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Studies of quality and nutrient use efficiency in vegetable crops grown under different sustainable cropping systems

Ph.D. Thesis

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Ph. D. Thesis
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“Ego borago gaudia semper ago”

Gaius Plinius Secundus

ABSTRACT

Keywords: biostimulant characterization; *Borago officinalis* L.; vegetables quality; nutrient use; sustainable agriculture; abiotic stress.

The purpose of the Ph. D. research project was to investigate the effects of biostimulant products on leafy vegetables (lettuce and rocket) and deepen the knowledge on their mode of action. The first part of the work regarded the further deepening of the effects of aqueous extracts obtained from leaves (LE) and flowers (FE) of *Borago officinalis* L. on lettuce, involving phenomic, agronomic, physiological, and biochemical aspects. Results showed that borage extracts enhanced the primary metabolism. Total flavonoids and phenols, as well as the total protein levels, the *in vitro* PAL specific activity, and the levels of PAL-like polypeptides increased by all borage extracts, with particular regards to FEs. FEs also proved efficient in preventing degradation and inducing an increase in photosynthetic pigments during storage. In conclusion, borage extracts, with particular regard to the flower ones, appear indeed to exert biostimulant effects on lettuce. Borage extracts were also applied on rocket plants, to investigate the influence of treatments on nitrate assimilation pathway and on the molecular responses. Gene expression analysis of the main enzymes involved in the nitrate

metabolism (*DtNR, DtNiR, DtGLU, DtGS1, DtNTR*) was evaluated. From the biochemical point of view, the most interesting result was surely the substantial reduction of nitrate level caused by both extracts, confirmed also by the increment of the NR *in vivo* activity. Borage treatments influenced also the gene expression, confirming that extracts have a role in the physiological processes in which the considered genes are involved.

In addition, work regarding borage extracts characterization was carried out. The auxin- and gibberellin-like activity of extracts on maize mutants was explored and, due to the multitude properties attributed to borage, the allelopathic effects of borage extracts on seeds germination of different plant species was investigated. LE treatment seems to possess a slight auxin-like activity. The bioassay on allelopathic properties of borage LE and FE demonstrated that they exert an effect on seeds germination (inhibition effect).

The work included also an activity carried out in collaboration with a private company to study the effectiveness of commercial biostimulants and prototypes on leafy vegetables quality and protection against abiotic stresses (salt stress).

Contents

INTRODUCTION

Biostimulants

Agricultural systems have been oriented for years to increase yield without considering the quality of the produce and the rational use of resources. In contrast, attention now is mainly focused on product quality and cultivations sustainability. Moreover, cultivation management takes into consideration the reduction of production costs by lowering inputs. Protected cultivation of vegetable crops usually requires high amounts of fertilizers and pesticides but it is not always true that high nutrient availability corresponds to a higher quality of the products. On the contrary, excessive fertilization, and especially high nitrogen supply, stimulates vegetative growth with a higher susceptibility to pathogens (Liebman & Davis, 2000). In leafy vegetables, the excessive availability of nitrates often induces an accumulation in leaves of these compounds with levels above the limits imposed by EU regulation N. 1258/2011. The accumulation of high levels of nitrates can impact adversely on human health since, in the organism, nitrate is reduced to nitrite that reacting with the free amines could form carcinogenic nitrosamines (Luo *et al*., 2006; Parks *et al*., 2008; Cavaiuolo & Ferrante, 2014; Bulgari *et al*., 2017). High rates of nitrogen fertilizers can have also detrimental

impacts on the environment, such as nitrate flows into waterways, and can increase greenhouse gas emissions of nitrous oxide (Mattner *et al*., 2013).

The need to practice a sustainable agriculture, maintaining good crop yield and quality, is favoring the expansion of biostimulants. They have increasingly been considered as production tools as demonstrated by the spread in scientific publications and by the constantly expansion of their market (Povero *et al*., 2016). France, Italy, and Spain are the leading EU countries in the production of biostimulants (Traon *et al*., 2014). They are products containing bioactive compounds able to improve water and nutrient use efficiency of crops, stimulate plant development, and counteract abiotic stresses (Van Oosten *et al*., 2017) by enhancing primary and secondary metabolism (Bulgari *et al*., 2015; Yakhin *et al*., 2017).

Biostimulants are obtained from organic raw materials; the most common components are mineral elements, vitamins, amino acids, chitin, chitosan, and poly- and oligosaccharides (Berlyn & Russo, 1990; Hamza & Suggars, 2001; Kauffman *et al*., 2007; du Jardin, 2015; Ertani *et al*., 2016).

Their composition usually is partly unknown; the complexity of the extracts and the wide range of molecules contained in the extracts it makes very difficult to understand which are the most active compounds. The isolation

and the study of a single component is almost impossible, moreover efficacy of a biostimulat is not due to a single compound but to the synergistic action of different bioactive molecules. The mechanisms activated by biostimulants are difficult to identify and still under investigation (Ertani *et al*., 2011, 2013; Guinan *et al.,* 2012). From a legal point of view, biostimulants can contain traces of natural plant hormones, but their biological action should not be ascribed to them, otherwise they should be registered as plant growth regulators. Biostimulants can act directly on the plant physiology and metabolism or by improving the soil conditions (Nardi *et al.,* 2009).

These products are usually applied in addition to standard fertilization treatments (Heckman, 1993). They differ from fertilizers because they have an effect on plant metabolism and their nutrient concentrations are negligible; moreover, they act at low concentrations (Zhang & Schmidt, 1999). Biostimulants can be soil- or leaf-applied, depending on their composition and on the desired results (Kunicki *et al*., 2010). They exert their action only if they penetrate into the plant tissue and is important to considered different species may have different leaf permeability to biostimulants. The leaf cuticle can represent a barrier for biostimulant adsorption and the chemical structure of bioactive compounds can be an

obstacle to their penetration in the inner part of the leaf. The absorbability depends also on field conditions (temperature, relative air humidity, wind speed for example); the application of surfactants can help in the penetration (Kolomaznik *et al*., 2012).

Categories

Biostimulants are classified in the following major groups:

Humic substances (*HSs*): they include humic acids, fulvic acids and humins. *HSs* are natural constituents of the soil organic matter, resulting from the decomposition processes of plants, animals, and microbial residues, but also from the metabolic activity of soil microbes (du Jardin, 2015). Treatments with humic substances can increase plants root growth, the uptake of nutrients, and enhance tolerance to abiotic stresses (Canellas *et al.*, 2015; Nardi *et al*., 2016). These positive effects could be mainly ascribed to the hormone-like activity (several hormones in the humus structure have been identified) (Nardi *et al*., 2000; Pizzeghello *et al*., 2001, 2002).

Seaweed extracts: seaweeds are a vast group of macroscopic, multicellular marine seaweeds that can be brown, red, and green. Seaweeds 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 enhance plant growth, abiotic stresses tolerance, photosynthetic activity, and resistance to fungi, bacteria and virus, improving the yield and productivity of several crops (Norrie & Keathley, 2006; Gajc-Wolska *et al*., 2013; Sharma *et al*., 2014). Seaweeds used for biostimulant production contain cytokinins and auxins or other hormone-like substances (Hamza & Suggars, 2001). 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.

Hydrolysed proteins and amino acids containing products: hydrolysed proteins are a mixture of amino acids, peptides, polypeptides and denatured protein and can be obtained by chemical, enzymatic and thermal hydrolysis of proteins from both plant and animal sources. Recent studies (Cerdán *et al.*, 2009; Lisiecka *et al*., 2011) reported that the applications of some commercial protein hydrolysate products from animal origin were phytotoxic causing negative effects on plant growth when compared to commercial protein hydrolysate of plant origin. They can induce plant defence responses and increase plant tolerance to many abiotic stresses.

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

A final 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. 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) (Ertani *et al*., 2011). Because of the diversity of source materials and extraction technologies, the mode of action of these products is not easily determined (Yakhin *et al*., 2017).

Application of biostimulants on vegetable crops

Biostimulants can be used in vegetables production to improve productivity and yield, and to enhance plant health and tolerance to stress factors. Biostimulants often increase the color of leaves by stimulating the chlorophyll content (Abbas & Akladious, 2013). This aspect is important for the visual appearance of the produce and consumer's appeal (in particular for leafy vegetables) and probably allows a greater photosynthetic activity of leaves. High concentration of leaf pigments resulting from biostimulant treatments in rocket was observed by Vernieri, Borghesi, *et al*. (2005, 2006), and in lettuce and endive by Bulgari *et al*. (2014). Inoculation with some plant growth promoting bacteria (PGPR) increased chlorophyll concentration, nutrient content, and yield of strawberry (*Fragaria ananassa*) plants growing in high saline soils (Karlidag *et al.*, 2013).

On carrot (*Dacus carota*), Aminoplant not only influenced productivity, but also the chemical composition of the roots. Aminoplant influenced yield of roots and leaf rosette biomass, increased the soluble sugars content in carrot roots and affected dry matter content. In general, different crops treated with this product showed a greater yield per hectare (Maini, 2006).

The use of Goe¨mar BM86 in the cultivation of broccoli (*Brassica oleracea* 'cymosa'), in open field, had a significant effect on the chemical quality of produce. The content of macro- and micronutrients increased, as well as the yield (Gajc-Wolska *et al*., 2013). Four different biostimulants, Radifarm, Megafol, Viva, and Benefit (Valagro S.p.A.) increased the yield of pepper (*C. annuum*) grown hydroponically and at the same time improved fruit quality during the hot summer season (Paradikovic´ *et al*., 2011). Petrozza *et al*. (2013a) showed that Radifarm treatments on tomato (*Solanum lycopersicum*) plants stimulated a greater root system and more secondary roots. Therefore, the treated plants had higher water use efficiency. The same authors demonstrated that Viva treatments on drought-stressed plants of *S. lycopersicum* 'Ikram' increased plant biomass and enhanced root development (Petrozza *et al*., 2013b).

Haider *et al*. (2012) studied the effect of foliar application of seaweed extract Primo on potato (*Solanum tuberosum* 'Sante') and showed a significant improvement of plant growth, yield and tuber quality. Moreover, it also improved nitrogen, total soluble solids and protein contents of the tubers. Mattner *et al.* (2013) demonstrated that kelp extract (Seasol) stimulates broccoli establishment and growth in the glasshouse and field; the leaf area, stem diameter and biomass of broccoli were significantly

increased. The application of Radifarm on lettuce (*L. sativa*) and tomato (*S. lycopersicum*) plants at nursery level had a positive effect on plant growth by increasing the shoot and roots development (Vernieri *et al*., 2002). Tarantino and colleagues (2015) evaluated the effects of three different commercial biostimulants on quali-quantitative yield characteristics of cauliflower, pepper, and fennel. Positive effects regarding fruits weights and soluble solid contents were obtained in pepper; with respect to fennel crops, a lower nitrate content was observed.

AIM

The purpose of the Ph. D. research project was to investigate the effects of biostimulants products on leafy vegetables (lettuce and rocket) and deepen the knowledge on their mechanism of action.

The first part of the work regarded the production and the study of aqueous extracts obtained from leaves and flowers of *Borago officinalis* L. as potential biostimulants. Borage was chosen since this plant is rich in bioactive compounds exploited in different field, but there are no studies on the application of its aqueous extract as biostimulant. Extracts were applied on lettuce (*Lactuca sativa* L. '*Longifolia*') and effects were evaluated through a multidisciplinary approach, involving phenomic, agronomic, physiological, and biochemical aspects, to set up a protocol to assess their effects.

Borage extracts were applied also on rocket (*Diplotaxis tenuifolia* L.), in order to evaluate the effects on another vegetable crop, and in particular to investigate the influence of treatments on nitrate levels. Rocket is in fact a hyper-accumulator of nitrate and so it is very interesting to reduce this compound in leaves. Moreover, the University of Milan has recently published the transcriptome of rocket under different abiotic stresses and

these informations are useful for studying different physiological and biochemical pathways.

The experiment was carried out studying in addition the molecular responses induced by borage. In particular, the gene expression analysis of the main enzymes involved in the nitrate metabolism (nitrate reductase, nitrite reductase, glutamine synthetase, glutamate synthase, nitrate transporter) was evaluated.

Moreover, the auxin- and gibberellin-like activity of extracts on maize mutants was explored and, due to the multitude properties attributed to borage, the allelopathic effects of borage extracts on seeds germination of different plant species was investigated.

The work included also a collaboration with a private company to study the effectiveness of commercial biostimulants and prototypes on leafy vegetables quality and protection against abiotic stresses.

CHAPTER 1

Evaluation of borage extracts as potential biostimulant using a phenomic, agronomic, physiological and biochemical approach

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Abstract

Biostimulants are substances able to improve water and nutrient use efficiency and counteract stress factors by enhancing primary and secondary metabolism. Premise of the work was to exploit raw extracts from leaves (LE) or flowers (FE) of *Borago officinalis* L., to enhance yield and quality of *Lactuca sativa* 'Longifolia', and to set up a protocol to assess their effects. To this aim, an integrated study on agronomic, physiological and biochemical aspects, including also a phenomic approach, has been adopted. Extracts were diluted to 1 or 10 mL L^{-1}, sprayed onto lettuce plants at the middle of the growing cycle and one day before harvest. Control plants were treated with water. Non-destructive analyses were conducted to assess the effect of extracts on biomass with an innovative imaging technique, and on leaf photosynthetic efficiency (chlorophyll *a* fluorescence and leaf gas exchanges). At harvest, the levels of ethylene, photosynthetic pigments, nitrate and primary (sucrose and total sugars) and secondary (total phenols and flavonoids) metabolites, including the activity and levels of phenylalanine ammonia lyase (PAL) were assessed. Moreover, a preliminary

study of the effects during postharvest was performed. Borage extracts enhanced the primary metabolism by increasing leaf pigments and photosynthetic activity. Plant fresh weight increased upon treatments with 10 mL L^{-1} doses, as correctly estimated by multi-view angles images. Chlorophyll *a* fluorescence data showed that FEs were able to increase the number of active reaction centers per cross section; a similar trend was observed for the performance index. Ethylene was 3-fold lower in FEs treatments. Nitrate and sugar levels did not change in response to the different treatments. Total flavonoids and phenols, as well as the total protein levels, the *in vitro* PAL specific activity, and the levels of PAL-like polypeptides were increased by all borage extracts, with particular regards to FEs. FEs also proved efficient in preventing degradation and inducing an increase in photosynthetic pigments during storage. In conclusion, borage extracts, with particular regard to the flower ones, appear to indeed exert biostimulant effects on lettuce; future work will be required to further investigate on their efficacy in different condition and/or species.

Keywords: *Borago officinalis* L., image analysis, *Lactuca sativa* L., non-destructive measurements, phenols, photosynthesis.

1.1 Introduction

In the last years, the use of biostimulants has been constantly increasing for sustainable agriculture, because these substances enhance nutrient use efficiency, reduce fertilizers consumption, stimulate plant development and growth (Kunicki *et al.*, 2010; Calvo *et al*., 2014; Halpern *et al*., 2015; Le Mire *et al*., 2016), and counteract stress factors, eventually enhancing crop quality and yield (Ziosi *et al*., 2013; Van Oosten *et al*., 2017). The interest in this sector is evidenced by the significant increase of research papers focused on it and by its economical relevance. The market of biostimulant products is projected to increase by 12% annually (Calvo *et al*., 2014), reaching $2,524.02 million by 2019 (Povero *et al*., 2016). Biostimulants are generally made of raw organic materials containing bioactive compounds. Their nature is heterogeneous, since they include humic acids, protein hydrolysates, plant growth-promoting *Rhizobacteria* and fungi, and extracts from seaweeds and higher plant species (du Jardin, 2015; Ertani *et al*., 2013, 2016). For this reason, also their chemical composition is highly heterogeneous, including mineral elements, vitamins, amino acids, chitin,

chitosan, and poly- and oligosaccharides, and therefore it is partly unknown. Moreover, their chemical complexity and the wide range of molecules present make very difficult to understand which are the most active compounds (du Jardin, 2015; Brown & Saa, 2015; Bulgari *et al*., 2015; Posmyk & Szafrańska, 2016; Yakhin *et al*., 2017).

Under a biochemical point of view, the increased plant growth induced by biostimulants can be associated with an increase in amino acid levels and enhanced protein biosynthesis, as well as in carbohydrate concentration in leaves (Abdalla, 2013). Higher sugar levels in plants treated with biostimulants have been found in several species, associated with higher chlorophyll accumulation, net photosynthesis (Abbas & Akladious, 2013; Abdalla, 2013), and quantum efficiency of photosystem II (Ferrini & Nicese, 2002; Amanda *et al.*, 2009; Ertani *et al.*, 2012). In lettuce, biostimulant application at the nursery level positively affects plant growth by increasing the development of shoots and roots, strongly stimulating root growth and increasing leaf area (Amanda *et al.*, 2009; Vernieri *et al.*, 2002). Moreover, a positive role on yield and quality of head lettuce has been reported, with particular regard to the reduction of nitrate that is an important issue for human health (Shehata *et al.*, 2016).

Biostimulants are capable to counteract plant stresses that are usually related to a burst in ethylene synthesis and sensitivity, and eventually affect produce quality by altering or accelerating tissue senescence (Saltveit, 1999). The activation of stress responses in plants is often accompanied by the synthesis of secondary metabolites (Ramakrishna & Ravishankar, 2011; Mazid *et al.*, 2011) that often act as antioxidants scavenging *in vivo* and *in vitro* (Cook & Samman, 1996) the free radicals (Halliwell, 2008) produced in stress-induced oxidative reactions (Sharma *et al.*, 2012), and counteracting the free radical-induced damages to cell components. In animal systems, and particularly in humans, several studies have shown that a diet rich in antioxidants from plant-derived foods may prevent the onset of a wide range of chronic-degenerative diseases (Manach *et al.*, 2004; Vauzour *et al.*, 2010; Martin *et al.*, 2013; Bertoia *et al.*, 2016).

The largest group of bioactive beneficial secondary plant metabolites is represented by phenolic compounds, ubiquitous in all tissues of higher plants, where they play an important role providing the plant with specific adaptations to changing environmental conditions and eliciting defense mechanisms (Caretto *et al.*, 2015 and references therein). Phenolic compounds are synthesized in the phenylpropanoid pathway, that produces an array of different substances including, amongst others, phenolic acids

and flavonoids (El Gharras, 2009), reported to possess powerful antioxidant activity *in vitro* (Kähkönen *et al.*, 1999). The first committed enzyme in the phenylpropanoid biosynthetic pathway is Phenylalanine Ammonia Lyase (PAL; E.C. 4.3.1.5), that catalyzes the non-oxidative deamination of phenylalanine to trans-cinnamic acid, common substrate for the biosynthesis of various phenylpropanoid compounds (Ferrer *et al.*, 2008), a step that represents a crucial link between primary and secondary metabolism. PAL activity is positively related with increased production of phenylpropanoids (Vogt, 2010). Vegetal extracts derived from red grape, blueberry fruits and hawthorn leaves or from oak, affect PAL activity and expression of PAL genes as well as the levels of polyphenols in maize or grape, respectively, when applied as biostimulant (Ertani *et al.*, 2011, 2016; Pardo-García *et al.*, 2014). To our knowledge, studies on PAL in lettuce have dealt mainly with its involvement in postharvest disorders (Ke and Saltveit, 1986, 1989), tissue browning (Campos *et al.*, 2004), and pigment biosynthesis under different temperature regimes (Chon *et al.*, 2012). In the last decade, the availability of relatively inexpensive and high-performance optical systems, digital cameras and associated software technologies has boosted the development of phenotyping systems, i.e., of semi-automatic or automatic devices enabling repeated and non-invasive measurement of macroscopic plant's

parameters related to growth, architecture features or to main tissue components (Fiorani & Schurr, 2013). These systems are typically based on RGB color cameras to compute leaf area, biomass volume or to count/quantify specific plant organs, but they can also include the use of VIS-NIR hyperspectral cameras, to estimate the levels of main tissue components as chlorophylls, anthocyanins and water, of fluorescence imaging devices, to map the efficiency of photosystems, or of thermal infrared cameras, to evaluate foliage temperature and leaf water status (Li *et al.*, 2014; Fahlgren *et al*., 2015).

Early applications of color imaging to monitor lettuce growth were aimed to investigate the possible use of sensed plant-projected area extracted from top-view images to identify nutrient stress in hydroponic cultivation (Giacomelli *et al.*, 1998). More recently, similar approaches have been applied to lettuce growth-rate data extracted from greenhouse imaging, to be used as state-variable to feedback control of nutrient solution in hydroponic system (Jung *et al.*, 2015) or for other crop management operations (Kim *et al.*, 2013). Bumgarner *et al.* (2012), by conducting an extensive study on imaging of lettuce plants grown in different environments, concluded that a top-view approach is an accurate method to indirectly measure lettuce biomass during the early stages of growth, while on canopy closure the

correlation is weakened by occlusions in plant's top-view due to leaves overlapping. This limitation, also reported by Jung *et al.* (2015) for lettuce plants at advanced stages of development, is encountered with any plant with erectophyle architecture (Stewart *et al.*, 2007; Tackenberg, 2007), and it has been addressed by deploying different approaches, as: by side-view imaging configurations, or, rarely, in combination with top-view (Pereyra-Irujo *et al.*, 2012); by the use of three dimensional (3D) measuring instrumentation such as LIDAR (Friedli *et al.*, 2016), stereoscopic or multi-view cameras (Rose *et al.*, 2015; Golbach *et al.*, 2016) or time-of-flight (ToF) cameras (Chéné *et al.*, 2012) .

Low-cost 3D imaging sensors are emerging as an alternative to expensive 3D measurement systems, especially interesting for experiments involving small-scale, custom-made phenotyping hardware (Azzari *et al.*, 2013; Paulus *et al.*, 2014). The Microsoft Kinect V1 is a popular example of such a device, able to acquire at real time rate (30 frames per second) RGB color images aligned and synchronized with a depth D images. It can operate under indoor (or protected) illumination conditions, in a recommended distance range of 1-3.5 m, with a nominal depth error of ±10 mm (Livingston *et al.*, 2012). A phenomic approach has recently been described in a study dealing with the evaluation of the efficacy of the biostimulant

Megafol® in reducing drought-stress related damage in tomato plants (Petrozza *et al*., 2014). Recently, an innovative method of selection and characterization of plant biostimulant matrices, involving a combination of technology, processes, and know-how, has been proposed (Povero *et al.*, 2016).

Borage (*Borago officinalis* L.; Boraginaceae) is native to the Mediterranean region (Baubaire & Simon, 1987). The beneficial properties of chemicals extracted from different organs of this plant are widely acknowledged (for a review, see Asadi-Samani *et al.*, 2014), and they are used in traditional medicine (Krishnaiah *et al.*, 2011; Asadi-Samani *et al.*, 2014), food preservation (Astiasaran, 2009; Garcia-Iñiguez de Ciriano *et al.*, 2009; Aliakbarlu & Tajik, 2012) and even for packaging purposes (Gómez-Estaca *et al.*, 2009). The antioxidant properties of borage extracts from defatted seeds, leaves or flowers can mainly be ascribed to the presence of phenolic compounds (Wettasinghe *et al.*, 2001; Aliakbarlu & Tajik, 2012). Borage leaves, that represent more than 60% of the plant matter, are considered also a low-cost crop by-product by some food processing industries (Garcia-Herreros *et al.*, 2010).

On these premises, it appeared worthwhile to explore the possibility of using borage as a cheap source of biostimulants. Aim of the present work was to

study the efficacy on lettuce plants (*Lactuca sativa* 'Longifolia') of foliar treatments with raw aqueous extracts obtained from leaves or flowers of *Borago officinalis* L. For this reason, a holistic approach has been adopted, including both traditional and innovative investigation techniques. Within the framework of this multidisciplinary study, a non-invasive measurement setup based on Kinect devices was implemented to evaluate plant growth (biomass) during time. Leaf functionality and stress responses were monitored by non-destructive measurements of chlorophyll *a* fluorescence, gas exchanges and by ethylene determination. Quality parameters such as concentrations of sugars, nitrates, and photosynthetic pigments were assessed, together with those of representative phenylpropanoid compounds (total phenols, flavonoids) and PAL activity and PAL-like polypeptide levels, by traditional biochemical methodologies. Eventually, a preliminary trial was set up in order to observe the effects of borage extracts integrated into packing films during storage of lettuce leaves.

1.2 Materials and methods

1.2.1 Preparation and chemical characterization of borage extracts

Borage plants were harvested in the flowering stage in open field in Lodi province during spring (April). Borage flowers or leaves were minced, macerated in deionized water (500 g in 1 L) for 25 d, in the dark, at room temperature (RT). The aqueous extracts were filtered and properly diluted in water (to 1 or 10 mL L^{-1}) to be used for treatments. For chemical characterization, borage extracts were digested in wet conditions (0.1 M HNO_3) and P, K, Ca, Fe, and Mn levels were measured by inductively-coupled plasma mass spectrometry (ICP-MS; Bruker Aurora M90; Giro & Ferrante, 2016). Total N was determined with the Dumas method by using an elemental analyser (ThermoQuest NA 1500 N; Thermo Electron, Milan, Italy). Total phenolic compounds in borage extracts were determined by the Folin-Ciocalteu's procedure (Singleton *et al.*, 1999; Kang & Saltveit, 2002). A 100 μL aliquot of extracts was diluted with 3.90 mL of double-distilled water and combined with 250 μL of 50% (v/v) Folin-Ciocalteu's reagent and 750 μL of saturated (20% w/v) Na_2CO_3. Samples were vigorously shaken and incubated for 2 h, at RT in the dark before absorbance measurement at 765 nm. Total phenolics were expressed as gallic acid equivalents (GAE; mg L^{-1}) upon comparison with a freshly prepared gallic acid standard curve. The pH values of aqueous extracts were measured by a Crison pH-Meter

GLP 21^{+}. The electrical conductivity was determined using a conductivity meter (Delta Ohm, Padova, Italy). Chemical characterization of extracts is reported as Supplementary Material (Table s1).

1.2.2 Plant material and treatments

Romain lettuce (*Lactuca sativa* 'Longifolia') was obtained from a local nursery. Two week-old plantlets were transplanted in 10 cm diameter plastic pots (nine pots per treatment), on a peaty substrate, in a greenhouse at the Faculty of Agricultural and Food Sciences of Milan, under controlled conditions. Environmental conditions in greenhouse during the experimental period were in average 20.3 °C and 67 % relative humidity. Treatment solutions were sprayed in the morning (between 09:00 and 10:00) onto lettuce leaves until run-off, at half cycle (13 d after transplanting) and one day before harvest (21 d after transplanting). The treatment conditions were: water (control plants); 1 or 10 mL L^{-1} of borage leaf extracts (LE); 1 or 10 mL L^{-1} of borage flower extracts (FE). Lettuce was harvested at commercial maturity stage. At harvest, after discarding the wrapper leaves from the lettuce heads, the next three non-injured leaves from four heads per treatment were carefully removed and 12 x 10 cm midrib sections were

excised, starting at ca. 7 cm from the basis of the leaf. The pooled leaf sections from each plant were gently rinsed with distilled water, blotted with paper towels, and immediately frozen in liquid N_2 and stored at -80 °C or at -20 °C until use for biochemical analyses.

1.2.3 Non-destructive determinations

During the growth cycle and at harvest non-destructive analyses were conducted on fresh leaf tissue.

Estimation of plant growth

To evaluate the lettuce head biomass during time, an *in vivo* measurement technique was applied, consisting in acquiring and processing images from multi-angle side views of undisturbed potted lettuce plants. Images were acquired with Kinect V1 (Microsoft, USA). Measurements of lettuce head volume were conducted in a 1.3 m x 1.3 m x 1.8 m controlled-light cabinet where two Kinect V1 units were installed, one acquiring images from top and one from side view. A motorized table holder rotated the potted plant around its vertical axis during imaging, enabling to acquire 11 side images of each lettuce head viewed at angle steps of 30°. Top-view was aimed to

monitor biomass growth during the very early stages of plant development. Since for this experiment, the quantitative analysis of the growth was focused on plants at advanced development stages, only the measurements from the side-view imaging device were considered, thanks the superior accuracy (i.e., reduced sensitivity on leaf occlusions) of this setup for at more advanced growth stages. To this aim, the head-projected area was automatically segmented from the background of the cabinet in each of the 11 images, and the head volume computed by composing the side areas into a solid of revolution around the vertical axis of the plant. Lettuce image acquisitions were repeated at three different time points at 5-d intervals approximately, i.e., at dates corresponding to: 2 d before treatment 1, 3 d after treatment 1, and the same day of treatment 2. From the computed head volume (Vh; cm^3) for each plant and each time point, an estimate of the corresponding fresh weight (FWh; g) was obtained through a linear model $FWh = a0 + a1 \times Vh$. This equation was calibrated using a dataset collected in a complementary experiment, separately conducted on 78 lettuce plants grown in pots according to the control protocol. After transplanting, every 4^{th} d a subset of six plants was imaged and destructively harvested to measure the FWh. A wide-range (from 2.5 g to 155.8 g) set of 78 known values of FW and their corresponding values of computed volume was

obtained. From a regression analysis computed with the Matlab 8.4 software package (MathWorks, Natick, USA), the coefficients a0= -1.97 g and a1=0.013 g × cm^{-3} were obtained with a root-mean-square error of calibration (RMSEC) of 2.2 g, to be used in the linear equation for non-invasive estimation of the lettuce heads biomass during growth by means of multi-angle side-view imaging of potted plants.

Chlorophyll *a* fluorescence and gas exchange

Chlorophyll *a* fluorescence was measured 1 d after each treatment (i.e., 14 d and 21 d after transplanting, respectively) using a hand-portable fluorometer (Handy PEA, Hansatech, Kings Lynn, UK). Leaves were dark-adapted for 30 min. Using a leaf clip (4 mm diameter), a rapid pulse of high-intensity light of 3000 μmol m^{-2} s^{-1} (600 W m^{-2}) was administered to the leaf inducing fluorescence. The fluorescence parameters were calculated automatically by the used device. Leaf gas exchange rates were measured using the portable infrared gas exchange system CIRAS-1 (PP Systems, Hitchin, UK), operated in open-configuration with controlled temperature, CO_2 concentration, and vapor pressure. Measurements were carried out on a fully expanded leaf between 09:00 and 13:00 hours IT time. In the cuvette,

during the recording time, light intensity was fixed to 1000 µmol·m^{-2}·s^{-1} and CO_2 concentration was set to 350 ppm.

1.2.4 Destructive determinations

Ethylene emission

Whole lettuce heads were harvested the day after the second treatment. Each plant was enclosed in a 1.7 L airtight jar at 20 °C. Ethylene was determined by withdrawing with a syringe, 3 h after jar sealing, a 1-mL headspace gas sample and injecting it into a Dani 3800 gas chromatograph (DANI Instruments S.p.A., Cologno M.se, Milan, Italy) equipped with a stainless steel column (100 cm long; 0.32 cm diameter) filled with Porapack Q at 100 °C and a flame-ionization detector at 210 °C. The carrier gas was N_2 at 0.8 bar.

Chlorophylls and carotenoids

Chlorophylls and carotenoids were determined in lettuce leaf tissue at harvest or after 7 d storage in plastic bags. Leaf tissue (30-50 mg) was extracted using 100% (v/v) methanol, for 24 h at 4 °C in a dark room; afterwards quantitative determination of chlorophylls was carried out.

Absorbance readings were measured at 665.2 nm and 652.4 nm for chlorophylls and 470 nm for total carotenoids. Pigment levels were calculated by Lichtenthaler's formula (Lichtenthaler, 1987) and expressed on the basis of fresh weight of the tissue.

Nitrate

Nitrate concentration was measured by the salicylsulphuric acid method (Cataldo *et al.*, 1975). One g of fresh leaf tissue was homogenized (mortar and pestle) in 3 mL of distilled water. The extract was centrifuged at 3000 x *g* for 15 min at RT (ALC centrifuge-model PK130R) and the recovered supernatant was used for the colorimetric determination. Twenty µL of sample were added to 80 µL of 5% (w/v) salicylic acid dissolved in H_2SO_4 plus 3 mL of 1.5 N NaOH. The samples were cooled at room temperature and absorbance at 410 nm was measured. Nitrate concentration was calculated referring to a KNO_3 standard calibration curve.

Sugars

About 1 g of leaf tissue was homogenized in 5 mL of distilled water and centrifuged at 3000 x *g* for 15 min at RT. Sucrose and total sugars were

assayed according to the resorcinol method and anthrone assay, respectively (Cocetta *et al.*, 2015). Absorbance was read at 500 nm for sucrose and at 620 nm for total sugars and the levels were calculated referring to sucrose or glucose calibration curves respectively.

Total antioxidant capacity

One g of the frozen pooled leaf tissue was ground (mortar and pestle) in the presence of liquid N_2 to a fine powder. Two volumes of 100% (v/v) methanol were added and the suspension was homogenized and centrifuged in a Sorvall RC-5B refrigerated centrifuge (10000 x *g*, 20 min, 4 °C). The supernatant was recovered and the resulting pellet, resuspended in 0.5 mL of 70% (v/v) methanol, was centrifuged again. The two pooled supernatants were kept at 4 °C until immediate use for spectrophotometric determinations. An aliquot of 0.1 mL of methanolic lettuce leaf extract was combined with 1 mL of reagent solution [0.6 M H_2SO_4, 28 mM NaH_2PO_4, 4 mM $(NH_4)_6Mo_7O_{24}$], and incubated at 95 °C for 90 min. After cooling to RT, the absorbance of the samples was measured at 695 nm in a UV-vis spectrophotometer (Secomam UviLine 9400). The levels of ascorbic acid-like substances were calculated from a standard curve obtained with a 10-150 µM freshly prepared ascorbic acid standard solution in 70% (v/v)

methanol and extracts antioxidant capacity was expressed as ascorbic acid equivalents g FW^{-1} (AAE; Prieto *et al.*, 1999).

Phenolic compounds

Total phenolic compounds were assayed in the methanolic leaf extracts by the Folin-Ciocalteu's procedure as described in the paragraph "Preparation and chemical characterization of borage extracts", and expressed as GAE (mg g^{-1} FW of the tissue) upon comparison with a standard curve obtained with freshly prepared gallic acid in 70% (v/v) methanol.

Total flavonoids

Total flavonoids were determined according to Floegel *et al.* (2011). An aliquot of 500 µL of leaf methanolic extracts or standard solution (freshly prepared rutin dissolved in 70% v/v methanol) were mixed with 3.2 mL of double-distilled water. One hundred and fifty µL of 5% (w/v) $NaNO_2$ solution were added and mixed, followed, after 5 min, by the addition of 150 µL of 10% (w/v) $AlCl_3$. After 6 min, 1 mL of 1 M NaOH was added and absorbance at 510 nm of the colored flavonoid-aluminum complex was measured immediately. Total flavonoid concentration was expressed as nmol rutin equivalents g^{-1} FW of the sample.

PAL extraction and *in vitro* activity assay

Extraction and *in vitro* assay of PAL activity were conducted as described by Chen *et al.* (2006) and Jhin and Hwang (2015) with slight modifications. Two g of frozen leaf tissue were homogenized (mortar and pestle) in the presence of liquid N_2 with four volumes of a buffer containing 100 mM Tris-HCl (pH 8.8), 2 mM Na_2-EDTA, 5 mM ascorbic acid, 1 mM PMSF, 5 mM MSH, 10% (w/w) PVPP. The samples were filtered through four layers of cheesecloth and centrifuged (15000 x *g*, 30 min, 4° C; Sorvall RC-5B); the supernatants, containing total soluble proteins, were used as crude enzyme extracts. The *in vitro* assay of PAL activity was conducted in a mix (1 mL total volume) containing 100 mM Tris-HCl buffer (pH 8.8), 20 mM (final concentration) phenylalanine and aliquots (100 µL and 200 µL) of crude enzyme extract, added to start the reaction. The mix was incubated at 38 °C and the reaction stopped, after 0 min (blank), 30 min and 60 min, by addition of 250 µL of 6 N HCl. After centrifugation, the absorbance at 290 nm of the recovered supernatants was read. One unit of PAL activity was defined as the amount of enzyme causing an increase of 0.01 in absorbance at 290 nm, equal to 3.09 nmol of cinnamic acid (CA) formed per hour. PAL specific activity was then expressed on the basis of the tissue soluble protein

concentration, determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard (Micro-Bio-Rad Protein Assay; Bio-Rad Laboratories, Segrate, Italy).

SDS-PAGE and Western blotting

Proteins denatured in sodium dodecyl sulfate (SDS) sample buffer (Laemmli, 1970) were analyzed by tricine-SDS-PAGE (10% total acrylamide/bis-acrylamide concentration; Schägger and von Jagow, 1987) in a MiniProtean™ apparatus (Bio-Rad Laboratories); gels were stained with Coomassie Blue R-250. Molecular weight markers were from Bio-Rad (Kaleidoscope Pre-Stained Standards).

Proteins were electro-blotted onto nitrocellulose membrane (0.2 µm, Amersham Life Science) in a Multiphor II Nova-Blot (Amersham Biosciences, Milan, Italy) apparatus (Morgutti *et al.*, 2006). Protein transfer was carried out at RT at 0.8 mA cm^{-2}. The membrane was blocked for 2 h in 3% (w/v) defatted milk in Tris-buffered saline-Tween buffer [TBS-T: 20 mM Tris-HCl, (pH 7.6), 200 mM NaCl and 0.05% (w/v) Tween-20] and incubated overnight at 4 °C with parsley anti-PAL polyclonal antisera (Dr. Imre E. Somssich) diluted (1:3000) in TBS-T. Blots, thoroughly washed with TBS-T, were incubated (2 h, RT) with alkaline phosphatase-conjugated

anti-rabbit IgG from goat (Sigma; 1:30000 dilution). The membrane was stained with 10 mL of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP®/NBT; SIGMAFAST™ tablets, Sigma-Aldrich).

1.2.5 Preparation of coated plastic bags and postharvest storage of lettuce

Pullulan (PI-20 grade, Mw ~200 kDa), an exopolysaccharide (EPS) produced by the yeast-like forms of the fungus *Aureobasidium pullulans*, was purchased from Hayashibara Biochemical Laboratories Inc. (Okayama, Japan). Oriented polypropylene (OPP, 30 μm), kindly provided by Bonduelle Srl (Milan, Italy), was used as a plastic substrate for the deposition of the active coating. Two different active coating solutions were prepared using the borage LEs and FEs, respectively. In both cases, a 10% (w/v) water solution was prepared. Six different pullulan solutions were prepared in water (10 wt.%, wet basis) under gentle stirring for 15 min at 25 °C. Before coating deposition, the OPP films were treated with a high-frequency corona treatment (Arcotec, Mönsheim, Germany) to increase the film surface energy, improving plastic substrate-coating adhesion. An aliquot (approx. 5 mL) of the active solution was placed on the corona-treated side of the OPP film. The deposition of the coating solution was

carried out by an automatic film applicator (ref 1137, Sheen Instruments, Kingston, UK), at a constant speed of 150 mm min^{-1} (ASTM D823-07-Practice C), using a horizontal steel rod with an engraved pattern, which yielded final coatings with comparable nominal thickness (1.0 μm) after water evaporation. Drying was performed using a constant and perpendicular air flux at 25.0±0.3 °C for 2 min at a 40 cm-distance from the applicator. Packaging of the lettuce leaves (about 20-25 g) was carried out using a Polikrimper TX/08 thermal heat sealer (Alipack, Pontecurone, Italy: 130 °C; dwell time: 0.5 s; 4.0 bar) equipped with smooth plates. The postharvest trials were conducted by storing lettuce leaves up to 7 d at 4 °C. Samples and conditions of packaging were: A) control leaves packed in uncoated plastic bag; B) control leaves packed in LE-coated plastic bag; C) control leaves packed in FE-coated plastic bag; D) 10 mL L^{-1} LE-treated leaves packed in uncoated plastic bag; E) 10 mL L^{-1} FE-treated leaves packed in uncoated plastic bag. Analyses of total chlorophylls and carotenoids were performed at the end of the storage time period (t=7 d) and compared to the levels measured at harvest (t=0).

1.3 Statistical analysis

All data were subjected to one way ANOVA and differences among means were determined by Bonferroni's post test. Data referred to chlorophyll *a* fluorescence parameters and to postharvest trial were subjected to two way ANOVA. Additional information is reported in the figure legends.

1.4 Results

1.4.1 Plant growth

Growth (fresh weight) of lettuce plants treated (LE or FE, at 1 mL L^{-1} or 10 mL L^{-1} each) or not with borage extracts, was measured (data not shown). The average fresh weight of the control plant heads at harvest (22 d after transplanting) was 55.9 g. All treatments enhanced growth, with a maximum effect (+16%) at the highest LE and FE concentration. The stimulating effect was minimum (+6.44%) at 1 mL L^{-1} FE.

Figure 1 shows an overview of biomass growth during time, as estimated from the head volume computed from multi-view images through the introduced regression linear equation. For the three considered time points (12 d, 17 d, 22 d after transplanting), the mean value of estimated head plant fresh weight per each treatment is shown. Twelve days after transplanting,

i.e., prior to any treatment, the estimated head mass ranged around 20-24 g, with no significant difference among groups, as expected. Seventeen days after transplanting, i.e., 4 d after the first treatment, the estimated average head weight (34 g) of plants treated with 1 mL L^{-1} of both LE and FE didn't deviate the control average (35 g), whereas some difference in the growth rate (average head weight 38 g) appeared in the groups treated with both LE and FE at the highest concentration (10 mL L^{-1}). Nevertheless, the ANOVA did not reveal a significant ($P<0.05$) difference between groups. After the second treatment (22 d after transplanting), i.e., just prior to harvest, the estimated head mass was significantly ($P<0.01$) affected by both LE and FE treatments at 10 mL L^{-1} (+15% and +18%, respectively, compared to the control). Similarly, the 1 mL L^{-1} FE treatment exerted a significant ($P<0.01$), albeit lower, stimulating effect (+13% over control). One mL L^{-1} LE exerted a very low stimulating effect (+4%). The values of head weight estimated from multi-view images at 22 d after transplanting were fairly related ($R^2=0.74$) to those of fresh weight measured immediately after harvest, even if with a relevant bias which led to a tendency to overestimate the absolute value of plant fresh weight.

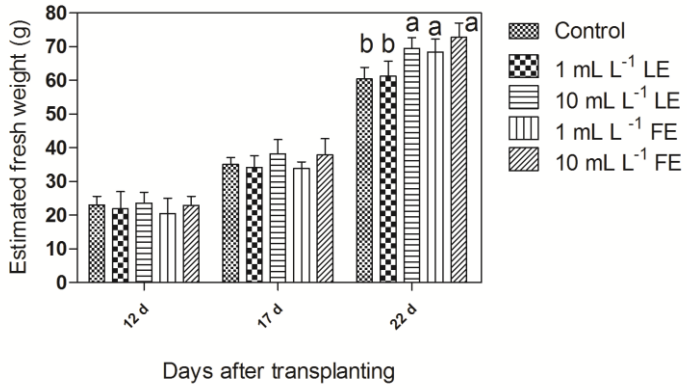

Fig 1. Estimated fresh weight of Romaine lettuce plants treated with water (control), 1 or 10 mL L^{-1} borage leaf (LE) or flower extract (FE). Data were obtained by processing of multi-view angles images from undisturbed lettuce potted plants at three time points of growth (days after transplanting). Values are means ± SE (n=9). Data were subjected to one way ANOVA. Different letters, where present, indicate significant differences among treatments.

1.4.2 Chlorophyll *a* fluorescence

The maximum quantum efficiency of PSII (Fv/Fm) (Figure 2 A) did not show any significant change in response to treatments; all samples yielded values higher than 0.83, commonly referred to as the threshold value between stressed and unstressed leaf tissue.

After the first treatment with borage extracts, the performance index (PI) did not show any significant change, even if the values were slightly lower in LE-treated plants, whereas FE-treated plants did not show any difference in comparison to the controls. After the second treatment, FE-treated samples showed a marked, even if not significant, increase in PI

compared to controls and to LE-treated plants (Figure 2 B). The positive effect of FE was confirmed by the higher number of reaction centers per cross section (RC/CS); in fact, the value of this parameter was significantly higher in FE-treated (10 mL L^{-1}) plants than in the controls already after the first treatment. The second treatment induced a more evident effect: FE-treated samples showed significantly higher values of RC/CS compared to both controls and LE-treated ones (Figure 2 C). Furthermore, the rate of energy dissipated by the PSII per reaction center (DIo/RC) was slightly lower in FE-treated plants compared to controls or LE-treated ones (Figure 2 D).

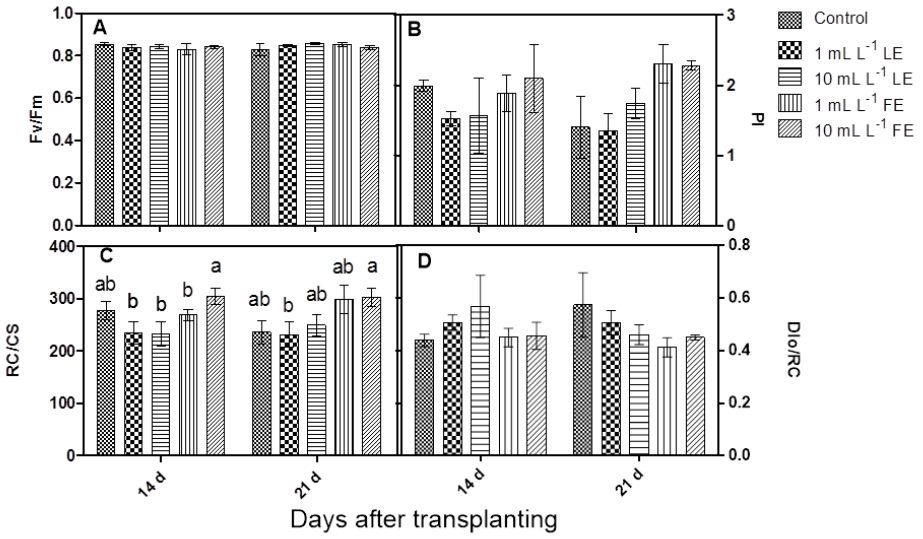

Fig 2. Chlorophyll *a* fluorescence parameters measured in lettuce treated with water (control), 1 or 10 mL L^{-1} borage LE or FE. A) maximum quantum efficiency of PSII, B) performance index, C) number of reaction centers per cross section, D) energy dissipated per reaction center. Values are means ± SE (n=3). Data were subjected to two way ANOVA. Different letters, where present, indicate significant differences among treatments or times.

1.4.3 Gas exchange measurements

The considered parameters in this trial were net photosynthesis (A), stomata conductance (gs), transpiration (E), photosynthetic water use efficiency (pWUE) and intrinsic water use efficiency (iWUE). All treatments with borage extracts enhanced net photosynthesis, even if significant differences were only observed between controls and 10 mL

L^{-1} FE-treated plants (Figure 3 A). The effects of borage extracts on gs and E values showed a similar trend (Figure 3 B, C) even if no significant difference among treatments could be observed. Similar results were found for the pWUE (Figure 3 D) and iWUE indexes (data not shown).

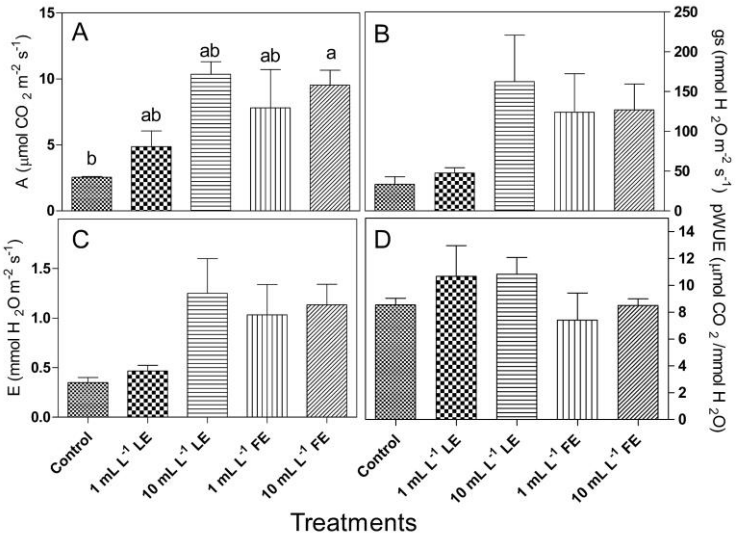

Fig 3. Leaf gas exchanges in lettuce plants treated with water (control), 1 or 10 mL L^{-1} LE or FE. A) Net photosynthesis, B) transpiration, C) stomata conductance, D) photosynthetic water use efficiency. Values are means ± SE (n=3). Data were subjected to one way ANOVA. Different letters, where present, indicate significant differences among treatments.

1.4.4 Plant ethylene production

The amount of hormone produced by both control and treated plants did not exceed 2.5 µL kg^{-1} h^{-1} (Figure 4). Lower amounts of ethylene production

were recorded after FE treatment, irrespective of the applied dose. Ethylene produced in both controls and 10 mL L^{-1} LE-treated plants was by about 9-10 fold greater than that produced in 1 mL L^{-1} FE-treated plants. However, the effects were not statistically relevant due to high data variability.

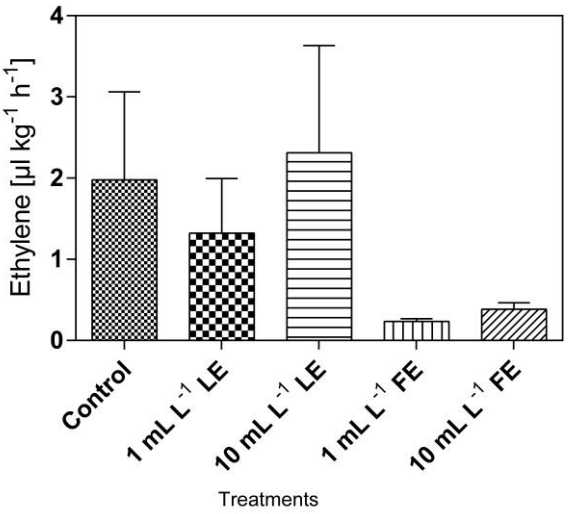

Fig. 4. Ethylene emission in Romaine lettuce head treated with water (control), 1 or 10 mL L^{-1} LE or FE. Values are means ± SE (n=3). Data were subjected to one way ANOVA.

1.4.5 Total chlorophylls and carotenoids

Lettuce leaf tissue treated with 1 mL L^{-1} LE showed the highest concentration of chlorophyll *a*+*b* (0.765 mg g^{-1} FW), and the same effect was observed for carotenoids (0.174 mg g^{-1} FW). In all cases, the concentrations of these pigments in plants treated with the borage

extracts were slightly higher, even if not significantly different, than in the controls (Figure 5 A, B).

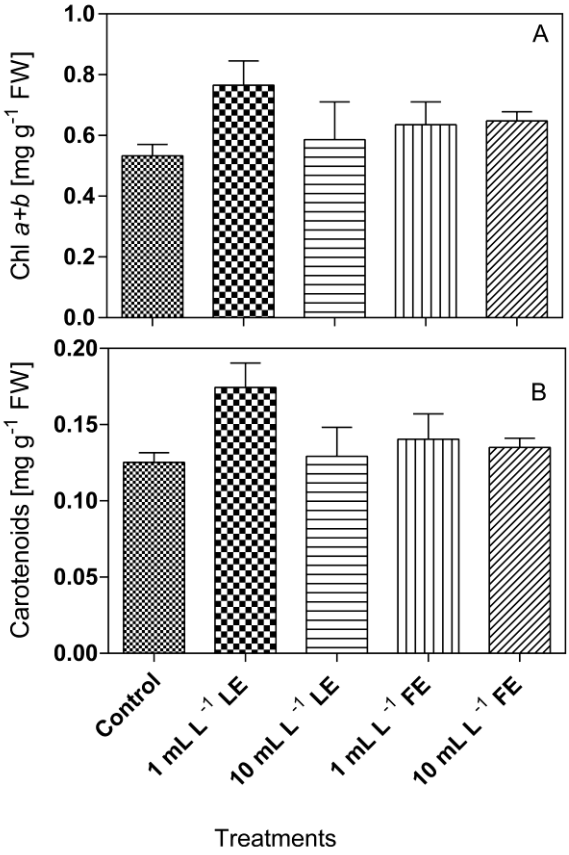

Fig 5. Chlorophyll a+b (A) and carotenoids (B) concentrations in Romaine lettuce leaf tissue treated with water (control), 1 or 10 mL L-1 LE or FE. Values are means ± SE (n=3). Data were subjected to one way ANOVA.

1.4.6 Nitrate

Table 1 shows the nitrate concentration in lettuce leaves treated or not with borage extracts. The absolute values of nitrate ranged from 138.9 mg kg^{-1} FW to 236.2 mg kg^{-1} FW. LE-treated plants showed slightly lower nitrate levels than the controls, whereas the FE-treated ones showed slightly higher nitrate levels.

1.4.7 Sucrose and total sugars

The highest concentration of sucrose (Table 1) was found in leaves of control plants (1885.2 mg kg^{-1} FW), while borage extracts (and particularly so FE) induced a decrease in this parameter, even if the observed differences among treatments were not statistically significant. Also for total sugars, control plants showed the highest value (2785.9 mg kg^{-1} FW), and 10 mL L^{-1} LE induced a decrease in this parameter (1551.6 mg kg^{-1} FW; Table 1).

	Nitrate [mg kg^{-1} FW]	Sucrose [mg kg^{-1} FW]	Total sugars [mg kg^{-1} FW]
Control	138.90±12.90	1885.20±316.48	2785.89±476.97
1 mL L^{-1} LE	164.43±13.93	1658.33±72.34	2538.42±405.17
10 mL L^{-1} LE	138.55±8.71	1489.67±203.49	1551.61±218.44
1 mL L^{-1} FE	195.27±49.71	1313.17±160.58	2517.43±52.62
10 mL L^{-1} FE	236.16±13.93	1283.62±200.00	1907.36±486.11

Tab 1. Nitrate and sugars concentrations of Romaine lettuce leaf tissue treated with water (control), 1 or 10 mL L^{-1} borage leaf (LE) or flower extract (FE). Values are means ± SE (n=3). Data were subjected to one way ANOVA.

1.4.8 Total phenols and flavonoids and total antioxidant capacity

The phenolic concentration (Figure 6 A) in the leaf tissue of control plants were 0.82 mg GAE g^{-1} FW and increased upon treatment with borage extracts. In particular, the values recorded were significantly increased by all treatments (+26.3%, +19.6%, +23.5%, +17.2% by 1 mL L^{-1} LE, 10 mL L^{-1} LE, 1 mL L^{-1} FE and 10 mL L^{-1} FE, respectively). Also the concentrations of total flavonoids (Figure 6 B) were increased upon treatments with borage extracts. In the leaves of control plants a value of 2.37 μmol rutin equivalents g^{-1} FW was observed, that was significantly increased by all treatments (+20.0%, +24.2%, +34.7%, +21.7% by 1 mL L^{-1} LE, 10 mL L^{-1} LE, 1 mL L^{-1} FE and 10 mL L^{-1} FE, respectively). The antioxidant capacity

was 8.01 AAE g^{-1} FW in the control plants, and it showed a general tendency to increase upon treatment with borage extracts (Figure 6 C).

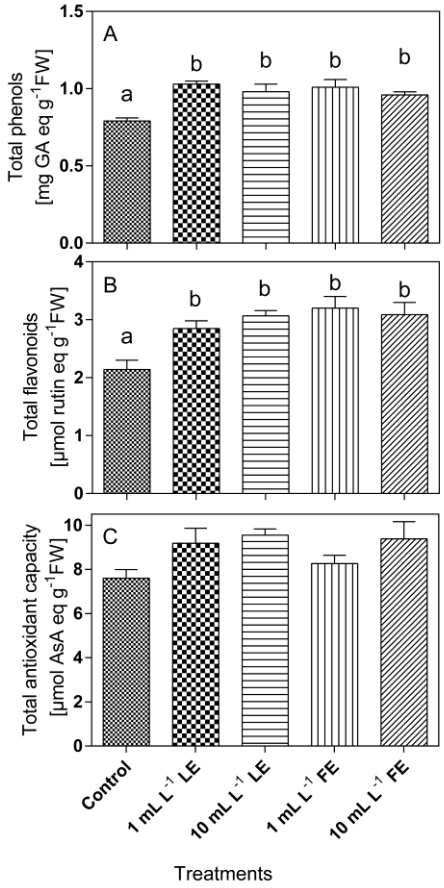

Fig. 6. Phenolics (A) and total flavonoids (B) concentrations, and antioxidant capacity (C) in Romaine lettuce leaf tissue treated with water (control), 1 or 10 mL L-1 borage LE or FE. Values are means ± SE (n=8). Data were subjected to one way ANOVA. Different letters, where present, represent significant differences among treatments.

1.4.9 Total soluble proteins

The levels of total soluble proteins in lettuce leaf tissue (Figure 7) were affected by the treatments with borage extracts. In fact, the lowest amount of soluble proteins (approx. 10 mg g^{-1} FW) was observed in the control plants; increases of +12%, +16%, +26% and +32% were induced by 1 mL L^{-1} LE, 10 mL L^{-1} LE, 1 mL L^{-1} FE and 10 mL L^{-1} FE, respectively. In particular, the highest and significant effect was induced by the treatment with 10 mL L^{-1} FE.

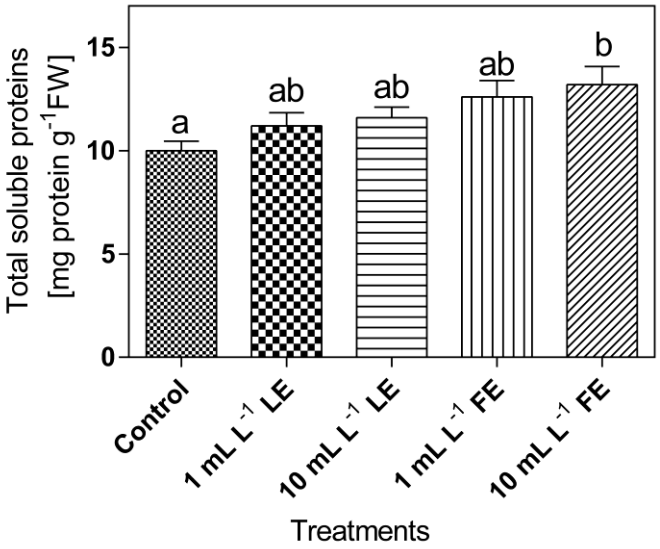

Fig. 7. Total soluble proteins in Romaine lettuce leaf tissue treated with water (control), 1 or 10 mL L-1 LE or FE. Values are means ± SE (n=8). Data were subjected to one way ANOVA. Different letters represent significant differences among treatments.

1.4.10 *In vitro* PAL activity and PAL-like polypeptide levels

Figure 8 A shows that the *in vitro* PAL specific activity in leaves of the control plants was 47.3 nmol CA h^{-1} mg^{-1} soluble protein. All treatments with borage extracts enhanced, albeit not significantly, the enzyme activity, with an average effect for the four treatments of about +17%. The levels of PAL-like polypeptides were also assessed in the same soluble protein extracts used for the determination of *in vitro* PAL activity. Figure 8 B shows that, in all soluble protein extracts of the lettuce leaves, the anti-PAL antibodies from parsley yielded a clear immunogenic signal against two polypeptides of approx. 71 kDa and 38 kDa, reacting also, even if only weakly, with a polypeptide of approx. 51 kDa. The signal against the three PAL-like polypeptides showed a tendency to increase upon all four borage treatments, and particularly so in the case of FEs (1 mL L^{-1} and 10 mL L^{-1}).

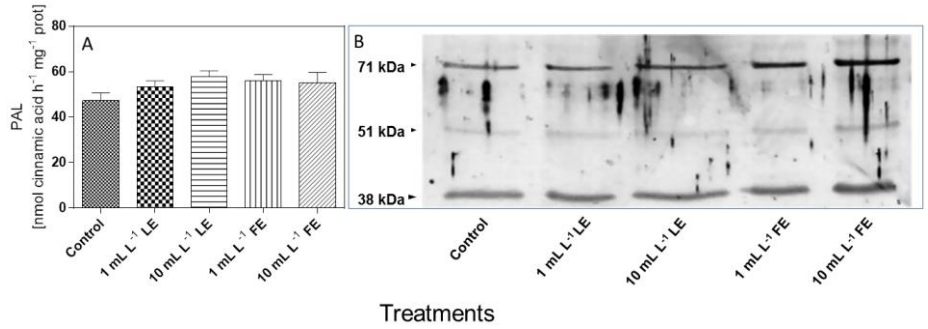

Fig. 8. *In vitro* PAL specific activity (A) and levels of PAL-like polypeptides (B) in Romaine lettuce leaf tissue treated with water (control), 1 or 10 mL L-1 borage LE or FE. *In vitro* PAL activity data are means ± SE (n=8). For immunoblotting, polyclonal antibodies raised against a PAL protein of *Petroselinum crispum*, (kind gift of dr. I.E. Somssich) were used. Loading was 10 µg protein per lane. The results of one experiment, representative of three, are shown.

1.4.11 Effect of borage extracts during storage

In general, a positive effect of borage extracts was observed on total chlorophylls and carotenoids concentrations during cold storage. In fact, leaves subjected to all kinds of treatment showed higher concentrations after 7 d at 4 °C compared to those at harvest, even though the observed increments were not significant. At the end of the storage period the only significant increment of both chlorophylls and carotenoids was observed in FE-treated lettuce leaves packed in uncoated bags (Table 2). After cold storage, no apparent decay symptoms were recorded in any sample.

Storage time/ coating type	*In vivo* plant treatment					
	Control	LE	FE	Control	LE	FE
	Chl *a* + *b* [mg g^{-1} FW]			Carotenoids [mg g^{-1} FW]		
0 d/-	0.53±0.04ab	0.59±0.12ab	0.65±0.03ab	0.13±0.01ab	0.13±0.02ab	0.14±0.01ab
7 d/-	0.48±0.01b	0.68±0.24ab	0.97±0.17 a	0.12±0.02ab	0.17±0.06ab	0.24±0.04 a
7 d/LE	0.86±0.24ab	-	-	0.22±0.05ab	-	-
7 d/FE	0.84±0.10ab	-	-	0.22±0.02ab	-	-

Tab 2. Effects of borage leaf (LE) and flowers (FE) extracts, administered *in vivo* to lettuce plants or as coatings of plastic films, on total chlorophylls and carotenoids concentrations of lettuce leaves at harvest (0 d) and after 7 d of storage at 4 °C. For the *in vivo* experiment, plants were treated with water (control) or 10 mL L-1 borage extracts, as described in "Materials and methods"; leaves were then packed in uncoated or LE- or FE-coated plastic bags. Values, subjected to two way ANOVA, are the means ± SE (n=3). Different letters indicate significant differences between treatments.

1.5 Discussion

Biostimulants act at different levels, increasing plant growth, photosynthetic, and metabolic activity and nutrient absorption (Bulgari *et al.*, 2015; Yakhin *et al.*, 2017). The production of a potential biostimulant begins with raw material characterization followed by the study of plant responses (Povero *et al*., 2016). The effect of raw material extracts has to be evaluated under normal or stress conditions and investigating the physiological and biochemical processes that are activated after treatments. Successful biostimulant candidates should increase biomass and yield or counteract the

negative effect of different stresses. Their use is nowadays becoming a common practice in crop production to improve productivity and yield. In leafy vegetables, the biostimulant Actiwave® (containing betaine, alginic acid, and caidrine) applied as an additional component to the nutrient solution increases yield of rocket grown in a floating system, even with reduced nutrient concentrations (Vernieri *et al.*, 2005); its effect was confirmed when administered as a spray on baby leaf lettuce grown in plastic tunnel (Amanda *et al.*, 2009). An extract of brown marine algae was reported to increase growth of spinach *in vitro* (Fan *et al.*, 2013). Dudaš *et al.* (2016) observed, on winter production of lettuce 'Four Seasons', that the plant head mass was by 30% higher after treatment with Bio-algeen S-90 compared to control plants. Sternecker and Balas (2014) observed in lettuce 'Mathilda' a head weight increase of 31% upon use of a biostimulant composed by a mixture of extracts from 21 plant species associated with *Lactobacillus* and yeast. The increment that we observed in Romaine lettuce growth is consistent, in spite of their high variability, with the cited literature reports, and supports the hypothesized role of borage extract in stimulating the biomass of treated plants.

Imaging methods have been successfully used for non-invasive estimation of plant growth (Tackenberg, 2007) also after biostimulant treatments (Povero

et al., 2016), but literature concerning their application to lettuce is scanty and relies upon a top-view imaging of plant heads. Due to leaf overlap after canopy closure, this approach revealed a generally weak correlation between image-based and destructive measurements of biomass, as growth progressed. For 'Outredgeous', Bumgarner *et al*. (2012) observed a decrease in correlation coefficient from r=0.87 at 10 d after sowing to r=0.22 at harvest (28 d). Similarly, Jung *et al.* (2015) reported a sharp decrease in the correlation when lettuce heads have a fresh weight above 25 g, even if they found an overall RMSEC of less than 5 g when estimating the biomass of samples with fresh weight ranging up to 70 g. In this study, the adopted multi-angle, side view approach enabled to define a linear model capable to estimate the lettuce biomass through image data with a RMSEC=2.2 g, for fresh weight values up to 155 g. This model was successfully applied to monitor non-invasively the growth of lettuce heads as affected by borage extracts application, and the multiple side view approach allowed capturing the subtle effects of the treatments during plant growth. The application of multi-angle, side view imaging, instead of classical top-view approach, allowed obtaining a fair correlation (R^2=0.74) with the destructive harvest data even for plants at advanced growth (i.e., commercial harvest stage). It must be noted that the multi-angle approach used in this work can be

successfully applied when conducting phenomic studies, but it does not appear suitable for on-the-go measurements in field or in greenhouse, where top-view imaging setup is the best option thanks to its much simpler implementation.

In lettuce, ethylene production is extremely low compared to other plant tissues (Burg, 2004). In lettuce 'Acephala' values of ethylene production lower than 10 μL kg^{-1} h^{-1} are reported (Diaz *et al.*, 2007). Concerning Romaine lettuce, to our knowledge, only scanty literature is available about ethylene production. Regarding the effects of borage extracts described in the present work, it is interesting to notice that, despite the high variability of the results, possibly due to the extremely low levels of ethylene emission, in three out of the five experimental conditions a decrease in ethylene production was induced by borage extracts, particularly evident upon FEs administration, suggesting a healthier physiological status in the FE-treated plants. This result could be explained considering the antioxidant activity due to the presence of radical scavenging components reported for crude *B. officinalis* extracts (Bandoniene & Murkovic, 2002; Bandoniene *et al.*, 2005), that may play a role in counteracting the effects of potential stress factors and the related ethylene production.

Biostimulants enhance plant growth and total photosynthesis determining higher dry matter accumulation in vegetable and ornamental crops (Khan *et al.*, 2009; Bulgari *et al.*, 2015; Massa *et al.*, 2016). Chlorophylls (also important for the visual appearance of the produce) and carotenoids (photoprotective molecules whose amount is related to chlorophyll) are involved in fundamental photochemical processes tightly associated with crop biomass production. Moreover, carotenoids and chlorophylls play an important role in preventing various human chronic-degenerative diseases associated with oxidative stress (Znidarcic *et al.*, 2011), contributing to the nutraceutical quality of plant produce (Yuan *et al.*, 2015). Biostimulant treatments are often able to increase leaf pigments concentration. In rocket, treatments with a *Moringa oleifera* extract increased chlorophyll and carotenoids levels (Abdalla, 2013); similar results were obtained with the biostimulant Actiwave® (Vernieri *et al.*, 2005). The commercial product ONE® had positive, dose-dependent effects, on the chlorophylls levels of lettuce and endive (Bulgari *et al.*, 2014). Consistently, borage extracts (in particular 1 mL L^{-1} LE) slightly increased the chlorophyll and carotenoids levels compared to controls.

Leaf functionality is also described by gas exchanges analysis or estimated by chlorophyll *a* fluorescence. These non-destructive methods can be applied to evaluate the health status of the photosynthetic apparatus or the different responses of plant tissues to stress factors or experimental treatments (Murchie & Lawson, 2013). In the present work, a positive effect of borage FEs may be suggested by the higher values of PI, a general index of the leaf health status. Moreover, the higher number of reaction centers and lower rates of energy dissipation confirmed the hypothesis of a direct positive effect of the treatment on PSII efficiency. In lettuce, significant changes in the Fv/Fm ratio (a good indicator of leaf stress) usually observed after a mid- or long-term exposure to a specific treatment or stressful condition (Stępień & Kłbus, 2006), are considered an index of irreversible photoinhibition of PSII reaction centers (Dias *et al.*, 2014). In our material, the Fv/Fm values did not show any significant change, suggesting a general positive effect of extracts on leaf functionality.

Biostimulant applications in coriander under cold stress were able to increase Fv/Fm ratio, transpiration, and stomatal conductance rates, but reduced intercellular carbon dioxide concentration (Pokluda *et al.*, 2016), suggesting that biostimulants may accelerate the adaptation to

chilling. The chlorophyll *a* fluorescence-derived parameters have been used for evaluating the vitality of transplant-sensitive tree species after transplanting, and the effects of biostimulants application, that increased leaf functionality as shown by higher values of PI (Fraser & Percival, 2003).

Our results showed that the highest doses of both types of borage extracts increased the net photosynthesis as revealed by gas exchange analyses. Similar to what observed in other horticultural crops. In strawberry, Actiwave® increased the photosynthetic activity by 27% compared with control (Spinelli *et al.*, 2010). Consistent results were found in ornamental plants treated with a municipal biowaste: hibiscus plants showed an increase of net photosynthesis by 24% (Massa *et al.*, 2016) and similar findings were observed in *Euphorbia x lomi* (Fascella *et al.*, 2015).

Biostimulants improve the primary metabolism of plants, increasing the levels of free-amino acids, protein, carbohydrates, pigments, and various enzymes as reported by Yakhin and colleagues (2017).

In our material, the leaf sucrose levels were not affected by any borage extract treatment, suggesting that neither the nutritional nor the sensorial quality of the produce were significantly altered. However, the tissue

levels of total sugars were diminished by all treatments, whereas the levels of some secondary metabolites, like total phenolics and flavonoids increased, as well as the antioxidant capacity. The opposite changes in the levels of total sugars and phenylpropanoid compounds would enhance their health-related characteristics, at the same time maintaining a high level of chemical defense capability (Neilson *et al.*, 2013) and, in turn, better performance in terms of plant growth. A better general status of the plants treated with borage extracts, as well as potential higher resistance to stress factors thanks to the presence of phenolic substances, is also suggested by the higher levels of total soluble proteins, indicative of the bulk of metabolic activity (Veerasamy *et al.*, 2007 and references therein). The observed tendency to lower ethylene production is consistent with this view. Several primary metabolites (like free amino acids, sugars or other molecules not immediately required for growth and development) are precursors of secondary compounds, among which polyphenols (Mazid *et al.*, 2011). In particular, the deamination of phenylalanine to trans-cinnamic acid catalyzed by PAL, links the primary metabolism to the production of a wide variety of secondary phenolic compounds, that serve diverse functions in plants, including protection against biotic and abiotic stresses, cellular signaling,

mechanical support (Mac Donald & D'Cunha, 2007). Our results on *in vitro* PAL activity and levels of PAL-like polypeptides are, in general, coherent with the results on total phenolic and flavonoids concentrations, even if we could not observe a tight correlation between the cited parameters. This result might be attributed to the very complex regulation of this enzyme, that involves several steps, from *PAL* (iso)genes transcription to assembling of the functional protein, and to enzyme turnover and mechanisms of activity regulation (phosphorylation-dephosphorylation); also feedback control by the levels of total phenolics/flavonoids is reported to regulate PAL protein turnover and catalytic activity (Zhang & Liu, 2015).

High dietary nitrate intake is hazardous for health, since in the human organism nitrate is reduced to nitrite that can react with the free amines deriving from protein digestion and form carcinogenic nitrosamines. For this reason, the European Union has posed limits in nitrate concentrations of commercialized leafy produce (Cavaiuolo & Ferrante, 2014). Nitrate accumulation in leafy vegetables is affected by several environmental factors like light intensity, photoperiod, and temperature (Lillo, 1994 and references therein). Biostimulants reduce the nitrate levels in several species of leafy vegetables (Vernieri *et al.*, 2005; Liu &

Lee, 2012; Dudaš *et al.*, 2016). The borage treatments applied in the present work did not significantly affect nitrate in the lettuce cultivar used. Nitrate concentration shows considerable variations in different lettuce cultivars (from 26 mg kg^{-1} to more than 2500 mg kg^{-1}; Cometti *et al.*, 2011). In our material, the nitrate levels were lower than approx. 250 mg kg^{-1} FW, possibly explaining the observed lack of effect of borage treatments, similar to what observed for Actiwave®-treated baby leaf lettuce (Amanda *et al.*, 2009). It should also be stressed that the effect of biostimulants on nitrate levels in leaves can be different depending on the species/cultivar and it is affected, in addition to environmental factors, by dose and time of application (Kunicki *et al.*, 2010).

The effect of borage extracts, containing themselves bioactive molecules or possibly releasing volatile compounds (VOCs), during postharvest of lettuce leaves, was also evaluated in a preliminary trial. The visual appearance (chlorophyll) of the produce and leaf carotenoids levels, both known to be affected by storage conditions (Bolin & Huxsoll, 1991; Bergquist *et al.*, 2007; Agüero *et al*., 2008) were assessed. Borage extracts administered either as *in vivo* treatment to plants or applied as coating on packaging films, exerted a positive effect on the photosynthetic pigments, preventing their degradation, and even

inducing their increase during storage. In particular, 10 mL L^{-1} FE proved capable to induce a significant increase in total chlorophylls and carotenoids after 7 d of storage as compared to the controls. However, further experiments will be necessary in order to investigate the mechanism of action of the bioactive compounds potentially present in borage extracts, when incorporated into a primary packaging. Moreover, a study of the release kinetics of such active molecules in the package headspace may also help to understand whether the effects of these treatments is linked to the production of active VOCs and/or is due to a direct contact between the produce and the inner surface of the packaging.

Taken as a whole, the multidisciplinary approach used in this work demonstrated that borage extracts do indeed exert biostimulant effects on lettuce plants. This result suggests a possible exploitation of borage extracts in vegetable production including different species, as well as in the commercialization, to improve quality and nutraceutical properties and thus adding value to produce. Moreover, the phenomic approach adopted proved capable to estimate with good accuracy plant growth and represented, in general, a fast and reliable method for the non-destructive screening of the efficacy of experimental treatments,

integrating well the biochemical–physiological approach. Aspects related to both primary and secondary metabolism were enhanced, suggesting a potential ability of these extracts to counteract possible stress factors. In particular, flower extracts proved more effective than leaf extracts on the plant physiological and biochemical parameters considered. These results may be validated at molecular level by studying the transcriptional profiles using high-throughput technological tools like microarrays and RNA-seq. The molecular mechanisms elicited by crude plant extracts acting as biostimulants were recently studied, in *Arabidopsis thaliana*, through a microarray-based genomic approach (Santaniello *et al*., 2013). Moreover, a transcriptional profiling of phenylpropanoid pathway genes in *Arabidopsis thaliana* as affected by application of microbial products has been recently published (Ali & McNear, 2014). These additional research activities will also allow describing more completely the efficacy of borage extracts in preserving and enhance crop performance.

1.6 Conclusions

Our results appear suitable to be fruitfully included in a larger, integrated framework where different approaches are systematically

combined (Povero *et al*., 2016) in order to study at different levels the potential positive effects of various natural extracts on plant performance and biochemical-physiological quality parameters, eventually characterizing and validating them as new biostimulant products exploitable in the field.

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SUPPLEMENTARY MATERIAL

	P (mg L^{-1})	K (mg L^{-1})	Ca (mg L^{-1})	Fe (mg L^{-1})	Mn (mg L^{-1})	N-NO_3 (mg L^{-1})	N-NH_4 (mg L^{-1})	PHENOLS (mg L^{-1} GAE)	pH	EC (mS)
LE	56762	-	190678	263	120	0.16	213	364	6.31	6.38
FE	17966	-	52322	399	70	0.29	88	242	9.23	4.31

Tab. s1. Chemical characterization of borage leaf (LE) and flower extract (FE).

CHAPTER 2

Effects of borage extracts on rocket quality and influence on nitrate metabolism

2.1 Introduction

Rocket is a fast growing crop that is widely cultivated as baby leaf. It is a low calories vegetable and it is considered a potential health promoting produce with diuretic, stimulant, depurative, and stomachic properties. In particular, it has a high content of health promoting compounds like ascorbic acid, flavonoids, carotenoids, and glucosinolates, with proven antioxidant, pharmaceutical, and anti-cancer properties (D'Antuono *et al*., 2009; Jakse *et al*., 2013; Cavaiuolo & Ferrante, 2014). Rocket is considered a hyper-accumulator of nitrate (Di Gioia *et al*., 2013), that forming compounds (nitrosamines) believed to be potentially toxic to human health (Santamaria *et al*., 2001; D'Anna *et al*., 2003; Ferrante *et al*., 2003). However, some researches showed that its conversion to nitrite plays an important antimicrobial role in the stomach (McKnight *et al*., 1999). Nitrate concentrations depend on several factors, like season, light intensity, temperature, fertilization, and storage of the crop (Premuzic *et al*., 2001; Frezza *et al*., 2005; Magnani *et al*., 2007; Kim & Ishii, 2007). In Europe, for the commercialization of leafy vegetables, there is a threshold of nitrate content (Reg. N° 1258/2011). The limits for rocket range from 7000 NO_3^-

[mg kg^{-1} FW] (harvest from October to March) to 6000 NO_3^- [mg kg^{-1} FW] (harvest from April to September).

2.1.1 Nitrate metabolism and enzyme involved

Nitrogen (N) is an essential macronutrient, required for the synthesis of amino and nucleic acids and is a fundamental nutrient for cellular metabolism (Parker & Newstead, 2014). Its absorption at the root level determines plant growth and consequently crop productivity (Krapp *et al*., 2014; O'Brien *et al.*, 2016). Nitrates are actively transported through the plasma membrane of the epidermal and cortical cells of the roots across the proton symporters (NO3- :2H+) or Cl- canal (2NO3- :H+); this active action exploits the driving force of 10 transmembrane different potential, create thanks to the ATP hydrolysis by H+ ATPase of the plasma membrane. The transport is regulated by a large family of nitrate transporters (NTR). NTR1 transports nitrate, histidine, and nitrite, and belongs to the subgroup of nitrate/nitrite transporters (Pao, 1998); NTR2 transports peptides, amino acids, nitrate, chlorate, and nitrite and belongs to the subgroup of proton dependent oligopeptide transporters (Galvan & Fernandez, 2001). After his uptake, NO3- can be loaded and stored inside the cell vacuoles to accomplish osmotic functions, go back in the soil via apoplast, translocate

via xylem and transported to other tissues, or it can be reduced with different redox reactions in order to assimilate it (Jakse *et al*., 2013). These redox reactions, catalyzed by specific enzymes, are energy dependent and generally exploit NAD(P)H+ as electron donor. Nitrate metabolism starts with the reduction of nitrate to nitrite in the cytosol, catalyzed by nitrate reductase, NR. Then nitrite is transported into the chloroplast of the leaf, or in the plastid of the root, to be reduced to ammonium by ferredoxin-nitrite reductase, NiR. Nitrite and ammonium ions are cytotoxic because lead to pH changes and induce an increase in reactive nitrogen species and oxidative damages, so they cannot be accumulated inside the cell (Chow, 2002). For these reasons, their incorporation into organic compounds must be relatively fast (Chow, 2002). Ammonium then triggers the "Glutamine Syhntetase/Glutamine Oxoglutarate Aminotransferase" cycle (GS/GOGAT). Glutamine oxoglutarate aminotransferase is also known as glutamate synthase GOGAT. Ammonium is transformed to have glutamine (Stitt, 1999) in the cytosol or in the chloroplasts/plastistids, by the enzyme glutamine synthetase, GS, which presents two active isoforms, one cytosolic and another cloroplastic/plastidial, respectively called GS1 and GS2 (Lancien *et al*., 2000). GS catalyzes a condensation of ammonium whit glutamate to obtain glutamine and this process requires ATP (Temple *et al*.,

1998). At this point, if α-chetoglutarate (or oxo-glutarate) and energy are available from photosynthesis, two amide groups of glutamine can be transferred, thanks to glutamate synthase, to α-chetoglutarate (or oxo-glutarate) (Temple *et al*., 1998). One of the two molecules of glutamate can accept NH4+ during another GS/GOGAT cycle, while the other can be organicated in amino acids by transaminases and then transformed in proteins to be effectively used by the plant (Sun *et al.*, 2010). Probably also other three enzymes participate to the ammonium assimilation process: cytosolic asparagine synthetase (AS), plastidial carbamoylphosphate synthase (CPSase) and mitochondrial NADH-glutamate dehydrogenase (GDH) (Masclaux-Daubresse *et al.*, 2010). AS, using ammonia as substrate, catalyses the transfer of the amide group of glutamine and a molecule of aspartate to generate glutamate and asparagine (Masclaux-Daubresse *et al*., 2010). Carbamoylphosphate synthase (CPSase) uses bicarbonate, ATP, ammonium or the amide group of glutamine to catalyze the formation of carbamoylphosphate, a precursor of citrulline and arginine (Masclaux-Daubresse *et al*., 2010). GDH can catalyze the de-amination of glutamate or, alternatively, incorporate ammonium into glutamate in the presence of high level of ammonium due to stress, (Masclaux-Daubresse *et a*l., 2010). In the mesophyll of the cells there is high activity of GS2, while GS is low in

leaves, being generally limited to the phloem; these two isoenzymes have an organ-specific expression pattern (Edwards & Coruzzi, 1990). Therefore, GS1 is the major form of GS in plant roots, it is very important for the primary nitrogen assimilation and its expression is metabolically regulated by Nitrogen and Carbon availability (Sun *et al*., 2010). GS2 plays a crucial role in re-assimilation of NH4 + released via photorespiration in plants. Glutamate synthase is present with two forms in plants: Fd-GOGAT, that uses ferredoxin as electron donor, and NADH-GOGAT, that uses NADH. The first one is generally localized in the chloroplasts, while NADH-GOGAT is found in the plastids of non-photosynthetic tissues (Masclaux-Daubresse *et al*., 2010). Generally, the reduction of nitrate is more efficient in leaves than in roots due to the close dependence on photosynthesis for reductants, energy, and carbon skeleton (Chen *et al*., 2004).

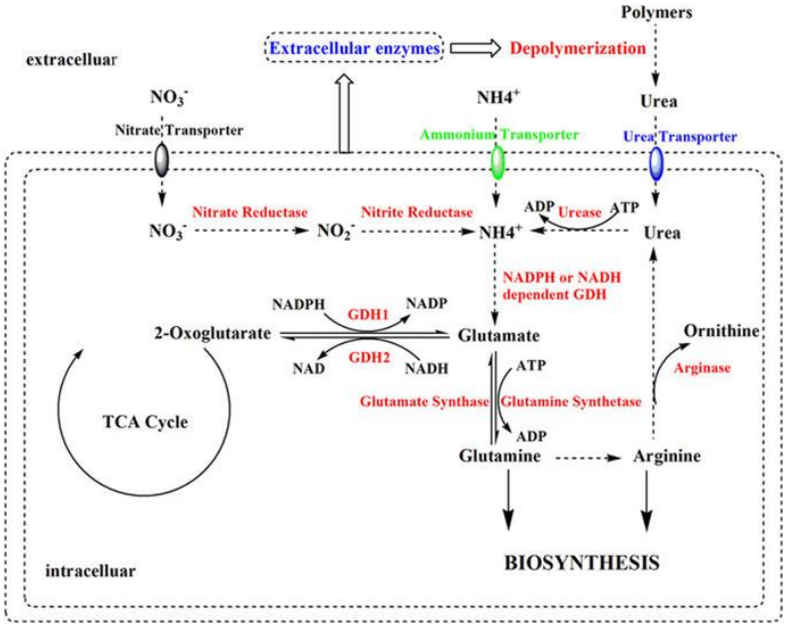

Fig. 9. Nitrate assimilation pathway.

Considering therefore the commercial importance of rocket, it would be very interesting to look for strategies aiming to reduce nitrate content in leaves (as affected by nitrate metabolism) and enhance the produce quality (influencing for example the levels of antioxidant compounds or chlorophyll concentration). For these reasons, foliar treatments with aqueous borage extracts, used as potential biostimulant, were performed. Biostimulants, in fact may influence plant metabolism (for example acting on the regulation of enzymes involved in N metabolism (Schiavon *et al.*, 2008; Ertani *et al.*, 2009)). They can also regulate enzymes of the TCA cycle, contributing to

the interplay of C and N metabolisms (Schiavon *et al*., 2008; Santi *et al*., 2017), which is strictly related to plant productivity.

To screen the effect of borage extracts on rocket, several biochemical analyses (among which nitrate concentration, sugars, chlorophyll, and carotenoids levels) were done on leaves at harvest. Moreover, a set of genes encoding for the key enzymes involved in nitrate metabolism were selected, to be used as markers, and their expression was measured by quantitative RT-PCR. Those genes showing significant changes in their expression after treatment, can thus be considered as targets and can be used to better understand the way of action of the observed biostimulant effect.

2.2 Materials and methods

2.2.1 Plant material, treatments, and sampling

Rocket (*Diplotaxis tenuifolia* L.) was grown in hydroponic system in a greenhouse at the Faculty of Agricultural and Food Sciences of the University of Milan. Seeds were sown in polystyrene trays on perlite substrate within tanks filled with a standard Hoagland' s solution (Table 3).

Hoagland' s solution	
Salts	**g/L**
$CaNO_3$	0.36
KNO_3	0.46
NH_4NO_3	0.31
K_2HPO_4	0.24
$MgSO_4$	0.1
K_2SO_4	0.19
Oligo Green	0.02
H_2SO_4	Up to pH 5.5 - 6.5

Tab. 3. Formulation of standard Hoagland' s solution used for rocket cultivation.

The treatment conditions were: water (control); 10 mL L^{-1} of borage leaf extract (LE); 10 mL L^{-1} of borage flower extract (FE). Treatments were sprayed between 09:00 and 10:00 a.m. onto leaves until run-off, 35 days after sowing and 1 day before harvest (45 days after sowing). Harvesting was performed when the baby leaf commercial stage was reached.

For the gene expression analysis, sampling was performed 2-4-6-9 and 24 hours (h) after the second treatments, to evaluate the genes response over time and to identify the peaks of expression. Leaves were gently rinsed with distilled water, blotted with paper towels, immediately frozen in liquid N_2 and stored at -80 °C. After that, sampling for biochemical determinations (chlorophyll and carotenoids, nitrates, sugars, abscisic acid) was done and samples were stored at -20 °C until used for analyses. The *in vivo* nitrate reductase activity was performed the day after the second treatment, at T0 (condition of dark), T1 (2 h of light exposure), and T2 (4 h of light exposure).

2.2.2 Biochemical analyses

Chlorophylls and carotenoids determination

Chlorophylls and carotenoids concentrations were determined at harvest. Leaf tissue (30-50 mg) was extracted using 100% (v/v) methanol, for 24 h at 4 °C in a dark room; afterwards quantitative determination of chlorophylls was carried out. Absorbance readings were measured at 665.2 nm and 652.4 nm for chlorophylls and 470 nm for total carotenoids. Pigment levels were

calculated by Lichtenthaler's formula (1987) and expressed on the basis of fresh weight of the tissue.

Chlorophyll content was estimated also *in vivo* with a chlorophyll meter (CL-01, Hansatech, UK) that provides an indicator of green color of leaves. This device determines relative chlorophyll content using dual wavelength optical absorbance (620 and 940 nm wavelength).

Sucrose and total sugars levels

About 1 g of leaf tissue was homogenized in 4 mL of distilled water and centrifuged at 3000 *x g* for 15 min at RT. Sucrose and total sugars were assayed according to the resorcinol method and anthrone assay (Cocetta *et al.*, 2015), respectively. Absorbance was read at 500 nm for sucrose and at 620 nm for total sugars and the levels were calculated referring to sucrose or glucose calibration curves respectively.

Nitrate reductase *in vivo* activity and nitrate levels

Nitrate reductase *in vivo* activity was performed as described by Aslam *et al*., 1984 modified. Fresh leaves were immediately put in ice at dark. Leaves were quickly cut in little disks of 5 mm of diameter and putted in 15 mL tubes to reach 0.8 mg of fresh weight. After that, tubes were closed and

placed in ice in order to maintain inactive the enzyme. The control samples were immediately boiled in water for 5 min in order to denature the enzyme. The incubation medium was composed of potassium phosphate 100 mM (pH 7.5); 5% v/v isopropanol and 30 mM potassium nitrate. 1 mL of the reaction buffer was added to the tubes placed in ice and then the tubes were transferred in a water bath at 30 °C for 30 min. After incubation, the reaction was stopped with 1 mL of 1% sulfanilamide in HCl 3.0 N, and 0.02% N naftin etilen diamide as indicator of nitrites content. Tubes were left in the dark for 30 min to wait the color development and then spectrophotometric readings were made at 540 nm. The calibration was carried out with a standard solution of sodium nitrite.

Nitrate concentration was measured by the salicylsulphuric acid method (Cataldo *et al*., 1975). One g of fresh leaf tissue was homogenized (mortar and pestle) in 4 mL of distilled water. The extract was centrifuged at 3000 *x g* for 15 min at RT (ALC centrifuge-model PK130R) and the recovered supernatant was used for the colorimetric determination. Twenty μL of sample were added to 80 μL of 5% (w/v) salicylic acid dissolved in H_2SO_4 plus 3 mL of 1.5 N NaOH. The samples were cooled at room temperature and absorbance at 410 nm was measured. Nitrate concentration was calculated referring to a KNO_3 standard calibration curve.

Abscisic acid concentration

ABA was determined by an indirect enzyme linked immuno-sorbent assay (ELISA) based on the use of DBPA1 monoclonal antibody, raised against S(+)-ABA (Vernieri *et al*., 1989). Rocket (1 g) was homogenized (mortar and pestle) in 4 mL of distilled water. The extract was centrifuged at 3000 *x g* for 15 min at RT (ALC centrifuge-model PK130R) and the recovered supernatant was used for the analysis. The ELISA was performed according to the method described by Borghesi *et al*. (2016).

2.2.3 RNA extraction and qRT-PCR

About 100 mg of grounded tissues were used for the extraction of total RNA using the Spectrum Plant Total RNA Kit with on-column DNase-treatment (Sigma) according to manufacture instructions. RNA concentration and integrity were assessed by NanoDrop N-1000 spectrophotometer (NanoDrop technologies). 3 µg of RNA were reversely transcribed to cDNA using the SuperScript® III cDNA Synthesis Kit according to the manufacturer's instruction (Invitrogen). qRT-PCR analysis was performed using the SYBR® Green PCR Master Mix (Applied Biosystems) in 20 µL reaction mix consisting of 2 µL of cDNA (1:20 dilution), 10 µL of 1 Master Mix, 0.4 µM

of forward and reverse primers, and sterile water up to 20 µL. Analysis was performed using a ABI7300 (Applied Biosystem) thermocycler. Temperature profiles consisted of an initial step at 50 °C for 2 min, followed by denaturation at 95 °C for 2 min, and by 40 cycles of denaturation (95 °C for 15 s) and annealing/extension (60 °C for 1 min).

Gene expression analyses were performed using gene-specific primers for: nitrate reductase (*DtNR*), nitrite reductase (*DtNiR*), glutamyne synthetase (*DtGS1*), glutamate synthase (*DtGLU*), nitrate transporter (*DtNTR*) (Table 4).

Primers were designed based on an EST library recently built by using the Illumina RNA-Seq technology and providing sequence information and expression levels (RPKM) of *Diplotaxis tenuifolia* L. transcriptome (Cavaiuolo *et al*., 2017).

Actin and elongation factor (*EF-1α*) were tested to be used as housekeeping gene (Table 4). Due to the highest stability in its expression levels, *EF1-α* was used for the calculations.

The expression levels were calculated using the delta–delta Ct (ΔΔCt) method. The reported values are averages of two independent runs performed on each sample.

Gene	Primers 5'>3'	Tm (°C)
DtNR	F: CGAAGGAGCGGAGGATCTTC	67.3
	R: TAACCATCCTTCCGCCGATG	69.0
DtNiR	F: GCTCAAGGGCTCATCTCCTC	65.3
	R: TGGGAGGAGAGTGTGAGGAG	64.6
DtGS1	F: GGATGCACACTACAAGGCCT	64.1
	R: TCCAGCTCCGTTCCAATCAC	67.5
DtGLU	F: ACCCTTGTGTTTGGACTGCA	65.6
	R: CACCAAGCGAGGAAGACACT	64.4
DtNTR	F: GAAAGAGAGGATCGCGGAGG	67.3
	R: CCCTCCACTAGTCCCCATCA	66.2
EF1-α	F: TCTTGGTAGACGCCTTCACG	65.3
	R: AGGAAGCGGTGTCATTGTTG	65.0
Actin	F: GCCAATCTACGAGGGTTATGC	64.4
	R: CAAGAGCGACATAGGCAAGC	64.9

Tab. 4 Primers

2.3 Statistical analysis

Statistical analysis was performed with GraphPad Prism 6. All data were subjected to one-way ANOVA and differences among means were determined by Bonferroni's post test ($P < 0.05$). Additional information is reported in the figure legends.

2.4 Results

2.4.1 Chlorophylls and carotenoids

The chlorophyll content determined *in vivo* with chlorophyll meter (Figure 10A) and the chlorophyll *a+b* concentration determined with destructive method (Figure 10B) show the same trend; borage treatments (in particular FE) slightly diminished pigments in rocket leaves than untreated control, but differences are not significant. Carotenoids concentration (Figure 10C) shows an opposite behavior; in fact, borage treatments enhance carotenoids, in particular LE, even if not significantly.

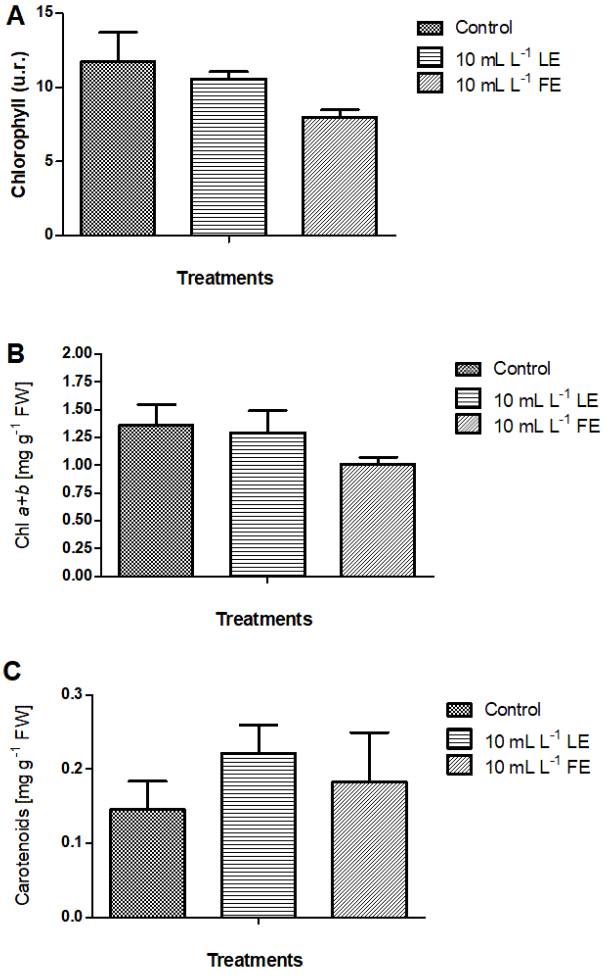

Fig. 10. Chlorophyll content determined *in vivo* (A), chlorophyll *a*+*b* (B), and carotenoids concentrations (C) in rocket leaves treated with water (control), 10 mL L^{-1} borage LE or FE. Values are means ± SE (n = 3). Data were subjected to one-way ANOVA.

2.4.2 Abscisic acid levels

FE determined a marked decrement of ABA in rocket leaves (Figure 11), with values more than halved. Despite this, the high variability of results does not allow to have significant differences among treatments.

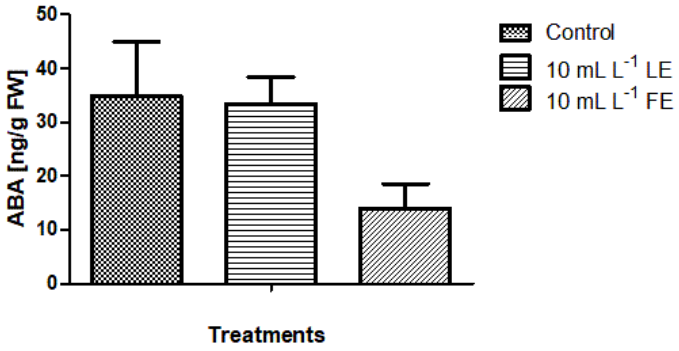

Fig. 11 Abscisic acid (ABA) levels in rocket leaves treated with water (control), 10 mL L^{-1} borage LE or FE. Values are means ± SE (n = 3). Data were subjected to one-way ANOVA.

2.4.3 Sugars concentration

Figure 12 shows that FE exerted a positive effect on sucrose concentration. Borage extracts slightly enhanced also total sugars concentrations than control, but in this case differences were not significant.

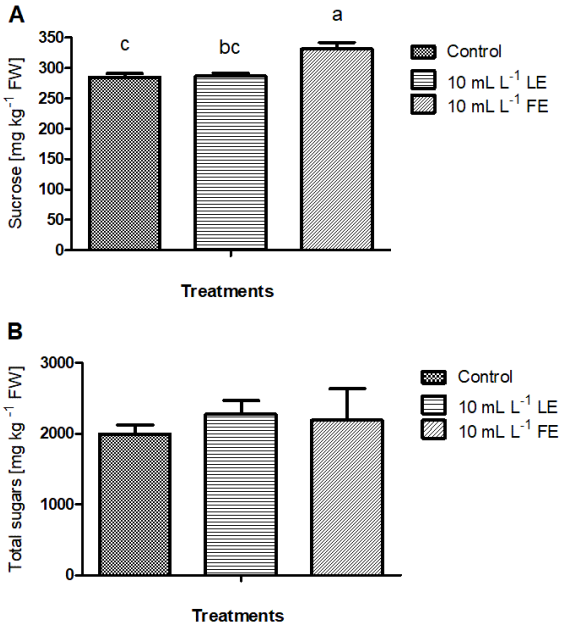

Fig. 12. Sucrose concentration (A) and total sugars (B) in rocket leaves treated with water (control), 10 mL L^{-1} borage LE or FE. Values are means ± SE (n = 3). Data were subjected to one-way ANOVA. Different letters, where present, represent significant differences among treatments.

2.4.4 Nitrate concentration and nitrate reductase *in vivo* activity

Figure 13 shows the nitrate concentration in rocket leaves treated or not with borage extracts. The absolute values of nitrate ranged from 2800 to 5500 mg kg^{-1} FW ± SE. It is interesting to observe that borage extracts treated plants showed nitrate levels halved than untreated control, and this difference was confirmed by statistical analysis.

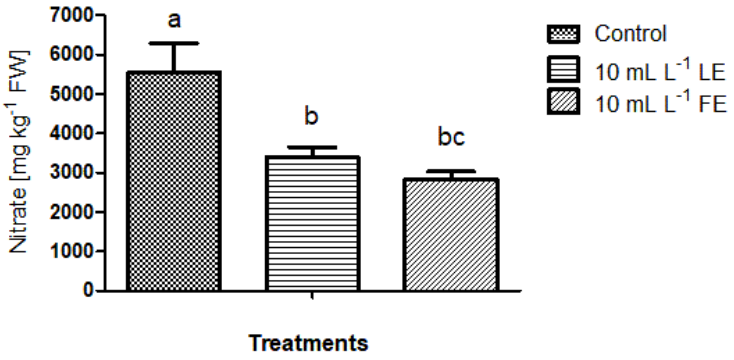

Fig. 13. Nitrate concentration of rocket leaves treated with water (control), 10 mL L^{-1} borage LE or FE. Values are means ± SE (n = 3). Data were subjected to one-way ANOVA. Different letters represent significant differences among treatments.

Figure 14 shows the activity of nitrate reductase *in vivo*, monitored at three different time points (0, 2, and 4 h of light exposure), the day after the second treatment. FE determined a significant increment of the activity, at 2 h and 4 h compared to control. LE presented an intermediate activity, with a peak at 4 h. Control showed the lowest values of activity. These results were coherent with the halved nitrate concentration observed above.

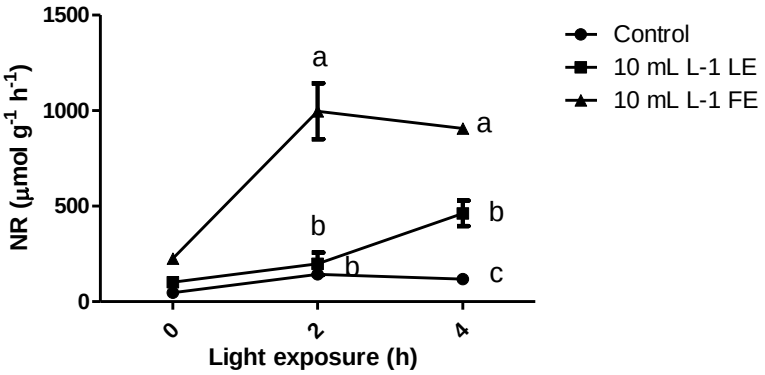

Fig. 14. Nitrate reductase *in vivo* activity measured in rocket leaves treated with water (control), 10 mL L^{-1} borage LE or FE at three different time points (0, 2, and 4 h of light exposure). Values are means ± SE (n = 3). Data were subjected to two-way ANOVA. Different letters represent significant differences among treatments.

2.4.5 Genes expression analysis

In order to verify the effect of borage extracts on nitrate metabolism, the expression of some key genes involved in this metabolic route was studied in response to the different treatments.

The expression levels of *DtNR* measured by qRT-PCR at each time point showed that LE induced an increment during the 4 h after treatment and then decreased (Fig. 15). FE instead caused a peak of *DtNR* expression after 6 h from the treatment. After 9 h, treatments effect tended to decrease.

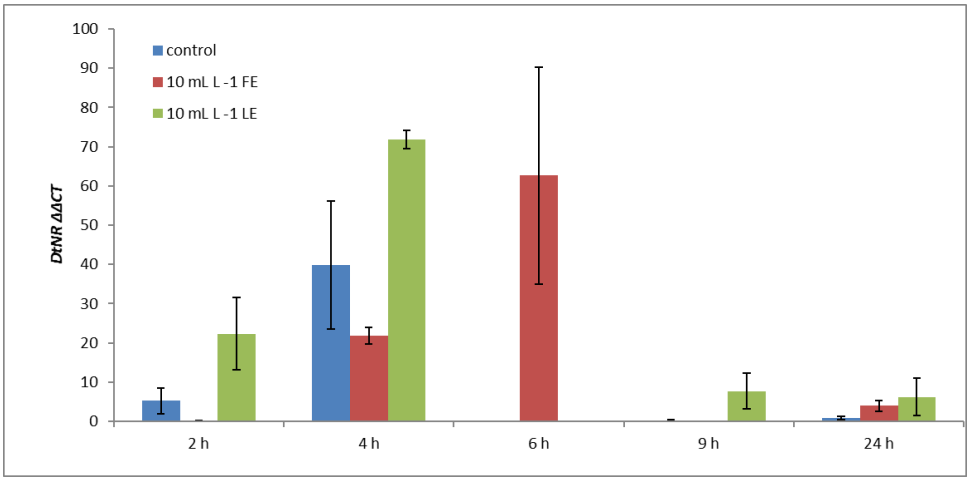

Fig. 15. Gene expression of *NR* in rocket leaves treated with water (control), 10 mL L^{-1} borage LE or FE. Sampling was performed 2-4-6-9-24 hours after the second treatments. Data are means of 3 replicates per each time point. The expression levels were calculated using the delta–delta Ct (ΔΔCt) method.

The different effectiveness of extracts is highlighted in Figure 16, where it is possible to notice an opposite gene regulation: if *DtNR* is up-regulated by LE, it is conversely down-regulated by FE, with the exception of 24 h after treatment where the gene was up-regulated by both borage treatments.

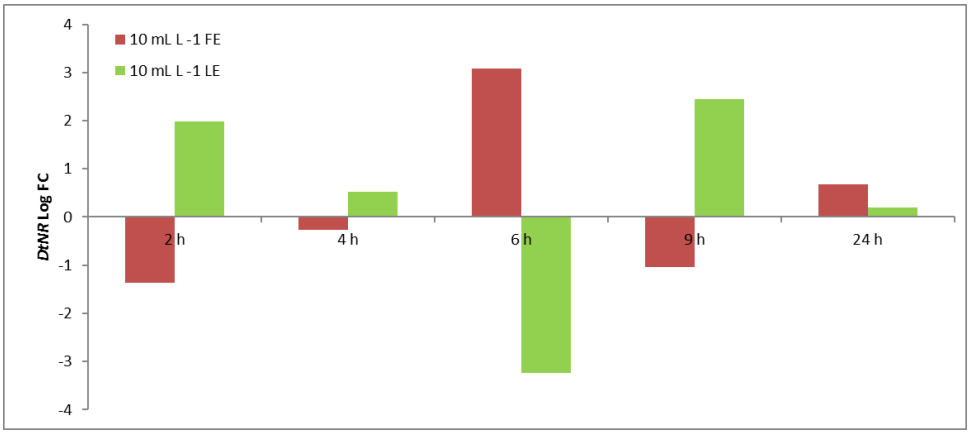

Fig. 16. Gene regulation in response to FE or LE treatment at 2-4-6-9-24 hours.

DtNiR expression was particularly influenced by FE at 2, 4, and 9 h after treatment. Both borage extracts induced a peack at 4 h, with an expression increased three times than untreated control (Fig. 17).

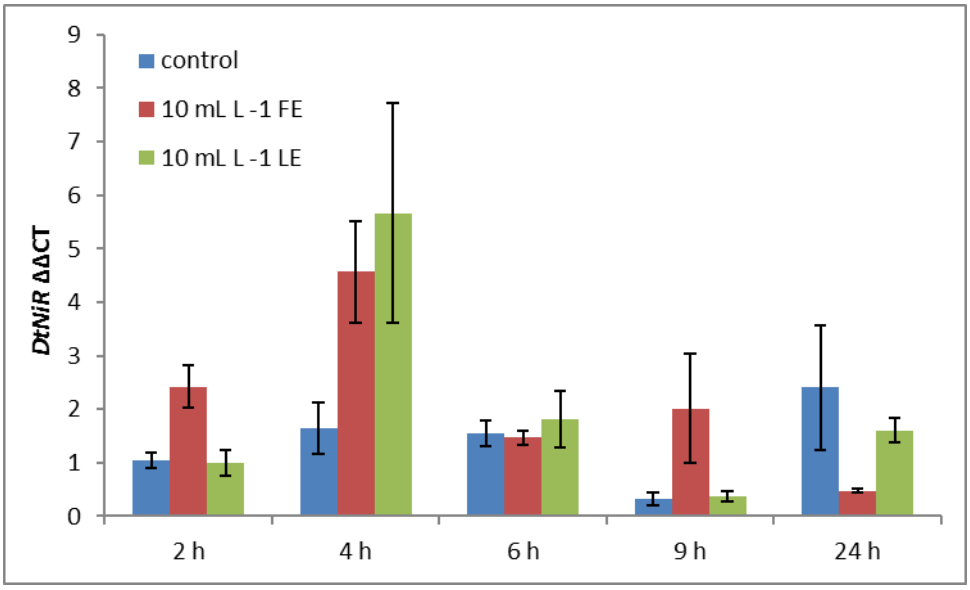

Fig. 17. Gene expression of *NiR* in rocket leaves treated with water (control), 10 mL L^{-1} borage LE or FE. Sampling was performed 2-4-6-9-24 hours after the second treatments. Data are means of 3 replicates per each time point. The expression levels were calculated using the delta–delta Ct (ΔΔCt) method.

Fig. 18 shows that FE induced an up-regulation of *DtNiR*, except at 6 and 24 h after treatment. LE influenced *NiR* expression mostly at 4 h, where it was possible to notice an increment in transcripts abundance. At 24 h both extracts induced a down-regulation of the gene.

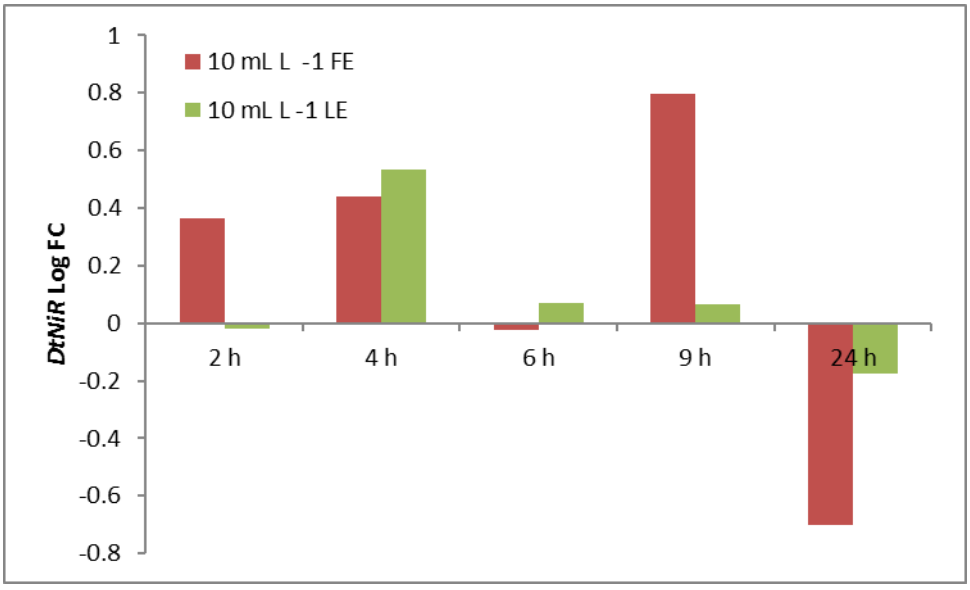

Fig. 18. Gene regulation in response to FE or LE treatment at 2-4-6-9-24 hours.

The expression of *DtGLU* was not enhanced by treatments than control in most of the time points considered (Fig. 19). Only at 4 h FE induced a slight increase of *DtGLU* expression than control, then the effect of treatment decrease.

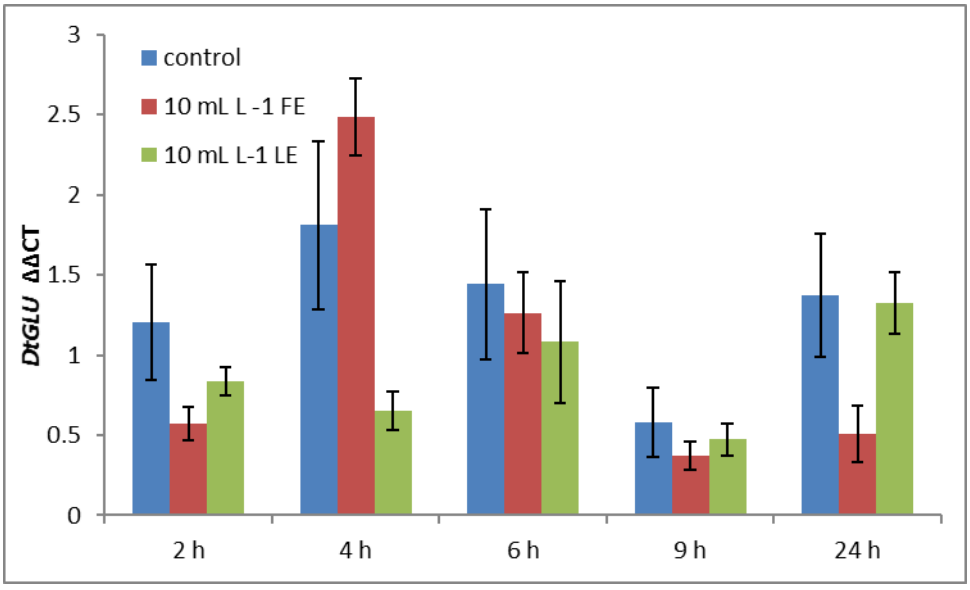

Fig. 19. Gene expression of *GLU* in rocket leaves treated with water (control), 10 mL L^{-1} borage LE or FE. Sampling was performed 2-4-6-9-24 hours after the second treatments. Data are means of 3 replicates per each time point. The expression levels were calculated using the delta–delta Ct (ΔΔCt) method.

It is interesting to notice that borage extracts caused a down-regulation of this gene at each time point considered (Fig. 20). The only exception was represented by FE at 4 h, point at which the gene was up-regulated.

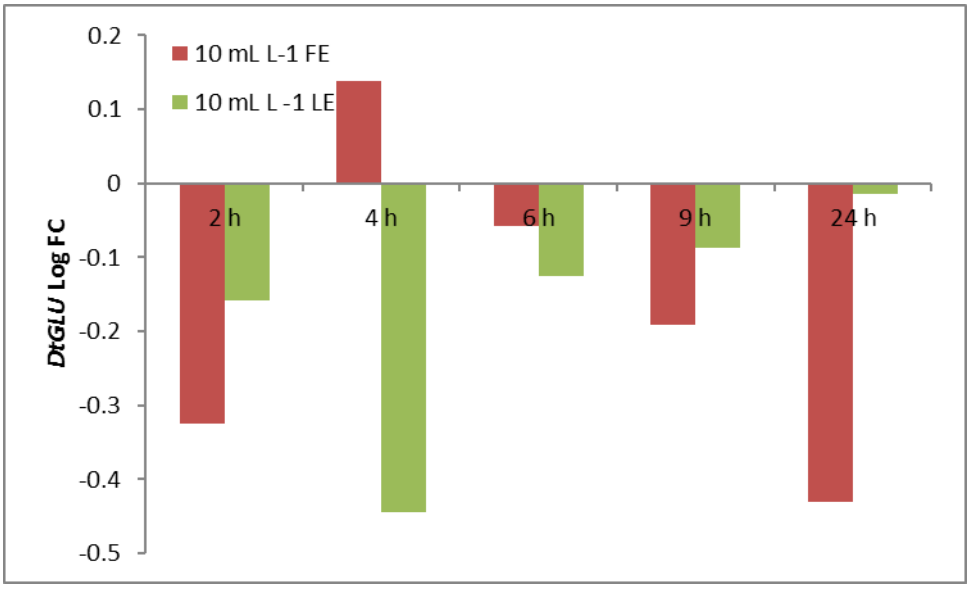

Fig. 20. Gene regulation in response to FE or LE treatment at 2-4-6-9-24 hours.

The influence of LE on *DtNTR* expression (Figure 21) is observable at 2, 4, 6, and 9 h. The highest level was reached at 4 h after treatment. FE showed an effectiveness in particular at 4 and 6 h after treatment.

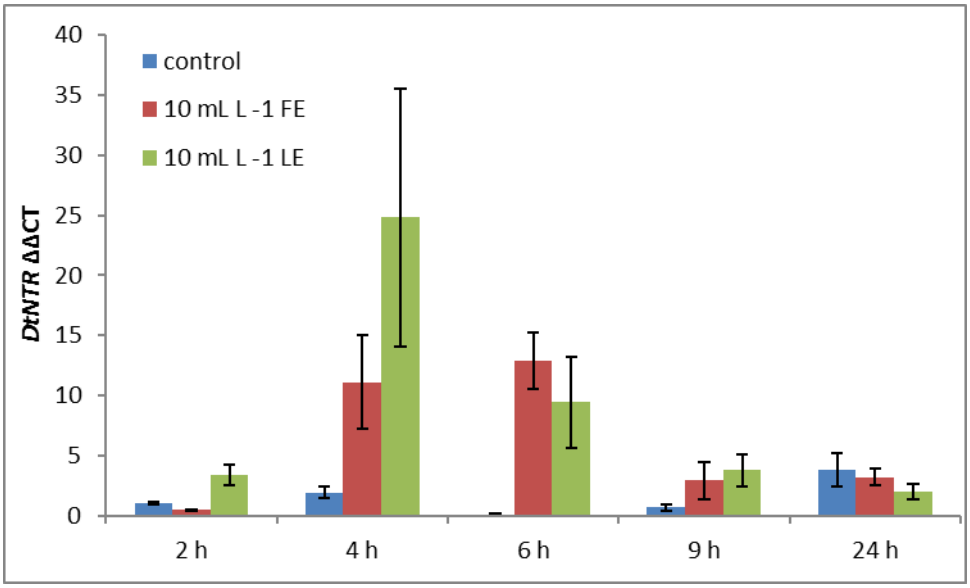

Fig. 21. Gene expression of *NTR* in rocket leaves treated with water (control), 10 mL L^{-1} borage LE or FE. Sampling was performed 2-4-6-9-24 hours after the second treatments. Data are means of 3 replicates per each time point. The expression levels were calculated using the delta–delta Ct (ΔΔCt) method.

Borage LE causes a *DtNTR* up-regulation at all the time point considered (Fig. 22). The only exception was represented by 24 h point, at which the gene was down-regulated. FE determined a down-regulation of the gene at 2 and 24 h; in the other time points, values were similar or slightly lower than LE.

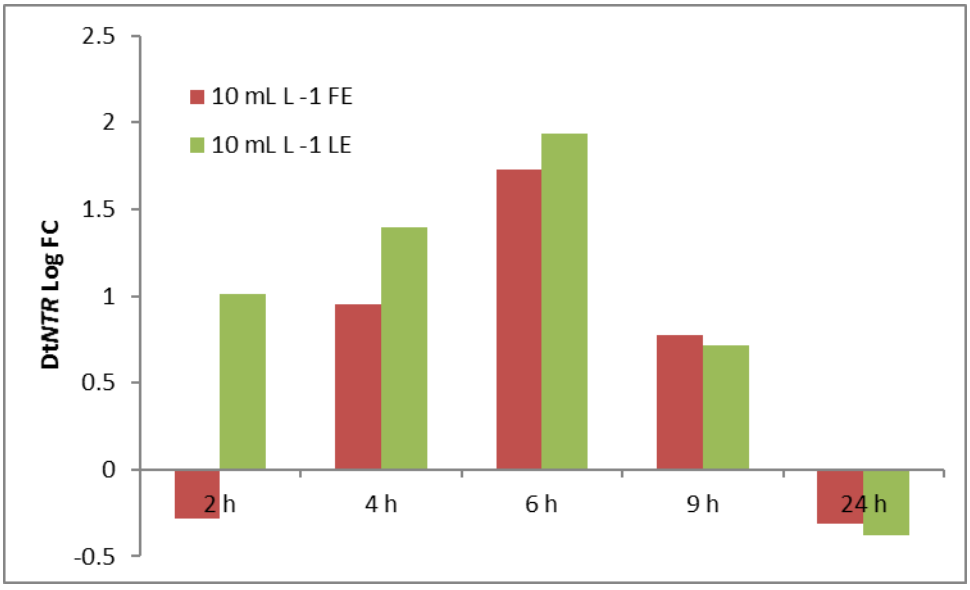

Fig. 22. Gene regulation in response to FE or LE treatment at 2-4-6-9-24 hours.

FE enhanced the *DtGS1* expression at 4 and 9 h than control (Figure 23). The influence of LE was observable at 9 h after treatment, point that corresponds to the highest expression level reached.

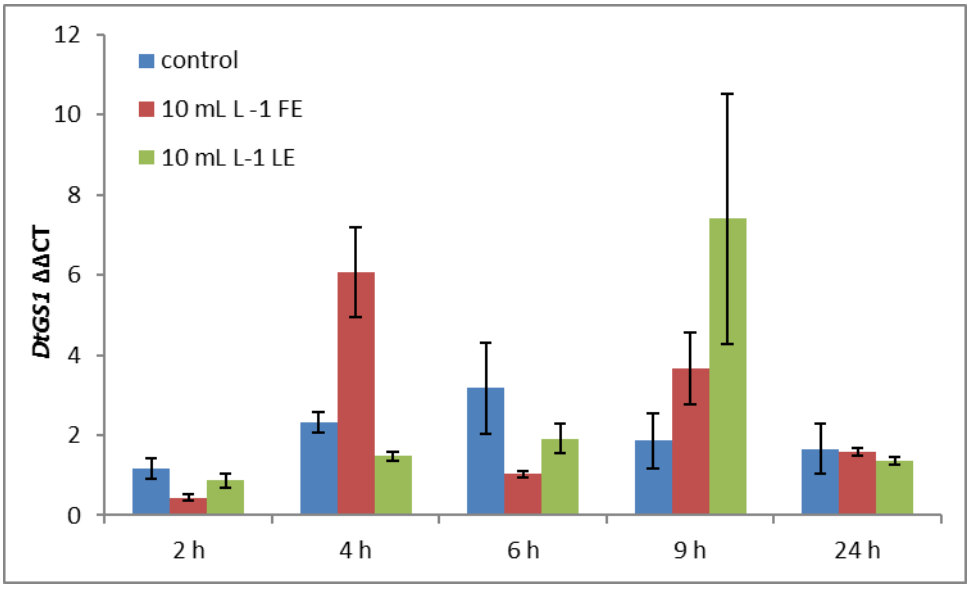

Fig. 23. Gene expression of *GS1* in rocket leaves treated with water (control), 10 mL L^{-1} borage LE or FE. Sampling was performed 2-4-6-9-24 hours after the second treatments. Data are means of 3 replicates per each time point. The expression levels were calculated using the delta–delta Ct (ΔΔCt) method.

DtGS1 results down-regulated after borage extracts application at 2, 6, and 24 h. At 9 h both extracts induced an up-regulation of gene. At 4 h, instead, borage FE and FE caused an opposite response in *DtGS1* expression (Figure 24).

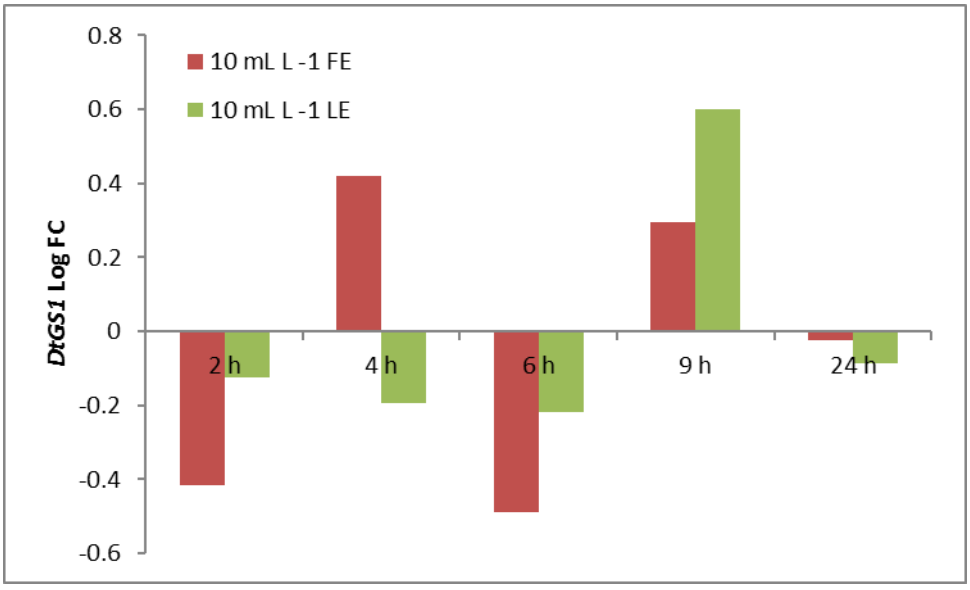

Fig. 24. Gene regulation in response to FE or LE treatment at 2-4-6-9-24 hours.

2.5 Discussion and conclusions

The main goal today, in vegetables production, is to obtain produce with high quality and nutraceutical characteristics (Tarantino *et al*., 2015), for an increasingly demanding market (Ragaert *et al*., 2004; Ramos *et al*., 2013). It was proven that leafy vegetables can get benefit from biostimulants application, for the reduction of nitrate levels (Vernieri *et al*., 2005; Liu *et al*., 2008 Liu & Lee, 2012; Dudaš *et al.*, 2016) and the increment of many antioxidant compounds with potential benefit for human health (Bulgari *et al*., 2015; Colla *et al*., 2015).

Treatments with borage extracts, on rocket, seem to exert these positive effects. From the biochemical point of view, it was possible to note that the mean of carotenoids concentration was slightly enhanced by both borage extracts, even if not significantly. Sucrose level was enhanced by FE, instead the concentration of total sugars was not significantly affected, even if an upward trend was observable. However, the most interesting result was surely the substantial reduction of nitrate level caused by both extracts, confirmed also by the increment of the NR *in vivo* activity. Regarding this aspect, FE seems to possess an effect more marked. A similar influence was observed on maize plantlets by Ertani and colleagues (2009), in consequence of treatment with proteins hydrolysed; an increase in the activity of nitrate reductase and glutamine synthetase, and a reduction of nitrate accumulation in roots and leaves occurred. Schiavon *et al*. (2008) demonstrated that the activity of enzymes involved in C metabolism and N reduction (among which nitrate and nitrite reductase) were positive affected by alfaalfa protein hydrolised. A lower nitrate content was observed in fennel (Tarantino *et al*., 2015) after biostimulant application. The decrease of nitrate levels in rocket leaves could be due to the fact that treated plants consume better and faster nitrate, for example to produce amino acids. So there is probably an action

of activation on N metabolism, as reported in several scientific papers (Ertani *et al*., 2009, 2013; Baglieri *et al*., 2014; Calvo *et al*., 2014). Similar results were obtained on spinach, on which an aminoacid based biostimulant increased nitrate reductase activity and lowered the nitrate content in leaves (Kunicki *et al*., 2010). Moreover, FE induced a significant increment of sucrose concentration; this increase may explain the enhanced nitrogen assimilation, remarking the positive effect on N metabolism, and probably C metabolism as well. The production of more C skeletons promotes nitrate assimilation (Schiavon *et al*., 2008; Colla *et al*., 2015) and more energy for aa and protein synthesis is available.

Furthermore, analogously to what observed with other several species like lettuce (Bulgari *et al*., 2017), rocket (Vernieri *et al*., 2005), tomato (Zodape *et al*., 2011), bean plants (Abbas, 2013) etc., biostimulants are able to increase the cholorphyll content and the photosynthetic activity. Hence, the nitrate organication is probably enhanced since the NR enzyme uses the electrons coming from the photosynthetic machinery.

Borage treatments affected the nitrate assimilation pathway also at molecular level. In fact, the gene expression of Dt*NR*, Dt*NiR*, partially of Dt*GLU*, and Dt*NTR* was influenced by extracts applications. These results confirm that borage extracts have a role in the physiological processes in which the

considered genes are involved. However, extracts showed a different effectiveness on genes up and down regulation, confirming that the different composition of borage FE and LE cause a variable response, due to their bioactive molecules content. A deep investigation on extracts composition it will be necessary for determining the molecules of interest of the two aqueous extracts. The results encourage further investigations on borage extracts, considering that they may improve N use efficiency in rocket.

CHAPTER 3

Hormone-like activity of borage extracts on maize mutants and influence on seeds germination of different plant species

3.1 Introduction

3.1.1 Hormone-like activity

Nowadays plant biostimulants are used to improve crop performance, tolerance against abiotic stresses, and increase yield. In addition to contain naturally plant growth biostimulatory compounds such as vitamins, oligosaccharides, and micronutrients, they also could contain phytohormones (Wally *et al*., 2013), which are compounds produced by plants, with low molecular weight, able to regulate all physiological and developmental processes as well as the responses to biotic and abiotic stresses. These compounds include, among others, auxins (IAA), gibberellic acid (GA), cytokinins (CK), abscisic acid (ABA), ethylene, jasmonic acid (JA), and salicylic acid (SA) (Peleg & Blumwald, 2011). Chemical analysis of biostimulant products revealed the presence of a wide variety of plant growth regulators such as auxins, gibberellins, and cytokinins, in varying amounts (Nardi *et al*., 2002; Canellas *et al*., 2002; Zhang & Ervin, 2004; Quaggiotti *et al*., 2004; Rayorath *et al*., 2008), which elicit strong physiological responses at low doses (Pramanick *et al*., 2013).

The hormone-like activity of borage extracts was evaluated by measuring auxin-like (IAA like) and gibberellin-like (GA like) activity on two maize mutants (the brachytic R3932 and the dwarf R4194, respectively). Maize seeds were kindly provided by Prof. R. Pilu (University of Milan), who isolated and characterized the mutation with his research group (Cassani *et al*., 2009). Mutants were chosen for the experiment because they possess the capacity to be restored to wild type (even if not completely) by exogenous application of hormone.

3.1.2 Allelopathic effects

Plant aqueous extracts are studied also for their potential allelopathic properties (Turk & Tawaha, 2003; Bogatek *et al*., 2006; Islam & Kato-Noguchi, 2013; Baziar *et al*., 2014), deriving from substances therein present. Allelopathy is "a direct or indirect effect of one plant upon another through the production of chemical compounds released into the environment" (Rice, 1984). In the last years, there is an interest in allelopathy, because may provide alternative tools to synthetic herbicides (Bhowmik, 2003; Jabran *et al*., 2015). Promising results were obtained with several allelopathic crop types, that actively influenced the germination and growth of surrounding plants (Azania *et al*., 2003; Weston & Duke, 2003).

Due to the multitude properties attributed to borage, we decided to investigate also this possible activity of extracts, under laboratory conditions.

3.2 Material and methods

3.2.1 Maize mutants – plant material and treatments

Three trials were performed in July 2016, March 2017, and May 2017. Maize seeds were sown on a peaty substrate. After 20 d, seedlings were transplanted in plastic pots, on a peaty substrate, in a greenhouse at the Faculty of Agricultural and Food Sciences of the University of Milan.

The mutants were sprayed every 4 d, for 3 weeks in the cycle of July 2016, every 3 d, for 2 weeks during the cycle of March 2017, and every 2 d for 2 weeks in May 2017. These timings were determined because, after the first experiment, we tried to enhance the effect on plantlets shortening the time between treatments. Foliar treatments were performed in the morning, with water (control), FE 10 mL L^{-1}, LE 10 mL L^{-1}, 0.1 mM GA_3 (mutant R4194) or 0.1 mM IAA solutions (mutant R3932), until run off. The plant height increase (cm) was monitored and measured during the trials.

3.2.2 Nitrate and total sugars concentrations in maize leaves

To evaluate the effect of treatments on maize plantlets, nitrate and total sugars concentrations of leaves were determined in the second and third experiment, at harvest.

Nitrate concentration was measured by the salicylsulphuric acid method (Cataldo *et al*., 1975). One g of fresh leaf tissue was homogenized (mortar and pestle) in 4 mL of distilled water. The extract was centrifuged at 3000 *x g* for 15 min at RT (ALC centrifuge-model PK130R) and the recovered supernatant was used for the colorimetric determination. Twenty µL of sample were added to 80 µL of 5% (w/v) salicylic acid dissolved in H_2SO_4 plus 3 mL of 1.5 N NaOH. The samples were cooled at room temperature and absorbance at 410 nm was measured. Nitrate concentration was calculated referring to a KNO_3 standard calibration curve.

About 1 g of leaf tissue was homogenized in 4 mL of distilled water and centrifuged at 3000 *x g* for 15 min at RT. Total sugars were assayed according to the anthrone assay (Cocetta *et al.*, 2015). Absorbance was read at 620 nm and the levels were calculated referring to glucose calibration curve.

3.2.3 Germination bioassay

Seeds of rocket (*Diplotaxis tenuifolia* L.), lettuce (*Lactuca sativa* L.), basil (*Ocimum basilicum* L.), barley (*Hordeum vulgare* L.), and alfaalfa (*Medicago sativa* L.) were selected as test plant species. Trials were performed in April 2015 and 2016, in laboratory, using microtiter plates. Undiluted borage FE and LE were used for the bioassay and distilled water was used as control. Seeds (24 for each plant species) were placed in 250 µL of LE, FE, or water (1 seed for each well). Plates were incubated in darkness at 20 °C. Germination was observed daily up to 7 d, in the first trial, and up to 9 d in the second one (time when no further seeds germinated). Germination percentage (GP), a commonly used index to measure the effects of phytotoxic substances (Haugland & Brandsaeter, 1996; Hoffman *et al*., 1996; Islam & Kato-Noguchi, 2014) was calculated. This index indicated the total germination percent of seeds after certain period of time when seed germination became constant.

3.3 Statistical analysis

Statistical analysis was performed with GraphPad Prism 6. All data (except GP data) were subjected to one-way ANOVA and differences among means

were determined by Bonferroni's post test ($P < 0.05$). Additional information is reported in each figure legends.

3.4 Results

3.4.1 Hormone-like activity of extracts on maize mutants

The plants height (cm) was monitored during the three trials. Measurements were performed before every treatment application. The reported data showed the plants height at the end of each experiment.

In the experiment of July 2016 (Fig. 25A) treatments on maize mutants R3932 induced a slightly height increase compared to control, in particular LE, even if not significant. The observed effect was greater than hormone (IAA) treatment. Maize mutants R4194 were not influenced by treatments. It is possible to note that wild control showed the higher height, as expected, and all the other treatments had height similar to dwarf control. Not even the hormone (GA_3) treatment induced a reconversion of mutant (Fig. 25B).

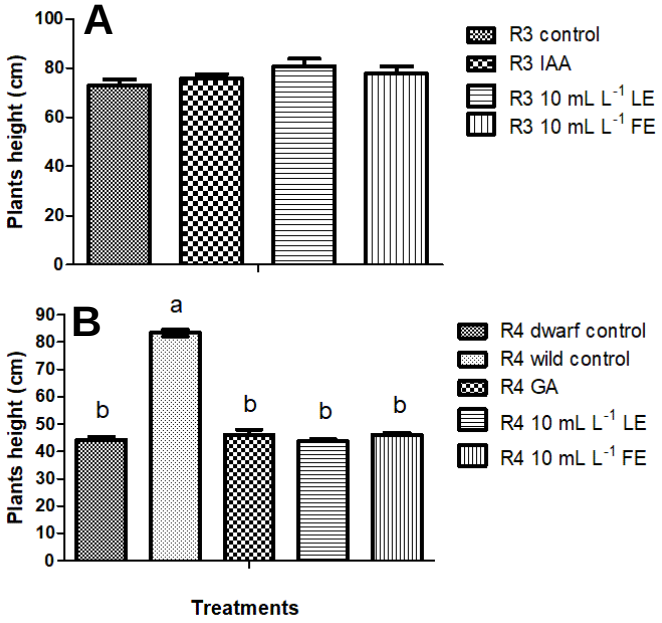

Fig. 25. Plants height, at harvest, of maize mutants R3932 (A) and R4194 (B) treated with water (controls), 10^{-5} M IAA (mutant R3932), 10^{-5} M GA_3 (mutant R4194),10 mL L^{-1} borage LE or FE. Treatments were performed every 4 d, for 3 weeks. Values are means ± SE (n = 6). Data were subjected to one-way ANOVA. Different letters, where present, represent significant differences among treatments.

Considering these results, in March 2017 treatments were intensified. Figure 26 shows that plants reached a lower height (< 50 cm), in general, compared to the other experiments. Maize mutants R3932 (Fig. 26A) did not show significant changes after treatments. However, the activity of borage extracts was similar (FE) or greater (LE) compared to IAA. R4194 mutants treated with hormone had height similar to wild control; LE and FE treatments reached instead values similar to dwarf control (Fig. 26B).

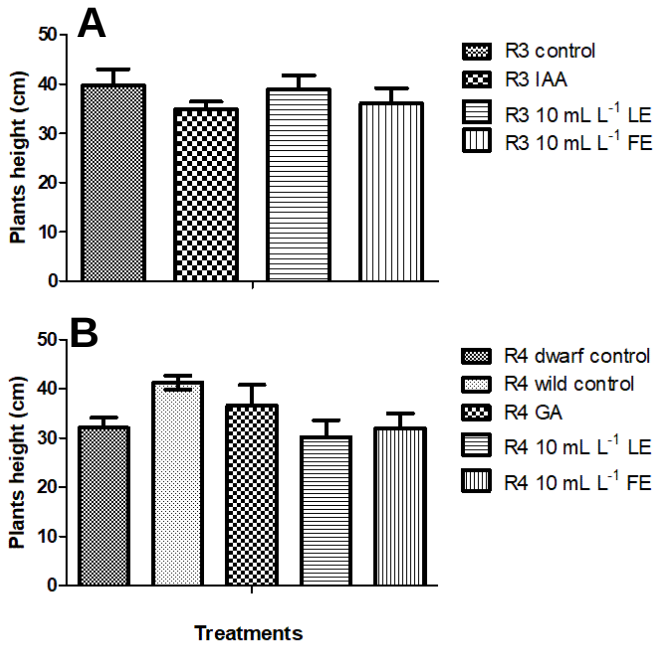

Fig 26. Plants height, at harvest, of maize mutants R3932 (A) and R4194 (B) treated with water (controls), 10^{-5} M IAA (mutant R3932), 10^{-5} M GA_3 (mutant R4194), 10 mL L-1 borage LE or FE. Treatments were performed every 3 d, for 2 weeks. Values are means ± SE (n = 5). Data were subjected to one-way ANOVA.

In the last of the three trials, R3932 mutants (Fig. 27A) were not significant affected by treatments. In fact, values were similar to control. However, the mean of the activity of LE was slightly higher than IAA treatment. The maize R4194 plants (Fig. 27B), defective for the GA biosynthesis, were used as bioassay for the evaluation of GA-like activity of borage extracts. At the end of the experiment, the plant height was significantly different between the mutant and wild type. LE, instead,

stimulated the plant growth and plant height was similar to GA_3 treatment. Plants treated with LE showed intermediate height between the dwarf and wild type control, even if data were not significant.

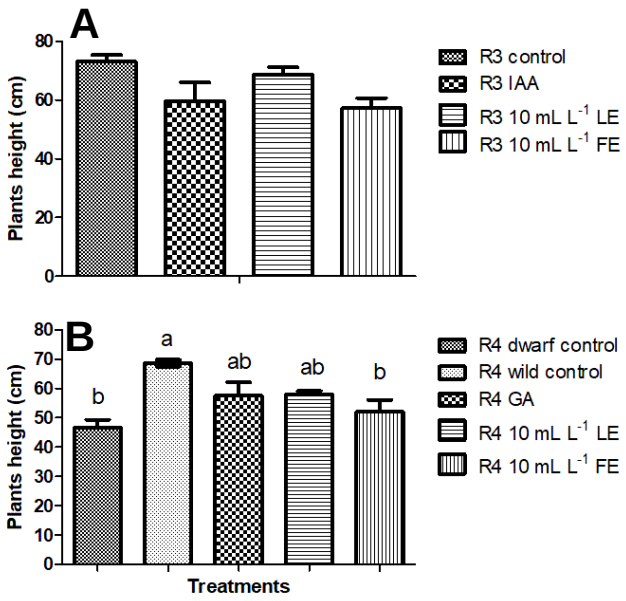

Fig 27. Plants height, at harvest, of maize mutants R3932 (A) and R4194 (B) treated with water (controls), 10^{-5} M IAA (mutant R3932), 10^{-5} M GA_3 (mutant R4194), 10 mL L^{-1} borage LE or FE. Treatments were performed every 2 d, for 2 weeks. Values are means ± SE (n = 6). Data were subjected to one-way ANOVA. Different letters, where present, represent significant differences among treatments.

3.4.2 Nitrate and total sugars concentrations in maize leaves

The leaves of maize mutants R3932 (Tab. 5) did not show significant differences in nitrate concentration after treatments. During March, nitrate levels were higher, in general, and borage treatments induced a greater

accumulation than control and IAA. In May, as expected in the summer period, concentrations were lower and the means of nitrate levels were slightly diminished by borage extracts and IAA treatment than control. Total sugars were lowered in March by FE, but this difference was not significant. During the experiment of May, it was possible to observe, instead, significant differences among treatments; LE caused a decrement in total sugars concentrations than IAA treatment.

	Nitrate [mg kg^{-1} FW]		Total sugars [mg kg^{-1} FW]	
	March	May	March	May
R3 control	761.14±133.36	913.96±241.60	9707.10±690.03	7279.75±263.57ab
R3 IAA	1243.04±18.85	342.54±76.69	10598.20±777.37	7643.37±766.17a
R3 10 mL L^{-1} LE	2167.10±318.81	463.25±36.29	6530.76±1533.87	4272.32±401.01b
R3 10 mL L^{-1} FE	2330.46±597.75	602.47±152.79	3676.16±775.37	4795.32±795.99ab

Table 5. Nitrate and total sugars concentrations of leaves of maize mutants R3932 treated with water (control), IAA, or 10 mL L^{-1} borage leaf (LE) or flower extract (FE). Values are means ± SE (n=3). Data were subjected to one way ANOVA. Different letters, where present, represent significant differences among treatments.

The leaves of maize mutants R4194 (Tab. 6) did not show significant differences in nitrate concentration in the cycle of March. The means of nitrate levels were slightly enhanced by borage extracts and GA_3 treatment. In May, as expected during summer, concentrations were lower and wild control plants showed higher nitrate levels than dwarf control, GA_3, and LE treated plants. Total sugars were not affected by LE and FE.

	Nitrate [mg kg^{-1}FW]		Total sugars [mg kg^{-1}FW]	
	March	May	March	May
R4 dwarf control	1274.23±103.12	194.97±43.57b	6553.94±1189.66	8447.33±70.03
R4 wild control	1590.34±294.62	501.34±97.66a	4351.98±216.86	10368.80±75.37
R4 GA_3	2469.79±329.04	169.89±28.97b	6080.88±1073.45	6634.58±1821.86
R4 10mLL^{-1}LE	2027.93±131.76	214.04±22.06b	2556.64±832.82	7624.71±1121.35
R4 10mLL^{-1}FE	2346.02±497.86	227.35±14.30ab	4464.39±545.819	10516.80±62.37

Table 6. Nitrate and total sugars concentrations of leaves of maize mutants R4194 treated with water (control), GA_3, or 10 mL L^{-1} borage leaf (LE) or flower extract (FE). Values are means ± SE (n=3). Data were subjected to one way ANOVA. Different letters, where present, represent significant differences among treatments.

3.4.3 Effect of borage extracts on seeds germination

GP index shows that borage LE and FE possess influence on seeds germination. An inhibitory effect was more evident in the first trial (Fig. 28A). Borage extracts totally inhibited the germination of lettuce, basil, and barley. In rocket, GP of seeds treated with FE was lower than LE and control. Instead, FE stimulated the germination of alfaalfa seeds.

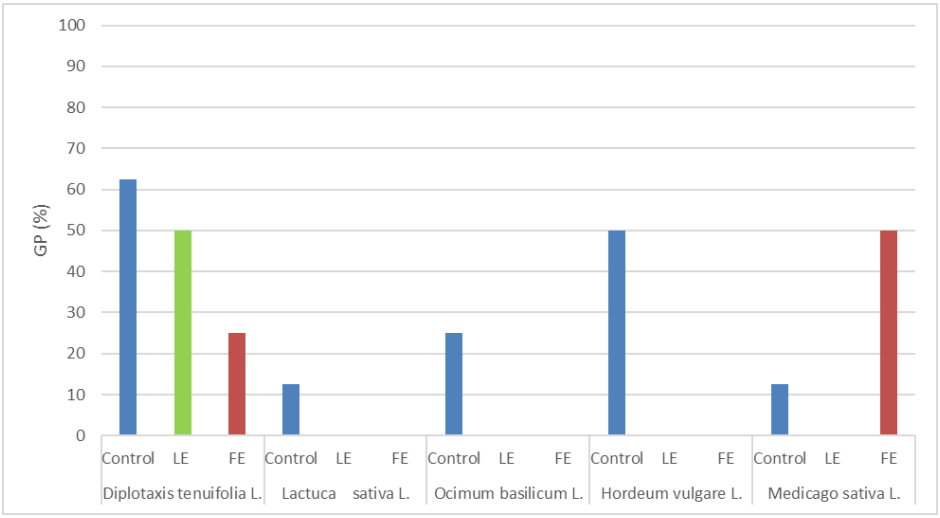

Fig 28A. Effect of borage LE and FE on GP of different plant species (rocket, lettuce, basil, barley, and alfaalfa). Data were referred to the experiment of April 2015.

In the second experiment (Fig. 28B), the percentage of seeds germination was generally higher and extracts caused, in all the cases examined, a decrement in GP, in particular by FE.

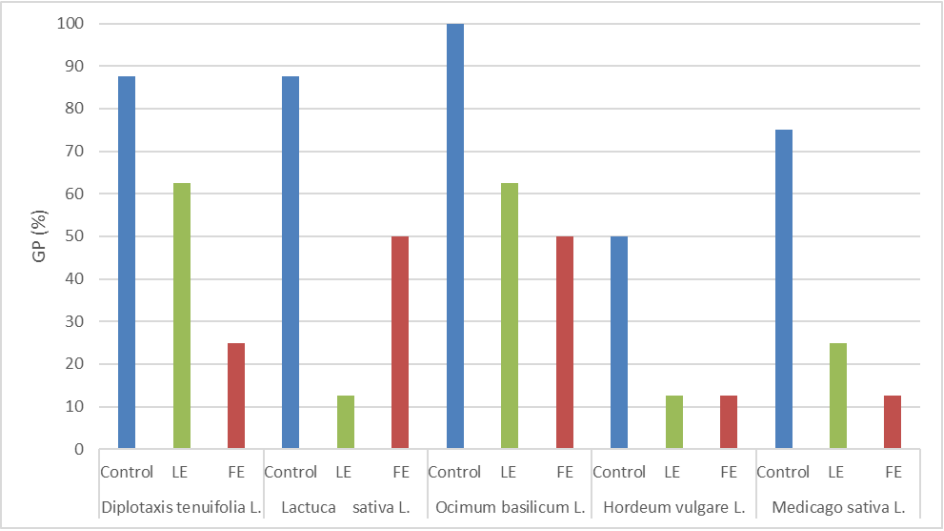

Fig 28B. Effect of borage LE and FE on GP of different plant species (rocket, lettuce, basil, barley, and alfaalfa). Data were referred to the experiment of April 2016.

3.5 Discussion and conclusions

Considering the results obtained during the three trials on maize mutants, not significant differences in plants height were observed after borage treatments. However, some considerations can be done. LE treatment seems to exert a slight auxin-like activity on the brachytic mutants R3932. In fact, in all the experiments, the mean of the height of plants treated with 10 mL L^{-1} LE was higher than IAA treated plants. The activity observed may be in part due to the presence in the extracts of small peptides and aa, which are

considered to be precursor of hormone biosynthesis (Schiavon *et al*., 2008; Colla *et al.*, 2014) or also of glycosides, polysaccharides, and organic acids that could act as activators of endogenous plant hormones (Paradikovic *et al.*, 2011). Many papers reported the auxin- or gibberellin-like activities of plant derived biostimulants (Ertani *et al*., 2009; Matsumiya & Kubo, 2011; Colla *et al*., 2015; Yakhin *et al.*, 2017).

Observing moreover that none of the treatments completely restored mutants to wild type (not even IAA), it is possible that more application, or an extended treatment period, could be necessary to better observe differences in treated plants.

From the biochemical point of view, the influence on maize mutants was more evident on the brachytic R3932, and in particular LE lowered the total sugars concentration in May.

These results confirm that LE exert both a physiological and biochemical influence on maize, even if not evident. Other authors highlighted that biostimulants are able to interact with a variety of biochemical mechanisms and physiological processes (Ertani *et al*., 2009 and references therein).

We can say that, under our experimental conditions, borage extracts effects do not depend on the activity of these two hormones.

GP index gives a global interpretation of germination (inhibition, stimulation, or no action) (Chiapusio *et al.*, 1997). The bioassay on allelopathic properties of borage LE and FE demonstrated that they exert an effect on seeds germination (inhibition effect) and that this activity depending on extract type and plant species tested. In the second experiment, perhaps for the longer period of observation (9 d instead of 7 d), the effect was more evident.

Phenolic acids are often considered as putative allelochemics and they are the most commonly investigated (Chon *et al*., 2005). The observed inhibition effect could be ascribed to the high phenolics content of borage extracts, as suggested in literature (Wettasinghe *et al*., 2001; Aliakbarlu & Tajik, 2012). Moreover, since undiluted borage extracts were used, a high concentrations of bioactive compounds may have occured. Results, once again, suggested that FE is more effective than LE.

CHAPTER 4

Evaluation of biostimulant prototypes against salt stress

4.1 Introduction

4.1.1 Biostimulant and abiotic stress

Biostimulants have been promoted for their ability to counteract abiotic stresses in plants and their mode of action is increasingly studied. These products are able to counteract environmental stress such as water deficit, soil salinization, and exposure to sub-optimal growth temperatures (du Jardin, 2015; Pokluda *et al*., 2016; Van Oosten *et al*., 2017). Abiotic stresses are among the primary causes of crop losses worldwide, reducing average yields for most major crops by more than 50% (Bray *et al*., 2000; La Pena & Hughes, 2007). Salinity, in particular, is one of the main factor that affects plant growth and metabolism in many Mediterranean areas, leading to severe damage and to a substantial loss of productivity (Lucini *et al*., 2015; Taibi *et al*., 2016; Borgognone *et al*., 2016; Rouphael *et al.*, 2017). It represents a serious problem for commercial horticulture (Xu & Mou, 2016), especially in the Mediterranean region where the electric conductivity of water is often higher and overcome the crop threshold sensitivity (Colla *et al*., 2010). Sodium chloride (NaCl) is the main salt presents in saline environments along the seaside production areas (Viegas et *al*., 2001). Exposure of plants

to salinity results in stunted growth, nutrient imbalance, and reduction in water potential (Munns & Termaat, 1986; Blaylock, 1994; Marschner, 1995; Maas & Grattan, 1999; Shaheen *et al*., 2013). Plants have different degree of tolerance that depends from the different adaptation methods and metabolic plasticity. Salt stress could also alter several metabolic processes in plants, such as photosynthesis (Agastian *et al.,* 2000; Sayyad-Amin *et al*., 2016), respiration (Moud & Maghsoudi, 2008), phytohormone regulation, protein synthesis, nitrogen assimilation, and can also generate secondary oxidative stress (Flowers, 2004; Van Breusegem & Dat, 2006; Colla *et al*., 2010). The plant defence mechanisms are oriented to reduce the water uptake to avoid salts loading in the cells. Therefore, salinity and water stress can induce similar physiological and biochemical alterations in plants.

To verify the effects deriving from the applications of biostimulants, trials on lettuce plants under salt stress were performed. Lettuce is in fact considered to be a moderately salt sensitive crop (Shannon & Grieve, 1998) and it is one of the most important leafy vegetable cultivated in the Mediterranean area, where saline water is frequently used for irrigation. The effect of biostimulants can be ascribed to the improvement of the osmotic adjustment in cells by the accumulation of osmotic metabolites and the sequestration of salts in vacuoles, interfering with other compounds.

4.2 Materials and methods

4.2.1 Plant material and treatments

Romaine lettuce (*Lactuca sativa* 'Longifolia') plants were obtained from a local nursery. Two-week-old plantlets were transplanted in 10 cm diameter plastic pots (five pots/treatment), on a peaty substrate, in a greenhouse at the Faculty of Agricultural and Food Sciences of Milan, under controlled conditions. Three NaCl solutions, with increasing concentration (0.8, 1.3, and 1.8 dS/m of electrical conductivity (EC)) were prepared in laboratory. The first saline solution can be considered not stressful for lettuce, the second one as a threshold of salinity tolerance, while the last one as stressful for the crop considered. Treatments conditions were: control (water), two biostimulant prototypes (52124 and 51266), provided by Valagro S.p.A., applied at two doses (10 or 20 L/ha), compared with a commercial competitor applied at the recommended dose of 20 L/ha. The biostimulants used in the experiment contain carboxylic acids, magnesium (Mg), and calcium (Ca). During the cycle, four treatments were performed and four events of stress, both administered as soil application. Lettuce plants were

harvested at commercial maturity stage. At harvest, non-destructive analyses were conducted on leaves and then fresh leaf tissues were immediately stored at -80°C or -20°C until use for biochemical analyses. Two trials were performed: the most significant results are shown.

4.2.2 Leaf gas exchange measurements – net photosynthesis

Leaf gas exchange rates were measured using the portable infrared gas exchange system CIRAS-1 (PP Systems, Hitchin, UK), operated in open-configuration with controlled temperature, CO2 concentration, and vapor pressure. Measurements were carried out on a fully expanded leaf between 09:00 and 13:00 hours IT time. In the cuvette, during the recording time, light intensity was fixed to 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and CO_2 concentration was set to 350 ppm.

4.2.3 Chlorophylls and carotenoids determination

Chlorophyll content was estimated *in vivo* with a chlorophyll meter (CL-01, Hansatech, UK) that provides an indicator of green color of leaves. This device determines relative chlorophyll content using dual wavelength optical absorbance (620 and 940 nm wavelength).

Chlorophylls and carotenoids concentrations were determined at harvest. Leaf tissue (30-50 mg) was extracted using 100% (v/v) methanol, for 24 h at 4 °C in a dark room; afterwards quantitative determination of chlorophylls was carried out. Absorbance readings were measured at 665.2 nm and 652.4 nm for chlorophylls and 470 nm for total carotenoids. Pigment levels were calculated by Lichtenthaler's formula (1987) and expressed on the basis of fresh weight of the tissue.

4.2.4 Total sugars determination

About 1 g of leaf tissue was homogenized in 3 mL of distilled water and centrifuged at 3000 *x g* for 15 min at RT. Total sugars were assayed according to the anthrone assay (Cocetta *et al.,* 2015). Absorbance was read at 620 nm and the levels were calculated referring to glucose calibration curve.

4.2.5 Nitrate levels

Nitrate concentration was measured by the salicylsulphuric acid method (Cataldo *et al*., 1975). One g of fresh leaf tissue was homogenized (mortar and pestle) in 3 mL of distilled water. The extract was centrifuged at 3000 *x g* for 15 min at RT (ALC centrifuge-model PK130R) and the recovered supernatant was used for the colorimetric determination. Twenty μL of sample were added to 80 μL of 5% (w/v) salicylic acid dissolved in H2SO4

plus 3 mL of 1.5 N NaOH. The samples were cooled at room temperature and absorbance at 410 nm was measured. Nitrate concentration was calculated referring to a KNO_3 standard calibration curve.

4.2.6 Proline concentration

Proline was determined with a colorimetric assay, as described by Abraham *et al.* (2010). Lettuce leaves (0.5 g) were ground in 10 mL of sulfosalicylic acid (3%). The tubes were kept on ice until finishing with all samples. Samples were centrifuged for 5 min, at RT, at 3800 *x g* for 10 min. In a separate tube was prepared the reaction mixture: 100 µL of 3% sulfosalicylic acid, 200 µL of glacial acetic acid, 200 µL of acidic ninhydrin. 5. Then 100 µL from the supernatant of the plant extract were added and the tubes were mixed well. Tubes were incubated at 96°C for 60 min. Then samples were put in ice. Subsequently, 1 mL of toluene was added to the reaction mixture and samples were vortexed for 20 s. Tubes were left on the bench for 5 min to allow the separation of the organic and water phases. The chromophore containing toluene was removed into a fresh tube. Absorbance readings were performed at 520 nm using toluene as reference. Proline concentration was determined using a standard concentration curve and calculated on fresh weight basis.

4.2.7 Abscisic acid assay

Abscisic acid (ABA) was determined by an indirect enzyme linked immuno-sorbent assay (ELISA) based on the use of DBPA1 monoclonal antibody, raised against S(+)-ABA (Vernieri *et al.*, 1989). Lettuce leaves (1 g) were homogenized (mortar and pestle) in 3 mL of distilled water. The extract was centrifuged at 3000 *x g* for 15 min at RT (ALC centrifuge-model PK130R) and the recovered supernatant was used for the analysis. The ELISA was performed according to the method described by Borghesi *et al*. (2016).

4.3 Statistical analysis

Statistical analysis was performed with GraphPad Prism 6. All data were compared by using one way ANOVA, with Dunnett's multiple comparison test. Additional information is reported in each figure legends.

4.4 Results

4.4.1 Fresh weight

The fresh weight of the whole lettuce plants was determined at harvest. The highest level of salinity determined a reduction of the fresh weight in controls, even if differences were not significant (Fig. 29). It is often

observable that biostimulant treatments increased significantly the fresh weight of lettuce plants compared to Control 0.8 dS/m (untreated and unstressed plants). The prototype 51266 at 20 L/ha dose increased more than double this parameter.

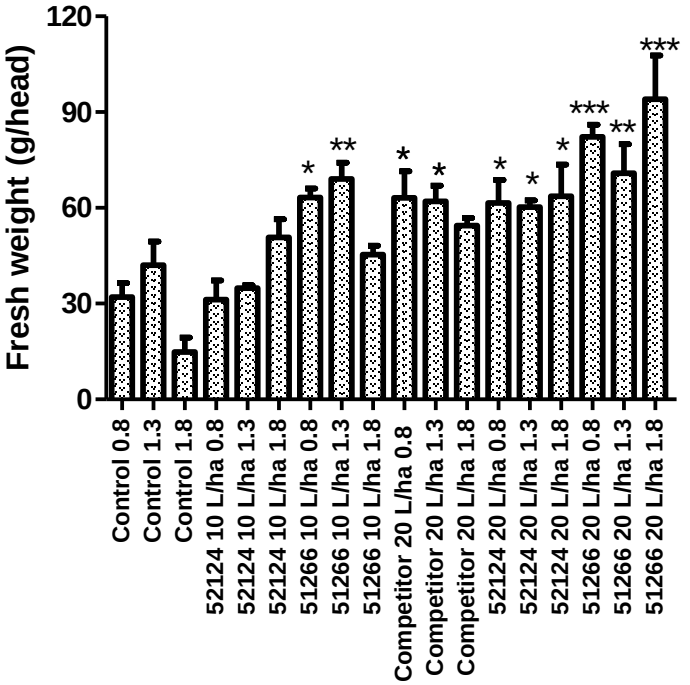

Fig. 29 Fresh weight of Romaine lettuce plants, at harvest, subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control) or with biostimulant prototypes at 10 or 20 L/ha dose. Values are means ± SE (n=3). Data were compared by using one way ANOVA, with Dunnett's multiple comparison test (*p<0.05, **p<0.01, ***p<0.001).

4.4.2 Net photosynthesis rate

Among the parameters considered in the gas exchange measurements, a decrement in the net photosynthesis rate (A) was noticeable in control plants under stressful conditions (Fig. 30). There was instead a general positive effect deriving from the application of the biostimulant products, even if these differences were not statistically significant.

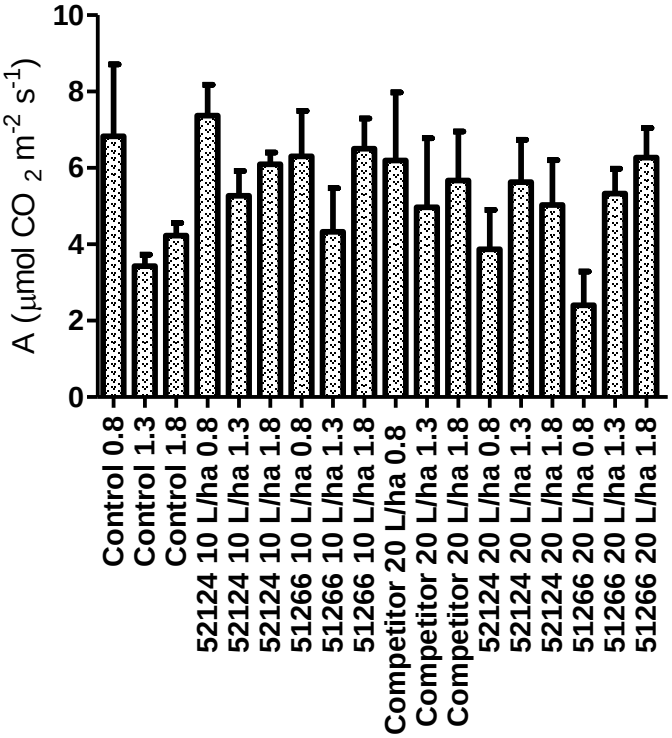

Fig. 30 Net photosynthesis measured *in vivo* in Romaine lettuce plants, at harvest. Plants were subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control), or with biostimulants at 10 or 20 L/ha dose. Values are means ±

SE (n=4). Data were compared by using one way ANOVA, with Dunnett's multiple comparison test (*p<0.05, **p<0.01, ***p<0.001).

4.4.3 Chlorophylls and carotenoids

Lettuce leaves treated with the prototype 51266, at 20 L/ha, and watered with the 1.8 dS/m saline solution showed the highest chlorophyll content measured *in vivo*, and this increment was confirmed by statistical analyses (Fig. 31). In general, it is possible to notice that all the biostimulants, except the product 52124, caused a chlorophylls increment than control, even if these data were not significant.

The destructive determinations showed the same pattern for chlorophylls *a+b* concentrations and carotenoids (Tab. 7). In fact, biostimulant prototypes caused a slightly increment of the considered pigments, however the effect was not statistically relevant compared to Control 0.8 dS/m (untreated and unstressed plants).

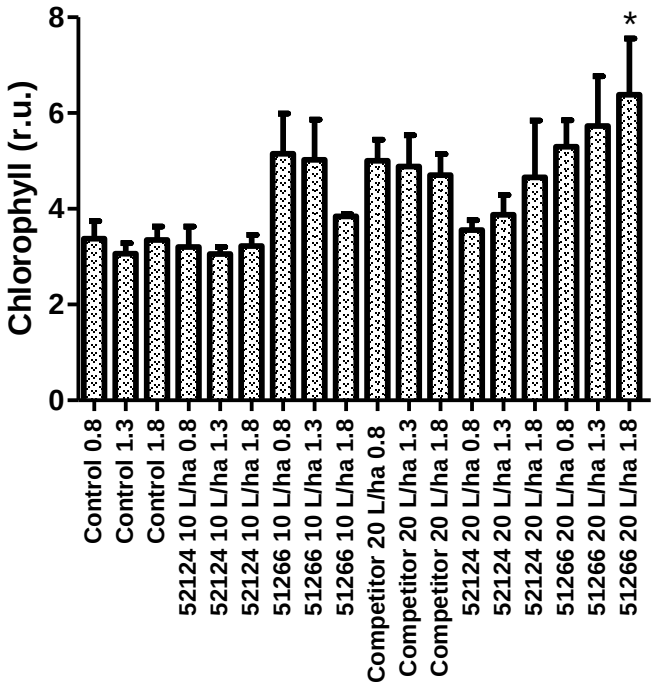

Fig. 31 Chlorophyll content of Romaine lettuce leaves, at harvest, subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control), or with biostimulants at 10 or 20 L/ha dose. Values are means ± SE (n=4). Data were compared by using one way ANOVA, with Dunnett's multiple comparison test (*p<0.05, **p<0.01, ***p<0.001).

	Chl *a+b* [mg g^{-1} FW]	Carotenoids [mg g^{-1} FW]
Control 0.8	0.39±0.10	0.07±0.02
Control 1.3	0.58±0.07	0.11±0.01
Control 1.8	0.51±0.01	0.09±0.01
52124 10 L/ha 0.8	0.57±0.04	0.11±0.01
52124 10 L/ha 1.3	0.48±0.04	0.10±0.01
52124 10 L/ha 1.8	0.54±0.02	0.11±0.01
51266 10 L/ha 0.8	0.71±0.13	0.13±0.02
51266 10 L/ha 1.3	0.63±0.07	0.13±0.01
51266 10 L/ha 1.8	0.63±0.13	0.12±0.02
Competitor 20 L/ha 0.8	0.64±0.06	0.13±0.01
Competitor 20 L/ha 1.3	0.58±0.09	0.11±0.01
Competitor 20 L/ha 1.8	0.61±0.08	0.11±0.01
52124 20 L/ha 0.8	0.58±0.03	0.11±0.01
52124 20 L/ha 1.3	0.62±0.06	0.12±0.01
52124 20 L/ha 1.8	0.66±0.04	0.12±0.01
51266 20 L/ha 0.8	0.51±0.07	0.10±0.01
51266 20 L/ha 1.3	0.63±0.03	0.13±0.01
51266 20 L/ha 1.8	0.72±0.15	0.14±0.03

Tab. 7 Chlorophylls *a+b* and carotenoids concentrations of Romaine lettuce leaves, at harvest, subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control), or with biostimulants at 10 or 20 L/ha dose. Values are means ± SE (n=3). Data were compared by using one way ANOVA, with Dunnett's multiple comparison test (*p<0.05, **p<0.01, ***p<0.001).

4.4.4 Total sugars concentration

Table 8 shows the total sugars concentrations in lettuce leaves treated or not with biostimulants and subjected to different levels of salinity. All plants showed similar values of total sugars, ranging from 6551.03 to 10528.26 mg kg^{-1} FW. No significant difference among treatments could be observed.

	Total sugars [mg kg^{-1} FW]
Control 0.8	8686.54±2845.07
Control 1.3	10528.26±1387.88
Control 1.8	9736.55±3787.24
52124 10 L/ha 0.8	8017.20±1753.12
52124 10 L/ha 1.3	9669.18±2954.03
52124 10 L/ha 1.8	8729.55±661.33
51266 10 L/ha 0.8	8226.37±133.10
51266 10 L/ha 1.3	7785.99±1047.18
51266 10 L/ha 1.8	8693.55±1899.03
Competitor 20 L/ha 0.8	7324.29±595.40
Competitor 20 L/ha 1.3	7971.40±297.09
Competitor 20 L/ha 1.8	7053.65±947.30
52124 20 L/ha 0.8	7415.40±1092.36
52124 20 L/ha 1.3	9947.23±2418.58
52124 20 L/ha 1.8	10440.35±637.91
51266 20 L/ha 0.8	7508.46±1563.27
51266 20 L/ha 1.3	6551.03±877.01
51266 20 L/ha 1.8	7915.06±1442.11

Tab. 8 Total sugars concentration of Romaine lettuce leaves, at harvest, subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control), or with biostimulants at 10 or 20 L/ha dose. Values are means ± SE (n=3). Data were compared by using one way ANOVA, with Dunnett's multiple comparison test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

4.4.5 Nitrate concentration

Nitrate values ranged from 83.7 to 248.7 mg kg^{-1} FW (Fig. 32). The graph shows that the increment of salinity caused a sensible increase of nitrate in leaves of control plants. Treated plants showed values similar to the Control 0.8 plants.

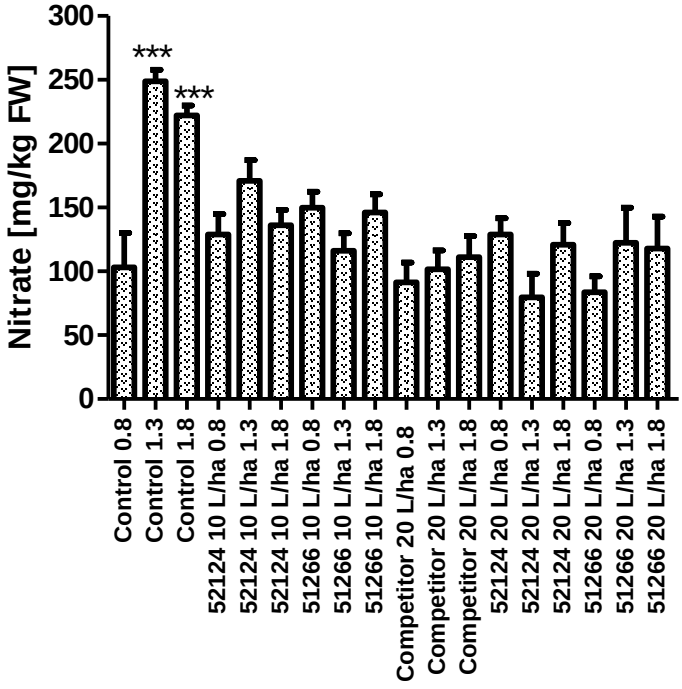

Fig. 32 Nitrate concentration in Romaine lettuce leaves, at harvest, subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control), or with biostimulants at 10 or 20 L/ha dose. Values are means ± SE (n=3). Data were compared by using one way ANOVA, with Dunnett's multiple comparison test (*p<0.05, **p<0.01, ***p<0.001).

4.4.6 Proline levels

In control plants it is possible to observe that the increasing levels of salinity caused a raise in proline concentration (Fig. 33). The highest concentration was found in leaves treated with the prototype 51266, at 10 L/ha and 0.8 dS/m. Biostimulant treatments at 20 L/ha dose, in general, allowed to maintain proline levels lower. So there were an apparent dose depending effect of treatments on lettuce.

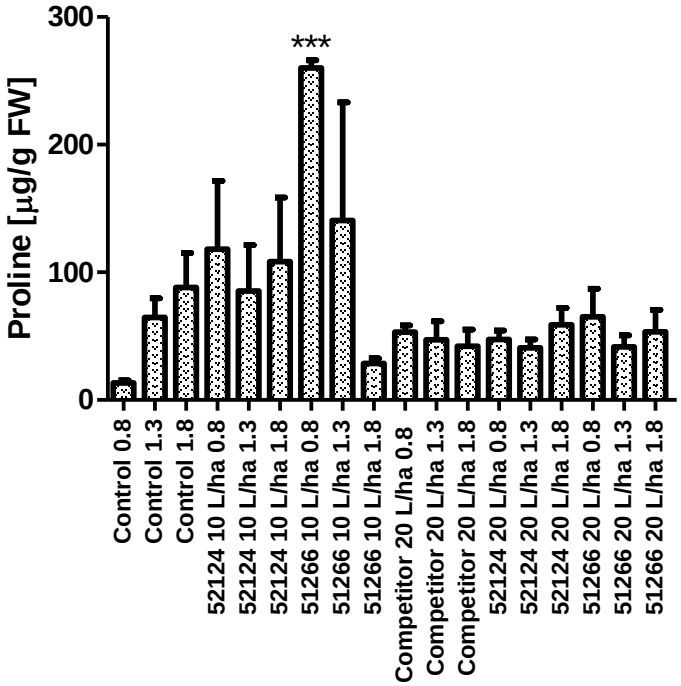

Fig. 33 Proline concentration in Romaine lettuce leaves, at harvest, subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control), or with biostimulants at 10 or 20 L/ha dose. Values are means ± SE (n=3). Data were compared by

using one way ANOVA, with Dunnett's multiple comparison test (*p<0.05, **p<0.01, ***p<0.001).

4.4.7 Abscisic acid

All plants treated with biostimulants showed lower value of ABA compared to controls (Fig. 34). The only exception was represented by the treatment 52124 at 10 L/ha dose, which caused an increment of ABA concentration in leaves, more marked at the level of salinity of 1.3 dS/m. However, the observed effects were not statistically relevant due to the high variability of some data.

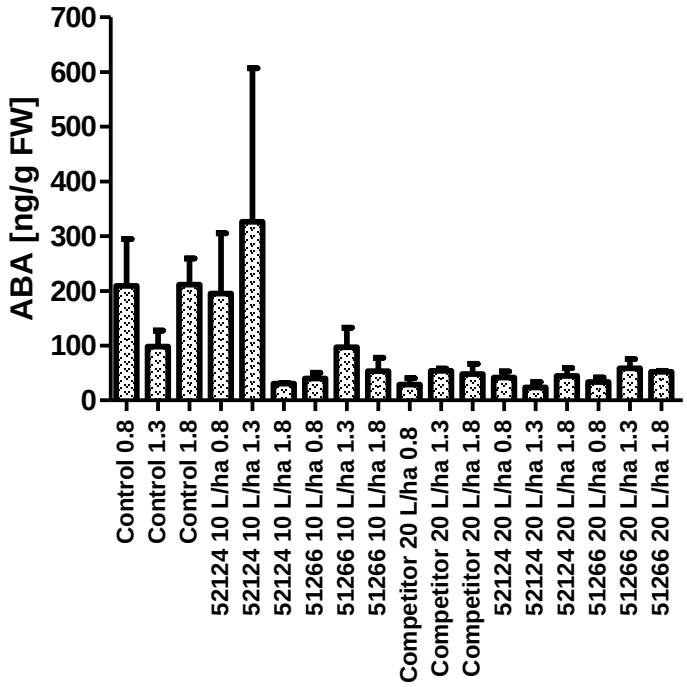

Fig. 34 Abscisic acid concentration in Romaine lettuce leaves, at harvest, subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control), or with biostimulants at 10 or 20 L/ha dose. Values are means ± SE (n=3). Data were compared by using one way ANOVA, with Dunnett's multiple comparison test (*p<0.05, **p<0.01, ***p<0.001).

4.5 Discussion and conclusions

The enhancement of plant tolerance toward abiotic stresses is increasingly being supported by biostimulants, as preferred alternatives to chemical fertilizers (du Jardin, 2015; Yakhin *et al*., 2017; Van Oosten *et al*., 2017). In scientific literature, numerous papers reported the positive effects of

biostimulants on drought, heat, salinity, chilling, oxidative, mechanical, and chemical stress (Yakhin *et al*., 2017 and references therein).

Focusing the attention on salt stress, a common negative effect was the growth reduction of plants (Santos & Caldeira, 1999; Shannon & Grieve, 1999; Akram *et al*., 2012). The biostimulants prototypes, tested in this work, increased significantly the fresh weight of lettuce plants compared to untreated and unstressed plants. The enhancement in the growth of lettuce plants, after treatments, could be attributed to an increased nutrient uptake, as reported by Turkmen *et al*., 2004, who used humic acids in combination with Ca to treat tomato seedlings. In recent years, the functions of Ca were studied in particular for its role as a second messenger in the signal conduction between environmental factors and plant responses, in terms of growth and development (Kaya *et al*., 2002; Hepler, 2005).

Lucini and colleagues (2015) observed that applications of plant-derived protein hydrolysate mitigated the deleterious effects of salt stress on lettuce. These results were consistent with a previous study of Ertani *et al.* (2013), who observed that a protein hydrolysate biostimulant derived from alfalfa increased maize plant biomass, even under salinity.

Salt stress was demonstrated to affect negatively also the leaves photosynthetic pigment contents (Santos, 2004; Turkmen *et al*., 2004). In the present work, results indicated that biostimulant treatments positively affected the chlorophyll content measured *in vivo* and they preserved leaves pigments, contributing to maintain a good produce visual appearance and nutraceutical properties. Biostimulants are often able to increase leaf pigments concentrations (Chbani *et al*., 2015; Bulgari *et al.*, 2015, 2017 and references therein).

To evaluate the health-status of the photosynthetic apparatus in response to stress factors, the gas exchange analysis is a useful non-destructive method. Results suggested that, under stressful conditions, a general positive effect deriving from the application of biostimulants was observable on net photosynthesis rate. Consistent results, regarding the effect of biostimulants on parameters of photosynthetic activity, were found, among others, in peas (Vasin & Lysak, 2015), rocket (Abdalla, 2013), strawberry (Spinelli *et al*., 2010), maize (Anjum *et al*., 2011), and also in ornamental plants (Massa *et al*., 2016). To sum up, biostimulant prototypes seems to protect the photosynthetic apparatus and to improve the gas exchange rates under salt conditions, thereby leading to growth improvement.

In general, soluble sugars tend to increase in leaves under salt stress, while starch content decreases (Chaves, 1991; Baki *et al*., 2000). In our material, the tissue levels of total sugars were not affected by treatments. In fact, all plants showed similar concentrations.

On the contrary, nitrate levels of lettuce leaves were affected by salinity; a sensible increase of nitrate was observable in control plants. Biostimulant treatments allowed maintaining nitrate concentration similar to the untreated and unstressed controls. The reduction of nitrate after biostimulant application was observed in several species of leafy vegetables (Vernieri *et al*., 2005; Liu & Lee, 2012; Dudas *et al*., 2016). The capability to keep nitrates under the limits imposed by EU regulations was very interesting in this commercial sector.

Proline accumulates in many plant species under to a broad range of adverse environmental conditions (Claussen, 2005; Rejeb *et al*, 2014; Xiong *et al*., 2014). Nowadays it is known that proline has multifunctional roles in plants (Szabados & Savouré, 2010). Other than being an osmoprotectant, proline can act as a potent non-enzymatic antioxidant. In our material, we can observe that the increasing levels of water salinity caused a raise in proline concentration in control plants. Hence, in response to stress, plants

accumulated proline, which attend to the osmotic adjustment and may participate to the scavenging of reactive oxygen species. Biostimulant treatments at 20 L/ha dose, in general, allowed maintaining lower the proline levels. On the contrary, the highest concentration was found in leaves treated with the prototype 51266, at 10 L/ha. These results prove a dose depending effect of treatments on lettuce and support the hypothesized positive role of biostimulants in protecting plants from salt stress.

Abscisic acid is an essential phytohormone that regulates various aspects of plant growth and development in response to abiotic stress (Fujita *et al*., 2011). In stressful conditions, such as salinity, ABA content increases dramatically and it triggers the expression of many genes encoding various proteins important for biochemical and physiological processes (Xiong *et al*., 2014 and references therein). Our results showed that all plants treated with biostimulants showed lower value of ABA compared to controls, despite the high data variability. Similar findings were observed in a field study with pistachio (*Pistacia vera*), in which biostimulant treatments ameliorated negative effects on plant growth resulting from irrigation with low to moderate rates of NaCl. This effect was related to a reduction in proline accumulation and decreased levels of ABA in leaves of treated plants compared to controls (Moghaddam & Soleimani, 2012).

The preliminary results of the application of biostimulants prototypes (containing carboxylic acids, magnesium (Mg), and calcium (Ca)) on lettuce revealed that they are able to accelerate the adaptation to salt stress, contrasting biochemical and physiological disorders. The prototype 51266, at maximum dose, seems to be the most effective in our experimental conditions, suggesting to study in depth its effects to get to the commercialization.

GENERAL CONCLUSIONS

The increase of world population and the reduction of chemical inputs in agriculture require more sustainable production systems. Nowadays biostimulants could contribute to make cropping systems more productive and efficient, with less negative impacts on the environment. These goals can be achieved since biostimulant products allow to enhance the efficiency of nutrient use in plants, reduce fertilizer application rates, improve produce quality, and increase plant stress tolerance.

The purpose of the present work was to investigate the effects of biostimulant products, in particular on leafy vegetables, and to study in depth their mechanisms of action, adopting both traditional and innovative techniques.

A new biostimulant obtained from borage plants has been developed and characterized during the Ph. D. project. The trials on extracts obtained from *Borago officinalis* L. showed that borage extracts enhanced both primary and secondary metabolism in lettuce plants. FE also proved to be efficient in preventing degradation during storage. So, borage extracts, with particular regard to the flower ones, appear indeed to exert biostimulant effects on lettuce. On rocket, the most interesting result was surely the substantial

reduction of nitrate level caused by both borage extracts, confirmed also by the increment of the NR *in vivo* activity. Treatments influence on nitrate metabolism was proved at molecular level as well, by analyzing the gene expression of the key enzymes involved in nitrate assimilation. Summarizing, it is possible to say that these extracts are able to improve the quality of leafy vegetables and to induce specific responses in the different species studied. The evaluation of the auxin- and gibberellin-like activity of borage extracts showed that LE seems to possess a slight auxin-like activity, but we can assume that the extracts efficacy, in our experimental conditions, was not due to these hormones. Finally, the inhibition effect on seeds germination suggested a high phenolics content in borage extracts or high concentrations of bioactive compounds.

The agronomic performance of biostimulant has to be correctly evaluated by monitoring different physiological pathways. Several commercial prototypes have been tested in collaboration with Valagro S.p.A. and results revealed that the tested prototypes seem to improve and accelerate the plant reaction against stress factors, playing a protective role on plants.

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