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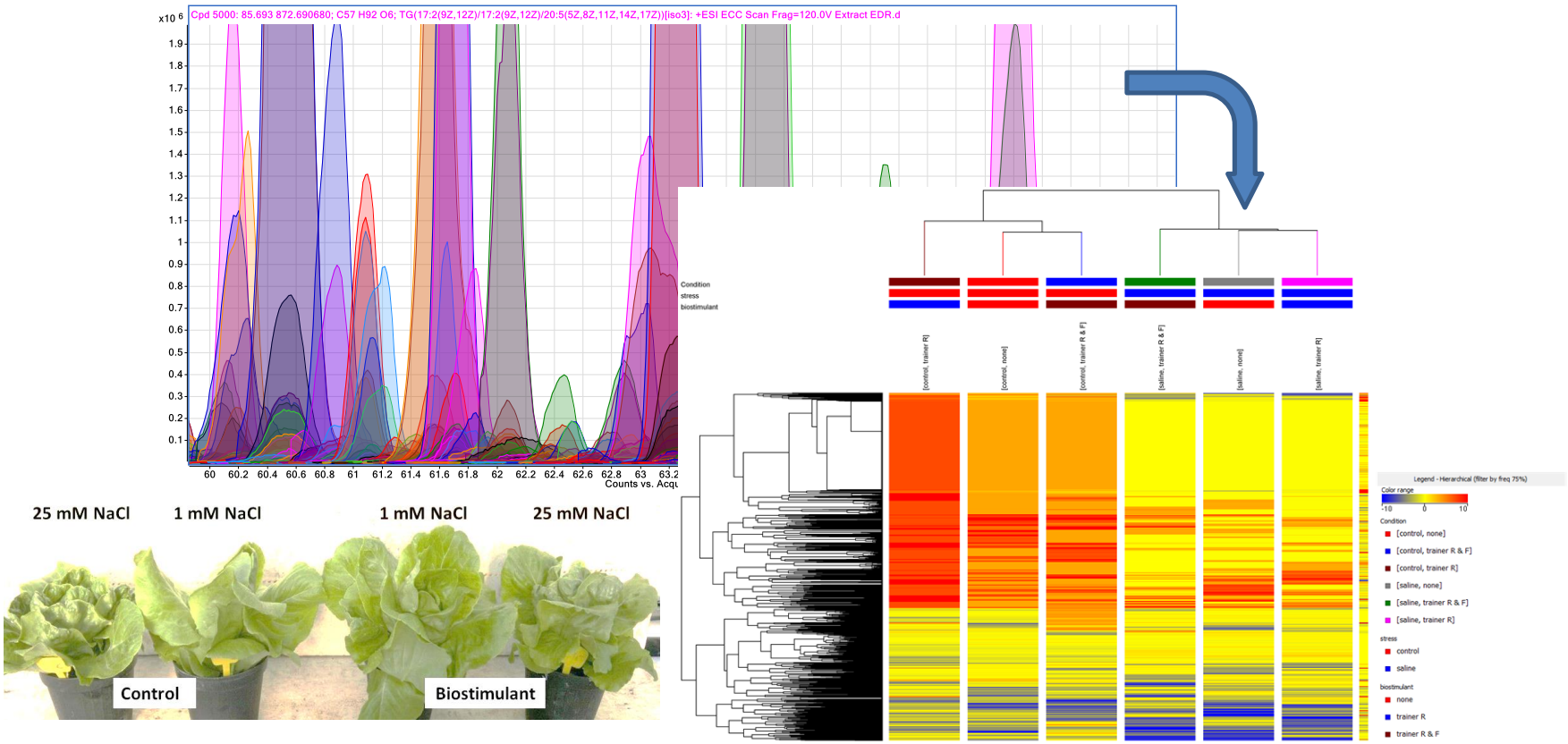
Graphical Abstract (for review)



Research Highlights

- Biostimulant effect on metabolic profile of salt-stressed lettuce plants was explored
- Biostimulant improved crop tolerance to salinity
- Biostimulant enhanced nitrogen metabolism and F_v/F_m-ratio efficiency
- Metabolomic analysis revealed significant changes as a result of biostimulant application

The effect of a plant-derived biostimulant on metabolic profiling and crop performance of lettuce grown under saline conditions

Luigi Lucini[a], Youssef Rouphael[b], Mariateresa Cardarelli[c], Renaud Canaguier[d], Pradeep Kumar[e], Giuseppe Colla[e*]

[a]*Institute of Environmental and Agricultural Chemistry, Università Cattolica del Sacro Cuore, 29122 Piacenza, Italy*

[b]*Department of Agricultural Sciences, University of Naples Federico II, 80055 Portici, Italy*

[c]*CRA-Centro di ricerca per lo studio delle relazioni tra pianta e suolo, 00184 Roma, Italy*

[d]*Nixe laboratory, 06905 Sophia Antipolis Cedex, France*

[e]*Department of Agriculture, Forestry, Nature and Energy, University of Tuscia, 01100 Viterbo, Italy*

*Corresponding author. Tel.: +39 (0)761 357536; fax: +39 (0)761 357453

E-mail address: giucolla@unitus.it (Giuseppe Colla)

Abstract

Plant-derived protein hydrolysates represent new biostimulant products able to improve crop tolerance to abiotic stresses. The aim of the study was to determine growth, root morphology, SPAD index, chlorophyll fluorescence, leaf mineral composition, and metabolic profiling of greenhouse lettuce either untreated or treated (root or leaf-root application) with a plant-derived protein hydrolysate. Lettuce were supplied with two nutrient solutions: non-salt control (1mM NaCl) or 25 mM NaCl. Salt stress decreased shoot and root dry biomass, SPAD index, chlorophyll fluorescence, leaf mineral composition and increased foliar proline concentration. Root and leaf-root application of the biostimulant increased fresh yield, dry biomass and root dry weight of lettuce under salinity conditions. This was associated with an improvement of plant nitrogen metabolism and an increase of the Fv/Fm-ratio efficiency in biostimulant-treated plants. Oxidative stress mitigation, increase in osmolytes, changes in sterols and terpenes composition, as well as the less expected increase in glucosinolates were also observed in biostimulant-treated plants grown under saline conditions. The present study proves that the application of plant-derived protein hydrolysate increases plant performance when plants are grown under salinity conditions. The most favourable metabolic profile was obtained when biostimulant was applied to both roots and leaves.

Keywords: *Lactuca sativa L.*; Chlorophyll fluorescence; Biostimulants; Metabolomics; Mineral compostion; Proline; Salt stress.

1. Introduction

Salinity in water or soil is a serious problem for commercial horticulture, especially in the Mediterranean region (Colla et al., 2010). Most of vegetables are glycophytes and, therefore, highly sensitive to salinity (Shannon and Grieve, 1999). Sodium chloride (NaCl), is the main salt in saline environments (Viegas et al., 2001). High levels of NaCl can be toxic for plants and cause stunted growth, nutrient imbalance, and reduction in water potential (Munns and Termaat, 1986; Marschner, 1995). Salt stress can also alter the major metabolic process in plants, such as photosynthesis (Agastian et al., 2000), protein synthesis (Giridara Kumar et al., 2003), nitrogen assimilation (Flores et al., 2004), and can also generate secondary oxidative stress (Colla et al., 2010). Any improvement in agricultural practices that increases nutrient uptake (e.g. nitrogen) and consequently crop performance under salinity conditions would be of great interest for vegetable growers. New strategies such as the use of biostimulants have been evaluated. As defined by Zhang and Schmidt (1997), biostimulants correspond to "materials, other than fertilizers, that enhance plant growth when they are applied in small quantities". They are present on the market in different formulation and are generally classified into three groups: amino acid containing products, marine bioactive substances, and humic substances (Kauffman III et al., 2007). Biostimulants have been reported to increase the crop performance of a number of agricultural and horticultural crops (Lisiecka et al., 2011). Besides, biostimulants, have also been shown to increase nitrogen and iron metabolism, water and nutrient uptake, resistance to abiotic and biotic stress, and enhancing fruit quality (Ruiz et al., 2000; Cerdán et al., 2009; Ertani et al., 2009; 2013; Lisiecka et al., 2011). However, the positive influence of biostimulants are dependent on plant species, cultivars, climatic conditions, dose, origin and time of application (Lisiecka et al., 2011).

According to Cavani et al. (2006), the amino acid containing products, consist of small peptides and amino acids obtained through chemical and/or enzymatic hydrolysis of several kinds of organic matrices (e.g. plant and animal by-products). Recent studies (Cerdán et al., 2009; Lisiecka et al., 2011) reported that the application of commercial protein hydrolysate product from animal origin was phytotoxic causing negative effect on plant growth when compared with commercial protein hydrolysate product from plant origin. Ertani et al. (2009), using two protein hydrolyzate from alfalfa and from meat flour, observed an increased in root dry weight, and leaf growth in maize seedlings. In another study conducted by those researchers the alfalfa hydrolysate-based biostimulant increases maize biomass even when plants were grown under different salinity levels (Ertani et al., 2013). Moreover, Ruiz et al. (2000) and Cerdán et al. (2009) reported several beneficial effects of plant-derived protein hydrolysates on plants including increased nutrient uptake in particular nitrogen and iron. In another study conducted by Botta et al. (2009), the use of a natural biostimulant based product containing peptides of low molecular weight, was able to overcome transplant stress and to increase yield and fruit quality in strawberry. The above findings are related to the direct use of amino acids in the plant metabolism, the improvement of nutrient availability due to the formation of mineral nutrients-amino acid chelates, and to the activity of signal peptides in regulating physiological processes such as rooting (Matsumiya and Kubo, 2011).

Metabolomics is a scientific discipline of chemical fingerprints that specific cellular processes leave behind, by considering the profile of low molecular weight metabolites which are the end products of metabolisms in various biological systems (Lindon et al., 1999; Davis, 2005). Metabolomics has been successfully applied to the study of molecular phenotypes of plants in response to environmental stress in order to find particular patterns associated to stress tolerance (Pedras and Zheng, 2010; Arbona et al., 2013). For instance,

the response to increased salt involves drastic changes in the activity of a number of genes and proteins which lead to changes in plant metabolism. The possibility of monitoring a complete set of metabolites can provide insights on many physiological processes under salt stress conditions to find particular patterns associated with the application of biostimulants.

Taking into consideration that lettuce (*Lactuca sativa* L.), which is considered to be moderately salt sensitive (Shannon and Grieve, 1999), is one of the most important vegetable crop grown in the Mediterranean area where saline water is frequently used for irrigation, it is of a great interest to know whether the use a plant-derived protein hydrolysate may represent a promising tool to alleviate the adverse effects of salinity on lettuce. The present study was aimed at verifying the influence of a plant-derived protein hydrolysate on lettuce tolerance to salinity, and at understanding the metabolic changes mediated by biostimulant application under saline conditions.

2. Materials and methods

2.1. Growth conditions, plant material and experimental design

An experiment was conducted in 2013 growing season in a 300 m^2 polyethylene greenhouse situated on the Experimental station of Tuscia University, Central Italy (42°25´N; 12°08´E; 310 m a.s.l.). Daily temperature was maintained between 18°C and 26°C. Night temperature was always higher than > 16°C, and relative humidity ranged from 55% to 85%.

Lactuca sativa L. cv. Regina di Maggio (La Semiorto Sementi, Sarno, Italy), were transplanted on September 11, into pots (diameter 14 cm, height 12 cm) containing 1.5 L of quartziferous sand. The pots were placed on 16 cm wide and 5 m-long troughs, with 30 cm between pots and 30 cm between troughs, giving a plant density of 11 plants m^{-2}.

The experiment was designed as a factorial combination of two nutrient solutions (non-salt control, or 25 mM NaCl) and three biostimulant application treatments (control, root application, or root and leaf application). Each experimental unit consisted of ten plants. The treatments were arranged in a randomized complete-block design with four replicates per treatment. The plant derived protein hydrolysate (Trainer, Italpollina S.p.A., Rivoli Veronese, Italy) contained 35.5% of organic matter, 5% of total nitrogen, and 27% of amino acids and soluble peptides. The plant derived protein hydrolysate was obtained through enzymatic hydrolysis of proteins from legume seeds. For foliar application, the plants were sprayed at weekly intervals with a solution containing 2.5 ml L^{-1} of plant derived protein hydrolysate using a stainless steel sprayer. For root application, 100 ml of solution with the same concentration of Trainer (2.5 ml L^{-1}) was applied to the growing medium at weekly intervals. Foliar and radical treatments started on September 16 while saline solution was applied from September 17 to the end of the trial.

2.2. Nutrient solution management

The basic nutrient solution was a modified Hoagland and Arnon formulation. All chemicals used were of analytical grade, and composition of the nutrient solution was: 8.0 mM N–NO_3^-, 1.0 mM S, 0.7 mM P, 2.5 mM K, 3.0 mM Ca, 0.7 mM Mg, 1 mM NH_4^+, 1 mM Na, 1 mM Cl, 20 μM Fe, 9 μM Mn, 0.3 μM Cu, 1.6 μM Zn, 20 μM B, and 0.3 μM Mo, with an electrical conductivity (EC) of 1.2 dS m^{-1}. The saline nutrient solutions had the same basic composition plus an additional 24 mM of NaCl, giving EC values of 3.5 dS m^{-1}. The pH of the nutrient solution for all treatments was 6.0 ± 0.3. All nutrient solutions were prepared using deionized water. Nutrient solution was pumped from independent tanks through a drip irrigation system, with one emitter per plant and an emitter flow rate of 2 L h^{-1}. Irrigation scheduling was per-formed using electronic low-tension tensiometers

(LT-Irrometer, Riverside, CA, USA) that controlled irrigation based on substrate matric potential (Norrie et al., 1994). In each treatment, four tensiometers were installed and located in different pots to provide representative readings of the moisture tension. Tensiometers were connected to an electronic programmer that controlled the beginning (−5 kPa) and end (−1 kPa) of irrigation, which correspond to the high and low tension set points for the major part of the media (Rouphael et al., 2004). Timing of the irrigations was increased to have at least 35% of the nutrient solution draining from the pots (Rouphael and Colla, 2005).

2.3. SPAD and fluorescence measurements

At the end of the experiment (35 days after transplanting, October 16) a chlorophyll meter (SPAD-502, Minolta corporation, Ltd., Osaka, Japan) was used to take readings from the fully expanded functional leaves. Twenty leaves were measured randomly per plot and averaged to a single SPAD value for each treatment.

Modulated chlorophyll fluorescence was measured in dark adapted (for at least 15 min) leaves in the same leaf leaflet in six plants per experimental unit, using a chlorophyll fluorometer Handy PEA (Hansatech Instruments Ltd, UK) with an excitation source intensity higher than 3000 µmol m^{-2} s^{-1} at the sample surface. The minimal fluorescence intensity (F_0) in a dark-adapted state was measured in the presence of a background far-red light to favor rapid oxidation of intersystem electron carriers. The maximal fluorescence intensities in the dark-adapted state (F_m) was measured by 0.8s saturating pulses (3000 µmol m^{-2} s^{-1}). The maximum quantum yield of open photosystem II (PSII) (F_v/F_m) was calculated as $(F_m - F_0)/F_m$ (Maxwell and ~~Jhonson~~, 2000).

164 *2.3. Yield, growth measurements, and root morphology*

165 At the same date of the SPAD and fluorescence measurements, the transplants of
166 lettuce were separated into stems, leaves, and roots. All plant tissues were dried in a
167 forced-air oven at 80°C for 72 h for biomass determination. Shoot biomass was equal to
168 the sum of aerial vegetative plant parts (leaves + stems). Root-to-shoot ratio was also
169 calculated. The dried material was used for mineral analysis, whereas three fresh leaves
170 were collected and frozen immediately in liquid nitrogen for proline analysis.

171 For the root morphology determination, four plants per experimental unit was selected.
172 The whole root system was collected by removing the pots. The samples were submerged
173 in bowls filled with distilled water, for 45 minutes. This procedure aimed at facilitating the
174 root washing process, which was performed using water jets, until the root systems were
175 free from any sand particles. The determination of the root system morphology was done
176 using a WinRHIZO Pro (Regent Instruments Inc., Canada), connected to a STD4800
177 scanner. A 400 (dpi) resolution was used for measuring roots morphology, as described by
178 Bouma et al. (2000) and Costa et al.(2002). The roots were arranged in a 20 cm wide and
179 30 cm long acrylic container filled with 1 cm of water. Three dimensional images were
180 acquired. The following root characteristics were determined: total root length (mm), mean
181 root diameter (mm) and total root surface area (cm).

182

183 *2.4. Mineral analysis*

184 The dried leaf tissues were ground in a Wiley mill to pass through a 20-mesh screen,
185 then 0.5 g samples were analyzed for the following macronutrients: N, P, K, Ca, Mg, and
186 Na. Nitrogen was determined by the Kjeldahl method (Bremner, 1965) after mineralization
187 with H_2SO_4. Phosphorus, K, Ca, Mg and Na were determined by dry ashing at 400 °C for
188 24 h, dissolving the ash in HNO_3 (1:20 w/v) and assaying the solution obtained using an

189 inductively coupled plasma emission spectrophotometer (ICP Iris, ThermoOptek, Milan,
190 Italy; Karla, 1998).
191
192 *2.5. Proline determination*
193 Free proline content was determined according to the method of Bates et al. (1973)
194 Around 0.5 g of leaf material was homogenized in 10mL of 30 g L^{-1} sulfosalicylic acid
195 (Sigma Aldrich) and the homogenate was filtered through Whatman No. 2 filter paper.
196 Then 2 mL of filtrate was reacted with 2 mL of acid-ninhydrin (1.25 g of ninhydrin in 30
197 mL of glacial acetic acid and 20 mL of 6 mol L^{-1} phosphoric acid) and 2 mL of glacial
198 acetic acid in a test tube at 100 °C for 1 h. The reaction was terminated in an ice bath and
199 then 4 mL of toluene was added and the product of the reaction was extracted by vortex
200 mixing. The absorption of the upper phase was read at 520 nm using toluene as a blank.
201 Proline concentration was calculated on a fresh weight (FW) basis using L-proline for the
202 standard curve.
203
204 *2.6. Metabolomics*
205 High resolution mass spectrometry (MS) analyses were performed on a hybrid
206 quadrupole-time-of-flight instrument, coupled to an UPLC chromatographic system
207 (UPLC/Q-TOF) to investigate metabolite profiling in the samples. The mass spectrometer
208 was run in the positive scan mode and was operated to acquire spectra in the range of 50–
209 1000 m/z after setting resolution at 30.000 FWHM. A 1290 liquid chromatograph system,
210 equipped with a binary pump and a Dual Electrospray JetStream ionization system, and
211 coupled to a G6550 mass spectrometer detector (all from Agilent technologies Santa Clara,
212 CA, USA) was used. Chromatographic separation was performed using an Agilent Zorbax
213 Extend-C18 RRHT column (50 x 2.1 mm i.d., 1.8 μm dp). The LC mobile phase A

consisted of water (Milli-Q grade, Millipore, Bedford, MA, USA), while mobile phase B was methanol (LCMS grade from Sigma Aldrich St. Louis, MO, USA). Formic acid 0.1% (v/v) and ammonium formate (5 mM) (both from Sigma) were added to both mobile phases. The gradient was initiated with 5% B and increased to 90% B within 15 min, then held for 3 minutes. The LC mobile phase temperature was set to 35 °C, the injection volume was 8 µl and the flow rate was 220 µl min^{-1}. Regarding samples preparation, specimens (3 g) were extracted in 80% methanol with 0.05% HCOOH using an Ultra Turrax (Ika T-25, Staufen, Germany), diluted 5 folds in 40% methanol, filtered on a 0.22 µm cellulose membrane and transferred to a vial for analysis.

Extracts were then analyzed by UPLC/Q-TOF, and a blank was run between each pair of analyses. Q-TOF conditions were as follows: sheath gas nitrogen 10 L min^{-1} at 350 °C; drying gas 8 L min^{-1} at 330 °C; nebulizer pressure 60 psig, nozzle voltage 300 V, capillary voltage 3.5 kV. Lock masses were continuously infused to correct accurate mass values; purine at m/z 121.0509 and HP-0921 at m/z 922.0098 were used.

Raw data, as provided by the time-of-flight analyzer, were processed by the MassHunter Qualitative Analysis B.05 software (from Agilent Technologies) using the "find-by-molecular-feature" algorithm. Confidence of compound identification was based on accurate mass and isotope pattern and expressed as overall identification score, computed as a weighted score gained from the isotopic pattern (exact masses, relative abundances, and m/z spacing having a weight of 100, 70 and 60 respectively).

Unidentified molecular features were subjected to a recursive analysis workflow using Mass Profiler Professional B12.05 (from Agilent Technologies) for features alignment and filtering after the initial deconvolution. Features that were not present in at least 75% of replications within a treatment were discarded. Filtered features were exported in MassHunter Qualitative Analysis, targeted using the "find-by-formula" algorithm and

identified using different databases, namely (i) the Metlin Metabolite PCDL library (version B.05.00 from Agilent technologies); (ii) the database exported from Phenol-Explorer 3.0 (Phenol-Explorer: an online comprehensive database on polyphenol contents in foods; doi: 10.1093/database/bap024; accessed January 2014); and (iii) the database exported from PlantCyc 8.0 (Plant Metabolic Network, http://www.plantcyc.org; released November 2013). The peak volume of each compound (identified after recursive analysis with a mass accuracy of 5 ppm maximum, and above 85/100 for overall identification score from isotopic pattern) was extracted from the total ions current and exported for statistics and data interpretation.

2.7. Statistical analysis

All data were statistically analyzed by ANOVA using the SPSS software package (SPSS 10 for Windows, 2001). Duncan's multiple range test was performed at P = 0.05 on each of the significant variables measured.

Interpretation of metabolomic analysis was carried out using Mass Profiler Professional B.12.05 (from Agilent technologies); the exported compounds were filtered (only those being above 5000 counts as peak volume and appearing in 100% of samples in at least one condition were considered) and then normalized at 75^{th} percentile for each filtered compound. Thereafter, compound abundance values in each sample were baselined to the median of each compound in all samples. Statistics and interpretations were then performed on this filtered dataset: Multivariate ANOVA analysis (P = 0.05, Benjamini-Hochberg multiple testing correction) and fold-change analysis (cut-off = 2) were combined into volcano plots. Unsupervised hierarchical cluster analysis on both compounds and treatments (setting similarity measure as Euclidean and Wards as linkage rule) and Principal Component Analysis (PCA) were finally done on the dataset.

3. Results

3.1. Fresh shoot yield, biomass production and root morphology

The lettuce shoot fresh yield was significantly affected by salinity ($P<0.001$) and biostimulant application ($P<0.001$), with significant salinity × biostimulant interaction ($P<0.05$; data not shown). In treated and untreated plants, the shoot fresh yield decreased in response to an increase of salinity concentration in the nutrient solution with more detrimental effect recorded in untreated plants (Fig. 1). In fact, increasing the NaCl concentration from 1 to 25 mM in the nutrient solution decreased the lettuce shoot fresh weight by 33% in the control treatment, whereas the fresh shoot reduction was significantly lower when the foliar (17%) and the root/foliar application (11%) of the commercial plant derived protein hydrolysate 'Trainer' was adopted (Fig. 1).

The shoot, root dry weight, and the total root surface were significantly affected by salinity and biostimulant application ($P<0.001$), with no significant salinity × biostimulant interaction. Moreover, the root-to-shoot ratio, total root length, and root diameter were highly influenced by biostimulant application, but not by salinity, there was no salinity × biostimulant interaction (Table 1). Increasing the nutrient solution salinity decreased the shoot, root dry weight and the total root surface by 7.1%, 5.7% and 6.7%, respectively (Table 1). When averaged over salt treatment, the shoot dry weight was higher by 16.6% in treated biostimulant than untreated plants with no significant difference observed between the two mode of applications (root versus root/foliar). The highest values of the root dry weight, root-to-shoot ratio, total shoot length and root surface were recorded in the root-foliar application treatment, followed by the root application treatment, whereas the lowest values were observed in the untreated lettuce plants (Table 1).

289 *3.2. SPAD index, chlorophyll fluorescence and proline concentration*

290 The maximum quantum use efficiency of PSII in dark-adapted state (F_v/Fm), was
291 significantly affected by salinity, biostimulant, with no salinity × biostimulant interaction,
292 whereas, the SPAD index and proline content, were highly influenced by salinity
293 application, but not by biostimulant, there was no salinity × biostimulant interaction (Table
294 2). Irrespective of the biostimulant treatment, increasing the NaCl concentration from 1 to
295 25 mM, decreased the SPAD index, the chlorophyll fluorescence by 14.0%, 34%,
296 respectively, while a strong increase (by 121%) in proline content in lettuce leaves was
297 observed (Table 2). Moreover, lowest efficiency of the PSII in dark-adapted leaves,
298 measured as the F_v/F_m ratio were recorded in the biostimulant treatment under both root
299 and root-foliar application (avg. 0.73) compared to untreated lettuce plants (0.58, Table 2).
300

301 *3.3. Mineral composition*

302 The macronutrient and sodium concentration as a function of biostimulant application
303 and salinity treatments are displayed in Table 3 and Fig. 2. The concentration of N in
304 leaves was significantly affected by salinity × biostimulant interaction. The reduction of
305 nitrogen in leaf tissue of plants treated with NaCl, with respect to control solution, was
306 significantly lower in plants treated with Trainer (10%, and 5%, for root and root/foliar
307 application, respectively) in comparison to that of untreated plants (19%) (Fig. 2). The P,
308 K, Ca, and Mg concentrations in lettuce leaves were highly affected by saline treatment,
309 since increasing the NaCl concentration from 1 to 25 mM significantly decreased the
310 concentration of these P, K, Ca, and Mg by 14.6%, 22.3%, 26.6%, and 54.1%, respectively
311 (Table 3). Finally, Leaf Na content was enhanced by the NaCl treatment, since the Na
312 concentration in lettuce leaves at 25 mM was significantly higher by 400% when compared
313 to the non saline treatment (Table 3).

314 *3.4. Metabolic profiling*

315 Accurate mass spectrometry is commonly used in plant metabolomics and processing
316 of raw data is crucial for identification of compounds with specific deconvolution
317 algorithms providing molecular formulae on the basis of both the mono-isotopic mass and
318 isotopic patterns (Kueger et al., 2012). Metabolites are typically identified by searching in
319 available databases, therefore the quality of the information strongly relies on the quality
320 identification and quality of the dataset.

321 In our experiment, sample extraction was carried out with minimal handling to limit
322 artifacts and to minimize the possibility of false negatives. The absence of false positives
323 was checked by analyzing blanks between samples. All the compounds were detected with
324 low mass error (nominally below 5 ppm, but in the sub-ppm range in most of the cases).
325 The results gained from Phenol-Explorer identification showed two clearly distinct clusters
326 of compounds, one early-eluting and comprising caffeoylquinic acids, and a second eluting
327 later comprising the flavonoids. The identification results from either Metlin ot PlantCyc
328 databases, however gave more comprehensive and homogenous profiles along the
329 UPLC/Q-TOF run. In this second case, a wide variety of compound classes were identified
330 as expected. The elution profile in the samples, together with their frequency of detection,
331 ~~is~~ provided in Fig. 3.

332 Overall, a total of 2120 compounds were detected in the samples, and a large number of
333 them could be identified using Metlin, Phenol-Explorer or PlantCyc databases. Recursive
334 analysis and the subsequent filtering in Mass Profiler Professional dramatically reduced the
335 number of compounds in the dataset, however statistics and data interpretation strongly
336 benefit from that reduction in size. Indeed, both cluster analysis and PCA in the initial
337 database gave mixed replications across treatments and therefore the clustering was

definitely not satisfactory, probably due to the high variability within the dataset (data not shown).

The interpretations regarding the dataset on phenolic compounds were based on the Phenol-Explorer identifications only; results did not evidence significant differences (neither in ANOVA nor in fold-change) between the treatments. Cluster analysis and PCA confirmed that this class of metabolites was not a high discriminant between the treatments, considering that replications were erroneously clustered among each other and that any cluster could be observed using three components in PCA (explaining less than 30% of variability). Therefore, these data were not included for further evaluations and thus were discarded to avoid redundancies.

Results from other identifications carried out using the more comprehensive databases Metlin and PlantCyc after recursion and filtering, gave better results. Between 30 and 40 compounds gave identification scores higher than 99%, and more than 100 higher than 90%. The compounds selected after multivariate ANOVA and fold-change analysis (volcano plots), using saline stress as interpretation (control versus stressed), were ascribed to carbohydrates (raffinose, maltotriose and α-ribose-1,5-diphosphate), glucosinolates (2-propenyl-glucosinolate), deoxycytidine triphosphate (dCTP), NAD^+, L-proline, 4'-phosphopantheine and hexadecanedioate. The covariance-based algorithm "find similar entities" in Mass Profiler Professional gave the same list as above.

Further chemometric interpretations were based on the analysis of covariance, to provide additional interpretation on metabolite profiles across the treatments. PCA and even more hierarchical cluster analysis gave interesting results. These results provided with a good separation of the salt-stressed replication from those not stressed and let to point out differences between the biostimulant. PCA and cluster analysis are represented in Figs. 4 and 5 respectively. As visually evidenced by the heat map in cluster analysis, few clusters

of compounds are responsible of most the difference between stressed and not stressed lettuce plants. Among others, these cluster were comprised of glucosinolates (such as 8-methylsulfinyloctyl- and methylsulfinylhexyl glucosinolate), compounds related to oxidative stress (L-proline, L-dehydro-ascorbate glutathione disulphide, selenodiglutathione, hydroxymethylglutathione, 4-hydroxy-2-nonenal-glutathione conjugate and other glutathione-related compounds, indolacetyl-conjugated forms), sterols, flavonoids, as well as several carbohydrates and their related compounds (mannitol-1-phosphate, 3-phospho-D-glicerate, raffinose, α-maltose, β-D-fructose and its phosphate derivative).

Unsupervised clustering was also performed on the sub-dataset comprising only salt-stressed samples, to better focus on the comparative effect of the different biostimulant treatments. This analysis was aimed to better understand the mitigation of saline stress in lettuce through application method of biostimulant rather than focusing on salt stress itself. The results evidenced that the application to both roots and leaves gave the highest distance from control, while biostimulant application to roots resulted in a metabolite profile closer to control. The heat map associated to hierarchical analysis let to identify sub-clusters of compounds contributing the most of difference between the treatments. These sub-clusters were extracted as compounds list and exported. Seven sub-clusters were chosen and the corresponding compounds classified in different classes according to their biochemical meaning. These results are reported in Table 4, while each detailed sub-cluster is provided in supplementary material. Few classes of compounds let to explain most of the difference between treatments, namely some hormones and related compounds, together with caffeoylquinics, flavonoids and some other compounds well known to be related to saline stress. Then carbohydrates, sterols, terpenes and glucosinolates were also evidenced.

Glucosinolates were those better represented, as both intermediates of the biosynthetic pathway together with end products could be found.

4. Discussion

Several studies have demonstrated that salinity induces osmotic stress by limiting water uptake, and ionic stress resulting from high concentrations of toxic ions (e.g. Na^+, Cl^-) within plant cells (Munns and Tester, 2008). Moreover, growth inhibition and reduction of biomass production are general responses of glycophytes to salt stress, and the decreasing in plant growth may change in relation to several interacting variables, including the phenological stage, the salt concentration and the time of exposure (Munns, 2002). Similarly, in the present study, significant depression in shoot fresh weight, shoot and root dry weight and also on total root surface area was observed (Table 1), in agreement with many greenhouse studies on mini-watermelon (Colla et al., 2006a), melon (Colla et al., 2006b), zucchini squash (Rouphael et al., 2006), and cucumber (Colla et al., 2012) grown hydroponically under greenhouse conditions. Reduced lettuce shoot weight under saline treatment could be attributed to sodium chloride increasing the osmotic potential as well as the activity of Na^+ and Cl^- ions in the root zone (Greenway and Munns, 1980) leading to nutritional imbalance (Pasternak, 1987).

However, when the lettuce plants were treated with biostimulant, the extent of yield (Fig. 1) and growth (Table 1) suppression was decreased and the treated plants exhibited greater shoot dry weight than untreated plants, indicating that both foliar application and foliar-root application of the plant-derived protein hydrolysate 'Trainer' can mitigate the deleterious effects of salt stress. These results are consistent with a previous study of Ertani et al. (2013), who observed that the application of a protein hydrolysate-based biostimulant derived from alfalfa (*Medicago sativa* L.) increased plant biomass, even when maize plants

were grown under salinity, likely because of its content in plant growth regulators, such as triacontanol and indole-3-acetic acid (IAA). Moreover, the presence of amino acids and small peptides have been extensively studied for their effect of increasing plant tolerance against abiotic stresses including salinity (Tuteja, 2007). The root dry weight, total root length and surface were significantly higher when leaf and root of lettuce plants were treated with 'Trainer' biostimulant in comparison to the control (Table 1). Our results indicated that the application of biostimulant can alter the morphology of the lettuce root system, yielding a more extensive absorbing area, which may considered a mechanism of salt tolerance (Tuteja, 2007).

Salt stress interferes with several aspects of plant biochemistry, including photosynthesis and pigment synthesis (Colla et al., 2010). The lower lettuce fresh yield, and dry biomass reduction recorded in plants treated with 'Trainer' biostimulant seems to be related to the better functioning of the photosynthetic apparatus. The reduction of photochemical activity is considered to be one of the non-stomatal factors that limit photosynthesis (Souza et al., 2004). In the current study, the maximum quantum use efficiency of PSII in dark-adapted state (F_v/F_m) decreased under salt stress (Table 2), suggesting that salinity induced an inhibition of PSII electron transport. These results are in agreement with those of Shu et al. (2013), and Cai et al. (2014) who reported that salt stress led to the decrease in the F_v/F_m, mainly due to an inhibition of electron transport at the acceptor side of the PSII reaction center. Moreover, when averaged over salinity treatment, F_v/F_m was significantly reduced by 20.5% in untreated plants, suggesting the occurrence of photoinhibition, and this could be a consequence of damage to PSII (Demmig-Adams and Adams, 1992). However, no change was detected in the F_v/F_m ratio of treated plants (Table 2), suggesting that the application of 'Trainer' biostimulant on leaf or on leaf and root can delay photoinhibition under salt stress. In addition to reduced

chlorophyll fluorescence, the SPAD index, which is indicative of chlorophyll content, decreased in salt treated plants (Table 2), in agreement with previous studies on cucumber (Colla et al., 2012) and maize (Ertani et al., 2013). This reduction could be considered a part of the senescence response occurring under salinity (Hörtensteiner, 2006)

Increasing the NaCl concentration from 1 to 25 mM in the nutrient solution, increased shoot proline accumulation (Table 3). Under salt stress conditions, accumulation of proline in plants, can alleviate symptoms of salinity injury by contributing to osmotic adjustment, protecting proteins and membranes and quenching ROS (Matysik et al., 2002; Heidari, 2009). However, changes in proline concentrations were not significant in plants treated with biostimulant. The concentrations of proline are always not high enough to adjust the osmotic potential in some plants under stress (Hoque et al., 2007). In fact, the inhibition of growth of lettuce was higher in untreated plants than in treated plants even if the levels of proline were similar for all plants under salinity. Thus, our result suggest that the accumulation of proline itself cannot confer salt tolerance in lettuce plants.

Many researchers have demonstrated that high concentrations of NaCl in the soil solution may increase the ratios of Na^+/K^+, Na^+/Ca^{2+}, Ca^{2+}/Mg^{2+}, and Cl^-/NO_3^-. As a result, the plant become susceptible to osmotic and specific ion injury (e.g. Na^+), as well as to nutritional imbalance that may result in reduced growth (Grattan and Grieve, 1999). This was the case in the present study, since increasing the NaCl concentration from 1 to 25 mM in the nutrient solution decreased significantly the leaf macronutrient (N, P, K, Ca, and Mg) composition (Table 3). The application of biosimulant containing amino acids and soluble peptides increased the N and P concentration by 10% and 16% respectively (Table 3), when compared to untreated plants, indicating a role of the biostimulant in promoting macronutrient uptake and assimilation. Protein hydrolysates can enhance nitrogen assimilation through an increase of glutamine synthetase and nitrate reductase activities as

462 observed in maize leaves by Ertani et al. (2009). Increased uptake of nitrogen could
463 account for improved photosynthetic activity and enhanced translocation of photosynthates
464 and other metabolites to the sinks, and consequently to the greater growth of plants
465 supplied with biostimulant.

466 Salt stress is complex and imposes a water deficit that affects most of the metabolic
467 activities. This osmotic effect leads then to an increased production of reactive oxygen
468 species (ROS), resulting in an oxidative stress to plants. Therefore, the metabolic response
469 to salinity must involve the synthesis of antioxidant compounds.

470 Some differential metabolites were expected to play a key role in differentiating the
471 treatments we tested (Table 4), being widely known to be related to oxidative stress.
472 Caffeoylquinics and flavonoids that can act as radical scavengers contrasting ROS, actually
473 were among these metabolites. Glutathione-related compounds can also be easily related to
474 the mitigation of oxidative damage, and therefore they were not surprisingly identified
475 among differential metabolites. A further confirmation of the different effect of
476 biostimulation on radical scavenging capabilities has been gained by the identification of
477 the end products of oxidative damage driven by ROS. Indeed, sulcatone is derived from
478 lycopene oxidation, while 4-hydroxynonenal results from lipid peroxidation.

479 Soluble metabolites such as L-proline, oligosaccharides and the polyols mannitol and
480 arabitol can be ascribed to low molecular weight cytoplasmic compounds that act to
481 counteract ionic strength in vacuole. Their accumulation under salt stress has widely been
482 documented in several species, and their function has been defined as osmotic adjustment
483 rather than osmoprotection (Parida and Das, 2005).

484 The involvement of sterols found in the current study are in agreement with previous
485 works: sterols are effective in the regulation of membrane stability and permeability;
486 salinity can alter sterol content as reported in literature (Navari-Izzo et al., 1988; Elkahoui

et al., 2004). Oxidative stresses also enhanced terpene levels (Loreto and Schnitzler, 2010) by promoting the expression of terpenoid synthase (Basyuni et al., 2009) in salt-stressed plants. Terpenes are also thought to promote direct and indirect defence by modulating the signaling that activates defence pathways(Basyuni et al., 2009).

Although the understanding of hormonal homeostasis is far from complete, our data suggest that the imbalance of plant hormones was triggered by salinity and modulated by biostimulant. The first intermediate in abscissic acid biosynthesis (1-deoxy-xylulose-5-phosphate) was reported among differential metabolites in our experiments, together with two cytokinins. Parida and Das (2005), in their review, actually reported the involvement of abscissic acid and cytokinins in salt stress response, together with jasmonates. This information is in agreement with our findings. In our study, the profile of gibberellins, the content of salicylic acid and conjugated auxins were also differentially measured. Phytohormone conjugation is considered as a part of the mechanism to control cellular levels of these compounds; therefore the increase of conjugated auxins could be also related to the altered trafficking induced by flavonoids (Peer and Murphy, 2007). Salicylic acid is also involved in cross talking with other hormones such as jasmonate, abscissic acid, auxins, cytokinins and gibberellins in a complex defense signaling network. However, it can also be postulated that the biostimulant possess hormone-like activity itself, being a protein hydrolizate of plant origin. In fact, a number of polypeptide hormones have been reported in plant and the discovery of several others is foreseen (Ryan et al., 2002).

The role of glucosinolates as compounds involved in the differential metabolic response to salt stress in lettuce was not expected at the beginning. However, glucosinolate contents are induced after pathogen attack as well as under abiotic stresses such as salt stress, UV radiation, and by plant signaling molecules (Variyar et al., 2014). The

aquaporin-mediated increase in glucosinolates was observed in broccoli (López-Berenguer et al., 2008) indicating the involvement of these compounds in the response to salinity.

Overall, the involvement of the differential metabolites we measured is in line with salt stressed plants, therefore justifications can be found in literature to support our findings. Interestingly, when looking at the heat maps in the sub-clusters gained from salt-stressed plants to which biostimulant was applied, we could find a correlation with the treatments. The fold-change of differentially measured metabolites in stressed samples to which the biostimulant was applied only to roots was intermediate between that gained from control (plants untreated with biostimulant and grown under 25 mM NaCl) and the one gained from plants to which biostimulant was applied to both roots and leaves. The same trend could be observed in almost all cases, suggesting proportionality in the mitigation effect toward salt stress. Finally, the stress modulation effect provided by biostimulant involved the main salinity-related metabolic processes as a whole, rather than acting on a specific target. The most favourable metabolomic profile in lettuce was observed when biostimulant was applied to both roots and leaves.

5. Conclusions

As a summary, the present study reveals substantial differences in the agronomical, physiological and metabolomics responses between treated and untreated plants in response to salinity. The percentage of yield and biomass reduction in comparison to control was significantly lower in the plants treated with ‘Trainer’ biostimulant. The application of the biostimulant to both leaf or leaf/root was capable of maintaining higher photochemical activity of PSII, and a better nutritional status (higher N, and P) in the shoot tissues leading to a higher crop performance. The plant response to salinity was affected by biostimulant, involving the processes related to oxidative stress mitigation, osmotic

adjustment, hormone network as well as sterols, terpenes and glucosinolate profile. The degree of mitigation seems also to be related to the application way, being the root and foliar application the most effective treatment.

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Table 1

Mean effects of biostimulant application and salinity level on dry weight of shoots and roots, root-to-shoot ratio, total root length, average root diameter, and total root surface of lettuce plants.

Treatments	Shoot dry weight (g plant^{-1})	Root dry weight (g plant^{-1})	Root to shoot ratio	Total root length (m plant^{-1})	Root diameter (mm)	Total root surface (m^2 plant^{-1})
Salinity (mM NaCl)						
1	7.16 a	1.59 a	0.22 a	322.5 a	0.29 a	0.30 a
25	6.65 b	1.50 b	0.23 a	317.6 a	0.28 a	0.28 b
Biostimulant						
Control	6.22 b	1.14 b	0.18 b	243.6 b	0.28 b	0.21 b
Root application	7.15 a	1.59 ab	0.22 ab	308.7 ab	0.30 a	0.29 ab
Root ad leaf application	7.35 a	1.90 a	0.26 a	407.2 a	0.29 ab	0.37 a
Significance[a]						
Salinity (S)	*	*	ns	ns	ns	*
Biostimulant (B)	**	**	**	**	*	**
S x B	ns	ns	ns	ns	ns	ns

[a]ns, *, **, nonsignificant or significant at $P \leq 0.05$, and 0.01, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test ($P = 0.05$).

Table 2

Mean effects of biostimulant application and salinity level on SPAD index, maximum quantum use efficiency of PSII in dark-adapted state (F_v/F_m), and proline content in leaves of lettuce plants.

Treatments	SPAD	F_v/F_m	Proline (µg g^{-1} f. wt.)
Salinity (mM NaCl)			
1	30.65 a	0.82 a	12.1 b
25	26.37 b	0.54 b	26.7 a
Biostimulant			
Control	28.10 a	0.58 b	24.5 a
Root application	28.18 a	0.71 a	17.2 a
Root ad leaf application	29.26 a	0.75 a	16.5 a
Significance[a]			
Salinity (S)	***	***	***
Biostimulant (B)	ns	*	ns
S x B	ns	ns	ns

[a]ns, *, ***, nonsignificant or significant at $P \leq 0.05$, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test ($P = 0.05$).

Table 3

Mean effects of biostimulant application and salinity level on macronutrients and sodium in leaves of lettuce plants.

Treatments	Major elements (g kg^{-1})					
	N	P	K	Ca	Mg	Na
Salinity (mM NaCl)						
1	36.2 a	4.1 a	39.4 a	7.9 a	2.4 a	2.7 b
25	32.0 b	3.5 b	30.6 b	5.8 b	1.1 b	13.5 a
Biostimulant						
Control	32.0 b	3.4 b	34.0 a	6.7 a	1.6 a	8.8 a
Root application	35.7 a	3.8 ab	35.4 a	7.0 a	1.7 a	7.7 a
Root ad leaf application	34.6 a	4.1 a	35.6 a	7.1 a	2.0 a	7.9 a
Significance[a]						
Salinity (S)	*	*	***	***	***	***
Biostimulant (B)	**	**	ns	ns	ns	ns
S x B	*	ns	ns	ns	ns	ns

[a]ns, *, **, *** nonsignificant or significant at $P \leq 0.05$, 0.01 and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test ($P = 0.05$).

Table 4

Unsupervised hierarchical cluster analysis from metabolomics: compounds giving the most contribution to metabolomic differences between the biostimulant-treated and control leaves in salt-stressed lettuce plants.

CAFFEOYLQUINICS	**HORMONES**	**STEROLS**
phloretate	methyl-jasmonate	brassicasterol
coumaraldehyde	jasmonoyl-isoleucine	episterol
rosmarinic acid	indol-3-pyruvate	desmosterol
	N-(3-indolylacetyl)-2-isoleucine	sitosterol
FLAVONOIDS	1-deoxy-xylulose-5-phosphate	campest-4-en-3-one
taxifolin	gibberellin A24	
dihydrotricetin	gibberellin A98	**TERPENES**
sativan	isopentenyladenine-N-glucoside	beta-mircene
2'-hydroxydihydrodaidzein	salicylic acid	beta-pinene
tetrahydrochalcone	trans-zeatin-O-glucoside-7-N-glucoside	limonene
tetrahydroisoflavanone		terpinolene
pelargonidin-3-glucoside	**STRESS RELATED**	pinoresinol
hesperidin	selenomethyl-selenocysteine	matairesinol
delphinidin	2,4-dinitrophenyl-S-glutathione	geranate
	homoglutathione	dehydrobetadiene-diol
CARBOHYDRATES	4-hydroxynonenal glutathione conjugate	
maltotriose	4-hydroxynonenal	**GLUCOSINOLATES**
rhamnose	sulcatone	6-methylthiohexyl-glucosinolate
6-phosphogluconate		indolylmethyl-glucosinolate
3-phosphoglicerate	**OTHER**	methylthiobutyl-glucosinolate
UDP-xylose	L-Proline	6-methylthiohexyl-glucosinolate
UDP-arabinose	itaconate	7-methylsulfinylheptyl-glucosinolate
UDP-apiose	3-hydroxybutanoyl-CoA	4-methylthiobutyl hydroxymate
GDP-fucose	arabitol	3-(7'-methylthio)heptylmalate
raffinose	3-octaprenyl-4-hydroxybenzoate	hypoglycin b

Figure(s)

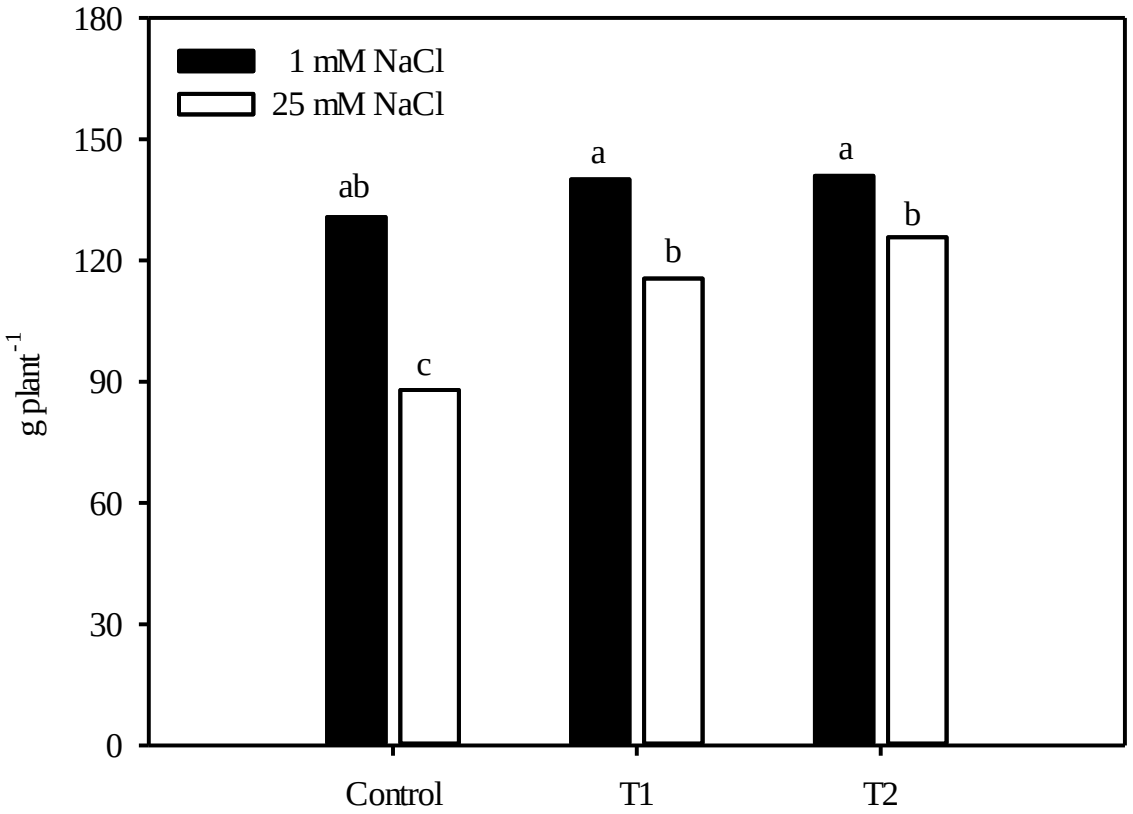

Fig. 1. Effect of biostimulant application to roots (T1) and to roots and leaves (T2) on shoot fresh weight of lettuce plants grown under two saline levels. Different letters indicate significant differences according to Duncan's test (P = 0.05). Values are the means of three replicate samples.

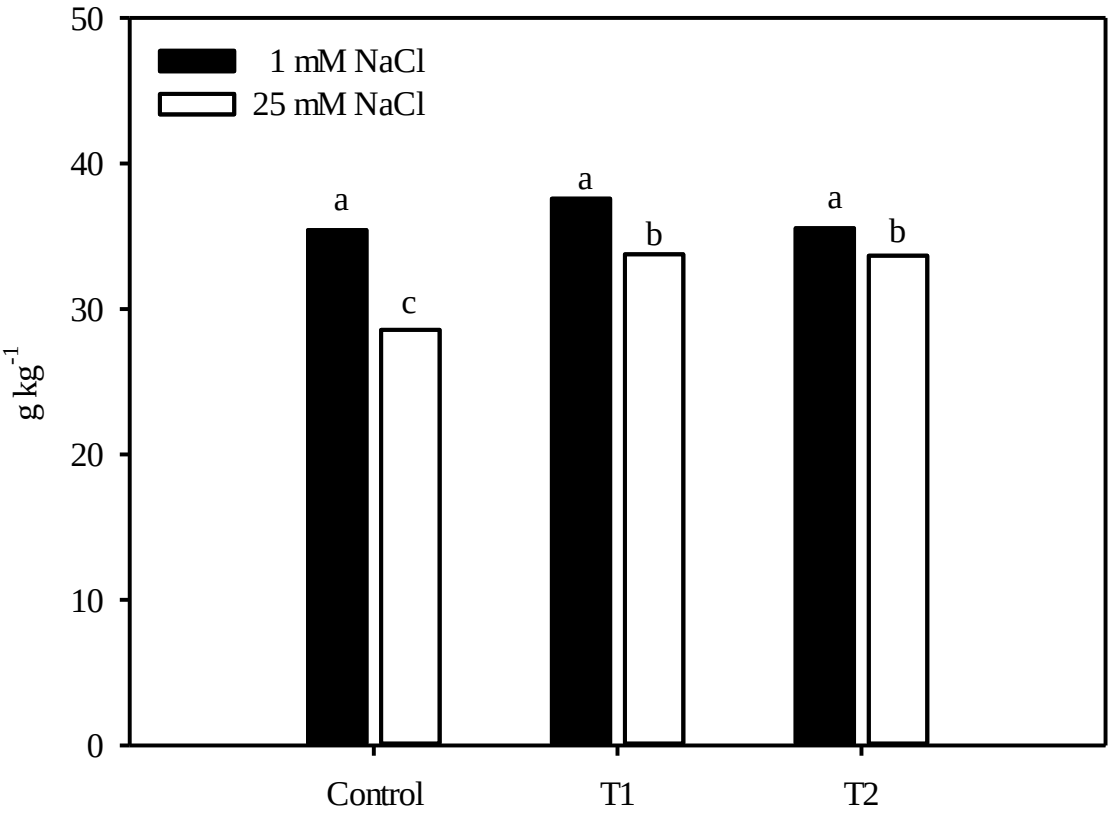

Fig. 2. Effect of biostimulant application to roots (T1) and to roots and leaves (T2) on leaf nitrogen content of lettuce plants grown under two saline levels. Different letters indicate significant differences according to Duncan's test (P = 0.05). Values are the means of three replicate samples.

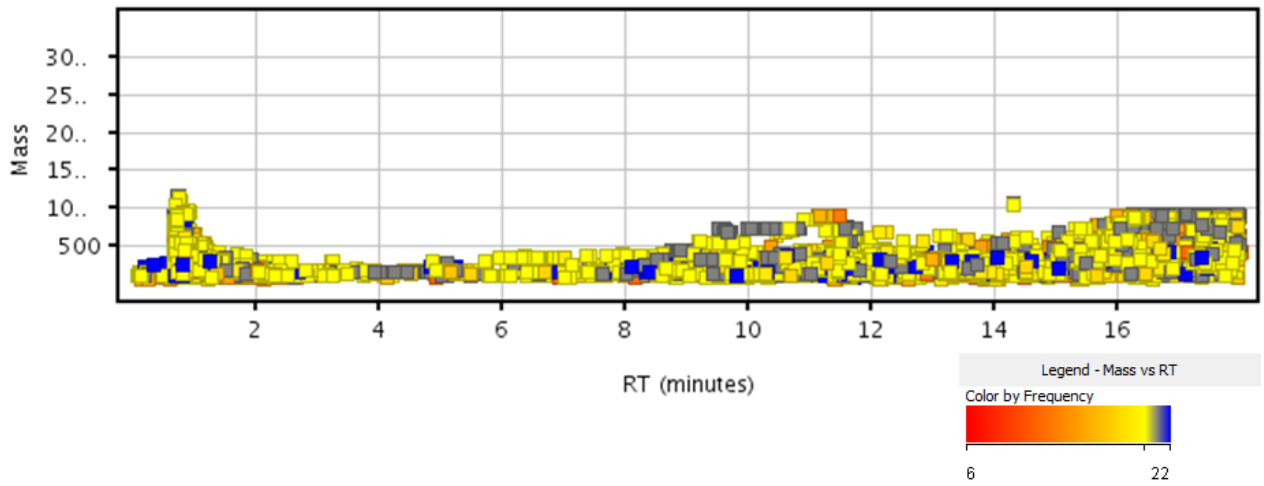

Fig. 3. Metabolomic analysis: compounds profile and frequency of detection for lettuce leaf samples.

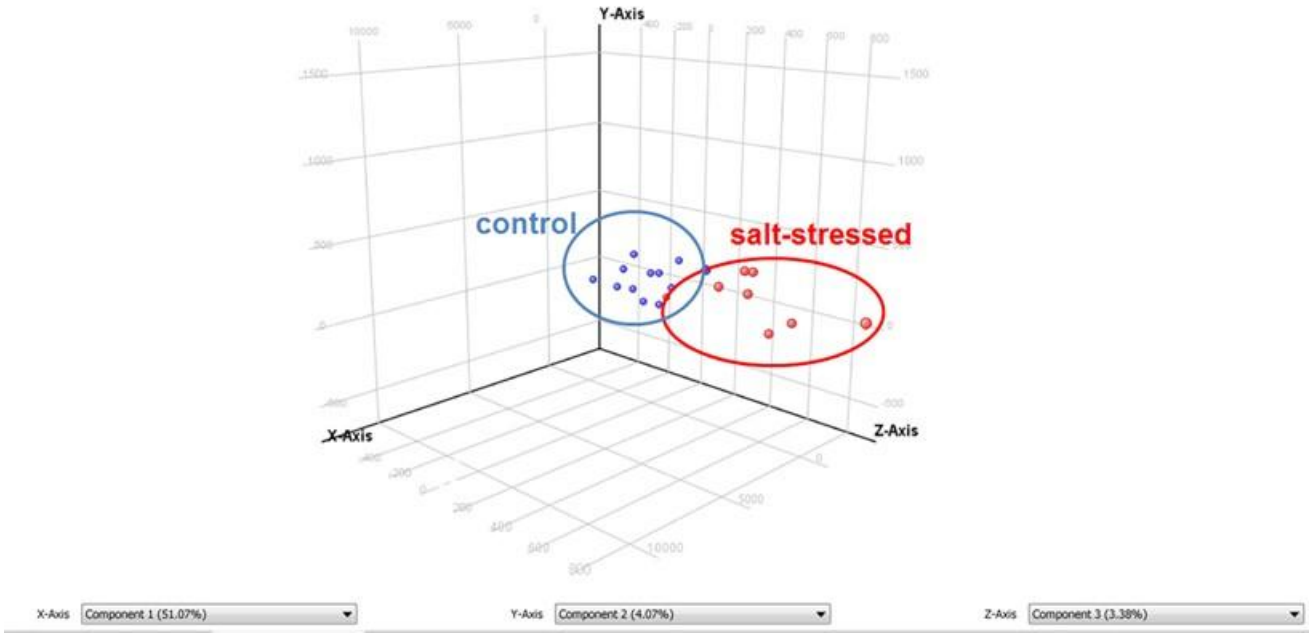

Fig. 4. Principal Component Analysis regarding leaf samples of lettuce plants grown either under normal (1 mM NaCl) or salt stress conditions (25 mM NaCl).

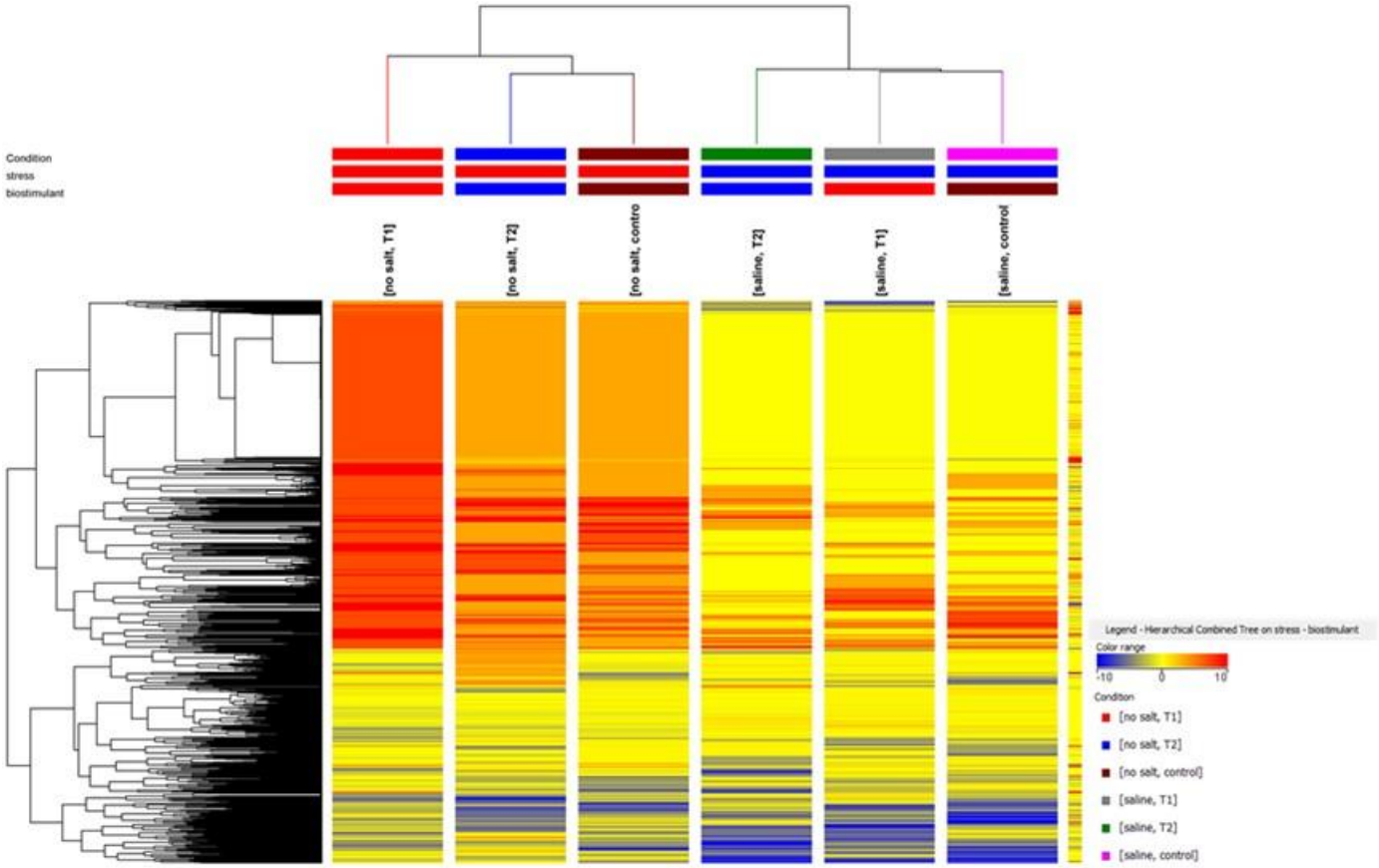

Fig. 5. Unsupervised hierarchical cluster analysis of leaf samples from lettuce plants grown under normal (1 mM NaCl) or saline conditions (25 mM NaCl), following root application (T1) or foliar and root application (T2) of biostimulant. Clustering was carried out on both conditions (treatments, vertical dendrogram) and compounds (metabolites, horizontal dendrogram).

Supplementary Material

Click here to download Supplementary Material: Supplementary figures - subclusters.pdf