with a low amount of water was significantly higher than control plants, both well irrigated and with less water. During the second growing cycle ϕ_{PSII} values were generally lower than those observed in the first one, whereas ETR

values were much higher than the first one, from three to seven time higher. The interaction between treatment and water level significantly affected both parameters. Moreover, plants grown with less water and treated with glutamic acid had the lowest ϕ_{PSII} value (0.411) and the highest ETR value (65.135).

Table 19. Effective PSII quantum efficiency (ϕ_{PSII}) and electron transport rate (ETR) measured in lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken and at the end of each trial. Values are means ± SE (n =5 1st trial or n=4 2nd trial). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

TREATMENT	WATER	1st TRIAL		2nd TRIAL	
		ϕ_{PSII}	ETR	ϕ_{PSII}	ETR
CONTROL	70	0.806 ± 0.002	7.213 ± 0.457 b	0.639 ± 0.020 ab	36.122 ± 6.506 ab
	100	0.811 ± 0.004	7.466 ± 0.386 bc	0.678 ± 0.030 a	26.217 ± 4.673 b
GLUTAMIC ACID	70	0.803 ± 0.002	9.101 ± 0.207 a	0.411 ± 0.101 b	65.135 ± 13.696 a
	100	0.802 ± 0.001	8.519 ± 0.483 abc	0.718 ± 0.008 a	30.706 ± 1.326 b
PROLINE	70	0.803 ± 0.003	7.891 ± 0.383 abc	0.687 ± 0.003 a	32.641 ± 3.872 b
	100	0.805 ± 0.003	7.710 ± 0.166 abc	0.687 ± 0.013 a	25.564 ± 3.805 b
GHI_16_VHL	70	0.806 ± 0.004	8.554 ± 0.442 abc	0.719 ± 0.017 a	32.234 ± 4.394 b
	100	0.807 ± 0.003	8.888 ± 0.171 ac	0.729 ± 0.007 a	28.583 ± 4.673 b
GHI_16_VHL + PROLINE	70	0.805 ± 0.002	9.031 ± 0.228 ac	0.725 ± 0.015 a	25.938 ± 1.604 b
	100	0.810 ± 0.003	8.609 ± 0.298 abc	0.550 ± 0.092 ab	44.406 ± 8.879 ab

1.7. Gas exchanges and water use efficiency

In the first growing cycle leaf conductance (gs) was not affected by treatment or water amount and values averaged between 200 and 400 mol H_2O m^{-2} s^{-1} (Figure 50 A). Plants treated with GHI_16_VHL alone or in combination with proline showed a slight increase in response to water reduction, while an opposite trend resulted in plants treated with glutamic acid. In the second experiment (Figure 50 B) all values were generally lower than 150 mol H_2O m^{-2} s^{-1}, the variability was higher and the interaction between water and treatment was statistically significant. In particular, in well-watered condition a slight decrease in gs appeared in response

to all treatments but only in plant treated with proline the decrease was significant. Moreover, water reduction decreased the gs value of almost all plants, except for plants treated with proline.

The same trend appeared in transpiration rate (E) (Figure 48) whereas net photosynthetic rate (Pn) (Figure 49) was significantly affected by the amount of water during the first trial and plants received less water had higher values if compared with the well-watered. Moreover, in the second trial, Pn values of plants treated with GHI_16_VHL alone or in combination with proline and irrigated with less water were significantly higher than control and plants treated with glutamic acid.

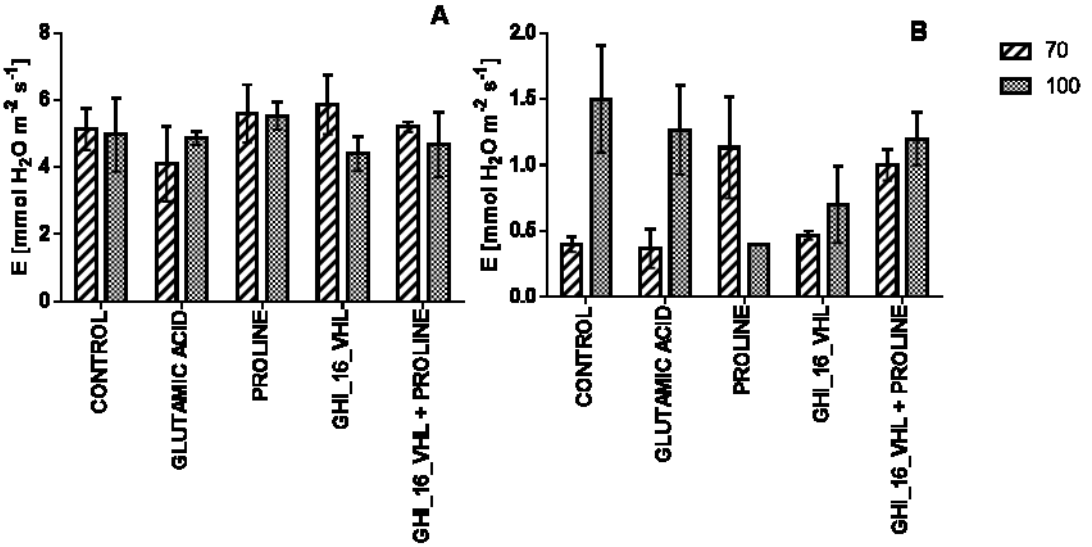


Figure 48. Transpiration rate (E) measured in lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken at the end of each growing cycle (**A**: 1st trial **B**: 2nd trial). Values are means ± SE (n =3). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

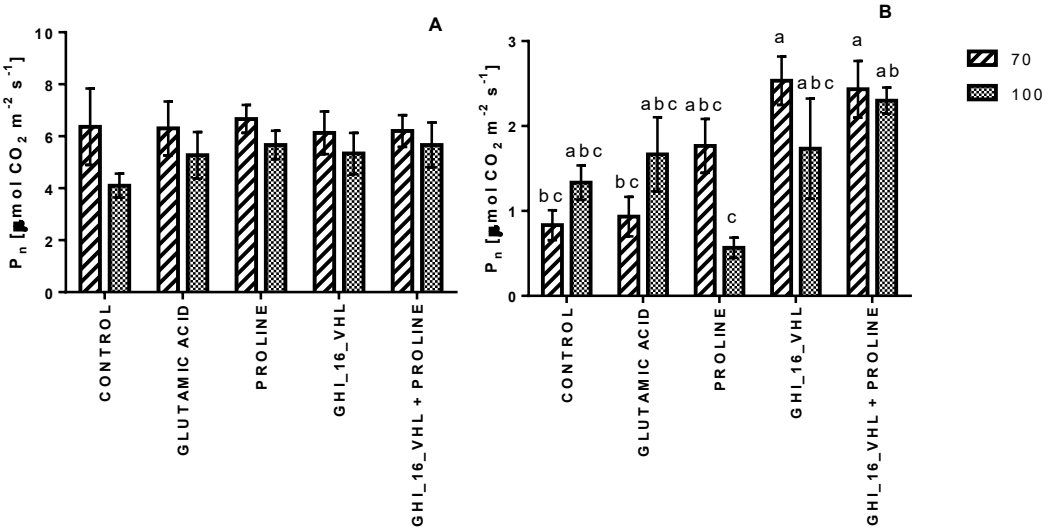


Figure 49. Net photosynthetic rate (Pn) measured in lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken at the end of each growing cycle (**A**: 1st trial **B**: 2nd trial). Values are means ± SE (n =3). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

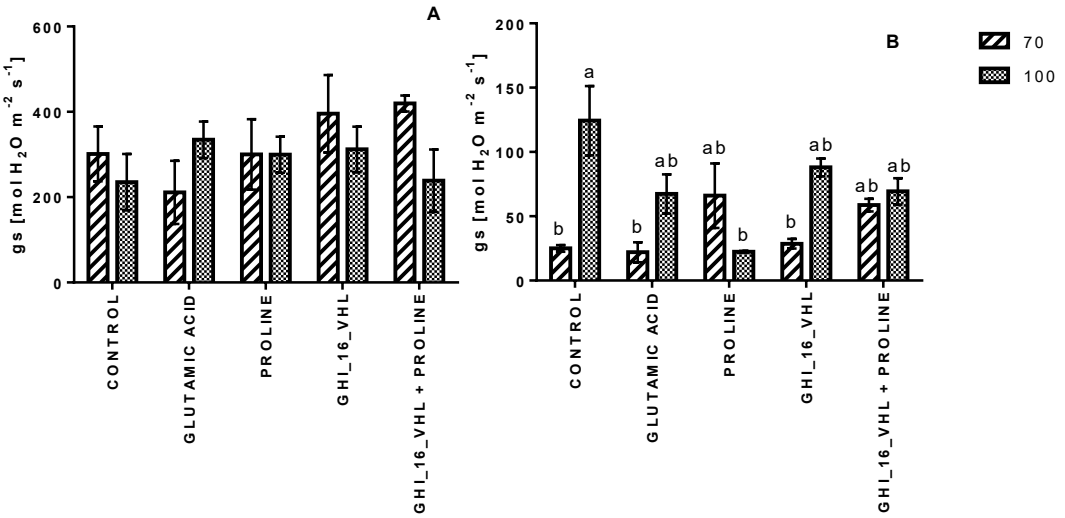


Figure 50. Leaf conductance (gs) measured in lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken at the end of each growing cycle (**A**: 1st trial **B**: 2nd trial). Values are means ± SE (n =3). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

Intrinsic water use efficiency calculated as the ratio between Pn and gs is reported in Figure 51. No significant effect resulted in the first growing cycle whereas in the second one the interaction between the amount of water and the treatments was statistically significant. In particular, highest value was reached in plants treated with GHI_16_VHL alone in combination with the reduction of irrigation.

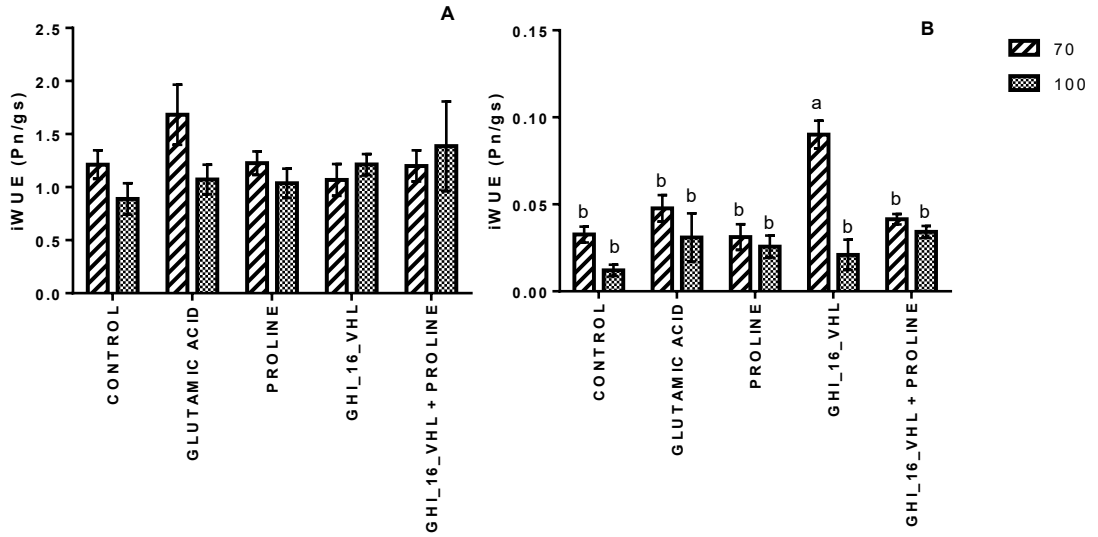


Figure 51. Intrinsic water use efficiency of lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken at the end of each growing cycle (**A**: 1st trial **B**: 2nd trial). Values are means ± SE (n =3). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

A similar trend resulted in the photosynthetic water use efficiency Figure 52. Unlike iWUE, pWUE values were higher in the second growing cycle than in the first one. During the first experiment all values were similar to the control, with the exception of plants treated with glutamic acid that received less water and well-watered plant treated with GH_16_VHL + proline. However, since the variability of the samples was high no significant differences appeared. As observed in iWUE, also in the second growing cycle GHI_16_VHL treatment significantly increased the pWUE of plants that received less water.

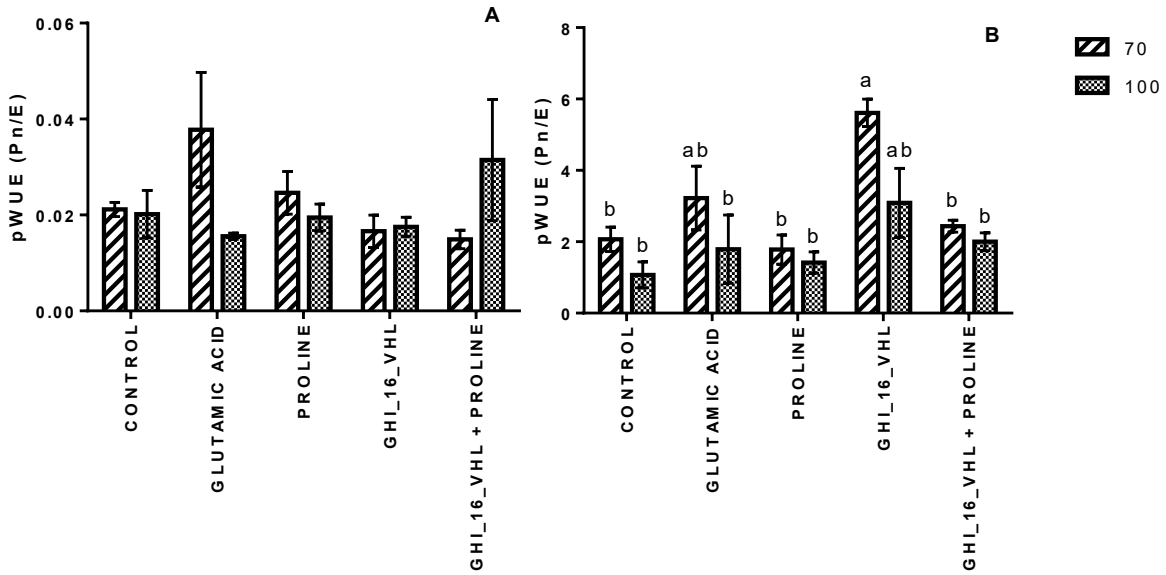


Figure 52. Photosynthetic water use efficiency of lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken at the end of each growing cycle (**A**: 1st trial **B**: 2nd trial). Values are means ± SE (n =3). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

1.8. Abscisic acid

The endogenous ABA concentrations in lettuce leaves are reported in Table 20.Treatments significatively ($P < 0.05$) affected ABA content in both growing cycles. In the first one ABA level increased after water reduction in control plants, in plants treated with glutamic acid and with GHI_16_VHL, while it decreased after the application of proline and GHI_16_VHL + proline. Moreover, control plants had the lowest values compared with the other treatments. An unexpected increase in ABA levels was measured in plants treated with GHI_16_VHL + proline.

Results obtained in the second experiment did not confirm the previous trend and control plants reached higher levels of ABA compared with almost all other treatments. The increased observed in response to GHi_16_VHL + proline treatment during the first trial was not detected in the second one. On the contrary, ABA concentration was significantly lower than control plants.

Table 20. Abscisic acid content in lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken at the end of each growing cycle. Values are means ± SE (n =4).

TRETMENT	WATER	ABA [ng g^{-1}]	
		1st TRIAL	2nd TRIAL
CONTROL	70	37.985 ± 10.683 b	68.850 ± 8.526 ab
	100	15.346 ± 7.969 b	92.653 ± 4.719 a
GLUTAMIC ACID	70	73.564 ± 10.623 b	15.219 ± 2.202 b
	100	49.192 ± 10.320 b	8.010 ± 3.216 b
PROLINE	70	47.621 ± 5.781 b	32.252 ± 3.632 ab
	100	66.059 ± 13.946 b	44.014 ± 25.179 ab
GHI_16_VHL	70	42.653 ± 9.489 b	23.599 ± 4.300 b
	100	36.190 ± 8.449 b	73.278 ± 33.473 ab
GHI_16_VHL + PROLINE	70	1461.683 ± 202.834 a	23.737 ± 0.818 b
	100	1618.181 ± 193.481 a	15.454 ± 8.029 b

1.9. Nitrate

Nitrate content in lettuce leaves measure was always below the EU thresholds (4000 mg kg-1 FW for the first growing cycle and 5000 mg kg-1 FW for the second one) both in the first and in the second growing cycles (Figure 53). In particular, in the first experiment the content of nitrate in lettuce leaves was much higher than second trial and they show a high variability between replication that probably did not allow to see any

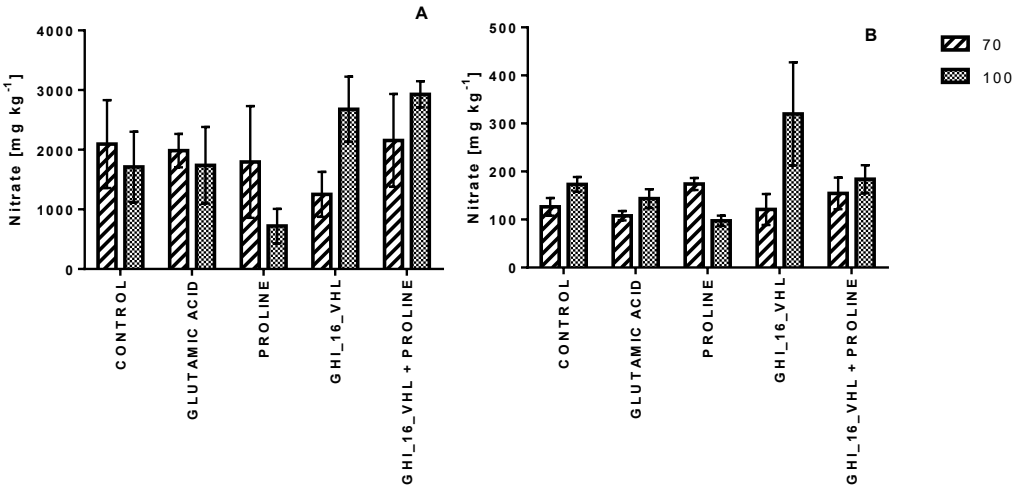


Figure 53. Nitrate concentration in lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken at the end of each growing cycle (**A**: 1st trial **B**: 2nd trial). Values are means ± SE (n =4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences (P < 0.05).

significant difference. Values averaged from 720 to 2929 mg kg^{-1} FW, detected in well-watered plants treated with proline and with GHI_16_VHL + proline, respectively. In plants grown under suboptimal condition nitrate concentration was similar to the control, regardless the treatment. On the contrary, in well-watered conditions plants treated with proline decreased the nitrate levels while GHI_16_VHL treatment alone or in combination with proline had an opposite effect.

In the second experiment water reduction induced a decrease in nitrate content in almost all treatment, with the exception of plants treated with proline where an opposite effect resulted. The highest nitrate content was observed in plants treated with GHI_16_VHL, however, the replications presented high variability.

1.10. Proline and osmolytes

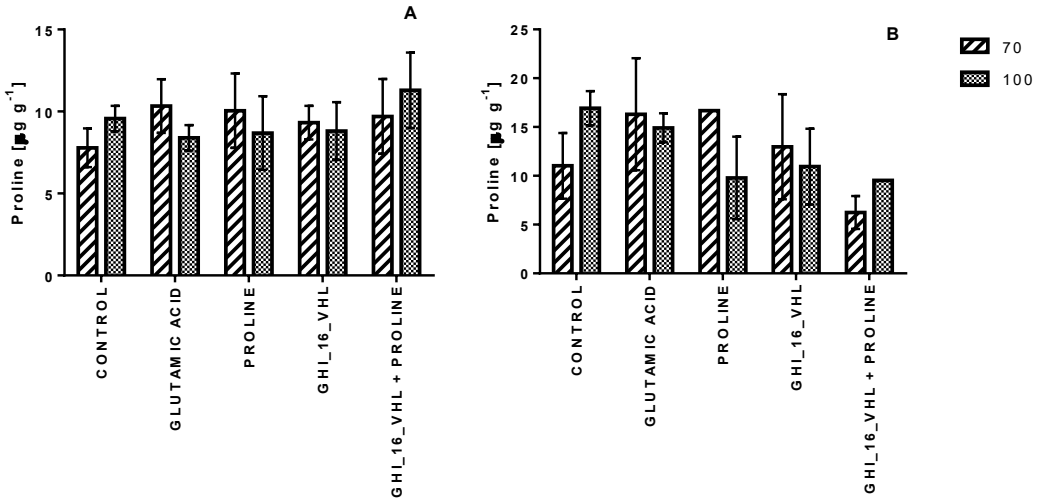


Figure 54. Proline concentration in lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken at the end of each growing cycle (**A**: 1st trial **B**: 2nd trial). Values are means ± SE (n =4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

The content of proline in lettuce leaves was not affected by the water reduction or by the different treatments (Figure 54). Average values ranged between 7.8 to 11.3 µg g^{-1} FW during the first growing cycle. In the second one the variability of the replicants did not allow highlighting any significant differences among samples, however, proline concentration was generally higher than the first trial, except for lettuce plants treated with GHI_16_VHL + proline.

The concentration of osmolytes in lettuce leaves was not affected by the water amount or by the treatment in both experiments and all values were around 0.09 mOsm kg^{-1} g^{-1} FW (Figure 55). A slight increase was observed in control plants and in plants treated with proline after the water reduction.

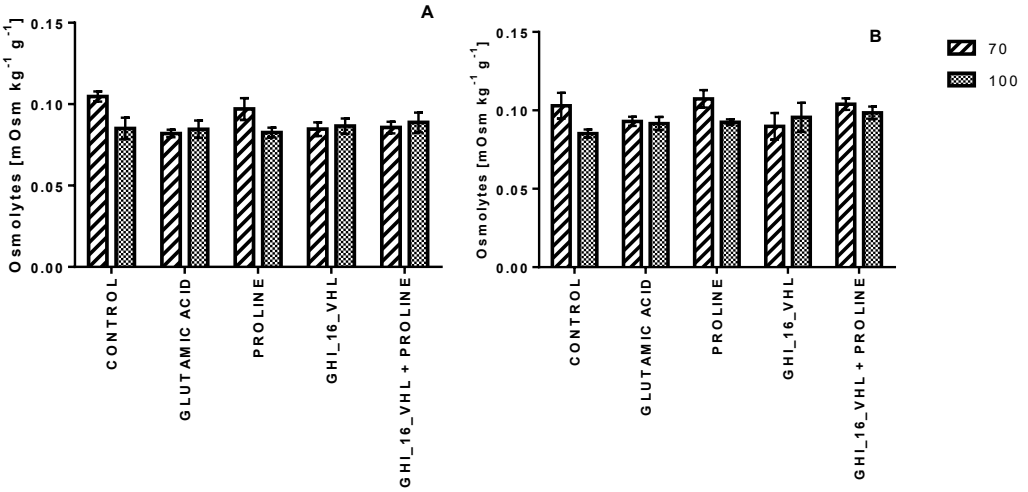


Figure 55. Osmolytes concentration in lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken at the end of each growing cycle (**A**: 1st trial **B**: 2nd trial). Values are means ± SE (n =4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

1.11. Sucrose and total sugars

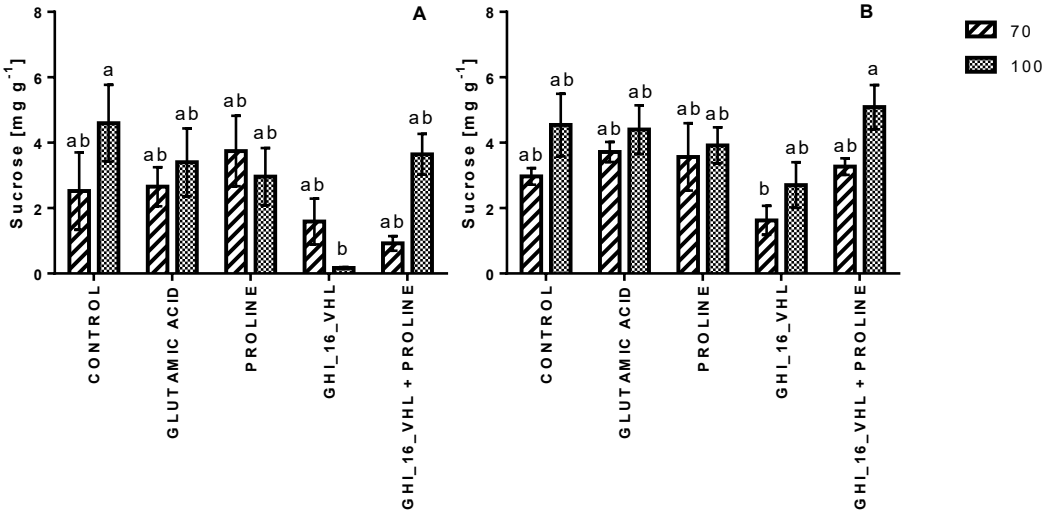


Figure 56. Sucrose concentration in lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken at the end of each growing cycle (**A**: 1st trial **B**: 2nd trial). Values are means ± SE (n =4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

The concentration of sucrose was affected by the treatments during the first cycle and both by the treatment and the water amount in the second one. In particular, in the first growing cycle GHI_16_VHL treatment induced a significant decrease in sucrose content in well-watered plants, if compared with the control. Except for this, the content of sucrose in well-watered plants was around 4 mg g^{-1} whereas in plants subjected to

water reduction the sucrose level was low. A similar trend was observed also in the second growing cycle; however, all values were generally higher than those measured in the first one.

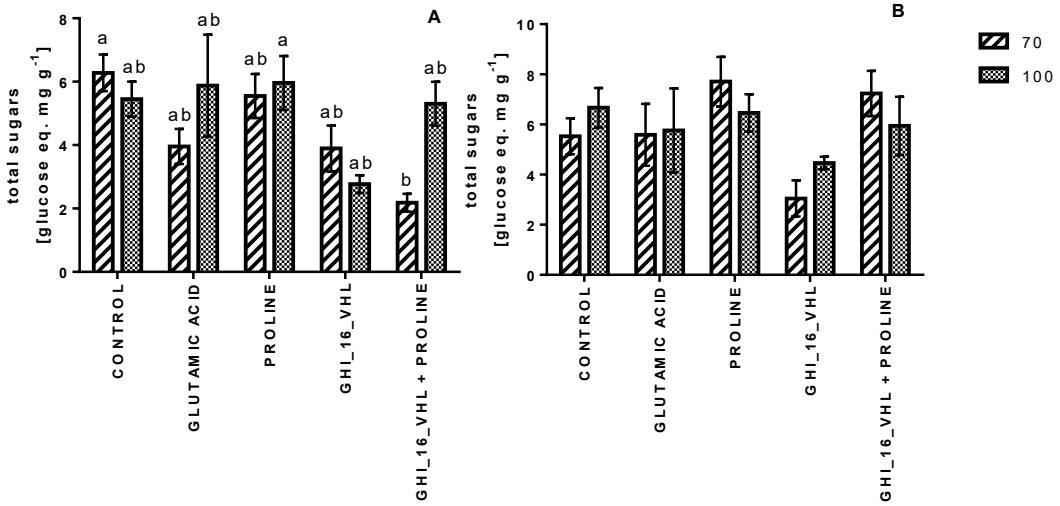


Figure 57. Total sugars concentration in lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken at the end of each growing cycle (**A**: 1st trial **B**: 2nd trial). Values are means ± SE (n =4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

The concentration of total sugars in lettuce plants was affected by the interaction between water amount and treatment, during the first growing cycle. In particular, in control plants and in plants treated with GHI_16_VHL alone, the water reduction slightly increased the concentration of the sugars. In all other condition an opposite tendency appeared. A significant difference resulted between control plants and plants treated with GHI_16_VHL + proline, and grown under water reduction. During the second trial, the application of the treatments resulted statistically significant. Even if no differences resulted among samples, plants treated with GHI_16_VHL alone had the lower sugar values, regardless the amount of water received.

1.12. Lipid peroxidation

In the present study, MDA content in lettuce leaves did not change during the first and the second growing cycle and all values were not significantly different from the control. In particular, in the first trial the average value was around 1.54 nmol g^{-1}, whereas in the second one all values were higher than 2 nmol g^{-1} and the interaction between water and treatment was significant.

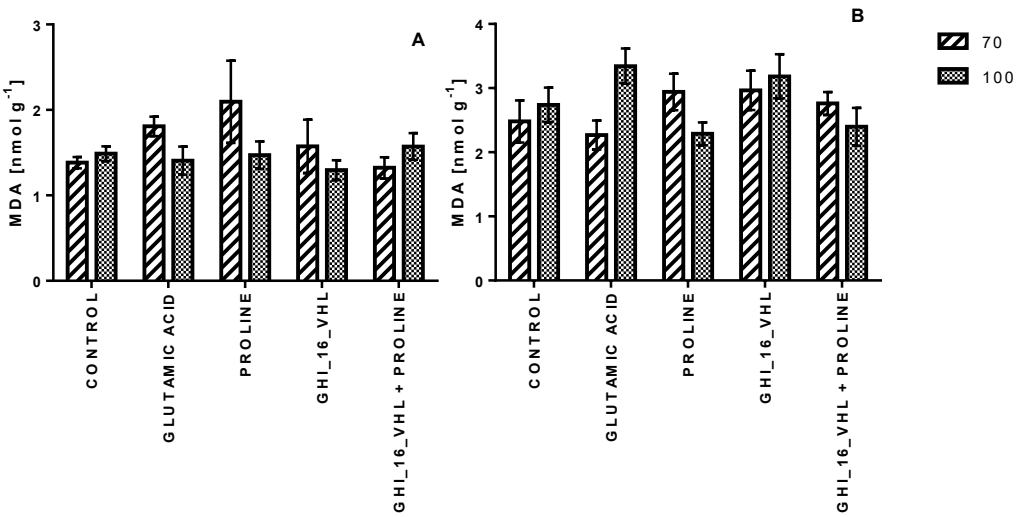


Figure 58. MDA concentration in lettuce leaves treated with water (CONTROL), glutamic acid, proline, GHI_16_VHL, GHI_16_VHL + proline and grown under two water regimes (well-watered: 100, water reduction: 70). Measures were taken at the end of each growing cycle (**A**: 1st trial **B**: 2nd trial). Values are means ± SE (n =4). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

2nd ACTIVITY

1.1. Yield, dry weight and dry matter

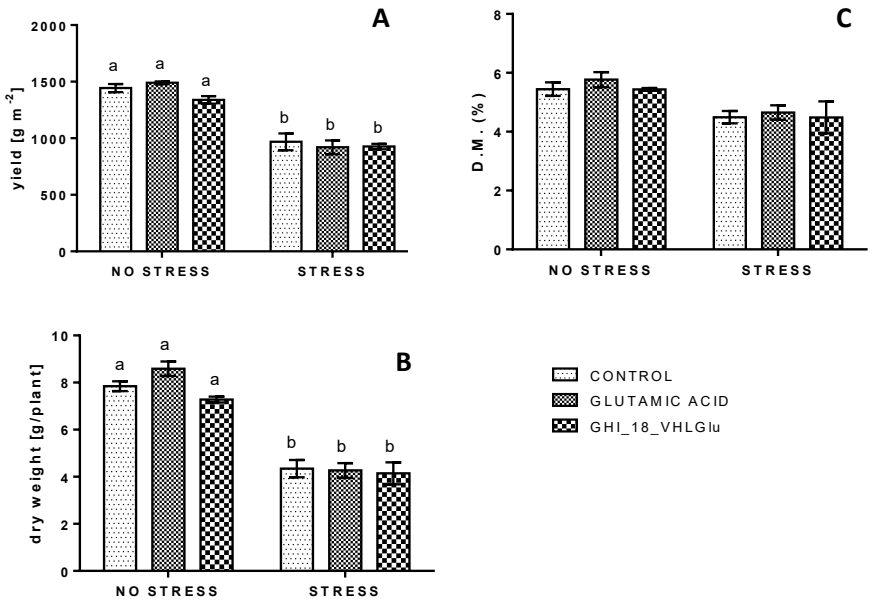


Figure 59. Yield (**A**), dry weight (**B**) and dry matter (**C**) of lettuce treated with water (CONTROL), glutamic acid and GHI_18_VHLGlu and grown under two water regimes (well-watered: **NO STRESS** and water stress and re-watering: **STRESS**). Measures were taken at the end of the growing cycle. Values are means ± SE (n =3). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

The yield of lettuce plants was significantly affected by the water stress period, in particular the average value of non-stressed plants was 1424 g m^{-2} whereas plants subjected to water deprivation had a value of 938 g m^{-2}. At the same time, any of the treatment applied had a significant effect during the recovery period after the water stress (Figure 59 A). The same trend resulted in the dry weight (Figure 59 B) and the average value of stressed plants (4 g) was half of the non-stressed weight (8 g). However, the difference of dry matter between stressed and non-stressed plants was not significant and values ranged from 4.4% to 5.7% (Figure 59 C).

1.2. Chlorophyll

The level of chlorophyll measured during the water stress event and after 24 hours from the third treatment is reported in Figure 60 A. Water stress had a significant effect on chlorophyll content and values observed in lettuce plants subjected to water deprivation were generally lower that those measure in non-stressful plants. However, statistical analyses did not reveal any significant difference among the treatments. At the ned of the cycle (Figure 60 B), after the recovery period chlorophyll content in stressed plants increased reaching the same values measured in plants grown with a constant water supply.

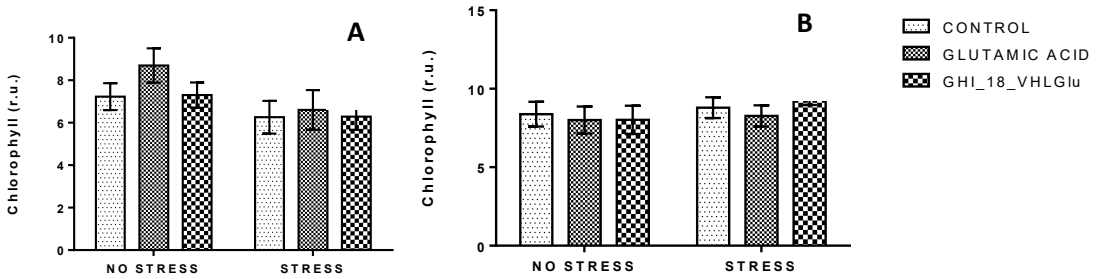


Figure 60. Chlorophyll content determined *in vivo* in lettuce leaves treated with water (CONTROL), glutamic acid and GHI_18_VHLGlu and grown under two water regimes (well-watered: **NO STRESS** and water stress and re-watering: **STRESS**). Measures were taken during the water stress (3/07) (**A**) and at the end of the cycle after the re-watering (12/07) (**B**). Values are means ± SE (n =15). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

1.3. Chlorophyll a fluorescence

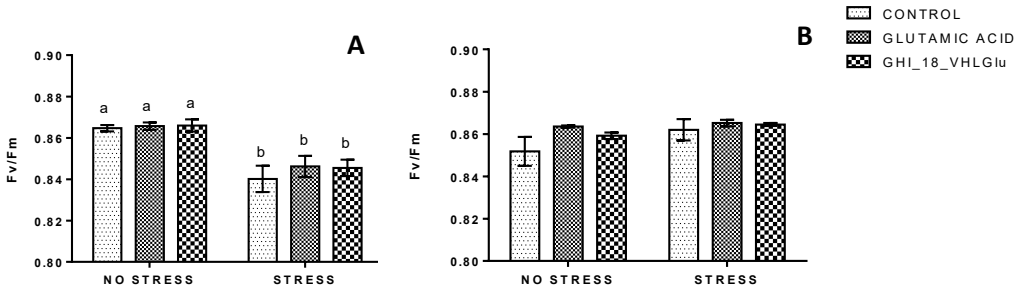


Figure 61. Maximum quantum efficiency of PSII (Fv/Fm) measured in lettuce leaves treated with water (CONTROL), glutamic acid and GHI_18_VHLGlu and grown under two water regimes (well-watered: **NO STRESS** and water stress and re-watering: **STRESS**). Measures were taken during the water stress (3/07) (**A**) and at the end of the cycle after the re-watering (12/07) (B). Values are means ± SE (n =6). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

The maximum quantum efficiency of PSII (Fv/Fm) measured during the water deprivation was significantly lower in stressed plants if compared with those grown under constant water supply (Figure 61 A). In particular, the average values were 0.86 in non-stressed plants and 0.84 in stressed plants. At the end of the cycle all values stabilised around 0.86, in both growing conditions and regardless the treatments applied (Figure 61 B). A similar result was obtained also in the measure of performance index (PI) of lettuce plants. This parameter gives us information about the functionality of the leaves and the water deprivation had a significant effect (Figure 62 A). After the re-watering PI increased in stressed plants reaching the same values of plants grown under non-stressed conditions (Figure 62 B).

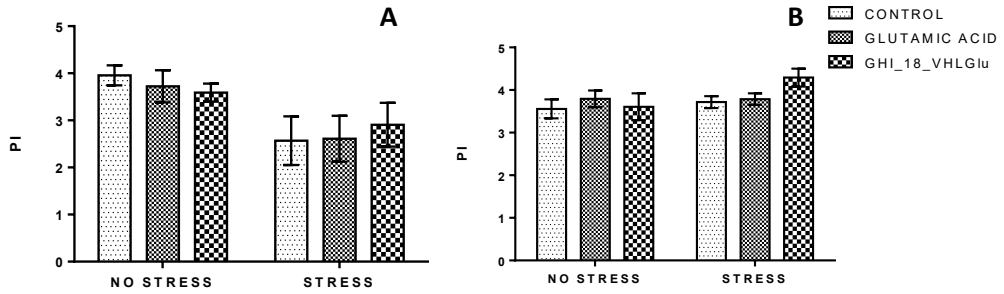


Figure 62. Performance index (PI) measured in lettuce leaves treated with water (CONTROL), glutamic acid and GHI_18_VHLGlu and grown under two water regimes (well-watered: **NO STRESS** and water stress and re-watering: **STRESS**). Measures were taken during the water stress (3/07) (**A**) and at the end of the cycle after the re-watering (12/07) (B). Values are means ± SE (n =6). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

The measurement of the chlorophyll *a* fluorescence in real condition showed similar results. Indeed, both the effective PSII quantum efficiency (ϕPSII) (Figure 63 A) and the electron transport rate (ETR) (Figure 63 B) measured at the end of the cycle showed that the photosynthetic apparatus functionality fully recovered after the re-watering. Moreover, a significant interaction between the stress and the treatment appeared in ETR analysis. In contrast, a significant difference appeared in the analysis of steady-state fluorescence (Fs).

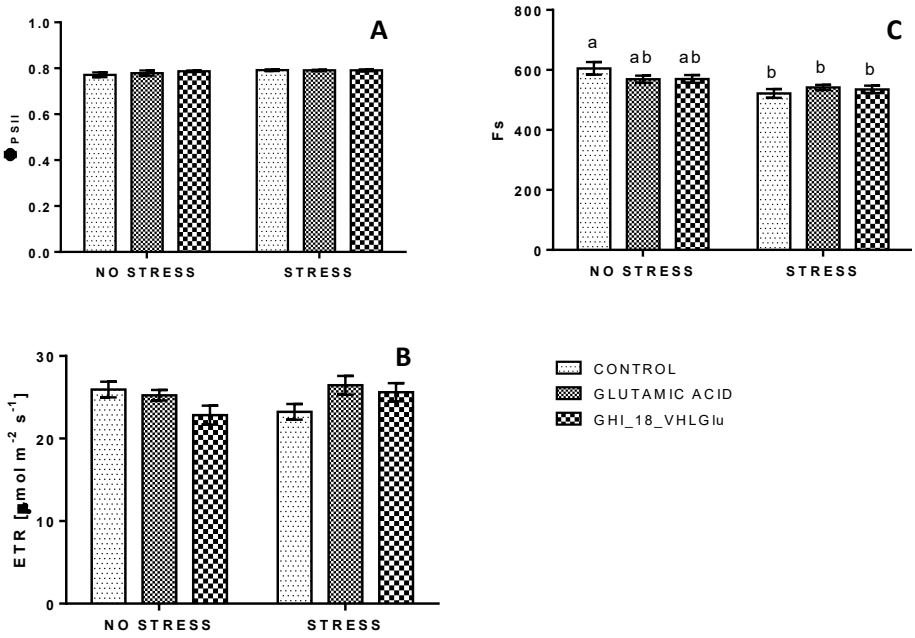


Figure 63. Effective PSII quantum efficiency (ϕ_{PSII}) (**A**) and electron transport rate (ETR) (**B**) and steady-state Chl fluorescence (Fs) (**C**) measured in lettuce leaves treated with water (CONTROL), glutamic acid and GHI_18_VHLGlu and grown under two water regimes (well-watered: **NO STRESS** and water stress and re-watering: **STRESS**). Measures were taken at the end of the cycle after the re-watering (12/07). Values are means ± SE (n =15). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

In particular, the value measured in non-stressed plants treated with water was significantly higher than those measured in stressed plants, regardless the treatment received (Figure 63 C).

1.4. Nitrate

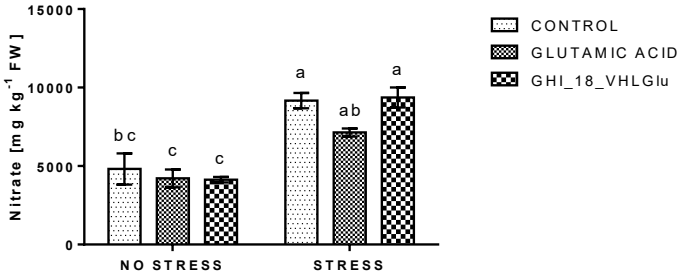


Figure 64. Nitrate content measured in lettuce leaves treated with water (CONTROL), glutamic acid and GHI_18_VHLGlu and grown under two water regimes (well-watered: **NO STRESS** and water stress and re-watering: **STRESS**). Measures were taken during the water stress (3/07). Values are means ± SE (n =3). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

The concentration of nitrate in lettuce leaves measured during the stress period was significantly affected by the water supply differently from the treatment applied 24 hours before (Figure 64). In particular, under non stressful conditions the average value was 4386 mg kg^{-1} FW whereas in stressed plants the nitrate concentration reached the value of 8559 mg kg^{-1} FW. Moreover, under stressed conditions a slight but not significant decrease in nitrate level appeared in plants treated with glutamic acid.

1.5. Water use efficiency

The water use efficiency of lettuce plants calculated as ratio between the fresh biomass at the end of the growing cycle and the total amount of water received from each plants is reported in Figure 65. Water stress significantly affected the WUE and in particular a significant decrease resulted in plants treated with glutamic acid. On the contrary the values calculated for plants treated with water or with the biostimulant prototype were not significantly different from those measured in non-stressed plants treated with the same product.

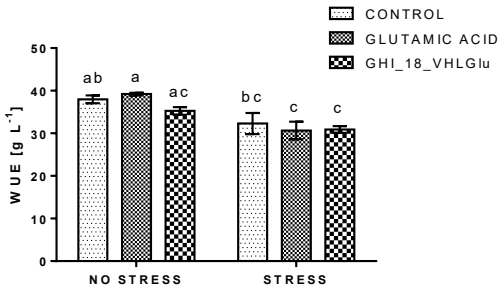


Figure 65. Water use efficiency of lettuce treated with water (CONTROL), glutamic acid and GHI_18_VHLGlu and grown under two water regimes (well-watered: **NO STRESS** and water stress and re-watering: **STRESS**). Measures were taken at the end of the growing cycle after the re-watering (12/07). Values are means ± SE (n =3). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences (P < 0.05).

1.6. Proline and osmolytes

The concentration of proline and osmolytes in lettuce leaves during the period of water deprivation was significantly affected by the stress but any effect resulted from the treatments applied 24 hours before (Figure 66 A and B). In particular, the average level of proline in non-stressed plants was about 18 µg g^{-1} whereas in those grown under water stress it reached the value of 451 µg g^{-1}. Similarly, the average concentration of osmolytes was 0.094 mOsm kg^{-1} g^{-1} in non-stressed plants and 0.194 mOsm kg^{-1} g^{-1} in stressed plants.

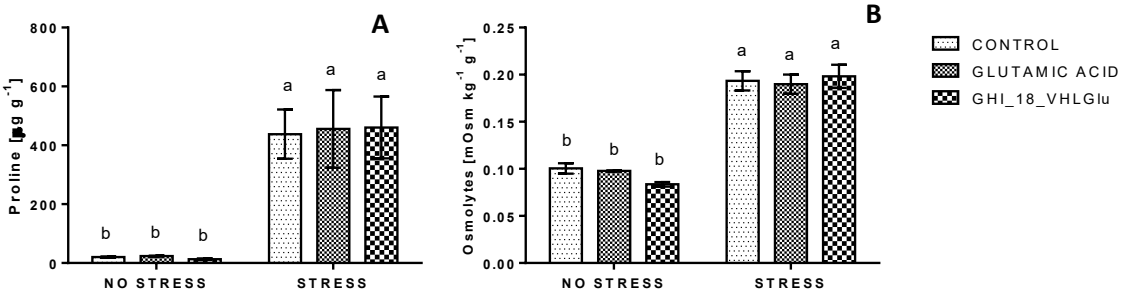


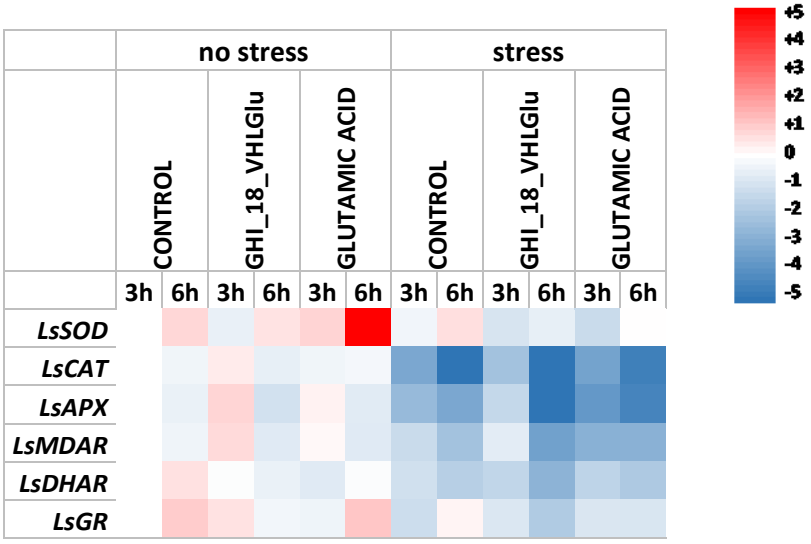
Figure 66. Proline and osmolytes concentrations in lettuce leaves treated with water (CONTROL), glutamic acid and GHI_18_VHLGlu and grown under two water regimes (well-watered: **NO STRESS** and water stress and re-watering: **STRESS**). Measures were taken during the water stress (3/07). Values are means ± SE (n =3). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences (P < 0.05).

1.7. Gene expression of LsSOD, LsCAT, LsAPX, LsMDAR, LsDHAR and LsGR

The changes in the expression of the genes involved in the ascorbate-glutathione cycle have been clustered into a heatmap (Table 21). Further on, a graph representing the expression analysis of each gene is presented (Figure 67). Different trends resulted in response to water stress, treatments and during time. Stressful

growing condition induced a general decrease in the expression levels of all genes, especially in *LsCAT* and *LsAPX* as shown by the blue colour shades in the heatmap.

Table 21. Heatmap showing temporal expression of selected genes in lettuce plants grown under water stress condition and treated with a water (CONTROL), GHI_18_VHLGlu and glutamic acid. Data are - ddCt calculated as - (Ct,Target - Ct,housekeeping)Time x - (Ct,Target - Ct,housekeeping)Time 0, where time x is any time point and time 0 is the expression of the target gene normalized to the housekeeping in non-stressed and non-treated samples after 3 hours. The rows represent the genes, and within each row the blue shaded areas indicate lower expression, whereas the red shaded areas indicate higher expression. No differences were visualized by white squares.



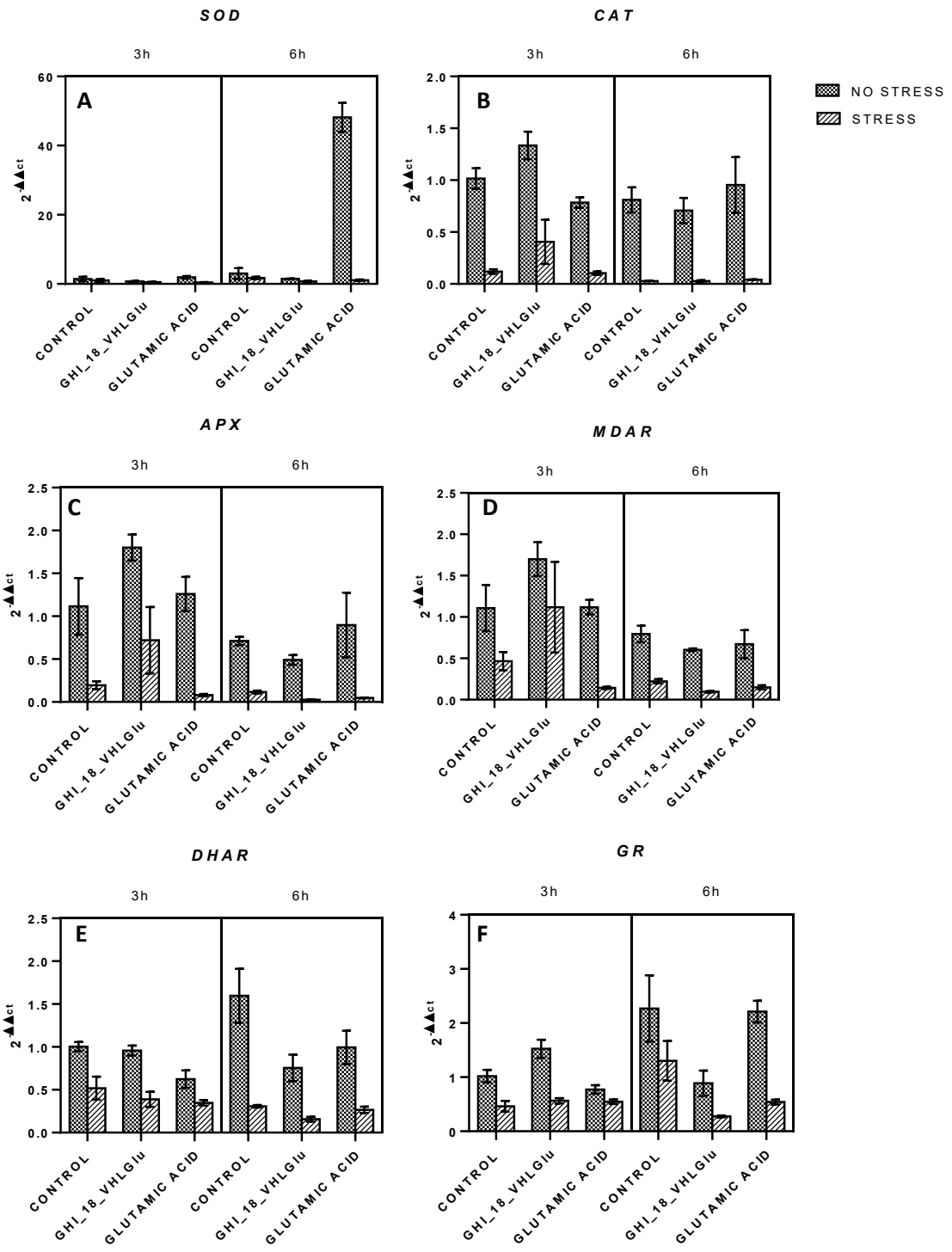


Figure 67. Changes in the expression of *LsSOD* (**A**), *LsCAT* (**B**), *LsAPX* (**C**), *LsMDAR* (**D**), *LsDHAR* (**E**), *LsGR* (**F**) in lettuce leaves treated with water (CONTROL), glutamic acid and GHI_18_VHLGlu and grown under two water regimes (well-watered: **NO STRESS** and water stress and re-watering: **STRESS**. Measures were taken 3 and 6 hours after the third treatment, before the re-watering. Values are means ± SE (n = 6). Data were subjected to three-way ANOVA.

3rd ACTIVITY

1.1. Yield

The yield of lettuce plants was significantly affected by the salt stress conditions as shown in the Figure 68. At the same time any effect resulted from the application of the glutamic acid or the biostimulant prototype. In particular, the average yields were about 668 g m^{-2} and 500 g m^{-2} in plants grown under optimal and non-stressful conditions, respectively.

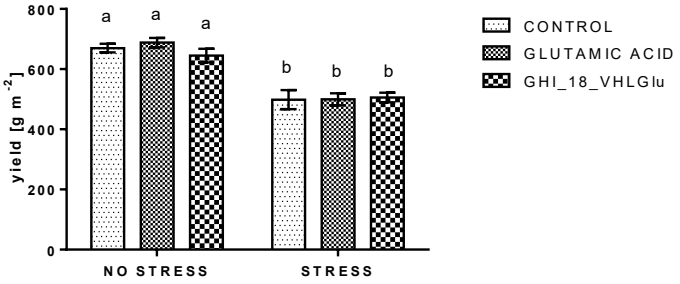


Figure 68. Yield of lettuce treated with water (CONTROL), glutamic acid and GHI_18_VHLGlu and grown under two salt levels (**NO STRESS** and 100 mM NaCl: **STRESS**). Measures were taken at the end of the growing cycle. Values are means ± SE (n =6). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

1.2. Chlorophyll

The levels of chlorophyll resulted significantly higher in plants subjected to salt stress if compared with those grown under optimal conditions (Figure 69) regardless the application of the glutamic acid or the biostimulant prototype.

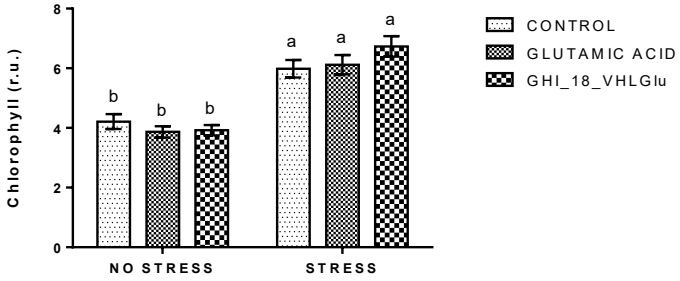


Figure 69. Chlorophyll content determined *in vivo* in lettuce leaves treated with water (CONTROL), glutamic acid and GHI_18_VHLGlu and grown under two salt levels (**NO STRESS** and 100 mM NaCl: **STRESS**). Measures were taken at the end of the growing cycle. Values are means ± SE (n =30). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

1.3. Chlorophyll a fluorescence

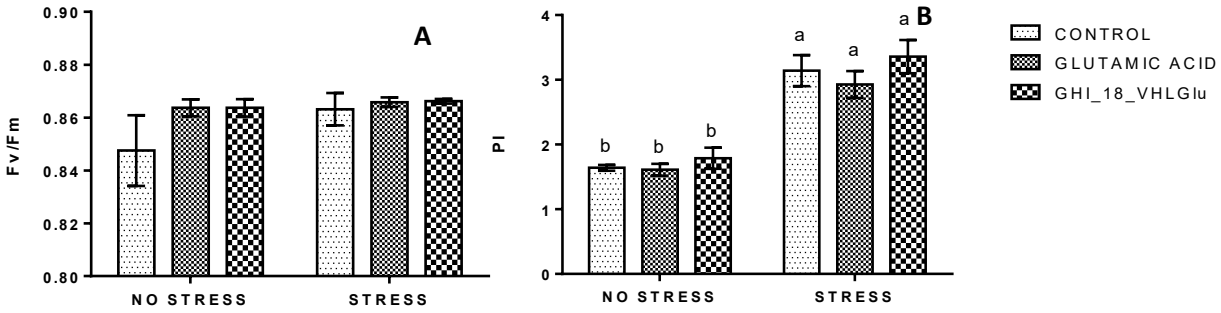


Figure 70. Maximum quantum efficiency of PSII (Fv/Fm) (**A**) and performance index (PI) (**B**) measured in lettuce leaves treated with water (CONTROL), glutamic acid and GHI_18_VHLGlu and grown under two salt levels (**NO STRESS** and 100 mM NaCl: **STRESS**). Measures were taken at the end of the growing cycle. Values are means ± SE (n =6). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

The maximum quantum efficiency of PSII (Fv/Fm) was not affected by the salinity of the growing conditions or by the treatments applied and almost all records were about 0.86 (Figure 70 A). A low average value and a high variability resulted in control plants grown under non-stressful conditions. On the contrary, the performance index (PI) was significantly increased in plants subjected to salt stress (Figure 70 B).

1.4. Gas exchanges

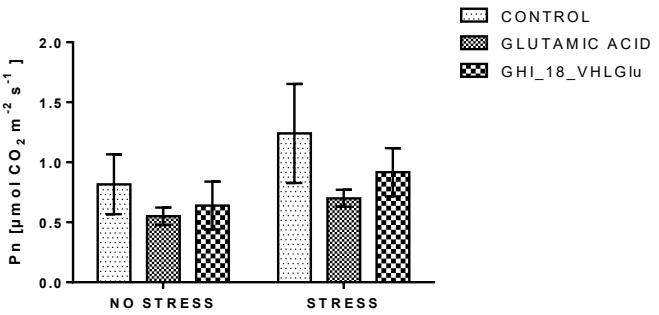


Figure 71. Net photosynthetic rate (Pn) measured in lettuce leaves treated with water (CONTROL), glutamic acid and GHI_18_VHLGlu and grown under two salt levels (**NO STRESS** and 100 mM NaCl: **STRESS**). Measures were taken at the end of the growing cycle. Values are means ± SE (n =5). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

The analysis of gas exchange and in particular the net photosynthetic rate (Pn) of lettuce leaves was slightly but not significantly higher in plants grown under salt stress conditions. However, the results obtained showed a high variability, in particular in response to water treatment (control). In both growing conditions the trends observed in response to the treatment were similar. The highest value was measured in control plants, the medium in plants treated with the biostimulant prototype, and the lowest in response to glutamic acid application.

1.5. Leaf nitrate

Salt stress has a significant effect on the concentration of nitrate in lettuce leaves. In particular, the levels measured in plants grown under high salinity (Figure 72). The application of glutamic acid and the prototype did not show any significant effect even if a slight increase resulted under stress conditions.

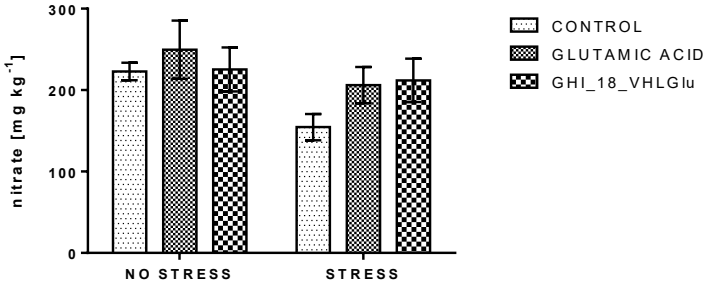


Figure 72. Nitrate content measured in lettuce leaves treated with water (CONTROL), glutamic acid and GHI_18_VHLGlu and grown under two salt levels (**NO STRESS** and 100 mM NaCl: **STRESS**). Measures were taken at the end of the growing cycle. Values are means ± SE (n =6). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

1.6. Proline and osmolytes

The concentration of proline in lettuce leaves was significantly affected by the salt stress (Figure 73 A). In particular, under non-stressful condition the average value was about 12 μg g^{-1} whereas in stressed plants values increased to reach a maximum of 234 μg g^{-1} in response to water treatments (control). However, the high variability resulted in control plants did not allowed to see any significant differences among the effect of the treatments under salt stress conditions.

The osmolytes concentration in lettuce leaves was significantly increased by the salt stress in plants treated with the glutamic acid and with the biostimulant prototype. No significant difference resulted in control plants (Figure 73 B).

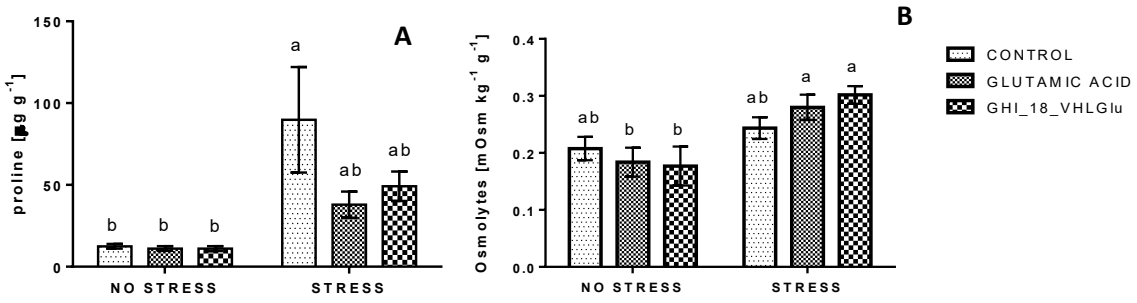


Figure 73. Proline (**A**) and osmolytes (**B**) concentration in lettuce leaves treated with water (CONTROL), glutamic acid and GHI_18_VHLGlu and grown under two salt levels (**NO STRESS** and 100 mM NaCl: **STRESS**). Measures were taken at the end of the growing cycle. Values are means ± SE (n =6). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

1.7. Abscisic acid

A significant effect of the salt stress resulted in the concentration of abscisic acid in lettuces leaves and ABA levels were generally low in plants grown under high salinity (Figure 74). Moreover, under non-stressful conditions the treatment with glutamic acid slightly increased the ABA accumulation. Due to the high variability of the results it was not possible to identify any significant difference.

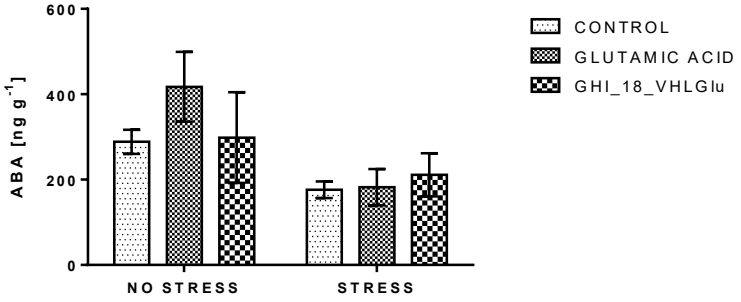


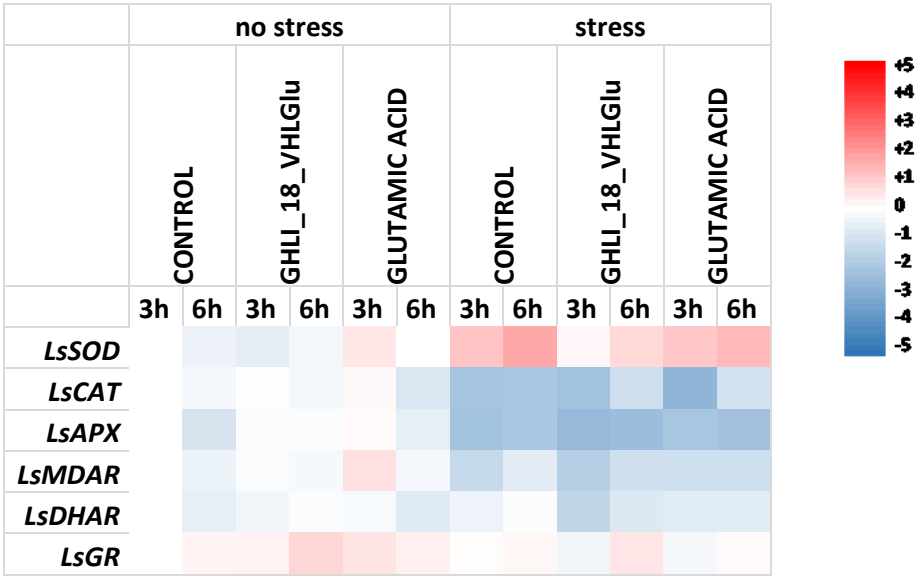
Figure 74. Abscisic acid concentration measured in lettuce leaves treated with water (CONTROL), glutamic acid and GHI_18_VHLGlu and grown under two salt levels (**NO STRESS** and 100 mM NaCl: **STRESS**). Measures were taken at the end of the growing cycle. Values are means ± SE (n =6). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

1.8. Expression analyses of LsSOD, LsCAT, LsAPX, LSMDAR, LsDHAR and LsGR

The changes in the expression of the genes involved in the ascorbate-glutathione cycle have been clustered into a heatmap (Table 22). Further on, a graph representing the expression analysis of each gene is presented (Figure 75). Different trends resulted in response to salt stress, treatments and during time. Stressful growing

condition induced a general decrease in the expression levels of *LsCAT*, *LsAPX*, *LsMDAR*, and *LsDHAR*. On the contrary, an increase in the expression resulted in *LsSOD* levels as shown by the colour shades in the heatmap.

Table 22. Heatmap showing temporal expression of selected genes in lettuce plants grown under salt stress condition and treated with a water (CONTROL), GHI_18_VHLGlu and glutamic acid. Data are - ddCt calculated as - (Ct,Target - Ct,housekeeping)Time x - (Ct,Target - Ct,housekeeping)Time 0, where time x is any time point and time 0 is the expression of the target gene normalized to the housekeeping in non-stressed and non-treated samples after 3 hours. The rows represent the genes, and within each row the blue shaded areas indicate lower expression, whereas the red shaded areas indicate higher expression. No differences were visualized by white squares



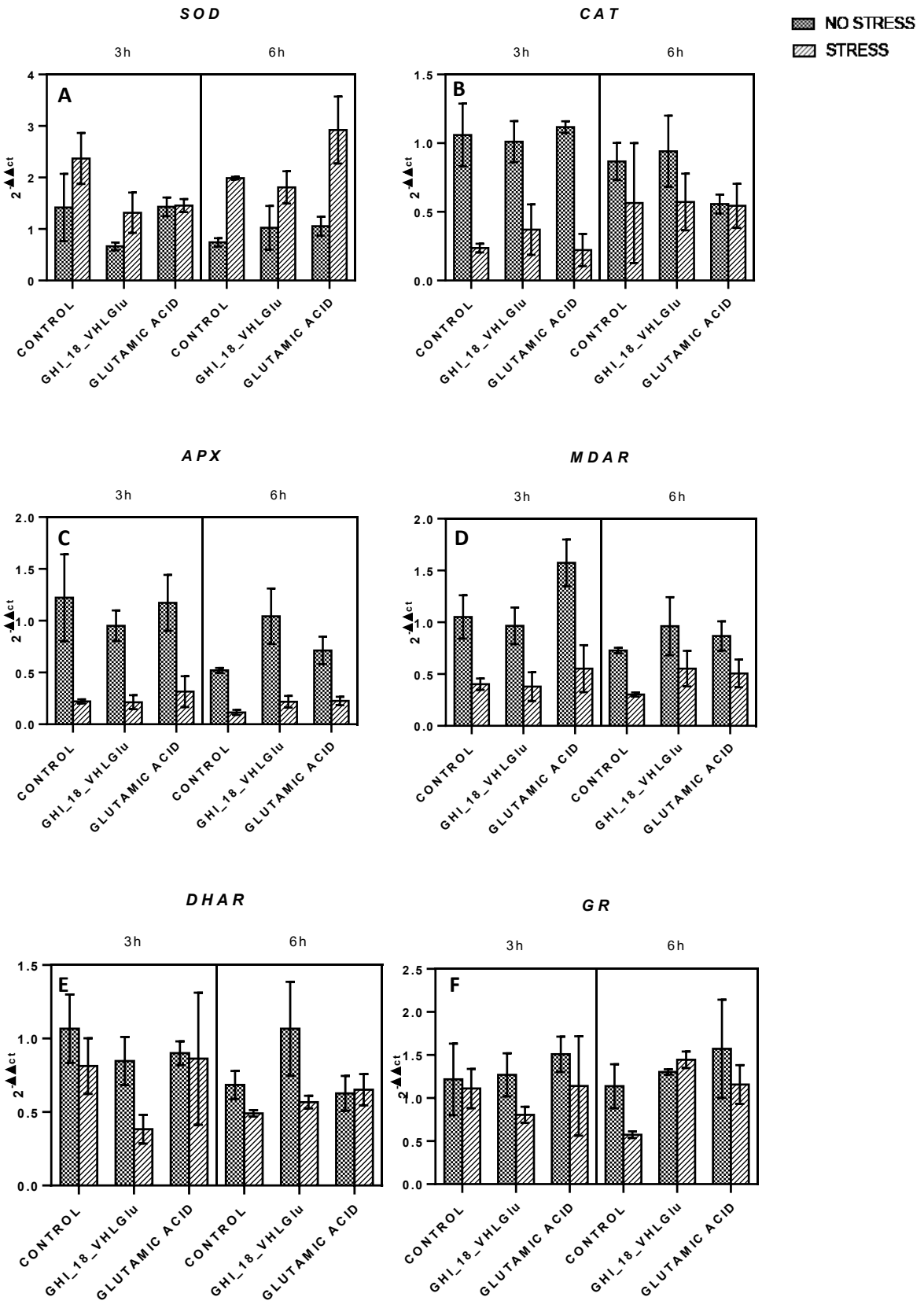


Figure 75. Changes in the expression of *LsSOD* (**A**), *LsCAT* (**B**), *LsAPX* (**C**), *LsMDAR* (**D**), *LsDHAR* (**E**), *LsGR* (**F**) in lettuce leaves treated with water (CONTROL), glutamic acid and GHI_18_VHLGlu and grown two salt levels (**NO STRESS** and 100 mM NaCl: **STRESS**). Measures were taken at the end of the growing cycle. Values are means ± SE (n =6). Data were subjected to three-way ANOVA.

DISCUSSION AND CONCLUSION

Among horticultural crops, leafy vegetables need constant soil moisture and they require high amount of water during all growing cycle. The aim of the work was the study of the efficacy of a biostimulant prototype on lettuce crop grown under stressful conditions. In the first activity we choose a baby leaf cultivar and the object was the study of plants response to a short period of water reduction in combination with the application of the biostimulant prototype GHI_16_VHL.

In common practice of baby-leaf production, during the week before the harvest and especially the evening before, irrigation is essential to maximize yield, increase the turgidity of the leaves and minimize postharvest losses. Indeed, one of the main quality traits of lettuce salad is the high water content [53]. Optimal substrate moisture tension was kept around -40 -50 kPa whereas in suboptimal conditions values were lowered to -110 kPa, in accordance to those reported by Hanson et al. [54]. These values were chosen in order to keep moisture deficit, without reaching a stressful condition for plants growth. Results obtained in this study demonstrated that plants are able to perceive a water reduction around 30% without showing the typical stress responses. Plants exposed to water stress generally increase osmolytes accumulation in their tissues in order to increase water uptake and maintain cell turgor pressure [55]. In our experiment, a slight but not significant accumulation of osmolytes was observed in control plants grown in suboptimal conditions, both in the first and in the second trial. A similar effect resulted in the final yields too and non-treated plants had lower values in response to water reduction if compared to those grown under optimal conditions. The decrease in final yield was more evident in control plants during the first growing cycle, and this make sense since water supply is particularly critical in summer season. Overall, no significant effect resulted in lettuce yield in response to treatments or irrigation levels. Agami, [56] reported that lettuce plants grown with a reduction in irrigation water regime around 40% of field capacity showed a significative decrease both in fresh and dry weight. Our results confirmed that a decrease of water around 30% for a limited period is not enough to affect lettuce growth, even in baby leaf production.

Total sugars and sucrose concentration showed different trends in response to treatments applications. In particular, treatments with the biostimulant prototype strongly decreased their levels, mostly during the first growing cycle. Since lettuce yield did not decrease in the same plants, it probably means that GHI_16_VHL did not interfere with plants growth metabolisms. Moreover, neither net photosynthetic rate nor chlorophyll content and the efficiency of PSII changed in response to the same treatment, meaning that the low levels of sugars are not due to a deficient biosynthesis machinery. The possible explanation for this trend of changes is that the biostimulant prototype is able to stimulate chemical reactions regulated by sucrose. In plants treated with GHI_16_VHL + proline the effect was less conspicuous. It might be related to the effect of proline as

reported also by Moustakas *et al*., [57]. They observed that exogenous proline positively affected sugars accumulation in *Arabidopsis thaliana* leaves under water stress conditions.

Photosynthetic pigments are frequently used as indicator of plants physiological status [58]. The high level of chlorophyll in plants treated with glutamic acid obtained in the first trial might have sense since this amino acid is a precursor in the biosynthesis of chlorophyll [59]. Unfortunately, the second growing cycle did not confirm the results. Moreover, changes in chlorophyll content have not been reflected in leaves colours, as resulted from leaves colour analyses.

The same trend observed in chlorophyll content, appeared also in the analysis of carotenoids concentration. The highest level of these pigments concurrently with the highest level of chlorophyll in plants treated with glutamic acid and grown with less water might be explained by their role in chloroplasts. Indeed, in addition to the activity as accessory pigments harvesting light, carotenoids are essential in photoprotection of photosynthetic apparatus [60].

There are conflicting reports concerning the effects of exogenous proline on chlorophyll and carotenoids; Some authors reported an increase of photosynthetic pigments in fennel [61], maize [62] or fava beans [63] after the application of 20, 25 or 30 mM proline but, for instance, in the same study on fava beans, a treatment with 50 mM proline caused significant decreases in chlorophyll and carotenoids content. In addition, Hare *et al*., [47] observed a dose-dependent damage to chloroplasts in *Arabidopsis thaliana*.

The analysis of chlorophyll *a* fluorescence provides useful information about the photosynthetic apparatus status and the efficiency of photosynthesis. Almost all chlorophyll fluorescence parameters did not show any statistical differences in response to treatments neither in response to water reduction. Fv/Fm is the maximum quantum yield of PSII and is normally used as indicator of the health status of plants even if it is not very sensitive to early changes of photosynthesis induced by water stress [64]. Optimal values in herbaceous crops are around 0.83 – 0.84 while lower values usually mean that plants are exposed to some stresses causing photoinhibition. Similar trend was observed in the effective PSII quantum efficiency of illuminated sample (ϕ_{PSII}). During the second growing cycle a low value resulted in plants treated with glutamic acid and grown with less water. Since the other analyses performed did not indicate any damages caused by glutamic acid treatments, the low value might be related to the high variability observed among replications.

A very sensitive parameter that correlate with stress and gives us information about the status of both photosystem I and II is the performance index (PI). PI values usually decline with the decrease of relative water content and so in water stress condition. At the same time, high PI values might be considered as a defensive mechanism of plant on moderate water stress [65–68]. In the present study, despite small changes in response to water amount and treatments, all PI values were not statistically different from the control plants grown in optimal conditions.

The dissipation of energy per reaction centre (DI_0/RC) showed an opposite trend in well-watered plants treated with glutamic acid comparing the two growing cycles. High values of this parameter are usually associated to photoinhibition stress [69], but it is also related with the number of RC. As observed in the results above, RC/CSm index was lower in plant grown without water reduction, thus the highest level of DI_0/RC in glutamic acid treated samples doesn't imply a stress condition. Indeed, Lv et al., [35] showed that the application of glutamic acid as foliar spray on non-stressed *Crataegus pinnatifida* positively affected chlorophyll fluorescence and dissipation energy index decreased.

The low ETR parameters obtained in the first growing cycle are due to the low PAR values measured. Moreover, both in the first and in the second growing cycle ETR levels were higher in plants treated with glutamic acid and subjected to water reduction if compare with control.

In addition to photosynthetic pigments, phenols compounds are usually investigated in plants. They are a group of secondary metabolites with several roles, they are involved in the colour of fruits and flowers, but they are also functional as antioxidants in scavenging peroxyl radicals in plant tissues [70]. In the second growing cycle a significant increase was observed in lettuce plants treated with proline. This is in accordance to those reported by Kwok and Shetty [71]. Indeed, they proposed that proline can stimulate the pentose phosphate, shikimate and phenylpropanoid pathways, and ultimately lead to an increase in phenolic synthesis. Moreover, Aksakal *et al*., [72] found that exogenous proline increases total phenolic concentration in the seedlings of lettuce exposed to UV-B radiation. Lettuce is a good source of several nutrients and it is considered to have a good antioxidant capacity, also due to its phenolic compounds composition [73].

Gas exchanges analysis is a useful non-destructive method frequently used to evaluate the health-status of the photosynthetic apparatus. Results obtained in the first cycle did not show any differences whereas in the second trial different trends appeared. Control plants, as expected, showed a decrease of E, gs and Pn after water reduction. Stomatal closure, indeed, is one of the earliest plant responses to changes in water amount since its role in the prevention of water loss. This phenomenon limits CO_2 diffusion in chloroplast and, subsequently affects plant photosynthesis. Plants treated with glutamic acid had the same trend of control while plants treated with proline showed an opposite response to water reduction and, gas exchange parameters increased. The same trend has been observed also in other species after water deprivation [74,75]. Results obtained in our study showed that glutamic acid treatments do not affect photosynthesis or transpiration. In contrast, Lv *et al*., [35] observed a significant increase in Pn, gs and E in response to 800 mg L^{-1} glutamic acid treatments on Hawthorn. This different response might be related to the lower concentration applied in our study compared with the mentioned experiment. The effects of proline treatments enhancing photosynthetic activities were also noticed by Zouari *et al*., [76] in palm tree grown in Cadmium stress condition, and by Ben Ahmed *et al.* [77] in olive tree grown under salt stress. Treatments with the biostimulant prototype alone or in combination with proline had a positive effect increasing the net photosynthetic rate in

plants grown under suboptimal conditions, whereas gs and E were similar to the control. A positive effect on net photosynthesis rate was found in response to several biostimulant products application on different species [78–81].

A direct consequence of increased net photosynthetic rate in plants treated with the biostimulant product was the enhanced water use efficiency. Similarly, other authors reported an increase in WUE after the application of biostimulants, such as extract of *A. nodosum*, leaves of *Moringa oleifera* and arbuscular mycorrhizal fungi [33,82–85].

Leafy vegetable market is subjected to strict regulation because of the harmful repercussion of dietary nitrate compounds on human health and their correlation with cancer incidence [86–88]. Actually, despite there are several evidences also about the positive effects [89] of nitrate consumption, the commercialization of some vegetable products is still limited [90]. The thresholds of nitrate concentration in lettuce have been established by the European Commission Regulations (EC) n° 1881/2006 and 1258/2011 [91]. In our study we referred to 4000 mg kg^{-1} FW for the first growing cycle and 5000 mg kg^{-1} FW for the second one according to the harvesting periods and the growing environment. Results obtained were always below the thresholds and levels measured in the second trial were almost ten times lower than those detected in the first one. Nitrate reduction in plants treated with proline is in accordance to those observed in *Phaseolus vulgaris* grown under salt stress conditions [45] and in pear leaves [92]. This might be related to the protective activity of proline on nitrate reductase observed in vitro by Sharma *et al*., [93]. Little information concerning the effects of exogenous application of glutamic acid on nitrate metabolism is available in literature. Haghighi, [41] showed that, when glutamic acid (100 mg L^{-1}) is added in a nutrient solution to replace a portion of Nitrogen source, the nitrate content in lettuce leaves does not significantly change. This means that glutamic acid stimulated NO_3 uptake and compensates the Nitrogen reduction. Moreover, glutamic acid increased the nitrate reductase activity.

As mentioned before, compatible osmolytes are usually accumulate during stress. They are amino acids, sugars, proline, and other small molecules. Plants exposed to water stress generally show an increase of osmolytes concentration in their tissues in order to increase water uptake and maintain cell turgor pressure [55]. In our experiment water deficit had a significant ($P < 0.05$) effect on osmolyte accumulation and, plants that received less water showed higher values than non-stressed ones. The increase was around 20% in control plants and 16% in plants treated with proline, in both cycles. No changes have been observed after glutamic acid treatment. It could be explained as a missed response to a non-optimal condition or as a better tolerance. Sometime is difficult to understand if the accumulation of a molecule related to a stress is a negative consequence or an adaptive response activated to counteract the adverse condition.

Exogenous proline application does not affect neither osmolytes nor free proline content. Moreover, water levels didn't cause any significant increase in proline concentration, regardless of the treatment received

during our experimentation. A similar result has been seen by Dawood *et al*., [63] in *Vicia faba* plants. In contrast, it is well known that proline accumulation in plant tissues is a common phenomenon observed in response to water stress since its activity both as osmoprotectant and ROS scavenging inducing abiotic stress tolerance [43,62,94–96] and Moustakas *et al*., [57] observed an increase of free proline in *Arabidopsis thaliana* after the application of proline in drought stress.

Lipid peroxidation of membranes is a common effect of several stresses and is measured as the level of malondialdehyde, a product of the breakdown of fatty acids. The extent of damage is commonly used as biochemical marker of oxidative stress in vegetable and fruits. Several studies showed that drought stress enhances the amount of ROS in plant tissues and that lipid peroxidation in leaves membrane is positively correlated with the level of water stress in different crops [97–100]. Basing on our results we can say that the amount of water deprivation in our work was not enough to cause membrane damages in lettuce leaves and measured values were similar to those found in another work on lettuce [101]. In contrast, a reduction of lipid peroxidation has been observed after proline application in several plant species [102–106].

Protective effect of exogenous proline has been linked to its ability detoxifying ROS induced by different stressful condition [107,108]. A few discordant information about the effect of glutamic acid treatment has been found in literature. Some authors observed that exogenous glutamic acid in combination with Zinc reduced the rate of lipid peroxidation induced by Cadmium toxicity in *Ceratophyllum demersum* [109]. Wang *et al*., [110] instead, did not seen any reduction of MDA concentration in rice leaves after the amino acid application.

ABA plays a central role regulating physiological responses in plants under several stressful and non-stressful conditions. It's well known that water deprivation triggers the production of abscisic acid since its involvement in the regulation of plant water status [111–113], thus, a slight but not significant increase of the concentration of this phytohormone in plants subjected to water reduction observed during the first growing cycle was expected. Only plants treated with proline showed an opposite trend. Results obtained in the second trial did not confirm those observed in the first one.

Results achieved in the first activity show that in our experimental conditions, a short period of water reduction even before the harvest does not impair the production of baby leaf. Any clear effect emerged from the application of the biostimulant prototype, alone or in combination with proline.

The differences observed between the two growing cycles might be due to the diverse environmental conditions during the growing period. Indeed, the first growing cycle was carried out in summer whereas the second one in autumn. Although plants grown in the same greenhouse under controlled conditions, plants growth was slower in autumn than in summer. Consequently, the period of water reduction was longer in the second growing cycle, and this might have affected the results. Another reason might be due to the complex

nature of biostimulant prototypes and their non-constant effect if tested in different environmental conditions or on different crops/varieties.

For all these reasons the formulation of the biostimulant prototype GHI_16_VHL was revised and the experimental plans for the following steps were adjusted. In particular, in cooperation with the private company we decided to increase the number of applications from 2 to 4. Thus, we choose a different lettuce cultivar with a longer growing cycle. Since during the first experiments the water reduction was not enough to stress the plants, in order to test the efficacy of the new formulation under water stress condition, we decide to suspend the water supply until plants showed the first symptoms of stress. Moreover, the comparison with proline was excluded from the new experimental plan. The treatments with the new prototype called GHI_18_VHLGlu and the glutamic acid were applied two time before the water stress, one time during the stress and the last time during the re-watering period in order to evaluate its efficacy also during the recovery. Indeed, it is known that some biostimulant products helps plants to better recovery from abiotic stresses [114].

At the end of the growing cycle the yield of plants subjected to a water stress did not reached the same levels of those grown under constant irrigation and the treatment did not increase the final production. The same result was confirmed also by the measurement of the dry weight. This might suggest that either the biostimulant prototype or the glutamic acid application do not alter the primary metabolism but at the same way they are not involved in the accumulation of the biomass ang growth processes. As mentioned above, glutamic acid is a precursor in the biosynthesis of chlorophyll and in the first growing cycle performed with the previous prototype product (GHI_16_VHL), the concentration of chlorophyll increased in plants treated with the glutamic acid solution. In response to the increased number of applications in the new experiment, we expected to see an increase in chlorophyll content. What we observed was that during the water stress the level of chlorophyll concentration decreased regardless the treatments. Similarly, at the end of the growing cycle the levels recovered at the same way of the plants treated with water. Glutamic acid has an essential role in amino acids metabolism and in the assimilation of ammonia in plants [115]. It is also known that a typical plant response to water stress is the accumulation of osmolytes such as soluble sugars, amino acids and other compatible solutes, aiming to protect the cellular machinery and to facilitate the osmotic adjustment [116–118].

In addition, Liu et al. [119] reported that the main pathway for the synthesis of proline under water stress is from glutamic acid. In our experiment the levels of proline and osmolytes increased and the amount of glutamic acid provided by the treatments might be have involved in the mechanisms to cope the negative effects of the water stress rather than the synthesis of chlorophyll.

The chlorophyll *a* fluorescence as well as the level of fluorescence was affected by the stress. Usually, the Fv/Fm ratio is used as marker of a stress and the optimal value is about 0.83. In this experiment the Fv/Fm of

stressed plants was 0.84 whereas in non -stressed plants the average value was 0.86 during the water deprivation. This could mean that the functionality of the photosynthetic apparatus was declining as effect of the shortage irrigation. Probably since the value was still higher than 0.83 and after 24 h the water supply was restored plants fully recovered as demonstrated from the same measured performed at the end of the growing cycle. This was confirmed also by the analysis of the effective quantum efficiency of PSII and by the performance index. Even though Fv/Fm and the PI completely restored to non-stressful value after the re-watering, the steady-state fluorescence (Fs) of control plants subjected to the stress was significantly lower than that measured in control plants grown under constant irrigation. The relation between water stress and Fs is currently exploited to have a rapid assessment of plant status, mostly at canopy level [120,121].

The high level of nitrate determined under water stress condition might be due to a decrease activity of the nitrate reductase. Indeed, it is known that the activity of this enzyme is inhibited when soil moisture decrease, as observed in several crops. Another reason of this increase could be related to the role of nitrate as osmotic regulator [122]. As reported above, the concentration of nitrate in leafy vegetables is subjected to regulation. As in the previous experiment, the threshold value we referred is 4000 mg kg-1 FW according to the harvesting periods and the growing environment. Results obtained were slightly higher in plants grown under non stressful conditions and two times higher in stressed plants. However, the measurement was taken during the water deprivation and not at the end of the growing cycle when plants were at harvest stage and nitrate accumulation is generally high in young leaves [123].

In order to evaluate the potential antioxidant activity of the biostimulant prototype, we also studied the expression of the genes for the biosynthesis of the enzymes involved in the ascorbate-glutathione cycle and in the ROS scavenging. Drought stress affect photosynthetic activity and leads to photoinhibition and is associated with enhanced levels of ROS. Since at high concentrations they are toxic for the cells, during stress conditions plants increase the antioxidant system in order to remove them and avoid oxidative stress.

Lettuce leaves for this analysis were sampled after 3 and 6 hours from the third treatment, applied during the water stress. From the results obtained in this experiment emerged that in stressed plants the expression of all genes was strongly decreased, in particular for *LsCAT* and *LsAPX* after 6 hours. The increase of CAT and APX activity in removing H_2O_2 produced through photorespiration during water stress has been reported in several plant species [124]. At the same time several authors also reported that the activity of the genes involved in ROS detoxification changes among plant species. Moreover, since the presence of several isoforms in cytosol, mitochondria and chloroplast, they showed how the cytosolic fraction were more active than the chloroplastic one in sorghum whereas an opposite situation was found in sunflower plants [125]. It has also been reported that in apple leaves severe drought stress decrease the activity of these enzymes and after a re-watering period it increase again [126]. In our experiment we observed that plant fully recovered at the end of the growing cycle, meaning that water stress period did not impair any essential process permanently.

Oh et al [127] observed an overall activation of genes involved in the secondary metabolism and antioxidant biosynthesis such as the phenylalanine ammonia-lyase (PAL), γ-tocopherol methyl transferase (γ-TMT) and l-galactose dehydrogenase (l-GalDH) in lettuce plants grown under protected condition and subjected to water stress. These genes are involved in the biosynthesis of phenolic compounds and flavonoids which exert antioxidant activity. It could be possible that in our experimental condition, despite the downregulation of the genes involved in the ascorbate-glutathione cycle, lettuce plants activated other antioxidant processes to protect from water stress damages.

It might be interesting to study the gene expression at different time points, to see if the expression will increase later in time or during the re-watering period.

Furthermore, the isoform of the genes chosen in this experiment were located in chloroplast or mitochondria, so it might be interesting to evaluate the expression of other isoforms located in different cell compartments.

Another experiment was conducted in parallel in order to test the biostimulant prototype also in under high salinity conditions. Salt stress together with water stress is one of the most severe environmental stress affecting plant growth and productivity. Especially in Mediterranean regions, where the use of water from the ground wells caused seawater intrusion, it represents a serious problem for commercial horticulture. Indeed, the high levels of EC in water used for irrigation can overcome the threshold tolerated by most of the plants [18,128]. The application of biostimulant products as a strategy to face the negative effect of salt stress has been evaluated from several authors [114,129–131]. Lucini et al. [132] observed that the application of a plant-derived protein hydrolysate on lettuce salad increased the fresh yield, dry biomass and plant performance under salinity conditions (NaCl 25 mM) if compared to untreated plants, probably related to a more extensive roots apparatus.

In our experiment the yield was significantly affected by the high salinity on the growing media regardless the application of the glutamic acid or the biostimulant prototype. It makes sense since one of the first effects of high salinity is a stunted growth due to the reduce ability of plants to absorb water from the growing media. Indeed, the first phase of salt stress is represented by the osmotic stress and it similar to those caused by drought [133]. Moreover, the low yield of lettuce plants grown under salt stress conditions could be attributed to a decrease in the nutrient uptake. The lack of effect observed in response to treatment could be due to the severe salt stress. Indeed, the NaCl concentration in the nutrient solution was 100 mM, much higher than the level tested in the paper mentioned before [132].

In our experimental conditions we observed that the level of chlorophyll measured *in vivo* and the PI were positively affected by the high salinity whereas the Fv/Fm was similar to those measured in the non-stressed plants. higher chlorophyll content might be associated to grater photosynthetic rates observed in the same plants. Indeed, even if the increase was not significant the values recorded in stressed plants were slightly higher than those measure in plants grown under optimal conditions. Similar results were observed in

Cucumis sp., *Salvinia auriculate*, *Dunaliella salina* and rice subjected to different levels of salt stress [134–137]. The measurement of chlorophyll *in vivo* is a measure that correlates the green colour of the leaves with the content of chlorophyll. It is known that chlorophyll *a* is the main pigment involved in the photosynthetic activity whereas chlorophyll *b* plays a role as accessory pigment. Moreover, chlorophyll *a* absorbs energy from wavelengths of blue-violet and orange-red light and it is responsible for the green colour of the leaves while chlorophyll *b* absorbs energy from wavelengths of green light. Gomes et al. [135] observed an increase in chlorophyll a and a decrease in chlorophyll b content in response to salt stress. It was in line with other studies reporting that salt stress affect more chlorophyll *b* than chlorophyll *a* [138]. Moreover, since the first step in the degradation of chlorophyll *b* is its conversion in chlorophyll *a* [139], this might explain the high levels of greenness measure in lettuce leaves in our experimental conditions. No significant effect resulted in response to glutamic acid or the biostimulant prototype. The concentration of nitrate in lettuce leaves at the end of the growing cycle was significantly affected by the stress and a general decrease in nitrate levels was observed in plants subjected to high salinity. This effect has been reported also by other authors and it is probably due to a limited nitrate uptake and nitrate reductase activity [140].

The concentration of osmolytes resulted significantly higher in stressed plants treated with glutamic acid and GHI_18_VHLGlu if compared with those grown under optimal condition. On the contrary the level measured in control plants unchanged. This might be related to the role of glutamic acid in amino acid metabolism and the amino acid content provided by the biostimulant prototype treatment and their role as compatible osmolyte in plants. This is a typical effect observe in response to salt stress and it might also mean a plant response to face the stressful condition.

Abscisic acid plays a central role in plant responses to stress, both in the regulation of several gene expression and in the mechanism of stress signal transduction [111,141,142], thus the low levels measure in lettuce leaves grown under salt stress were unexpected.

Similar to those observed in the previous experiment with the new biostimulant prototype, also in the present study, the expression of the genes involved in ascorbate-glutathione cycle was generally low in plants subjected to the high salinity. The only exception was the expression of *SOD* which catalysed the reaction transforming the superoxide anion to hydrogen peroxide and oxygen. Results achieved showed that salt stress and water stress conditions applied in our experiments strongly affected the lettuce production and even after the modification of the prototype formulation and the increase in the number of applications the biostimulant prototype did not show any clear effect. The experimental approach based on the comparison between the effect of the biostimulant prototype and the effect of the single compounds present in the product turned is a useful strategy to understand the biostimulant activity. Moreover, it confirms the complexity of biostimulants properties and that the effect of a product may not be ascribed to the sum of the effects of their components but, more likely, to their interaction.

REFERENCES

1. Acharya, S.K.; Shukla, Y.R.; Khatik, P.C. Effect of Water Regime on Growth and Yield of Lettuce (Lactuca Sativa L .). *N Save Nat. to Surviv.* **2013**, *8*, 201–206.
2. de Pascale, S.; Costa, L.D.; Vallone, S.; Barbieri, G.; Maggio, A. Increasing water use efficiency in vegetable crop production: From plant to irrigation systems efficiency. *Horttechnology* **2011**, *21*, 301–308.
3. Oh, M.-M.; Carey, E.E.; Rajashekar, C.B. Regulated Water Deficits Improve Phytochemical Concentration in Lettuce. *J. Am. Soc. Hortic. Sci.* **2019**, *135*, 223–229.
4. Bozkurt, S.; Mansuroğlu, G.S.; Kara, M.; Önder, S. Responses of lettuce to irrigation levels and nitrogen forms. *African J. Agric. Res.* **2009**, *4*, 1171–1177.
5. Capra, A.; Consoli, S.; Russo, A.; Scicolone, B. Integrated agro-economic approach to deficit irrigation on lettuce crops in Sicily (Italy). *J. Irrig. Drain. Eng.* **2008**, *134*, 437–445.
6. Capra, A.; Consoli, S.; Scicolone, B. *Deficit irrigation: Theory and practice*; 2008; ISBN 9781604565799.
7. Fereres, E.; Soriano, M.A. Deficit irrigation for reducing agricultural water use. *J. Exp. Bot.* **2007**, *58*, 147–159.
8. Malejane, D.N.; Tinyani, P.; Soundy, P.; Sultanbawa, Y.; Sivakumar, D. Deficit irrigation improves phenolic content and antioxidant activity in leafy lettuce varieties. *Food Sci. Nutr.* **2018**, *6*, 334–341.
9. Kang, S. Controlled alternate partial root-zone irrigation: its physiological consequences and impact on water use efficiency. *J. Exp. Bot.* **2004**, *55*, 2437–2446.
10. LUO, H.H.; ZHANG, Y.L.; ZHANG, W.F. Effects of water stress and rewatering on photosynthesis, root activity, and yield of cotton with drip irrigation under mulch. *Photosynthetica* **2016**, *54*, 65–73.
11. Huang, B.; Tu, J.; Acharya, K.; Yan, D. Effects of water stress and rewatering on growth and photosynthetic parameters of Typha orientalis Presl in coexistence conditions. *Desalin. Publ.* **2018**, *122*, 78–84.
12. Mancuso, S.; Azzarello, E.; Mugnai, S. Marine bioactive substances (IPA extract) improve foliar ion uptake and water stress tolerance in potted Vitis vinifera plants. **2006**.
13. Goñi, O.; Quille, P.; O'Connell, S. Ascophyllum nodosum extract biostimulants and their role in enhancing tolerance to drought stress in tomato plants. *Plant Physiol. Biochem.* **2018**, *126*, 63–73.
14. Petrozza, A.; Santaniello, A.; Summerer, S.; Di Tommaso, G.; Di Tommaso, D.; Paparelli, E.;

Piaggesi, A.; Perata, P.; Cellini, F. Physiological responses to Megafol® treatments in tomato plants under drought stress: A phenomic and molecular approach. *Sci. Hortic. (Amsterdam).* **2014**, *174*, 185–192.

15. Munns, R. Genes and salt tolerance: bringing them together. *New Phytol.* **2005**, *167*, 645–663.
16. Shannon, M.C.; Grieve, C.M. Tolerance of vegetable crops to salinity. *Sci. Hortic. (Amsterdam).* **1998**, *78*, 5–38.
17. Colla, G.; Rouphael, Y.; Leonardi, C.; Bie, Z. Role of grafting in vegetable crops grown under saline conditions. *Sci. Hortic. (Amsterdam).* **2010**, *127*, 147–155.
18. Miceli, A.; Moncada, A.; D'Anna, F. Effect of salt stress in lettuce cultivation. *Acta Hortic.* **2003**, *609*, 371–375.
19. Ray, P.D.; Huang, B.-W.; Tsuji, Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell. Signal.* **2012**, *24*, 981–990.
20. Lushchak, V.I. Free radicals, reactive oxygen species, oxidative stress and its classification. *Chem. Biol. Interact.* **2014**, *224*, 164–175.
21. Mittler, R. ROS Are Good. *Trends Plant Sci.* **2017**, *22*, 11–19.
22. Vranova, E. Signal transduction during oxidative stress. *J. Exp. Bot.* **2002**, *53*, 1227–1236.
23. Halliwell, B. Reactive Species and Antioxidants. Redox Biology Is a Fundamental Theme of Aerobic Life. *Plant Physiol.* **2006**, *141*, 312–322.
24. Mittler, R.; Vanderauwera, S.; Gollery, M.; Van Breusegem, F. Reactive oxygen gene network of plants. *Trends Plant Sci.* **2004**, *9*, 490–498.
25. Boguszewska, D.; Zagdask, B. ROS as Signaling Molecules and Enzymes of Plant Response to Unfavorable Environmental Conditions. In *Oxidative Stress - Molecular Mechanisms and Biological Effects*; InTech, 2012.
26. Noctor, G.; Foyer, C.H. ASCORBATE AND GLUTATHIONE: Keeping Active Oxygen Under Control. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 249–279.
27. *Ascorbate-Glutathione Pathway and Stress Tolerance in Plants*; Anjum, Nas.; Springer Netherlands: Dordrecht, 2010; ISBN 978-90-481-9403-2.
28. Pandey, P.; Singh, J.; Achary, V.M.M.; Reddy, M.K. Redox homeostasis via gene families of ascorbate-glutathione pathway. *Front. Environ. Sci.* **2015**, *3*.
29. Wahba, H.E.; Motawe, H.M.; Ibrahim, A.Y. Growth and chemical composition of Urtica pilulifera L. plant as influenced by foliar application of some amino acids. *J. Mater. Environ. Sci.* **2015**, *6*, 499–506.
30. Tsouvaltzis, P.; Koukounaras, A.; Siomos, A.S. Application of Amino Acids Improves Lettuce

Crop Uniformity and Inhibits Nitrate Accumulation Induced by the Supplemental Inorganic Nitrogen Fertilization. *Int. J. Agric. Biol.* **2014**, *16*, 951–955.

31. Moreira, A.; Moraes, L.A.C. Yield, nutritional status and soil fertility cultivated with common bean in response to amino-acids foliar application. *J. Plant Nutr.* **2017**, *40*, 344–351.
32. Rai, V.K. Role of amino acids in plant responese to stresses. *Biol. Plant.* **2002**, *45*, 481–487.
33. Calvo, P.; Nelson, L.; Kloepper, J.W. Agricultural uses of plant biostimulants. *Plant Soil* **2014**, *383*, 3–41.
34. Nardi, S.; Pizzeghello, D.; Schiavon, M.; Ertani, A. Scientia Agricola Plant biostimulants: physiological responses induced by protein hydrolyzed-based. *Sci. Agric. v* **2016**, *73*, 18–23.
35. Lv, D.G.; Yu, C.; Yang, L.; Qin, S.J.; Ma, H.Y.; Du, G.D.; Liu, G.C.; Khanizadeh, S. Effects of foliar-applied L-glutamic acid on the diurnal variations of leaf gas exchange and chlorophyll fluorescence parameters in hawthorn (Crataegus pinnatifida Bge.). *Eur. J. Hortic. Sci.* **2009**, *74*, 204–209.
36. Röder, C.; Mógor, Á.F.; Szilagyi-Zecchin, V.J.; Gemin, L.G.; Mógor, G. Potato yield and metabolic changes by use of biofertilizer containing L-glutamic acid. *Comun. Sci.* **2018**, *9*, 211–218.
37. Fabbrin, E.G. dos S.; Mógor, Á.F.; Margoti, G.; Fowler, J.G.; Bettoni, M.M. PURPLE CHICORY 'PALLA ROSSA' SEEDLINGS GROWTH ACCORDING TO THE FOLIAR APPLICATION OF L-GLUTAMIC ACID. *Sci. Agrar.* **2013**, *14*, 91–94.
38. Serna-Rodríguez, J.R.; Castro-Brindis, R.; Colinas-Leon, M.T.; Sahagún-Castellanos, J.; Rodríguez-Pérez, J.E. APLICACIÓN FOLIAR DE ÁCIDO GLUTÁMICO EN PLANTAS DE JITOMATE (Lycopersicon esculentum Mill.). *Rev. Chapingo Ser. Hortic.* **2011**, *17*, 9–13.
39. Beale, S.I.; Gough, S.P.; Granick, S. Biosynthesis of delta-aminolevulinic acid from the intact carbon skeleton of glutamic acid in greening barley. *Proc. Natl. Acad. Sci.* **1975**, *72*, 2719–2723.
40. Cao, Y.P.; Gao, Z.K.; Li, J.T.; Xu, G.H.; Wang, M. Effects of Extraneous Glutamic Acid on Nitrate Contents and Quality of Chinese Chive. *Acta Hortic.* **2010**, *856*, 91–98.
41. Haghighi, M. The effect of humic and glutamic acids in nutrient solution on the N metabolism in lettuce. *J. Sci. Food Agric.* **2012**, *92*, 3023–3028.
42. Lee, H.J.; Kim, J.S.; Lee, S.G.; Kim, S.K.; Mun, B.; Choi, C.S. Glutamic Acid Foliar Application Enhances Antioxidant Enzyme Activities in Kimchi Cabbages Treated with Low Air Temperature. *korean J. Hortic. Sci.* **2017**, *35*.

43. Liang, X.; Zhang, L.; Natarajan, S.K.; Becker, D.F. Proline Mechanisms of Stress Survival. *Antioxid. Redox Signal.* **2013**, *19*, 998–1011.
44. Oukarroum, A.; El Madidi, S.; Strasser, R.J. Exogenous glycine betaine and proline play a protective role in heat-stressed barley leaves (Hordeum vulgare L.): A chlorophyll a fluorescence study. *Plant Biosyst.* **2012**, *146*, 1037–1043.
45. Abdelhamid, M.T.; Rady, M.M.; Osman, A.S.; Abdalla, M.A. Exogenous application of proline alleviates salt-induced oxidative stress in Phaseolus vulgaris L. plants. *J. Hortic. Sci. Biotechnol.* **2013**, *88*, 439–446.
46. Ashraf, M.; Foolad, M.R. Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environ. Exp. Bot.* **2007**, *59*, 206–216.
47. Hare, P.D.; Cress, W.A.; van Staden, J.; Botha, C.E.J. Disruptive effects of exogenous proline on chloroplast and mitochondrial ultrastructure in Arabidopsis leaves. *South African J. Bot.* **2002**, *68*, 393–396.
48. Lichtenthaler, H.K. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. In; 1987; pp. 350–382.
49. Cataldo, D.A.; Maroon, M.; Schrader, L.E.; Youngs, V.L. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun. Soil Sci. Plant Anal.* **1975**, *6*, 71–80.
50. Bates, L.S.; Waldren, R.P.; Teare, I.D. Rapid determination of free proline for water-stress studies. *Plant Soil* **1973**, *39*, 205–207.
51. Du, Z.; Bramlage, W.J. Modified thiobarbituric acid assay for measuring lipid oxidation in sugar-rich plant tissue extracts. *J. Agric. Food Chem.* **1992**, *40*, 1566–1570.
52. Yemm, E.W.; Willis, A.J. The estimation of carbohydrates in plant extracts by anthrone. *Biochem. J.* **1954**, *57*, 508.
53. Gil, M.; Tudela, J.; Martínez-Sánchez, A.; Luna, M. Harvest maturity indicators of leafy vegetables. *Stewart Postharvest Rev.* **2012**, *8*, 1–3.
54. Hanson, B.R.; Orloff, S.; Peters, D. Monitoring soil moisture helps refine irrigation management. *Calif. Agric.* **2000**, *54*, 38–42.
55. Bray, E.A. Plant responses to water deficit. *Trends Plant Sci.* **1997**, *2*, 48–54.
56. Agami, R.A. Salicylic acid mitigates the adverse effect of water stress on lettuce (Lactuca sativa L .). *J. Appl. Sci. Res.* **2013**, *9*, 5701–5711.
57. Moustakas, M.; Sperdouli, I.; Kouna, T.; Antonopoulou, C.-I.; Therios, I. Exogenous proline induces soluble sugar accumulation and alleviates drought stress effects on photosystem II functioning of Arabidopsis thaliana leaves. *Plant Growth Regul.* **2011**, *65*, 315–325.

58. Penuelas, J.; Baret, F.; Filella, I. Semi-empirical indices to assess carotenoids/chlorophyll a ratio from leaf spectral reflectance. *Photosynthetica* **1995**, *31*, 221–230.
59. Beale, S.I. Enzymes of chlorophyll biosynthesis. *Photosynth. Res.* **1999**, *60*, 43–73.
60. Young, A.J. The photoprotective role of carotenoids in higher plants. *Physiol. Plant.* **1991**, *83*, 702–708.
61. Gholami Zali, A.; Ehsanzadeh, P. Exogenous proline improves osmoregulation, physiological functions, essential oil, and seed yield of fennel. *Ind. Crops Prod.* **2018**, *111*, 133–140.
62. Ali, Q.; Ashraf, M.; Athar, H.U.R. Exogenously applied proline at different growth stages enhances growth of two maize cultivars grown under water deficit conditions. *Pakistan J. Bot.* **2007**, *39*, 1133–1144.
63. Dawood, M.G.; Taie, H.A.A.; Nassar, R.M.A.; Abdelhamid, M.T.; Schmidhalter, U. The changes induced in the physiological, biochemical and anatomical characteristics of Vicia faba by the exogenous application of proline under seawater stress. *South African J. Bot.* **2014**, *93*, 54–63.
64. Živčák, M.; Brestič, M.; Olšovská, K.; Slamka, P. Performance index as a sensitive indicator of water stress in Triticum aestivum L. *Plant soil Environ.* **2008**, *2008*, 133–139.
65. Shao, H.; Liang, Z.; Shao, M.; Sun, S.; Hu, Z. Investigation on dynamic changes of photosynthetic characteristics of 10 wheat (Triticum aestivum L.) genotypes during two vegetative-growth stages at water deficits. *Colloids Surfaces B Biointerfaces* **2005**, *43*, 221–227.
66. KOVACEVIC, J. Photosynthetic efficiency and quantitative reaction of bread winter wheat to mild short-terms drought conditions. *TURKISH J. Agric. For.* **2013**.
67. Huseynova, I.M.; Suleymanov, S.Y.; Rustamova, S.M. Response of photosynthetic apparatus and antioxidant defense systems in Triticum aestivum L. genotypes subjected to drought stress. *Proc. ANAIS.(Biological Sci.* **2010**, *65*, 49–59.
68. Balouchi, H.R. Screening wheat parents of mapping population for heat and drought tolerance, detection of wheat genetic variation. *World Acad. Sci. Eng. Technol.* **2010**, *37*, 1122–1132.
69. Falqueto, A.R.; Silva, F.S.P.; Cassol, D.; Magalhães Júnior, A.M.; Oliveira, A.C.; Bacarin, M.A. Chlorophyll fluorescence in rice: probing of senescence driven changes of PSII activity on rice varieties differing in grain yield capacity. *Brazilian J. Plant Physiol.* **2010**, *22*, 35–41.
70. Shetty, K. Role of proline-linked pentose phosphate pathway in biosynthesis of plant phenolics for functional food and environmental applications: a review. *Process Biochem.*

2004, *39*, 789–804.

71. KWOK, D.; SHETTY, K. EFFECTS OF PROLINE AND PROLINE ANALOGS ON TOTAL PHENOLIC AND ROSMARINIC ACID LEVELS IN SHOOT CLONES OF THYME (Thymus vulgaris L.). *J. Food Biochem.* **1998**, *22*, 37–51.
72. Aksakal, O.; Tabay, D.; Esringu, A.; Icoglu Aksakal, F.; Esim, N. Effect of proline on biochemical and molecular mechanisms in lettuce (Lactuca sativa L.) exposed to UV-B radiation. *Photochem. Photobiol. Sci.* **2017**, *16*, 246–254.
73. Zeipiņa, S.; Alsiņa, I.; Lepse, L.; Horticultural, P.; Dūma, M. The Effect of Irrigation on the Biochemical Content of Leafy Vegetables. **2015**, 209–213.
74. Hura, T.; Hura, K.; Grzesiak, M.; Rzepka, A. Effect of long-term drought stress on leaf gas exchange and fluorescence parameters in C3 and C4 plants. *Acta Physiol. Plant.* **2007**, *29*, 103–113.
75. Ohashi, Y.; Nakayama, N.; Saneoka, H.; Fujita, K. Effects of drought stress on photosynthetic gas exchange, chlorophyll fluorescence and stem diameter of soybean plants. *Biol. Plant.* **2006**, *50*, 138–141.
76. Zouari, M.; Ben Ahmed, C.; Zorrig, W.; Elloumi, N.; Rabhi, M.; Delmail, D.; Ben Rouina, B.; Labrousse, P.; Ben Abdallah, F. Exogenous proline mediates alleviation of cadmium stress by promoting photosynthetic activity, water status and antioxidative enzymes activities of young date palm (Phoenix dactylifera L.). *Ecotoxicol. Environ. Saf.* **2016**, *128*, 100–108.
77. Ben Ahmed, C.; Magdich, S.; Ben Rouina, B.; Sensoy, S.; Boukhris, M.; Ben Abdullah, F. Exogenous proline effects on water relations and ions contents in leaves and roots of young olive. *Amino Acids* **2011**, *40*, 565–573.
78. V.G., V.; O.V., V.; O.N., L. EFFECT OF BIO-STIMULANTS ON THE PARAMETERS OF PHOTOSYNTHETIC ACTIVITY AND PRODUCTIVITY OF PEAS. *Fed. Sci. Cent. Legum. Groat Crop.* **2015**, *14*, 26–34.
79. Spinelli, F.; Fiori, G.; Noferini, M.; Sprocatti, M.; Costa, G. A novel type of seaweed extract as a natural alternative to the use of iron chelates in strawberry production. *Sci. Hortic. (Amsterdam).* **2010**, *125*, 263–269.
80. Abdalla, M.M. The potential of Moringa oleifera extract as a biostimulant in enhancing the growth, biochemical and hormonal contents in rocket (Eruca vesicaria subsp. sativa) plants. *Int. J. Plant Physiol. Biochem.* **2013**, *5*, 42–49.
81. Anjum, S.A.; Wang, L.; Farooq, M.; Xue, L.; Ali, S. Fulvic Acid Application Improves the Maize Performance under Well-watered and Drought Conditions. *J. Agron. Crop Sci.* **2011**, *197*, 409–417.

82. Ruzzi, M.; Aroca, R. Plant growth-promoting rhizobacteria act as biostimulants in horticulture. *Sci. Hortic. (Amsterdam).* **2015**, *196*, 124–134.
83. Canellas, L.P.; Olivares, F.L.; Aguiar, N.O.; Jones, D.L.; Nebbioso, A.; Mazzei, P.; Piccolo, A. Humic and fulvic acids as biostimulants in horticulture. *Sci. Hortic. (Amsterdam).* **2015**, *196*, 15–27.
84. Abd El-Mageed, T.A.; Semida, W.M.; Rady, M.M. Moringa leaf extract as biostimulant improves water use efficiency, physio-biochemical attributes of squash plants under deficit irrigation. *Agric. Water Manag.* **2017**, *193*, 46–54.
85. Spann, T.M.; Little, H.A. Applications of a Commercial Extract of the Brown Seaweed Ascophyllum nodosum Increases Drought Tolerance in Container-grown 'Hamlin' Sweet Orange Nursery Trees. *HortScience* **2011**, *46*, 577–582.
86. Lundberg, J.O.; Carlström, M.; Weitzberg, E. Metabolic Effects of Dietary Nitrate in Health and Disease. *Cell Metab.* **2018**, *28*, 9–22.
87. Reddy, B.S. *Diet, Nutrition, and Cancer: A Critical Evaluation*; Reddy, B.S., Cohen, L.A., Eds.; CRC Press, 2018; ISBN 9781351071383.
88. Eichholzer, M.; Gutzwiller, F. Dietary Nitrates, Nitrites, and N-Nitroso Compounds and Cancer Risk: A Review of the Epidemiologic Evidence. *Nutr. Rev.* **2009**, *56*, 95–105.
89. Weitzberg, E.; Lundberg, J.O. Novel Aspects of Dietary Nitrate and Human Health. *Annu. Rev. Nutr.* **2013**, *33*, 129–159.
90. Cavaiuolo, M.; Ferrante, A. Nitrates and glucosinolates as strong determinants of the nutritional quality in rocket leafy salads. *Nutrients* **2014**, *6*, 1519–1538.
91. Statement on possible public health risks for infants and young children from the presence of nitrates in leafy vegetables. *EFSA J.* **2010**, *8*, 2010–2012.
92. Takeuchi, M.; Arakawa, C.; Kuwahara, Y.; Gemma, H. EFFECTS OF L-PROLINE FOLIAR APPLICATION ON THE QUALITY OF "KOSUI" JAPANESE PEAR. *Acta Hortic.* **2008**, 549–554.
93. Sharma, S.S.; Schat, H.; Vooijs, R. In vitro alleviation of heavy metal-induced enzyme inhibition by proline. *Phytochemistry* **1998**, *49*, 1531–1535.
94. Verbruggen, N.; Hermans, C. Proline accumulation in plants: a review. *Amino Acids* **2008**, *35*, 753–759.
95. Hong, Z.; Lakkineni, K.; Zhang, Z.; Verma, D.P.S. Removal of Feedback Inhibition of Δ 1 -Pyrroline-5-Carboxylate Synthetase Results in Increased Proline Accumulation and Protection of Plants from Osmotic Stress. *Plant Physiol.* **2000**, *122*, 1129–1136.
96. Yoshiba, Y.; Kiyosue, T.; Nakashima, K.; Yamaguchi-Shinozaki, K.; Shinozaki, K. Regulation of Levels of Proline as an Osmolyte in Plants under Water Stress. *Plant Cell Physiol.* **1997**,

38, 1095–1102.

97. GE, T.; SUI, F.; BAI, L.; LU, Y.; ZHOU, G. Effects of Water Stress on the Protective Enzyme Activities and Lipid Peroxidation in Roots and Leaves of Summer Maize. *Agric. Sci. China* **2006**, *5*, 291–298.
98. Pandey, H.C.; Baig, M.J.; Chandra, A.; Bhatt, R.K. Drought stress induced changes in lipid peroxidation and antioxidant system in genus Avena. *J. Environ. Biol.* **2010**, *31*, 435–440.
99. Sayyari, M.; Ghavami, M.; Ghanbari, F.; Kordi, S. Assessment of salicylic acid impacts on growth rate and some physiological parameters of lettuce plants under drought stress conditions. *Int. J. Agric. Crop Sci.* **2013**, *5*, 1951–1957.
100. Sahin, U.; Ekinci, M.; Kiziloglu, F.M.; Yildirim, E.; Turan, M.; Kotan, R.; Ors, S. Ameliorative Effects of Plant Growth Promoting Bacteria on Water-yield Relationships, Growth, and Nutrient Uptake of Lettuce Plants under Different Irrigation Levels. *HortScience* **2015**, *50*, 1379–1386.
101. Eraslan, F.; Inal, A.; Savasturk, O.; Gunes, A. Changes in antioxidative system and membrane damage of lettuce in response to salinity and boron toxicity. *Sci. Hortic. (Amsterdam).* **2007**, *114*, 5–10.
102. Jain, M.; Mathur, G.; Koul, S.; Sarin, N. Ameliorative effects of proline on salt stress-induced lipid peroxidation in cell lines of groundnut (Arachis hypogaea L.). *Plant Cell Rep.* **2001**, *20*, 463–468.
103. MEHTA, S.K.; GAUR, J.P. Heavy-metal-induced proline accumulation and its role in ameliorating metal toxicity in Chlorella vulgaris. *New Phytol.* **1999**, *143*, 253–259.
104. Posmyk, M.M.; Janas, K.M. Effects of seed hydropriming in presence of exogenous proline on chilling injury limitation in Vigna radiata L. seedlings. *Acta Physiol. Plant.* **2007**, *29*, 509–517.
105. Shevyakova, N.I.; Bakulina, E.A.; Kuznetsov, V. V. Proline antioxidant role in the common ice plant subjected to salinity and paraquat treatment inducing oxidative stress. *Russ. J. Plant Physiol.* **2009**, *56*, 663–669.
106. Huang, Y.; Bie, Z.; Liu, Z.; Zhen, A.; Wang, W. Protective role of proline against salt stress is partially related to the improvement of water status and peroxidase enzyme activity in cucumber. *Soil Sci. Plant Nutr.* **2009**, *55*, 698–704.
107. Tripathi, B.N.; Gaur, J.P. Relationship between copper- and zinc-induced oxidative stress and proline accumulation in Scenedesmus sp. *Planta* **2004**, *219*.
108. Wang, F.; Zeng, B.; Sun, Z.; Zhu, C. Relationship Between Proline and Hg2+-Induced Oxidative Stress in a Tolerant Rice Mutant. *Arch. Environ. Contam. Toxicol.* **2009**, *56*, 723–

731.

109. Aravind, P.; Prasad, M.N.V. Cadmium-induced toxicity reversal by zinc in Ceratophyllum demersum L. (a free floating aquatic macrophyte) together with exogenous supplements of amino- and organic acids. *Chemosphere* **2005**, *61*, 1720–1733.

110. Wang, W.; Cang, L.; Zhou, D.-M.; Yu, Y.-C. Exogenous amino acids increase antioxidant enzyme activities and tolerance of rice seedlings to cadmium stress. *Environ. Prog. Sustain. Energy* **2017**, *36*, 155–161.

111. Zhang, J.; Jia, W.; Yang, J.; Ismail, A.M. Role of ABA in integrating plant responses to drought and salt stresses. *F. Crop. Res.* **2006**, *97*, 111–119.

112. Shinozaki, K.; Yamaguchi-Shinozaki, K. Gene networks involved in drought stress response and tolerance. *J. Exp. Bot.* **2006**, *58*, 221–227.

113. Seki, M.; Umezawa, T.; Urano, K.; Shinozaki, K. Regulatory metabolic networks in drought stress responses. *Curr. Opin. Plant Biol.* **2007**, *10*, 296–302.

114. Van Oosten, M.J.; Pepe, O.; De Pascale, S.; Silletti, S.; Maggio, A. The role of biostimulants and bioeffectors as alleviators of abiotic stress in crop plants. *Chem. Biol. Technol. Agric.* **2017**, *4*, 1–12.

115. Forde, B.G.; Lea, P.J. Glutamate in plants: metabolism, regulation, and signalling. *J. Exp. Bot.* **2007**, *58*, 2339–2358.

116. Sharma, A.; Shahzad, B.; Kumar, V.; Kohli, S.K.; Sidhu, G.P.S.; Bali, A.S.; Handa, N.; Kapoor, D.; Bhardwaj, R.; Zheng, B. Phytohormones Regulate Accumulation of Osmolytes Under Abiotic Stress. *Biomolecules* **2019**, *9*, 285.

117. Wang, W.; Vinocur, B.; Altman, A. Plant responses to drought, salinity and extreme temperatures: Towards genetic engineering for stress tolerance. *Planta* **2003**, *218*, 1–14.

118. Iqbal, N.; Nazar, R. *Osmolytes and Plants Acclimation to Changing Environment: Emerging Omics Technologies*; Iqbal, N., Nazar, R., A. Khan, N., Eds.; Springer India: New Delhi, 2016; ISBN 978-81-322-2615-4.

119. Liu, C.; Zhao, L.; Yu, G. The Dominant Glutamic Acid Metabolic Flux to Produce γ-Amino Butyric Acid over Proline in Nicotiana tabacum Leaves under Water Stress Relates to its Significant Role in Antioxidant Activity. *J. Integr. Plant Biol.* **2011**, *53*, 608–618.

120. DOBROWSKI, S.; PUSHNIK, J.; ZARCOTEJADA, P.; USTIN, S. Simple reflectance indices track heat and water stress-induced changes in steady-state chlorophyll fluorescence at the canopy scale. *Remote Sens. Environ.* **2005**, *97*, 403–414.

121. Flexas, J.; Briantais, J.-M.; Cerovic, Z.; Medrano, H.; Moya, I. Steady-State and Maximum Chlorophyll Fluorescence Responses to Water Stress in Grapevine Leaves. *Remote Sens.*

Environ. **2000**, *73*, 283–297.

122. Burns, I.G.; Zhang, K.; Turner, M.K.; Edmondson, R. ISO-OSMOTIC REGULATION OF NITRATE ACCUMULATION IN LETTUCE. *J. Plant Nutr.* **2010**, *34*, 283–313.
123. Hikosaka, K.; Terashima, I.; Katoh, S. Effects of leaf age, nitrogen nutrition and photon flux density on the distribution of nitrogen among leaves of a vine (Ipomoea tricolor Cav.) grown horizontally to avoid mutual shading of leaves. *Oecologia* **1994**, *97*, 451–457.
124. Sofo, A.; Scopa, A.; Nuzzaci, M.; Vitti, A. Ascorbate Peroxidase and Catalase Activities and Their Genetic Regulation in Plants Subjected to Drought and Salinity Stresses. *Int. J. Mol. Sci.* **2015**, *16*, 13561–13578.
125. Zhang, J.; Kirkham, M.B. Enzymatic responses of the ascorbate-glutathione cycle to drought in sorghum and sunflower plants. *Plant Sci.* **1996**, *113*, 139–147.
126. Ma, Y.-H.; Ma, F.-W.; Wang, Y.-H.; Zhang, J.-K. The responses of the enzymes related with ascorbate–glutathione cycle during drought stress in apple leaves. *Acta Physiol. Plant.* **2011**, *33*, 173–180.
127. Oh, M.-M.; Trick, H.N.; Rajashekar, C.B. Secondary metabolism and antioxidants are involved in environmental adaptation and stress tolerance in lettuce. *J. Plant Physiol.* **2009**, *166*, 180–191.
128. Xu, C.; Mou, B. Responses of Spinach to Salinity and Nutrient Deficiency in Growth, Physiology, and Nutritional Value. *J. Am. Soc. Hortic. Sci.* **2016**, *141*, 12–21.
129. Ertani, A.; Schiavon, M.; Muscolo, A.; Nardi, S. Alfalfa plant-derived biostimulant stimulate short-term growth of salt stressed Zea mays L. plants. *Plant Soil* **2013**, *364*, 145–158.
130. Battacharyya, D.; Babgohari, M.Z.; Rathor, P.; Prithiviraj, B. Seaweed extracts as biostimulants in horticulture. *Sci. Hortic. (Amsterdam).* **2015**, *196*, 39–48.
131. Yakhin, O.I.; Lubyanov, A.A.; Yakhin, I.A.; Brown, P.H. Biostimulants in Plant Science: A Global Perspective. *Front. Plant Sci.* **2017**, *7*.
132. Lucini, L.; Rouphael, Y.; Cardarelli, M.; Canaguier, R.; Kumar, P.; Colla, G. The effect of a plant-derived biostimulant on metabolic profiling and crop performance of lettuce grown under saline conditions. *Sci. Hortic. (Amsterdam).* **2015**, *182*, 124–133.
133. Machado, R.; Serralheiro, R. Soil Salinity: Effect on Vegetable Crop Growth. Management Practices to Prevent and Mitigate Soil Salinization. *Horticulturae* **2017**, *3*, 30.
134. Kuşvuran, Ş.; Yaşar, F.; Abak, K.; Ellialtıoğlu, Ş. Changes occur in lipid peroxidation, chlorophyll and ion contents of some salt tolerant and sensitive Cucumis sp. genotypes grown under salinity stress. *J. Agric. Sci.* **2008**, *18*, 13–20.
135. Gomes, M.A. da C.; Pestana, I.A.; Santa-Catarina, C.; Hauser-Davis, R.A.; Suzuki, M.S.

Salinity effects on photosynthetic pigments, proline, biomass and nitric oxide in Salvinia auriculata Aubl. *Acta Limnol. Bras.* **2017**, *29*.

136. Asch, F.; Dingkuhn, M.; Dorffling, K. Salinity increases CO2 assimilation but reduces growth in field-grown, irrigated rice. *Plant Soil* **2000**, *218*.
137. Sedjati, S.; Santosa, G.; Yudiati, E.; Supriyantini, E.; Ridlo, A.; Kimberly, F. Chlorophyll and Carotenoid Content of Dunaliella salina at Various Salinity Stress and Harvesting Time. *IOP Conf. Ser. Earth Environ. Sci.* **2019**, *246*, 012025.
138. Houimli, S.I.M.; Denden, M.; Mouhandes, B.D. Effects of 24-epibrassinolide on growth, chlorophyll, electrolyte leakage and proline by pepper plants under NaCl-stress. *EurAsian J. Biosci.* **2010**, 96–104.
139. Fang, Z.; Bouwkamp, J.C.; Solomos, T. Chlorophyllase activities and chlorophyll degradation during leaf senescence in non-yellowing mutant and wild type of Phaseolus vulgaris L. *J. Exp. Bot.* **1998**, *49*, 503–510.
140. Meloni, D.A.; Gulotta, M.R.; Martínez, C.A.; Oliva, M.A. The effects of salt stress on growth, nitrate reduction and proline and glycinebetaine accumulation in Prosopis alba. *Brazilian J. Plant Physiol.* **2004**, *16*, 39–46.
141. Sah, S.K.; Reddy, K.R.; Li, J. Abscisic Acid and Abiotic Stress Tolerance in Crop Plants. *Front. Plant Sci.* **2016**, *7*, 1–26.
142. Fricke, W.; Akhiyarova, G.; Veselov, D.; Kudoyarova, G. Rapid and tissue-specific changes in ABA and in growth rate in response to salinity in barley leaves. *J. Exp. Bot.* **2004**, *55*, 1115–1123.

CHAPTER 4

Evaluation of VOCs profile of rocket salad in response to glutamic acid treatment and salt stress

INTRODUCTION

1. Volatile organic compounds biosynthesis

Plants produce a huge variety of secondary metabolites that are not directly involved in plants growth processes, but they are essential for successful competitive strategies. Among them, volatile organic compounds (VOCs) play an important role and about 1,700 compounds have been isolated and identified from plants. These molecules are characterised by a low molecular weight and a high vapour pressure at ambient temperature. These proprieties allow them to cross the cell membranes and be vaporised into the environment [1,2]. Despite they are classified as secondary metabolites, VOCs production is connected to the primary metabolism and in particular to the availability of sulfur, carbon, and nitrogen. Considering the biosynthetic pathways, VOCs can be separated into different classes such as fatty acids derivates, isoprenoids, terpenoids, amino acids derivates, methyl jasmonate, green leaf volatiles, and phenylpropanoids.

VOCs are emitted by almost all kind of tissues, by flowers, leaves, stems, fruits and roots and they can be normally released by health tissues or induced by several stimuli like mechanical damage, herbivore or pathogen infection. VOCs that are produced in response to a stimulus are defined "induced VOCs" and it has been reported that they can be emitted not only by the damaged tissues by also by the healthy ones. In addition to spatial regulation, VOCs production is also regulated during time according to the developmental stage. Indeed, the emission generally increase in young tissues and decrease thereafter, or it is typical at specific developmental stages like flowering, fruit ripening or leaf maturation. In addition, IVOCs emission is related to the stressful event and it has been observed that they can be released with a different timing, from hours to days after the trigger event. This temporal regulation is particularly important to optimize the energy and carbon use in plants. In fact, the emission of VOCs reduces the availability of carbon and affects plant performance. Even though their regulation is similar in different plants, VOCs profile is strictly related to the species.

Plant VOCs take part in a wide range of ecological functions, mediating the interaction with other plants or animals, and affecting the composition of the air and the climate. VOCs are released as defence from insects or herbivores, as protection from pathogens or to attract pollinators. Moreover, they are also involved in plant protection against different abiotic stresses, removing reactive oxygen species or in the mechanism of

adaption [3,4]. Moreover, VOCs such as NO and ethylene play a role as stress messengers and stimulate plants to activate several stress defence mechanisms. Abiotic stress such as mechanical damage, nutrient deficiency, high salinity, high light, water stress and non-optimal temperatures are well known to alter the level of the VOCs emission. The enhanced emission of VOCs as stress response is partially due to the increase in the temperature of stressed tissues, resulting in a higher vapour pressure. Nevertheless, the release of VOCs often increases more than due to a change in vapor pressure, suggesting the presence of other regulatory mechanisms.

The study of changes of plant VOCs profile in response to different stressful conditions might be a useful strategy to obtain information about the health status of crops. By monitoring the presence or the level of specific VOCs, they can be use as biological markers of a particular stress conditions. Moreover, current technologies allow us to easily measure the concentration of VOCs through a non-invasive screening or with a minimal tissue disruption [5–7].

2. VOCs and salt stress

Some of the effects of salt stress are similar to those are caused by water stress, in particular on photosynthesis and stomatal conductance. The reduction of gas exchanges observed is reported to have a negative impact not only on plants growth and development but also on VOCs emission. This is due to the reduced availability of photosynthates, carbon and energy that are necessary for their biosynthesis. To date, very few studies have investigated the effect of salinity on VOCs profile. Among them particular attention has been directed to isoprene emission and few other molecules. Loreto and Delfine [8] evaluated the effect of salt stress on the release of isoprene from Eucalyptus leaves and they found out that even though photosynthesis and stomatal conductance are reduced, the isoprene emission was not significantly affected probably due to the activation of non -photosynthetic pathways of isoprene biosynthesis. In contrast, in grey poplar leaves salt stress led to a reduction of dimethylallyl diphosphate level, the substrate for isoprene [9]. It has been observed that salt stress affects the VOCs profile according to its severity. A study on sage showed that the concentration of monoterpenes increased with increasing salinity until a threshold point, after which it declines [10]. Moreover, the composition of the monoterpenes as well as their concentration was affected by the level of salt stress applied. Similar results have been observed in Origanum majorana [11]. Another example of salt stress affecting the quantity and the quality of volatilome and inter-plant communication through VOCs release has been reported by Lee and Seo [12]. They observed that salt stress induced the emission of VOCs to alert other plants and elicit stress responses.

A few classes of VOCs are suggested to be involved in plant responses to salt stress, such as terpenes, oxylipins, methanol, methane, and ethylene [13]. Terpenes exert antioxidant activity and are able to neutralize ROS probably due to their conjugate double bonds. Moreover, due to their lipophilic nature, they play a role protecting and stabilizing cellular membranes.

Green leaf volatiles, derived from oxylipins, are reported to be induced by several stressful condition, including salt stress. Their sensory properties suggest a contribution in salt stress adaptation strategies.

3. VOCs and aroma

Besides all the roles reported above, VOCs are also particularly important in the definition of the aroma and flavour of fruits and vegetables. The aroma is the results of the perception of a complex mixture of compounds that are present at high concentration but also in trace amount [14]. This mixture is usually defined with the term VOCs bouquet. Food aroma is perceived by the consumer and influence repurchase choices [15,16].

Unfortunately, plant breeding aimed to increase growth and yield negatively affected the secondary metabolism leading to the development of crop cultivars deficient in VOCs production. This resulted not only in a higher susceptibility to pest or disease, but also to a less flavour [17–19].

Rocket is a leafy vegetable commonly consumed as ready to eat fresh product alone or in salad mixtures. It is particularly appreciated for its characteristic flavour described as pungent, sharp, spicy, and peppery. Several studies focused on the origin of its aroma and it is reported that the VOCs released just after the disruption of the tissues derive from glucosinolates and isothiocyanates [20–23]. As reported by Jirovetz et al. [24] and Blazevic et al. [25,26] other compounds are important in rocket aroma, such as Hexanal, trans-2-hexenal, cis-3-hexen-1-ol, trans-2-hexen-1-ol, benzaldehyde and trans, trans-2,4-heptadienal.

AIM

Based on previous experience using glutamic acid as foliar spray treatment and since little is known about the effects of its application on plants aroma profile, the aim of this work was the investigation of VOCs from rocket salad treated with this amino acid. Moreover, the analysis was performed on plants subjected to salt stress in order to evaluate the changes in the VOCs in response to high concentration of NaCl and to the combination of stress and treatment.

MATERIALS AND METHODS

1. Plant material, growth conditions and stress treatment

The trial was carried out at the School of Biosciences of Cardiff University in 2019. Experiment timesteps are reported in Figure 76. Timelines of the experiment, with indication of treatment application and sampling.Figure 76. Rocket plants (*Diplotaxis tenuifolia* L. var. Frastagliata) were grown hydroponically into plastic tank (34 x 22.5 cm) with 10 L of a standard Hoagland medium and the concentrations of nutrients in the solution are reported in Table 23. Seeds of rocket were manually sown into polystyrene trays filled with an agri-perlite substrate. Cultivation took place in a growth room under controlled conditions.

Figure 76. Timelines of the experiment, with indication of treatment application and sampling.

Plants were treated with 20 mL of water (control) and 20 mL of glutamic acid [5,4 mM]. The concentration used in this experiment has been chosen since the positive results obtained by Lv et al.,[27] in hawthorn plants. Treatments were applied as foliar spray onto leaves until run-off 24 hours before the beginning of the stress. Salt stress was imposed by transferring plants to a fresh nutrient solution containing 200 mM NaCl, 40 days after sowing at 10:00. The nutrient solution of control plants was also changed. Each experimental unit consisted of two tanks.

Table 23. Composition of the Hoagland nutrient solution used for rocket plants cultivation.

Compounds	Concentration [mM]
$Ca(NO_3)_2$	2.19
KNO_3	4.55
NH_4NO_3	3.87
K_2HPO_4	1.38
$MgSO_4$	0.83
K_2SO_4	1.09
Oligo green*	0.02 (g L^{-1})
H_2SO_4	Up to pH 5.5 – 6.5

*Oligogreen: is a mineral water-soluble powder fertilizer that provides the plant with micronutrients, essential for the most important bio-chemical reactions. Green Has Italia.

2. Non-destructive analyses

2.1. Chlorophyll and chlorophyll a fluorescence

Leaf chlorophyll content was colorimetrically determined in vivo using a portable chlorophyll meter (SPAD-502 Plus, Konica Minolta. Minolta, Osaka, Japan), placing the leaves toward the emitting window of the instrument and avoiding the major veins. The results were express such as SPAD units. Chlorophyll a fluorescence was measured in vivo using a portable fluorometer (MINI-PAM-II, Photosynthesis Yield Analyzer. Walz). Before all measurement leaves were dark-adapted with the leaf clips for 30-40 minutes. Then were exposed to a saturating light and information about the structural and functional status of photosynthetic apparatus was provided by the maximum quantum of photosystem II (Fv/Fm). Measures were performed at the end of the trial.

3. Destructive analysis

3.1. Collection and analysis of Volatile organic compounds (VOCs)

For each condition, 3 g of rocket leaves were sampled, placed into a multi-purpose roasting bag (25 cm × 38 cm, TJM Ltd.) and sealed, using an elastic band and an Eppendorf tube with the end cut off. The Eppendorf tube served as a sampling port for the SafeLok™ thermal desorption (TD) tubes (Tenax TA & Sulficarb, Markes International Ltd., Llantrisant, UK). Leaves were then disrupted manually within the bags for 10 s by crushing the leaves between the hands and making a vigorous rubbing motion. Care was taken not to perforate the bags and inadvertently release VOCs. A 'blank' sample of atmosphere within empty bags to rule out any possible contaminating VOCs was taken at the same time. The bags were stored at room temperature (20 °C) for 1 h to equilibrate the headspace (1 L) that was collected with an EasyVOC manual pump (Markes International Ltd., Llantrisant, UK) onto TD tubes. Each sampling point was collected in triplicate mixing the leaves from the two agronomical replicates. A retention standard was prepared by loading 1 µL C8-C20 alkane standard mixture directly onto a TD tube and analysed under the same conditions as the samples.

All tubes were desorbed by a TD100 thermal desorption system (Markes International Ltd., Llantrisant, Wales, UK), using the following settings for tube desorption: 2 min at 100 °C, followed by 5 min at 280 °C, trap flow of 40 ml/min and trap desorption and transfer: 20 °C/s to 300 °C, split flow of 20 ml/min into GC (7890A; Agilent Technologies, Inc., Stockport, UK).

VOCs were separated over 60 m, 0.32 mm ID, 0.5 µm film thickness Rxi-5ms (Restek) at 2 ml continuous flow of helium, using the following temperature programme: initial temperature 40 °C for 2 min, 5 °C/min to 240

°C and 20 °C/min to 300 °C. The BenchTOF-dx mass spectrometer (Almsco International, Cincinnati, OH, USA) was operated in EI mode at an ion source temperature of 250 °C and a mass range of 35–450 m/z.

Data from GC–MS measurements were initially processed using MSD ChemStation software (E.02.01.1177; Agilent Technologies, Inc, Santa Clara, CA, U.S.A.), deconvoluted and integrated with AMDIS (NIST14) using a retention-indexed mass spectral library. The identification of each peak has been performed comparing the mass spectrum against a rocket library previously prepared in the same laboratory. If compounds could not be matched to the library they were search against a database (NIST) and added to the rocket library in order to build an update version of it. This step has been done assessing the quality of the NIST identification by choosing a minimum score value of 80 % and a retention index range of +/- 30. To validate AMDIS outputs and correct errors that sometimes are generated, all data were analysed with GaVIn software (GC–MS Assignment Validator and Integrator) implemented in Matlab.

4. Statistical analysis

Data of chlorophyll and chlorophyll *a* fluorescence were subjected to a two-way ANOVA and differences among means were determined by Tuckey post-test ($P < 0.05$). Statistics were performed using GraphPad Prism version 6 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Additional information is reported in each figure's legend.

VOC data from GaVIn were processed using StaCEy (STAtistics for gC-ms Experimental analysis) using R (R version 3.6.1). After normalisation of areas (GaVIn outputs) and square root transformation to reduce weight of large components, Permutational multivariate analysis of variance (PerMANOVA) and Canonical Analysis of Principal coordinates (CAP) analysis were performed [28] using the 'vegan' package 2.5-5 [29] and 'BiodiversityR' package 2.11-1 [30].

RESULTS

1. Non-destructive analyses

1.1. Chlorophyll and chlorophyll a fluorescence

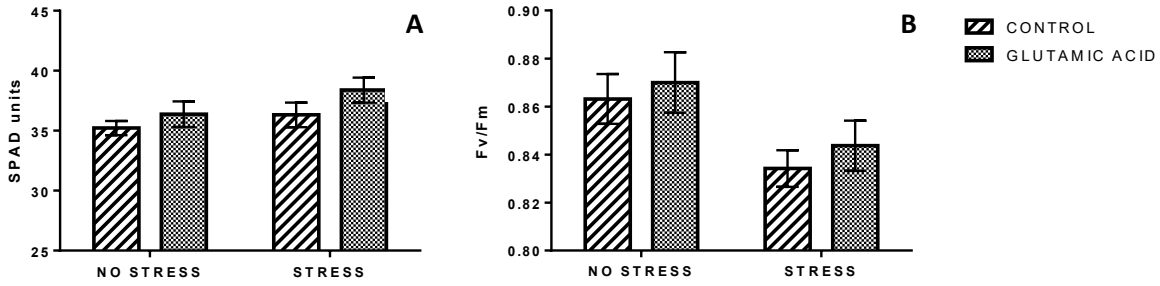


Figure 77. Chlorophyll content expressed as SPAD units (**A**) and maximum quantum efficiency of PSII (Fv/Fm) (**B**) measured *in vivo* in rocket leaves treated with water (CONTROL) and with glutamic acid and subjected to salt stress (200 mM) (**A**). Measures were taken at the end of the growing cycle. Values are means ± SE (n = 14) (**A**), (n = 6) (**B**). Data were subjected to two-way ANOVA. Different letters, where present, represent significant differences ($P < 0.05$).

The content of chlorophyll was not significantly affected by the stress and by the application of glutamic acid even if a slight increase appeared in treated plants (Figure 77 A). On the contrary, the maximum quantum efficiency of PSII (Fv/Fm) was significantly lowered by the salt stress regardless the glutamic acid treatment (Figure 77 B). Fv/Fm values were around 0.85 and 0.86 in non-stressed plants, and indicating a good status of leaf apparatus whereas under salt stress conditions the values were 0.83 and 0.84 in control and treated plants respectively. In general, stressful conditions induce a decrease of Fv/Fm values under the value of 0.83.

2. Destructive analysis

2.1. Volatile organic compounds

The VOCsof all rocket leaves samples have been identified for a total of 191. Aroma volatile organic compounds belonged to different families such as esters, alkanes, furans, terpenes, aromatic compounds, and nitriles.

Analysis using PerMANOVA indicated that the overall VOCs profile changed in response to salt stress ($P < 0.05$, $R^2 = 0.142$). Linear discrimination plots based on CAP analysis did not show a clear separation of profiles of plants grown with or without salt stress (percentage of correct classification of 83.3% and a significance of 0.07) (Figure 79) neither between treated and untreated samples (percentage of correct classification of 83.3% and a significance of 0.08) (Figure 78).

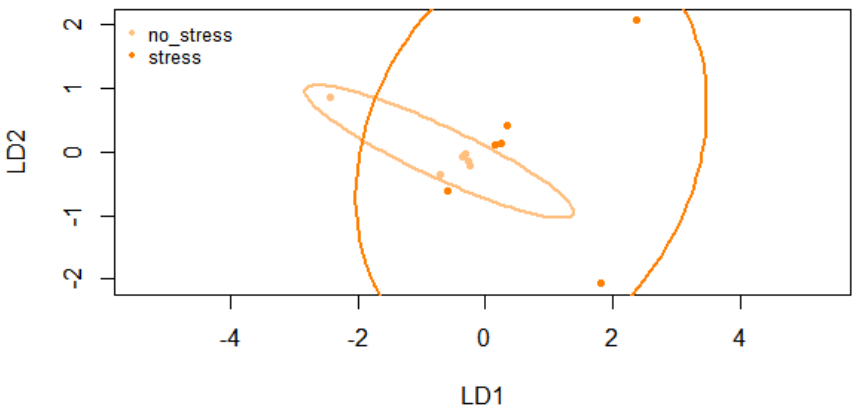


Figure 79. Canonical Analysis of Principal ordinates related to salt stress growing conditions based on all 191 rocket VOCs using TD-GC-TOF-MS: Each ellipse represents the 95% confidence interval. The plots use linear discriminants LD1 and LD2 (n = 6)

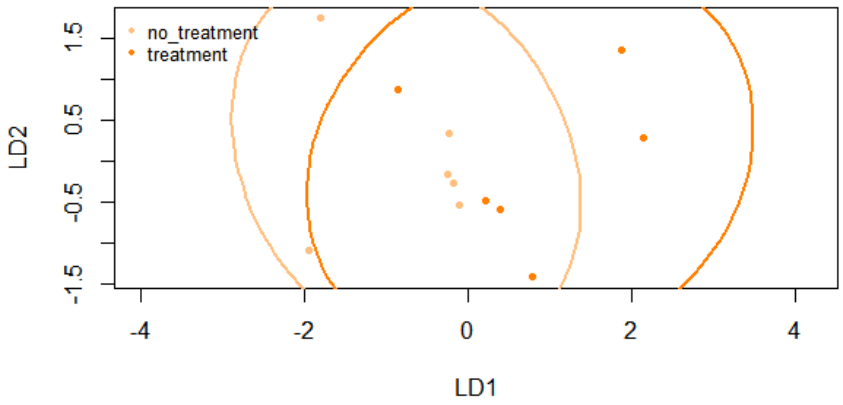


Figure 78. Canonical Analysis of Principal ordinates related to the treatment based on all 191 rocket VOCs using TD-GC-TOF-MS: Each ellipse represents the 95% confidence interval. The plots use linear discriminants LD1 and LD2 (n = 6)

However, there was no discrimination between treated and non-treated plants neither between stressful and non-stressful conditions.

A closer inspection of VOCs revealed that an average of 9 compounds account for 50% of the total area in all the samples (Figure 80, Table 24). Five of them (3-Hexen-1-ol (Z)-, Acetic acid hexyl ester, 1-Penten-3-ol, Butanoic acid 3-hexenyl ester (Z)-, 3-Hexen-1-ol acetate, (E)) were common to all of the examined conditions. In particular, 3-Hexen-1-ol acetate, (E) and 3-Hexen-1-ol (Z)-, were the two most abundant compounds in all samples, with a percentage average of 23.81 and 10.23, respectively.

Sulfur dioxide was abundant in plants grown under optimal conditions whereas 2-Hexen-1-ol acetate was found in plants subjected to salt stress, regardless the glutamic acid treatment. Two compounds (1,2-Benzenedicarboxylic acid butyl 2-ethylhexyl ester and 3-Pentanone) account for 7.35% of total area only in non-treated plants grown without stress whereas five compounds (2(5H)-Furanone 5-ethyl-, 2-Hexenal, Benzene, Ethanol, Furan, 2-ethyl-) were found in response to the combination of stress and glutamic acid treatment.

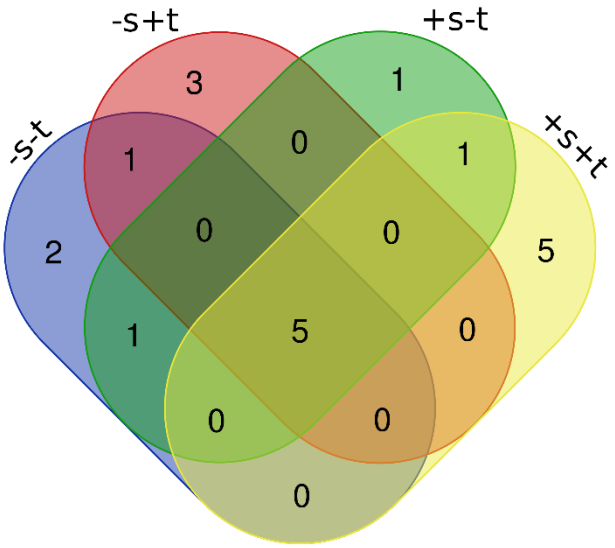


Figure 80. Shaded Venn diagram circles indicate number of VOCs representing the 50% of the total area. "s" means the salt stress, "t" means the glutamic acid treatment, "+/-" means the presence or the absence of the condition expressed by the followed letter.

Table 24. VOCs identified (expressed as percentage of the peak area of each compound compared to the total area) in rocket by TD-GC–MS-TOF and representing the 50% of the total area. "s" means the salt stress, "t" means the glutamic acid treatment, "+/-" means the presence or the absence of the condition expressed by the followed letter.

Compound name	-s-t	-s+t	+s-t	+s+t
1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester	5.85	-	-	-
1-Penten-3-ol	2.21	1.93	2.49	2.83
2(5H)-Furanone, 5-ethyl-	-	-	-	1.77
2-Hexen-1-ol, acetate	-	-	3.31	1.47
2-Hexenal	-	-	-	2.72
3-Hexen-1-ol, (Z)-	10.08	10.28	10.84	9.72
3-Hexen-1-ol, acetate, (E)-	23.34	26.66	25.55	19.67
3-Pentanone	1.50	-	-	-
Acetic acid, hexyl ester	1.55	1.79	3.07	2.04
Benzene	-	-	-	4.44
BRANCHED ALKANE 20	-	1.46	-	-
Butanoic acid, 3-hexenyl ester, (Z)-	2.31	1.92	1.94	2.13
Dimethyl sulfone	2.00	-	1.43	-

Ethanol	-	-	-	1.46
Furan, 2-ethyl-	-	-	-	1.84
Octane	-	2.85	-	-
Phenylmaleic anhydride	-	-	2.10	-
Sulfur dioxide	1.83	2.27	-	-
Undecane	-	1.45	-	-

About 115 compounds account for almost 95% of the total area in all samples and they are divided as reported in the Figure 81. Most of the compounds (86) were present in all conditions examined and some of the molecules found only in control or in stressed plants whit the previous analysis has been detected also in other condition in smaller amount. However, 4 compounds (Diphenyl sulfone, Hexadecane, 3-Pentanol and 2,6,10,14-tetramethyl-) were identified only in untreated plants grown under optimal conditions whereas 12 compounds were found exclusively in stressed plants treated with glutamic acid (2,4-Hexadienal, (E,E)- 2-Pentenal, (E)- (NS2) Thieno[3,2-b]thiophene (Z),(Z)-2,4-Hexadiene Thiophene, 3-ethyl- 2-Hexanone 2-Penten-1-ol, (Z)- 1,3-Cyclopentadiene, 1-methyl- p-Xylene 2,4-Hexadiene Thiophene, 2,5-dimethyl- D-Limonene). Moreover, 5 compounds (Furan, 2-ethyl- n-Pentyl isothiocyanate 3-Hexen-1-ol, (E)- 2(5H)-Furanone, 5-ethyl- (?) 2-Hexen-1-ol, (E)-) has been found in response to salt stress regardless the treatment.

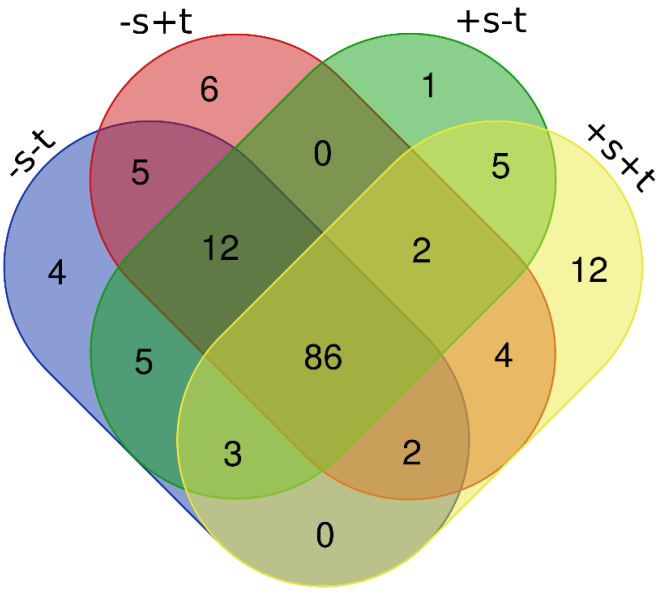


Figure 81. Shaded Venn diagram circles indicate number of VOCs representing the 95% of the total area. "s" means the salt stress, "t" means the glutamic acid treatment, "+/-" means the presence or the absence of the condition expressed by the followed letter.

DISCUSSION AND CONCLUSION

Glutamic acid is a central amino acid in plants, it is involved in protein biosynthesis, nitrogen metabolism, and it may also function as signalling molecule. Moreover, it is the precursor for chlorophyll synthesis in leaves [31]. Foliar application of glutamic acid solutions at different concentrations were tested on *Crataegus pinnatifida* in order to study their effect on the gas exchange and chlorophyll [27]. Authors observed that the highest concentration of glutamic acid tested in their experiment (800 mg L^{-1}) increased the net photosynthetic rate, the chlorophyll content and the maximum quantum efficiency of PSII (Fv/Fm) if compared with the control plants treated with water. In our experiment the content of chlorophyll measured with a non-destructive instrument and Fv/Fm of rocket leaves did not change in plants treated with the same concentration of glutamic acid. This might be due to the different number of applications between our experiment and the one reported in literature, indeed in the present work glutamic acid treatment has been sprayed only once. The analysis of chlorophyll fluorescence parameters is a common tool to evaluate the status of photosynthetic apparatus and the plant response to environmental conditions [32]. The maximum quantum efficiency of PSII of rocket plants was significantly decreased by the imposition of salt stress, as typical plant response to high salinity conditions [33,34]. A slight but not significant increase of chlorophyll content in response to salt stress was previously observed also by Cocetta et al. [35] in rocket salad.

Wild rocket is a good source of phytonutrients and it is particularly appreciated for its distinct flavour. Regardless its importance as vegetable mostly in ready to eat salad, there are only a few reports of VOCs and most of them are focused on changes in the whole VOC bouquet during post-harvest storage [36–38]. Abiotic stresses have several effects on plant metabolisms and among these also in the emission of VOCs [3,13]. Some of them are reported to increase in response to abiotic stress whereas others decrease. In this experiment, the overall VOCs profile was significantly affected by the salt stress conditions imposed and several differences in the most abundant compounds emerged from a deeper analysis.

Within those molecules contributing to the 50% of the total area, the two most abundant compounds found in all samples were 3-hexen-1-ol acetate (e)- and 3-hexen-1-ol, (Z). The first one is a typical plant volatile involved in the attraction of moths [39] whereas the second one is a leaf alcohol emitted by green plants upon mechanical damage [40] and previously found in rocket and radish [7,16,26]. In addition to these, 1-penten-3-ol, acetic acid hexyl ester and butanoic acid, 3-hexenyl ester, (Z)- were abundant in all plants regardless the growing conditions or the treatment. 1-penten-3-ol is reported to have a pungent green vegetable nuance and was identified in kale [41] and both in *D. tenuifolia* and *E. sativa* [7,16,38] while butanoic acid, 3-hexenyl ester, (Z)- was found in the essential oil of *Pistacia lentiscus* [42] and *Averrhoa carambola* [43].

The 3-pentanone is a ketone compound that was significantly correlated with green odour, flavour, and has been defined to have an 'ether' odour [44]. Initially, it was detected only in control plants while, expanding

the analyses to the compounds accounting for the 95% of the total peak area it was found also in the other samples. However, even by the extended analysis several compounds were identified only in some conditions. For example, diphenyl sulphone, 3-pentanol and phytane were found only in control plants grown under non-stressful conditions. Diphenyl sulphone is a sulfone compound that has been found in plants like *Gnidia glauca* and *Dioscorea bulbifera* and it has a role as plant metabolite. It has been reported to have antioxidants properties and it is used in treatment of rheumatoid arthritis [45]. Phytane is an acyclic isoprenoids is usually considered a petroleum residue contamination but it was also detected in fruit waxes, probably generated from the side chain of chlorophyll *a, b* or carotenoid pigments [46]. 3-Pentanol is an active organic compound produced by plants that has been recently reported to trigger induced resistance by priming SA and JA signaling pathways [47]. At the same way 2, 5-Dimethylthiophene, 2-penten-1-ol (Z), 2-pentenal (E), 2,4-hexadienal (E,E)- were identified only among the compounds responsible for the 95% on the aroma in plants treated with glutamic acid and subjected to salt stress. 2, 5-Dimethylthiophene is a volatile sulfur-containing compound that has been identified in the essential oil of onion and is formed via Maillard reaction/Strecker degradation of cysteine with furaneol [48,49]. 2-pentenal (E) was previously detected in kale [41] and rocket leaves [7,37,44] and together with 2-penten-1-ol (Z) was significantly correlated with sweet attributes. Interestingly, ethanone, 1,2-diphenyl- was fond only in control plant subjected to salt stress. It belongs to deoxygenises compounds that are intermediates in the synthesis of isoflavones.

The results of this study showed that 24 hours of high salinity affects VOC bouquets of wild rocket without causing any significant damage on chlorophyll content. At the same time, any clear effect of glutamic acid application emerged, even though some volatile compounds were identified only in treated plants. More then one hundred compounds of several nature were found in each samples and further analyses are needed to better understand their biological roles. Salt stress has been shown to increase the production of several important volatile molecules in Salvia [50]. Therefore, since rocket salad is particularly appreciated for its aroma and its high content of several biological active compounds, it might be interesting to indagate changes generated by stress or different treatments through metabolic and molecular analyses. Moreover, since environmental stresses affect the VOCs profile both in terms of quantity but also in terms of composition, it might be useful to elucidate the roles of individual compounds.

REFERENCES

1. Vivaldo, G.; Masi, E.; Taiti, C.; Caldarelli, G.; Mancuso, S. The network of plants volatile organic compounds. *Sci. Rep.* **2017**, *7*, 11050.
2. Dudareva, N.; Klempien, A.; Muhlemann, J.K.; Kaplan, I. Biosynthesis, function and metabolic engineering of plant volatile organic compounds. *New Phytol.* **2013**, *198*, 16–32.
3. Holopainen, J.K.; Gershenzon, J. Multiple stress factors and the emission of plant VOCs. *Trends Plant Sci.* **2010**, *15*, 176–184.
4. Spinelli, F.; Cellini, A.; Marchetti, L.; Mudigere, K.; Piovene, C. Emission and Function of Volatile Organic Compounds in Response to Abiotic Stress. In *Abiotic Stress in Plants - Mechanisms and Adaptations*; InTech, 2011.
5. Brilli, F.; Loreto, F.; Baccelli, I. Exploiting Plant Volatile Organic Compounds (VOCs) in Agriculture to Improve Sustainable Defense Strategies and Productivity of Crops. *Front. Plant Sci.* **2019**, *10*.
6. Niederbacher, B.; Winkler, J.B.; Schnitzler, J.P. Volatile organic compounds as non-invasive markers for plant phenotyping. *J. Exp. Bot.* **2015**, *66*, 5403–5416.
7. Bell, L.; Spadafora, N.D.; Müller, C.T.; Wagstaff, C.; Rogers, H.J. Use of TD-GC–TOF-MS to assess volatile composition during post-harvest storage in seven accessions of rocket salad (Eruca sativa). *Food Chem.* **2016**, *194*, 626–636.
8. Loreto, F.; Delfine, S. Emission of Isoprene from Salt-Stressed Eucalyptus globulus Leaves. *Plant Physiol.* **2000**, *123*, 1605–1610.
9. Teuber, M.; Zimmer, I.; Kreuzwieser, J.; Ache, P.; Polle, A.; Rennenberg, H.; Schnitzler, J.-P. VOC emissions of Grey poplar leaves as affected by salt stress and different N sources. *Plant Biol.* **2008**, *10*, 86–96.
10. Ben Taarit, M.; Msaada, K.; Hosni, K.; Hammami, M.; Kchouk, M.E.; Marzouk, B. Plant growth, essential oil yield and composition of sage (Salvia officinalis L.) fruits cultivated under salt stress conditions. *Ind. Crops Prod.* **2009**, *30*, 333–337.
11. JELALI, N.; DHIFI, W.; CHAHED, T.; MARZOUK, B. SALINITY EFFECTS ON GROWTH, ESSENTIAL OIL YIELD AND COMPOSITION AND PHENOLIC COMPOUNDS CONTENT OF MARJORAM (ORIGANUM MAJORANA L.) LEAVES. *J. Food Biochem.* **2011**, *35*, 1443–1450.
12. Lee, K.; Seo, P.J. Airborne signals from salt-stressed Arabidopsis plants trigger salinity tolerance in neighboring plants. *Plant Signal. Behav.* **2014**, *9*, e28392.
13. Possell, M.; Loreto, F. The Role of Volatile Organic Compounds in Plant Resistance to Abiotic Stresses: Responses and Mechanisms. In; 2013; pp. 209–235.

14. Paravisini, L.; Guichard, E. Interactions between aroma compounds and food matrix. In *Flavour*; John Wiley & Sons, Ltd: Chichester, UK, 2016; pp. 208–234.
15. Ragaert, P.; Verbeke, W.; Devlieghere, F.; Debevere, J. Consumer perception and choice of minimally processed vegetables and packaged fruits. *Food Qual. Prefer.* **2004**, *15*, 259–270.
16. Bell, L.; Methven, L.; Wagstaff, C. The influence of phytochemical composition and resulting sensory attributes on preference for salad rocket (Eruca sativa) accessions by consumers of varying TAS2R38 diplotype. *Food Chem.* **2017**, *222*, 6–17.
17. Young, A.M.; Severson, D.W. Comparative analysis of steam distilled floral oils of cacao cultivars (Theobroma cacao L., Sterculiaceae) and attraction of flying insects: Implications for aTheobroma pollination syndrome. *J. Chem. Ecol.* **1994**, *20*, 2687–2703.
18. Tamiru, A.; Bruce, T.J.A.; Woodcock, C.M.; Caulfield, J.C.; Midega, C.A.O.; Ogol, C.K.P.O.; Mayon, P.; Birkett, M.A.; Pickett, J.A.; Khan, Z.R. Maize landraces recruit egg and larval parasitoids in response to egg deposition by a herbivore. *Ecol. Lett.* **2011**, *14*, 1075–1083.
19. Rodriguez-Saona, C.; Vorsa, N.; Singh, A.P.; Johnson-Cicalese, J.; Szendrei, Z.; Mescher, M.C.; Frost, C.J. Tracing the history of plant traits under domestication in cranberries: potential consequences on anti-herbivore defences. *J. Exp. Bot.* **2011**, *62*, 2633–2644.
20. Bennett, R.N.; Mellon, F.A.; Botting, N.P.; Eagles, J.; Rosa, E.A..; Williamson, G. Identification of the major glucosinolate (4-mercaptobutyl glucosinolate) in leaves of Eruca sativa L. (salad rocket). *Phytochemistry* **2002**, *61*, 25–30.
21. Bennett, R.N.; Rosa, E.A.S.; Mellon, F.A.; Kroon, P.A. Ontogenic Profiling of Glucosinolates, Flavonoids, and Other Secondary Metabolites in Eruca sativa (Salad Rocket), Diplotaxis erucoides (Wall Rocket), Diplotaxis tenuifolia (Wild Rocket), and Bunias orientalis (Turkish Rocket). *J. Agric. Food Chem.* **2006**, *54*, 4005–4015.
22. D'Antuono, L.F.; Elementi, S.; Neri, R. Exploring new potential health-promoting vegetables: glucosinolates and sensory attributes of rocket salads and related Diplotaxis and Eruca species. *J. Sci. Food Agric.* **2009**, *89*, 713–722.
23. Pasini, F.; Verardo, V.; Cerretani, L.; Caboni, M.F.; D'Antuono, L.F. Rocket salad (Diplotaxis and Eruca spp.) sensory analysis and relation with glucosinolate and phenolic content. *J. Sci. Food Agric.* **2011**, *91*, 2858–2864.
24. Jirovetz, L.; Smith, D.; Buchbauer, G. Aroma Compound Analysis of Eruca sativa (Brassicaceae) SPME Headspace Leaf Samples Using GC, GC−MS, and Olfactometry. *J. Agric. Food Chem.* **2002**, *50*, 4643–4646.
25. Blažević, I.; Mastelić, J. Free and bound volatiles of rocket (Eruca sativa Mill.). *Flavour Fragr. J.* **2008**, *23*, 278–285.

26. Blažević, I.; Mastelić, J. Glucosinolate degradation products and other bound and free volatiles in the leaves and roots of radish (Raphanus sativus L.). *Food Chem.* **2009**, *113*, 96–102.
27. Lv, D.G.; Yu, C.; Yang, L.; Qin, S.J.; Ma, H.Y.; Du, G.D.; Liu, G.C.; Khanizadeh, S. Effects of foliar-applied L-glutamic acid on the diurnal variations of leaf gas exchange and chlorophyll fluorescence parameters in hawthorn (Crataegus pinnatifida Bge.). *Eur. J. Hortic. Sci.* **2009**, *74*, 204–209.
28. Anderson, M.J.; Willis, T.J. Canonical analysis of principal coordinates: a useful method of constrained ordination for ecology. *Ecology* **2003**, *84*, 511–525.
29. Oksanen, J.; Blanchet, F.G.; Kindt, R.; Legendre, P.; O'Hara, R.B.; Simpson, G.L.; Solymos, P.; Stevens, M.H.H.; Wagner, H. Vegan: Community ecology package. R package version 2.0-8 2013.
30. Kindt, R.; Coe, R. *Tree diversity analysis: a manual and software for common statistical methods for ecological and biodiversity studies*; World Agroforestry Centre, Ed.; 2005; ISBN 929059179X, 9789290591795.
31. Beale, S.I. Enzymes of chlorophyll biosynthesis. *Photosynth. Res.* **1999**, *60*, 43–73.
32. Kalaji, H.M.; Jajoo, A.; Oukarroum, A.; Brestic, M.; Zivcak, M.; Samborska, I.A.; Cetner, M.D.; Łukasik, I.; Goltsev, V.; Ladle, R.J. Chlorophyll a fluorescence as a tool to monitor physiological status of plants under abiotic stress conditions. *Acta Physiol. Plant.* **2016**, *38*, 102.
33. Hniličková, H.; Hnilička, F.; Martinková, J.; Kraus, K. Effects of salt stress on water status, photosynthesis and chlorophyll fluorescence of rocket. *Plant, Soil Environ.* **2017**, *63*, 362–367.
34. Lucini, L.; Rouphael, Y.; Cardarelli, M.; Canaguier, R.; Kumar, P.; Colla, G. The effect of a plant-derived biostimulant on metabolic profiling and crop performance of lettuce grown under saline conditions. *Sci. Hortic. (Amsterdam).* **2015**, *182*, 124–133.
35. Cocetta, G.; Mishra, S.; Raffaelli, A.; Ferrante, A. Effect of heat root stress and high salinity on glucosinolates metabolism in wild rocket. *J. Plant Physiol.* **2018**, *231*, 261–270.
36. Luca, A.; Bach, V.; Edelenbos, M. Optimization of Headspace Solid-Phase Microextraction and Static Headspace Sampling of Low-Boiling Volatiles Emitted from Wild Rocket (Diplotaxis tenuifolia L.). *Food Anal. Methods* **2015**, *8*, 1185–1196.
37. Spadafora, N.D.; Amaro, A.L.; Pereira, M.J.; Müller, C.T.; Pintado, M.; Rogers, H.J. Multi-trait analysis of post-harvest storage in rocket salad (Diplotaxis tenuifolia) links sensorial, volatile and nutritional data. *Food Chem.* **2016**, *211*, 114–123.

38. Mastrandrea, L.; Amodio, M.L.; Pati, S.; Colelli, G. Effect of modified atmosphere packaging and temperature abuse on flavor related volatile compounds of rocket leaves (Diplotaxis tenuifolia L.). *J. Food Sci. Technol.* **2017**, *54*, 2433–2442.
39. Zhang, J.; Liu, C.C.; Yan, S.W.; Liu, Y.; Guo, M.B.; Dong, S.L.; Wang, G.R. An odorant receptor from the common cutworm (Spodoptera litura) exclusively tuned to the important plant volatile cis-3-Hexenyl acetate. *Insect Mol. Biol.* **2013**, *22*, 424–432.
40. Ruther, J.; Kleier, S. Plant–Plant Signaling: Ethylene Synergizes Volatile Emission In Zea mays Induced by Exposure to (Z)-3-Hexen-1-ol. *J. Chem. Ecol.* **2005**, *31*, 2217–2222.
41. Fernandes, F.; Guedes de Pinho, P.; Valentão, P.; Pereira, J.A.; Andrade, P.B. Volatile Constituents throughout Brassica oleracea L. Var. acephala Germination. *J. Agric. Food Chem.* **2009**, *57*, 6795–6802.
42. Magiatis, P.; Melliou, E.; Skaltsounis, A.-L.; Chinou, I.; Mitaku, S. Chemical Composition and Antimicrobial Activity of the Essential Oils of Pistacia lentiscus var. chia. *Planta Med.* **1999**, *65*, 749–752.
43. LIU, S.; WEI, C.; LI, W.; ZANG, X. Analysis of the aromatic constituents in 3 starfruit cultivars [J]. *J. Fruit Sci.* **2008**, *1*.
44. Bell, L.; Methven, L.; Signore, A.; Oruna-Concha, M.J.; Wagstaff, C. Analysis of seven salad rocket (Eruca sativa) accessions: The relationships between sensory attributes and volatile and non-volatile compounds. *Food Chem.* **2017**, *218*, 181–191.
45. Ghosh, S.; Derle, A.; Ahire, M.; More, P.; Jagtap, S.; Phadatare, S.D.; Patil, A.B.; Jabgunde, A.M.; Sharma, G.K.; Shinde, V.S.; et al. Phytochemical Analysis and Free Radical Scavenging Activity of Medicinal Plants Gnidia glauca and Dioscorea bulbifera. *PLoS One* **2013**, *8*, e82529.
46. Velcheva, M.P.; Donchev, C. Isoprenoid hydrocarbons from the fruit of extant plants. *Phytochemistry* **1997**, *45*, 637–639.
47. Song, G.C.; Choi, H.K.; Ryu, C.-M. Gaseous 3-pentanol primes plant immunity against a bacterial speck pathogen, Pseudomonas syringae pv. tomato via salicylic acid and jasmonic acid-dependent signaling pathways in Arabidopsis. *Front. Plant Sci.* **2015**, *6*.
48. Boelens, M.; De Valois, P.J.; Wobben, H.J.; Van der Gen, A. Volatile flavor compounds from onion. *J. Agric. Food Chem.* **1971**, *19*, 984–991.
49. Zheng, Y.; Brown, S.; Ledig, W.O.; Mussinan, C.; Ho, C.-T. Formation of Sulfur-Containing Flavor Compounds from Reactions of Furaneol and Cysteine, Glutathione, Hydrogen Sulfide, and Alanine/Hydrogen Sulfide. *J. Agric. Food Chem.* **1997**, *45*, 894–897.
50. Valifard, M.; Mohsenzadeh, S.; Kholdebarin, B.; Rowshan, V. Effects of salt stress on volatile

compounds, total phenolic content and antioxidant activities of Salvia mirzayanii. *South African J. Bot.* **2014**, *93*, 92–97.

GENERAL CONCLUSION

During the cultivation period, crops are often exposed to abiotic stresses, acting individually or in combination, which could dramatically reduce the yield and quality of products. Biostimulants are agronomic tools that could represent an effective and sustainable strategy to enhance plant growth and productiveness, improving tolerance against abiotic stresses. In fact, biostimulants are defined as "fertilizing product the function of which is to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant and the plant rhizosphere: (a) nutrient use efficiency; (b) tolerance to abiotic stress; (c) crop quality traits, (d) availability of confined nutrients in the soil and rhizosphere", according to the EU regulation 1009/2019.

It is important to consider that the complex and variable nature of raw materials used for their production and the heterogeneous mixture of components of the final product can make it difficult to attribute a specific mode of action to each biostimulant. The situation is further complicated by the high number of plants, bacteria and in general, substances included into the category of plant biostimulants. For example, two products obtained by two different plants would fall in the same category, but their effects and their mode of action might be completely different. Moreover, the opposite situation may occur; the same product may produce different effects when applied on different plants. This could be related to the genetic variability among species, variety or cultivars. In addition, the biostimulant activity of a product may also depend on the nature and severity of the abiotic stress. Several examples of these situations emerged from the experiments performed during the Ph.D.. For instance, both the borage extract tested in the first activities and the biostimulant prototype provided by the private company induced different responses according to the plant species, the cultivar and also the season. The biostimulants can act by influencing the transcription factors network and through these induce specific crop responses. These transcription factors play an important role immediately after the biostimulants application and are able to modulate the transcription profiles under stress conditions. It must also be considered that trying to link a specific mode of action only to the main component of a product might be a mistake because it would be like excluding the effect of the molecules that are presents in small quantities or in traces, but it is known that the efficacy of biostimulant products is the result of a synergistic or antagonistic effect of many components. This aspect emerged in particular from the work done in collaboration with the private company. Indeed, despite the abundance of glutamic acid in the formulation, plant responded in different ways.

The combined study of the physiological, biochemical and molecular responses of plants to biostimulant application, together with the availability of innovative research tools will surely improve the knowledge about their activity. Additionally, another aspect emerged from this work is the difficulty and the time required in the process to develop a new biostimulant product.