

# Alfalfa plant-derived biostimulant stimulate short-term growth of salt stressed *Zea mays* L. plants

Andrea Ertani · Michela Schiavon ·  
Adele Muscolo · Serenella Nardi

Received: 29 March 2012 / Accepted: 25 June 2012 / Published online: 11 July 2012  
© Springer Science+Business Media B.V. 2012

## Abstract

**Background & Aims** The effects of an alfalfa plant (*Medicago sativa* L.) hydrolysate-based biostimulant (EM) containing triacontanol (TRIA) and indole-3-acetic acid (IAA) were tested in salt-stressed maize plants.

**Methods** Plants were grown for 2 weeks in the absence of NaCl or in the presence (25, 75 and 150 mM). On the 12th day, plants were supplied for 48 h with 1.0 mg L<sup>-1</sup> EM or 11.2 μM TRIA.

**Results** EM and TRIA stimulated the growth and nitrogen assimilation of control plants to a similar degree, while NaCl reduced plant growth, SPAD index and protein content. EM or TRIA increased plant

biomass under salinity conditions. Furthermore, EM induced the activity of enzymes functioning in nitrogen metabolism. The activity of antioxidant enzymes and the synthesis of phenolics were induced by salinity, but decreased after EM treatment. The enhancement of phenylalanine ammonia-lyase (PAL) activity and gene expression by EM was consistent with the increase of flavonoids.

**Conclusion** The present study proves that the EM increases plant biomass even when plants are grown under salinity conditions. This was likely because EM stimulated plant nitrogen metabolism and antioxidant systems. Therefore, EM may be proposed as bioactive product in agriculture to help plants overcome stress situations.

---

Responsible Editor: John McPherson Cheeseman.

**Electronic supplementary material** The online version of this article (doi:10.1007/s11104-012-1335-z) contains supplementary material, which is available to authorized users.

---

A. Ertani · M. Schiavon · S. Nardi (✉)  
Dipartimento di Agronomia Animali Alimenti Risorse  
Naturali e Ambiente, Università degli  
Studi di Padova, Agripolis,  
Viale dell'Università 16,  
35020 Legnaro, Padova, Italy  
e-mail: serenella.nardi@unipd.it

A. Muscolo  
Dipartimento di Gestione dei Sistemi Agrari e Forestali,  
Facoltà di Agraria Università "Mediterranea"  
di Reggio Calabria,  
Feo di Vito,  
89060 Reggio Calabria, Italy

**Keywords** Biostimulant · Salt stress · *Zea mays* L. ·  
Triacontanol · Indole-3-acetic acid · Phenylpropanoids

## Introduction

The salinization of land is a matter of concern for agricultural production and represents a major abiotic source of stress for plants in arid and semi-arid regions (Greenway and Munns 1980). Salts are common components of soils and some are indispensable plant nutrients (Marschner 1995). Sodium chloride (NaCl), in particular, is the main salt in saline environments (Viégas et al. 2001). Sodium (Na<sup>+</sup>) is required by plants that perform C4 photosynthesis (Brownell and

Crossland 1974; Ohnishi et al. 1990), and a number of halophytic plant species to survive in their natural habitat store high levels of this element in vacuoles and use it as osmoticum (Flowers and Colmer 2008; Shabala and MacKay 2011). Low  $\text{Na}^+$  concentrations can exert a beneficial effect on plant growth, especially in natrophilic species and under  $\text{K}^+$  limited conditions or moderate drought stress (Garcia-Sanchez et al. 2000). However, high levels of  $\text{NaCl}$  can be toxic for plants and cause reduction of water potential (Hernandez et al. 1999), ion imbalance (Parida et al. 2004) and stunted growth (Arshi et al. 2002; Zhao et al. 2010). Salinity can also alter the major metabolic processes of plants, like photosynthesis (Agastian et al. 2000), protein synthesis (Giridara et al. 2003), and nitrogen assimilation (Soussi et al. 1998; Flores et al. 2000).

A common response to salinity is the generation of reactive oxygen species (ROS) that trigger oxidative stress (Blokhina et al. 2003; De Azevedo Neto et al. 2005). Plants control the concentrations of ROS by an array of non-enzymatic and enzymatic antioxidants, these last including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and glutathione reductase (GR). SODs catalyze the dismutation of the superoxide anion ( $\text{O}_2^-$ ) to yield hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). The level of  $\text{H}_2\text{O}_2$  is further tightly regulated by the concerted activity of CAT and peroxidases (PODs) (Blokhina et al. 2003). The activity of antioxidant enzymes was shown to increase after application of exogenous proline to salt-stressed plants (Hoque et al. 2007). Proline is one of the most accumulated osmolytes in plants under salinity conditions (Ochoa-Alfaro et al. 2008; Ozden et al. 2009) and its synthesis has been reported to improve the adaptation of several plant species, such as maize, to excess  $\text{NaCl}$  by inducing the expression of salt-stress responsive proteins (Khedr et al. 2003; Carpci et al. 2010). Other plant species prefer to control  $\text{Na}^+$  uptake, as the most salt tolerant genotypes were found to accumulate less organic osmolytes, including proline (Lutts et al. 1999).

Plants may cope with salt stress also through stimulation of the secondary metabolism that leads to the synthesis of phenolic compounds (phenylpropanoids) (Ouyang et al. 2007). In plants of *Jatropha curcas* L. (Gao et al. 2008) and *Ilex oblonga* (Ouyang et al. 2007) salinity increased the activity of the phenylalanine (tyrosine) ammonia-lyase (PAL) enzyme, which catalyzes the first step in the biosynthesis of phenolics.

A recent study suggests that active molecules contained in biostimulant products can induce the pathway of phenylpropanoids (Schiavon et al. 2010). On this account, the use of biostimulants during agricultural practices may help plants to overcome stress situations, including salinity (de Vasconcelos et al. 2009). Basically, biostimulants function as positive growth regulators at low doses by enhancing plant nutrition and metabolism (Chen et al. 2003; Schiavon et al. 2008, 2010; Ertani et al. 2009, 2011; Khan et al. 2009). They are commercially available in different formulations and are generally classified into three major groups on the basis of their original matrix: humic substances, marine bioactive substances, and amino acid containing products (Kauffman et al. 2007). These last consist of oligo and polypeptides and free amino acids obtained through chemical and/or enzymatic hydrolysis of organic matrix from plant (alfalfa) or animal sources (Cavani et al. 2006).

Among the amino acid containing products, a alfalfa plant hydrolysate-based biostimulant (EM), previously characterized and with proven biological activity (Schiavon et al. 2008), was used in this study to test its capacity of ameliorating the negative effects of salt stress in maize. The contents of triacontanol (TRIA) and indole-3-acetic acid (IAA) were determined in the biostimulant, as both compounds are abundant in fabaceae (Ries and Violet 1977; Kumaravelu et al. 2000) and are known to act as plant growth regulators (Chen et al. 2002). TRIA, in particular, has been shown to ameliorate salt-stress damages in some plant species (Çavuşoğlu et al. 2008).

The present study was aimed at verifying if the addition of EM to maize plants grown under salinity conditions could help plants to maintain their performance. For this purpose, a number of parameters associated with crop productivity were evaluated, as well as the activity of enzymatic and non-enzymatic antioxidants.

## Materials and methods

### Characterization of the biostimulant

The protein hydrolysate (EM) tested in this study was produced by a fully controlled enzymatic hydrolysis by ILSA S.p.A. (Arzignano, VI, Italy) using vegetal material from alfa-alfa (*Medicago sativa* L.) plants.

The elemental composition, organic matter content and physical properties of EM have already been reported in Schiavon et al. (2008).

The concentration of triacontanol (TRIA) in EM, determined by mass spectrometry following the procedure of Ries and Violet (1977), was 11.2  $\mu\text{M}$ . The same concentration of triacontanol was used when plants were grown hydroponically in the presence of pure TRIA at different NaCl concentrations.

Indole-3-acetic acid (IAA) in EM was quantified by using an enzyme linked immuno-sorbent assay (ELISA) standardized with methylated IAA (Phytodetek-IAA, Sigma, St. Louis, MO, USA). The ELISA test utilized a monoclonal antibody to IAA and was sensitive in the range of 0.05–100 picomoles. The tracer and standard solutions were prepared following the manufacturer's instructions, and the absorbance values were read at  $\lambda=405$  nm with a Biorad micro-plate reader (Hercules, CA, USA). The IAA content in EM was 18.46 nmol mg  $\text{C}^{-1}$ .

#### Plant material and growth conditions

Seeds of *Zea mays* L. (var. DKc 6286, DeKalb, Italy) were soaked in distilled water overnight and then surface-sterilized in 5 % (v/v) sodium hypochlorite for 10 min, while shaking. Seeds were left to germinate for 60 h in the dark, at 25 °C, on a filter paper wetted with 1 mM  $\text{CaSO}_4$  (Nardi et al. 2000). Germinated seedlings were transplanted into 3 L pots containing an aerated complete culture solution, at a density of 24 plants per pot. The nutrient solution was renewed every 48 h and had the following composition ( $\mu\text{M}$ ):  $\text{KH}_2\text{PO}_4$  (40),  $\text{Ca}(\text{NO}_3)_2$  (200),  $\text{KNO}_3$  (200),  $\text{MgSO}_4$  (200),  $\text{FeNaEDTA}$  (10),  $\text{H}_3\text{BO}_3$  (4.6),  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$  (0.036),  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  (0.9),  $\text{ZnCl}_2$  (0.09),  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$  (0.01). Sodium chloride (NaCl) was added to the nutrient solution at different concentrations: 0 (control), 25, 75 and 150 mM. Plants were cultivated for 14 days inside a climate chamber with a 14 h light/10 h dark cycle, air temperature of 27/21 °C, relative humidity of 70/85 % and a photon flux density of 280 mol  $\text{m}^{-2} \text{s}^{-1}$ . Twelve days after transplanting, a subsample of control and NaCl treated plants was supplied with either 1.0 mg  $\text{L}^{-1}$  EM or 11.2  $\mu\text{M}$  TRIA for 48 h. EM and TRIA were directly added to the complete culture solution.

Plants were randomly harvested from three pots of each treatment, carefully washed and dried with

blotting paper. A sub-sample of plant material was immediately frozen with liquid nitrogen and kept at  $-80$  °C for physiological and molecular analyses. For fresh weight measurement, thirty plants of each treatment were used (ten per pot). Plants were divided into roots and leaves, and weighed separately.

#### SPAD index

The measurement of the SPAD index was performed using the SPAD-502 Leaf Chlorophyll Meter (Minolta Camera Co., Ltd., Osaka, Japan) on the last expanded leaf of maize plants.

#### Elemental analysis

Quantification of  $\text{K}^+$  and  $\text{Na}^+$  in roots and all leaves of maize plants was obtained after an acid digestion procedure using a microwave (Milestone Ethos model 1600, Milestone, Shelton, CT).

All digestion reactions were carried out in closed Teflon vessels of 120 mL volume using 500 mg plant material and 10 mL of 30 % (v/v) HCl as a solvent. Digested samples were then diluted in 10 mL ultrapure water and assayed via Inductively Coupled Plasma Atomic Emission Spectroscopy (Spectrum CirosCCD, Kleve, Germany).

#### Proline determination

The proline content was quantified according to Ozden et al. (2009). Leaf tissues (0.3 mg) were homogenized with 3 % (w/w) sulphosalicylic acid. The extracts were centrifuged at 10,000 g for 10 min at 4 °C and aliquots of the supernatant (2 mL) had a 3 % (v/v) acid-ninhydrin solution added. Samples were incubated at 100 °C for 45 min, followed by cooling for 5 min to stop the reaction. Toluene (5 mL) was added and they were vortexed. Samples were then maintained for 15 min in the dark at room temperature to allow the separation of the toluene-containing phase from the aqueous phase. The toluene phase was collected and the absorbance was measured at  $\lambda=520$  nm. Proline concentration was expressed as  $\mu\text{g}$  proline  $\text{g}^{-1}$  fresh weight.

#### Protein extraction

For the extraction of soluble proteins, frozen foliar and root tissues (500 mg) were ground in liquid nitrogen,

vortexed with 5 mL extraction buffer (100 mM Tris HCl pH 7.5, 1 mM Na<sub>2</sub>EDTA, 5 mM DTT), and centrifuged at 14,000 g for 15 min. The supernatants were mixed with 10 % (w/v) trichloroacetic acid and centrifuged. The pellets obtained were re-suspended in 0.1 N NaOH. The protein concentration was analyzed at  $\lambda=595$  nm according to Bradford (1984) using a UV/VIS spectrophotometer (Lambda 1, Perkin-Elmer, Monza, Italy) and expressed in mg protein g<sup>-1</sup> fresh weight.

#### Enzyme extraction and assay conditions

For the extraction of glutamine synthetase (GS, EC 6.3.1.2) and glutamate synthase (GOGAT, EC 1.4.7.1) fresh leaf tissue (1 g) was manually crushed in a mortar with a solution added containing 100 mM Hepes-NaOH pH 7.5, 5 mM MgCl<sub>2</sub> and 1 mM dithiothreitol. The ratio of plant material to mixture solution was 1:3 (w/v). The enzyme extracts were filtered through two layers of muslin and clarified by centrifugation at 20,000 g for 15 min.

For the assay of GS, the extract was added to a mixture containing 90 mM imidazole-HCl (pH 7.0), 60 mM hydroxylamine (neutralized), 20 mM Na<sub>2</sub>HSO<sub>4</sub>, 3 mM MnCl<sub>2</sub>, 0.4 mM ADP, 120 mM glutamine. The assay was performed in a final volume of 750  $\mu$ L. The enzymatic reaction was developed for 15 min at 37 °C. The  $\alpha$ -glutamyl hydroxamate was determined colorimetrically by adding a mixture (250 mL) of (1:1:1) 10 % (w/v) FeCl<sub>3</sub> 6H<sub>2</sub>O in 0.2 M HCl, 24 % (w/v) trichloroacetic acid and 50 % (w/v) HCl. The optical density was recorded at  $\lambda=540$  nm (Canovas et al. 1991).

The glutamate synthase assay contained 25 mM HEPES-NaOH (pH 7.5), 2 mM glutamine, 1 mM  $\alpha$ -ketoglutaric acid, 0.1 mM NADH, 1 mM Na<sub>2</sub>EDTA, and 100 mL of enzyme extract. GOGAT was assayed spectrophotometrically by monitoring NADH oxidation at  $\lambda=340$  nm (Avila et al. 1987).

The catalase (CAT; EC 1.11.1.6) activity was measured by the method of Chance and Maehly (1955). The extraction mixture contained 50 mM phosphate buffer (pH 7.0), 20 mM Polivinilpirrolidone (PVP), 250  $\mu$ L Triton X-100. The reaction mixture contained 64 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM H<sub>2</sub>O<sub>2</sub> and 50  $\mu$ L of enzyme extract. The reaction was initiated by adding the enzyme extract. CAT activity was determined by following the consumption of H<sub>2</sub>O<sub>2</sub> (extinction coefficient 39.4 mM cm<sup>-1</sup>) at  $\lambda=240$  nm over a 2 min interval.

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined by following the decrease of ascorbate (extinction coefficient 2.8 mM cm<sup>-1</sup>) and measuring the change in absorbance at  $\lambda=290$  nm over a 2 min interval. The extraction mixture contained 62.5 mM KH<sub>2</sub>PO<sub>4</sub>. The reaction mixture contained 50 mM K-phosphate buffer (pH 7.0), 1 mM EDTA-Na<sub>2</sub>, 0.5 mM ascorbic acid, 0.1 mM H<sub>2</sub>O<sub>2</sub> and 50  $\mu$ L of crude enzyme extract (Nakano and Asada 1981).

Guaiacol peroxidase (GPX; EC 1.11.1.7) activity was determined by measuring the oxidation of guaiacol in the presence of H<sub>2</sub>O<sub>2</sub> (extinction coefficient, 26.6 mM cm<sup>-1</sup>) at  $\lambda=470$  nm over a 2 min interval. The extraction mixture contained 62.5 mM KH<sub>2</sub>PO<sub>4</sub> and the reaction mixture contained 0.05 mL of guaiacol (20 mM), 2.9 mL of K-phosphate buffer (10 mM, pH 7.0) and 50  $\mu$ L of enzyme. The reaction was initiated by adding 2 mL of 40 mM H<sub>2</sub>O<sub>2</sub> to the mixture (Osswald et al. 1992).

For the phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) assay, fresh leaf tissues (1 g) were homogenized for 15 min on ice with a glass homogenizer using 0.10 g polyvinylpyrrolidone (PVP) and 5 mL of 100 mM potassium-phosphate buffer (pH 8.0) containing 1.4 mM 2-mercaptoethanol. The homogenate was centrifuged at 15,000 g for 15 min at 4 °C. The supernatant was chromatographed using a Sephadex<sup>®</sup> G-25 (GE Healthcare UK, Buckinghamshire, England) containing column equilibrated with the buffer described above. The eluate was the enzyme extract used for the assay. A mixture of 0.4 mL of 100 mM Tris-HCl buffer (pH 8.8), 0.2 mL of 40 mM phenylalanine, and 0.2 mL of enzyme extract was incubated for 30 min at 37 °C and stopped with 0.2 mL 25 % (v/v) TCA (Mori et al. 2001). After centrifuging at 10,000 g for 15 min at 4 °C, the absorbance of the supernatant was measured at  $\lambda=280$  nm relative to the control. PAL activity was expressed as nmol cinnamic acid mg<sup>-1</sup> protein min<sup>-1</sup>.

The enzyme activity of all the enzyme extracts was calculated relatively to the protein concentration measured according to the Bradford method (1984).

#### Extraction and measurement of soluble phenols and flavonoids

Soluble phenolic acids were extracted by crushing 1 g of fresh leaves in a mortar with pure methanol (1:3, w/v). The extracts were maintained in an ice bath for 30 min and centrifuged at 5,000 g for 30 min at 4 °C. The supernatants

were stored at 20 °C until use. Total phenols were measured according to Arnaldos et al. (2001). One mL of 2 % Na<sub>2</sub>CO<sub>3</sub> and 75 µL of Folin-Ciocalteu reagent (Sigma-Aldrich) were added to 100 mL of phenolic extract. After 15 min of incubation at 25 °C in the dark, the absorbance at  $\lambda=725$  nm was measured. Gallic acid was used as standard according to Meenakshi et al. (2009). Flavonoids were extracted from 1 g of fresh leaf tissue in 50 mL of acidified methanol solution. The extracts were kept at 4 °C for 16 h and the absorbance was then measured at  $\lambda=300$  nm. Flavonoids were expressed as gallic acid equivalents (Meenakshi et al. 2009).

### Semi-quantitative RT-PCR

RNA isolation was performed in leaves of *Z. mays* plants using the Nucleon PhytoPure kit (GE Healthcare UK, Buckinghamshire, England) according to the protocol provided by the manufacturer. First-strand cDNA was synthesized from 5 µg of RNA, after Dnase treatment with Dnase RQ1 (Promega, Milan, Italy), using 200 U of ImProm-II™ Reverse Transcriptase (Promega, Milan, Italy) and oligodT as primers in 20 µL reactions (Schiavon et al. 2008).

RT-PCR experiments were performed with specific primers (forward: 5'-CGCATCAACACCCTCCTC-3'; reverse: 5'-GATGTAGGAGAGCGGGACCA-3') to evaluate the gene expression level of PAL (*ZmPAL1*: GenBank Accession number L77912). For all PCR reactions, 1 µl of the cDNA was used in a 20 µl reaction mixture with 2 µl of a 0.025 U/µl Taq-polymerase (Amersham-Pharmacia-Biotech, Piscataway, NJ, USA). Each PCR cycle consisted of: 3 min initial denaturation at 95 °C, 30 s denaturation at 95 °C, 30 s annealing at 61 °C, 30 s extension at 72 °C, 7 min final extension at 72 °C. The constitutively expressed *Z. mays* actin gene (J0128) was used as the internal control to normalize the obtained gene expression results (primers: forward, 5'-TGTTTCGCCTGAAGATCACCTGTG-3'; reverse, 5'-TGAACCTTTCTGACCCAATGGTGATGA-3'). For *ZmPAL1* and *Zmact* the number of cycles were 24 and 20, respectively.

The RT-PCR analysis was performed using the Gen Amp PCR system 9700 (PE Biosystems, Branchburg, NJ, USA) and the DNA fragments were quantified through the ImageJ program (ImageJ 1.23 J, Wayne Rasband, National Institute of Health, Bethesda, MD, USA). To confirm the expression analysis results,

PCR reactions were carried out on cDNAs obtained from two different RNA extractions performed on roots of seedlings of two independent experiments, and were repeated at least 4 times for each cDNA.

### Statistical analysis

Data on SPAD index, proline, phenolics, proteins and enzyme activity represent the means of measurements from three different pots per treatment. Five plants were used ( $\pm$  *std*) for each measurement. Analysis of variance (ANOVA) was performed using the SPSS software, and was followed by pair-wise post-hoc analyses (Student-Newman-Keuls test) to determine which means differed significantly at  $p<0.05$ .

## Results

### Effect of NaCl, TRIA and EM on growth of *Zea mays* plants

Salinity significantly affected maize plant growth (Table 1). Leaves, in particular, were more sensitive to NaCl treatment than roots, and the maximum decrease of fresh weight was observed in plants treated with 150 mM NaCl (−49 % and −55 % for roots and leaves, respectively). On the opposite, the provision of either TRIA or EM to control plants improved the fresh weight of roots (+74 % and +77 %, respectively) and leaves (+13 % and +19 %, respectively). EM and TRIA also stimulated short-term growth of maize even in the presence of NaCl. Indeed, the root and leaf fresh weight of plants supplied NaCl was significantly increased by EM and TRIA, in most cases to a similar degree (Table 1 of Supplementary Material).

### Effect of NaCl, EM and TRIA on SPAD index and protein content

Salinity decreased the SPAD index values and the protein content in maize, especially when NaCl was supplied to plants at the highest concentration (150 mM) (Table 2). On the contrary, EM and TRIA significantly enhanced the SPAD index and the protein amount in control plants.

In salt-treated plants added with EM or TRIA, the SPAD index values were comparable to those recorded in control plants. Furthermore, the treatment

**Table 1** Root and leaf fresh weight of *Z. mays* plants grown for 14 days in a complete culture solution with 0 (control), 25, 75 and 150 mM NaCl. A sub-sample of 12 d-old plants was additionally treated for 48 h with 1 mg L<sup>-1</sup> EM or 11.2 μM TRIA. Data represent the means of three measurements per treatment with ten plants in each (± *std*). Different letters in the same column indicate significant differences between treatments ( $p < 0.05$ ) according to Student-Newman-Keuls test

Treatment	Roots		Leaves	
	Fresh weight (g)	%	Fresh weight (g)	%
Control	1.98±0.05b	(100)	4.66±0.12b	(100)
TRIA	3.45±0.01a	(174)	5.27±0.13a	(113)
EM	3.51±0.10a	(177)	5.54±0.16a	(119)
25 mM NaCl	1.41±0.13c	(71)	3.02±0.13d	(65)
25 mM NaCl + TRIA	1.95±0.02b	(98)	3.52±0.12c	(76)
25 mM NaCl + EM	1.87±0.08b	(94)	3.88±0.13c	(69)
75 mM NaCl	1.24±0.10 cd	(63)	2.59±0.15e	(56)
75 mM NaCl + TRIA	1.32±0.02 cd	(67)	3.31±0.11d	(71)
75 mM NaCl + EM	1.84±0.13b	(93)	3.15±0.24 cd	(68)
150 mM NaCl	1.01±0.10d	(51)	2.10±0.12f	(45)
150 mM NaCl + TRIA	1.62±0.09c	(82)	2.87±0.14e	(62)
150 mM NaCl + EM	1.59±0.11c	(80)	2.66±0.17e	(57)

with EM or TRIA increased the content of proteins in plants exposed to 25 or 75 mM NaCl to values higher than those measured in control plants. In plants grown in the presence of 150 mM NaCl and supplied with either EM or TRIA, the content of proteins was slightly lower compared to control plants.

#### Effect of NaCl, EM and TRIA on K<sup>+</sup> and Na<sup>+</sup> concentrations

The concentration of K<sup>+</sup> in control plants was higher than in NaCl-treated plants, while that of Na<sup>+</sup> was extremely

low (Table 3). The treatment with EM or TRIA enhanced the level of K<sup>+</sup> in leaves of control plants, while no effect on Na<sup>+</sup> concentration was evident. In plants supplied with NaCl, Na<sup>+</sup> was significantly accumulated in roots and leaves, while the concentration of K<sup>+</sup> and K<sup>+</sup>/Na<sup>+</sup> ratio decreased. The rates of increase in Na<sup>+</sup> content were higher in leaves than in roots, whereas K<sup>+</sup> accumulation was more reduced in roots.

The application of EM or TRIA to salt-stressed plants determined a reduction of Na<sup>+</sup> in roots and leaves and, on the other hand, an increase of K<sup>+</sup> concentration. However, EM and TRIA were not able to restore the

**Table 2** SPAD index and protein content in roots and leaves of *Z. mays* plants grown for 14 days in a complete culture solution with 0 (control), 25, 75 and 150 mM NaCl. A sub-sample of 12 d-old plants was additionally treated for 48 h with 1 mg L<sup>-1</sup> EM or 11.2 μM TRIA. Data represent the means of five measurements per treatment with three plants in each (± *std*). Different letters in the same column indicate significant differences between treatments ( $p < 0.05$ ) according to Student-Newman-Keuls test

Treatment	SPAD index	Protein	
		Root mg (g fresh weight) <sup>-1</sup>	Leaf
Control	32.66±0.13b	1.01±0.02b	2.90±0.06c
EM	37.33±0.10a	1.15±0.03a	3.40±0.11a
TRIA	35.11±0.11a	1.20±0.01a	3.54±0.01a
25 mM NaCl	29.54±0.07c	1.00±0.03b	2.72±0.12 cd
25 mM NaCl + TRIA	30.95±0.05bc	1.20±0.02a	3.04±0.11b
25 mM NaCl + EM	30.93±0.09bc	1.16±0.11ab	3.22±0.07b
75 mM NaCl	29.01±0.07c	0.82±0.03c	2.19±0.11e
75 mM NaCl + TRIA	32.28±0.03b	0.99±0.01b	3.10±0.06b
75 mM NaCl + EM	31.36±0.14b	1.02±0.10b	3.16±0.02b
150 mM NaCl	28.14±0.12d	0.79±0.03c	1.83±0.14f
150 mM NaCl + TRIA	31.11±0.02b	0.95±0.11b	2.60±0.11d
150 mM NaCl + EM	30.15±0.19bc	0.98±0.02b	2.72±0.13cd

**Table 3** K<sup>+</sup>, Na<sup>+</sup> concentration and K<sup>+</sup>/Na<sup>+</sup> in roots and leaves of *Z. mays* plants grown for 14 days in a complete culture solution with 0 (control), 25, 75 and 150 mM NaCl. A subsample of 12 d-old plants was additionally treated for 48 h 1 mg L<sup>-1</sup> EM or 11.2 μM TRIA. Data of K<sup>+</sup> and Na<sup>+</sup>

concentrations are expressed as percent of dry matter. All data represent the means of five measurements per treatment with three plants in each ( $\pm$  *std*). Different letters in the same column indicate significant differences between treatments ( $p < 0.05$ ) according to Student-Newman-Keuls test

Treatment	Roots			Leaf		
	K <sup>+</sup> (%)	Na <sup>+</sup> (%)	K <sup>+</sup> /Na <sup>+</sup>	K <sup>+</sup> (%)	Na <sup>+</sup> (%)	K <sup>+</sup> /Na <sup>+</sup>
Control	1.238±0.011a	0.408±0.090e	3.03±0.141a	2.501±0.051c	0.040±0.001 g	62.52±3.210c
TRIA	1.130±0.072a	0.472±0.081 g	2.11±0.10b2	3.722±0.030ab	0.048±0.005 g	77.54±2.181b
EM	1.120±0.090a	0.488±0.080 g	2.29±0.060b	4.203±0.062a	0.051±0.004 g	82.41±2.810a
25 mM NaCl	0.915±0.008c	0.972±0.015c	0.94±0.044e	2.320±0.150c	1.102±0.070e	0.210±0.012 h
25 mM NaCl + TRIA	0.982±0.017b	0.513±0.034f	1.91±0.012c	3.302±0.210b	0.914±0.070f	3.61±0.018d
25 mM NaCl + EM	0.990±0.014b	0.502±0.021f	1.97±0.011c	3.312±0.121b	0.921±0.038f	3.58±0.022d
75 mM NaCl	0.751±0.011e	1.120±0.008b	0.67±0.023 g	2.002±0.110 cd	2.652±0.034c	0.754±0.022f
75 mM NaCl + TRIA	0.765±0.016de	0.872±0.056d	0.87±0.020f	2.340±0.100c	2.041±0.070d	1.14±0.072e
75 mM NaCl + EM	0.789±0.020d	0.723±0.090d	1.09±0.011d	2.357±0.101c	2.018±0.143d	1.16±0.033e
150 mM NaCl	0.432±0.060f	1.209±0.045a	0.35±0.012i	1.791±0.122d	3.702±0.121a	0.483±0.021 g
150 mM NaCl + TRIA	0.480±0.022f	0.891±0.052d	0.53±0.015 h	1.918±0.131d	2.987±0.132b	0.64±0.042f
150 mM NaCl + EM	0.495±0.024f	0.794±0.034d	0.62±0.030 g	1.926±0.091d	2.970±0.109b	0.64±0.021 g

values of K<sup>+</sup> and Na<sup>+</sup> concentrations in roots. The only exception was observed at 25 mM NaCl, as the level of Na<sup>+</sup> was similar between control and salt-treated plants added with EM. In leaves of salt-treated plants, a reduction of Na<sup>+</sup> ranging from 17 % to 23 % was observed after the treatment with EM or TRIA, but the levels of this element remained still much higher than those measured in the control plants. On the contrary, the concentration of K<sup>+</sup> was comparable between control and salt-treated plants at 25 mM NaCl, and significantly increased after EM or TRIA addition. At 75 mM NaCl concentration, both EM and TRIA restored the concentration of K<sup>+</sup>, while at 150 mM NaCl the effect of EM or TRIA was not significant.

#### Effect of NaCl and EM on GS and GOGAT activities

The activity of glutamine synthetase (GS) and glutamate synthase (GOGAT) was evaluated in foliar tissues as the nitrogen metabolism in maize plants takes place mainly in leaves (Table 4). The activity of both enzymes decreased in NaCl-treated plants compared to the control. The reduction was more pronounced for GS than for GOGAT activity, especially when plants were grown in the presence of 75 mM or 150 mM NaCl.

The supply of EM to control and NaCl-treated maize plants stimulated the activity of both GS and GOGAT in some respects. Specifically, the biostimulant restored the activity of GOGAT in plants supplied with 25 and 150 mM NaCl, as well GS activity in plants exposed to 75 and 150 mM NaCl. The activity

**Table 4** Glutamine synthetase (GS), glutamate synthase (GOGAT) activities in leaves of *Z. mays* grown for 14 days in a complete culture solution with 0 (control), 25, 75 and 150 mM NaCl. A subsample of 12 d-old plants was additionally treated for 48 h with EM at 1 mg L<sup>-1</sup>. Data represent the means of five measurements per treatment with three plants in each ( $\pm$  *std*). Different letters in the same column indicate significant differences between treatments ( $p < 0.05$ ) according to Student-Newman-Keuls test

Treatment	GS (μmol <sup>-1</sup> g <sup>-1</sup> fw)	GOGAT
Control	4.27±0.12b	14.46±0.32c
EM	6.11±0.32a	16.82±0.18a
25 mM NaCl	3.13±0.10c	12.50±0.11d
25 mM NaCl + EM	5.50±0.17a	14.90±0.23c
75 mM NaCl	3.81±0.11c	11.90±0.20e
75 mM NaCl + EM	5.00±0.28b	15.52±0.17b
150 mM NaCl	3.72±0.11c	12.57±0.35d
150 mM NaCl + EM	4.75±0.30b	14.78±0.33c

of GOGAT was higher (+ 28 %) in salt-treated plants cultivated with EM than in the control at 75 mM NaCl. EM enhanced at a minor extent the GS activity in plants exposed to 25 mM NaCl.

#### Effect of NaCl and EM on antioxidant enzyme activity

Salinity stimulated the activity of antioxidant enzymes, mainly in plants supplied with 150 mM NaCl (Table 5). At this salt concentration, the enzymes were induced to a similar degree in roots (roughly +27 %), but CAT and APX activities were enhanced more than GPX activity in foliar tissues. The maximum increment percentage of enzyme activity was observed for APX in leaves (+ 71 %).

EM reduced the activity of CAT, APX and GPX in NaCl-treated plants to values often comparable to those determined in the control, especially when plants were grown in the presence of 25 mM or 75 mM NaCl. However, the EM treatment had no effect on the activity of antioxidant enzymes in plants cultivated without NaCl.

#### Effect of NaCl and EM on proline, phenol compounds and PAL enzyme activity

The content of free proline increased in maize leaves after plant exposure to NaCl (Table 6). The supply of EM to plants added with 150 mM NaCl caused a further significant accumulation of this osmolyte,

**Table 5** Catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX) activities, in roots and leaves of *Z. mays* plants grown for 14 days in a complete culture solution with 0 (control), 25, 75 and 150 mM NaCl. A sub-sample of 12 d-old plants was additionally treated for 48 h with EM at

while in control plants the content of proline did not vary after EM treatment.

Salt-treated plants accumulated more phenols and flavonoids than control plants (Table 6). The application of EM to plants exposed to NaCl caused a more remarkable enhancement of flavonoid amount, but decreased the level of phenols to values comparable to the ones measured in the control. In plants grown without NaCl, the addition of EM reduced the content of phenols, but it did not affect the level of flavonoids.

The activity of the phenylalanine ammonia-lyase (PAL) enzyme significantly increased after NaCl application to maize plants (Table 6). This increase was more pronounced when EM was added to NaCl-treated plants, while no effect on PAL activity was observed in control plants supplied with the biostimulant.

#### Effects of NaCl, EM and TRIA on *ZmPAL1* gene expression

Salinity influenced the PAL enzyme also at the level of gene expression as the *ZmPAL1* transcript was up-regulated in plants treated with 150 mM NaCl (Fig. 1). The supply of EM did not cause significant variation of *ZmPAL1* transcript accumulation in control plants, but strongly induced the accumulation of *ZmPAL1* transcripts in plants grown with NaCl. In contrast to EM, TRIA had no significant effect on *ZmPAL1* transcript abundance in both control and salt-treated plants (Fig. 2).

1 mg L<sup>-1</sup>. Data represent the means of five measurements per treatment with three plants in each ( $\pm$  *std*). Different letters in the same column indicate significant differences between treatments ( $p < 0.05$ ) according to Student-Newman-Keuls test

Treatment	CAT		APX		GPX	
	(μmol H <sub>2</sub> O <sub>2</sub> min <sup>-1</sup> mg <sup>-1</sup> protein)					
	Root	Leaf	Root	Leaf	Root	Leaf
Control	201.22±10.10c	232.13±6.71d	15.01±0.15c	5.26±0.22d	1022.89±30.11c	12.02±0.12c
EM	202.78±6.02c	221.53±7.99d	14.23±0.16c	5.12±0.54d	1022.63±31.20c	11.01±0.26c
25 mM NaCl	218.55±6.15b	260.33±5.15c	16.94±0.25b	6.41±0.29c	1185.36±22.12b	12.33±0.34b
25 mM NaCl + EM	193.08±11.03c	225.82±7.85b	15.32±0.18c	5.32±0.34d	1034.26±26.22c	11.58±0.33c
75 mM NaCl	228.16±4.13b	283.54±6.24d	17.98±0.12ab	7.06±0.32b	1289.86±62.09a	12.93±0.32ab
75 mM NaCl + EM	210.05±7.16c	256.38±8.32c	15.57±0.17b	5.39±0.18d	1043.61±23.00c	12.73±0.48c
150 mM NaCl	256.12±3.12a	315.23±10.06a	19.07±0.24a	9.01±0.63a	1291.16±48.05a	13.77±0.52a
150 mM NaCl + EM	228.42±5.17b	275.55±7.82b	17.36±0.16b	6.52±0.33e	1092.79±20.35a	13.12±0.38c



**Table 6** Effect of EM treatment on leaf proline, phenol, flavonoid content and phenylalanine ammonia-lyase (PAL) activity of *Z. mays* plants grown for 14 days in a complete culture solution with 0 (control), 25, 75 and 150 mM NaCl. A subsample of 12 d-old plants was additionally treated for 48 h with

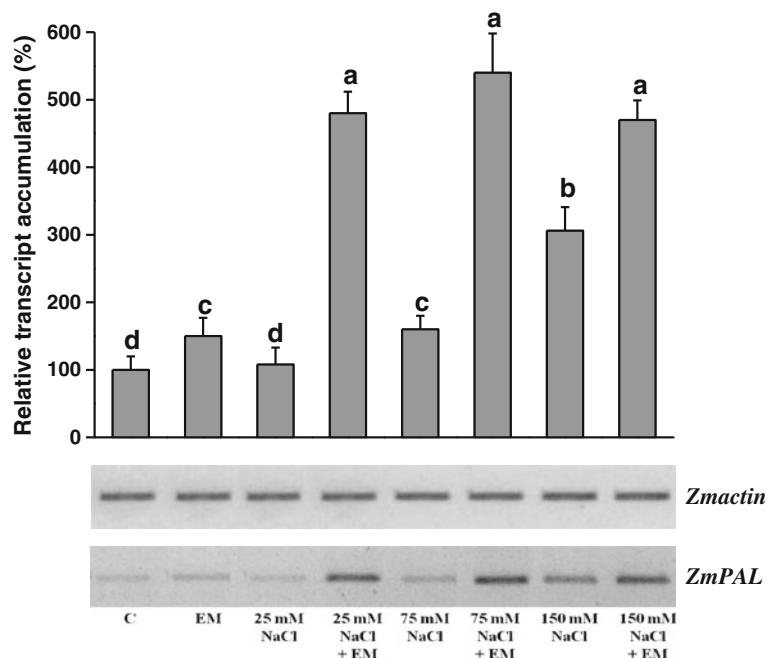
EM at 1 mg L<sup>-1</sup>. Data represent the means of five measurements per treatment with three plants in each ( $\pm$  *std*). Different letters in the same column indicate significant differences between treatments ( $p < 0.05$ ) according to Student-Newman-Keuls test

Treatment	Proline $\mu\text{g g}^{-1}$ f. wt.	Phenols (mg gallic acid g <sup>-1</sup> f.wt)	Flavonoids	PAL (nmol cinn acid mg <sup>-1</sup> prot min <sup>-1</sup> )
Control	315.01 $\pm$ 23.04 c	1.020 $\pm$ 0.06 e	0.084 $\pm$ 0.002 e	3.94 $\pm$ 0.31 f
EM	310.12 $\pm$ 34.11 c	0.820 $\pm$ 0.02 f	0.075 $\pm$ 0.013 e	3.04 $\pm$ 0.29 f
25 mM NaCl	377.03 $\pm$ 24.09 b	2.080 $\pm$ 0.12 c	0.171 $\pm$ 0.005 d	8.03 $\pm$ 0.20 e
25 mM NaCl + EM	422.16 $\pm$ 23.12 b	1.101 $\pm$ 0.22d	0.275 $\pm$ 0.011 c	12.92 $\pm$ 0.15 c
75 mM NaCl	388.18 $\pm$ 34.23 b	3.110 $\pm$ 0.14 b	0.263 $\pm$ 0.020 cd	12.34 $\pm$ 0.09 d
75 mM NaCl + EM	392.33 $\pm$ 19.05 b	1.122 $\pm$ 0.40 d	0.326 $\pm$ 0.031b	15.27 $\pm$ 0.22 b
150 mM NaCl	410.22 $\pm$ 32.13 b	4.152 $\pm$ 0.24 a	0.270 $\pm$ 0.021 c	12.69 $\pm$ 0.18 d
150 mM NaCl + EM	492.20 $\pm$ 26.16 a	1.138 $\pm$ 0.22 d	0.372 $\pm$ 0.022 a	17.47 $\pm$ 0.33a

## Discussion

Plants are frequently subjected to adverse environmental conditions, such as salinity. To date different strategies have been employed to maximize plant growth under saline conditions. One of them consists in generating salt tolerant genotypes of different crop species. This strategy,

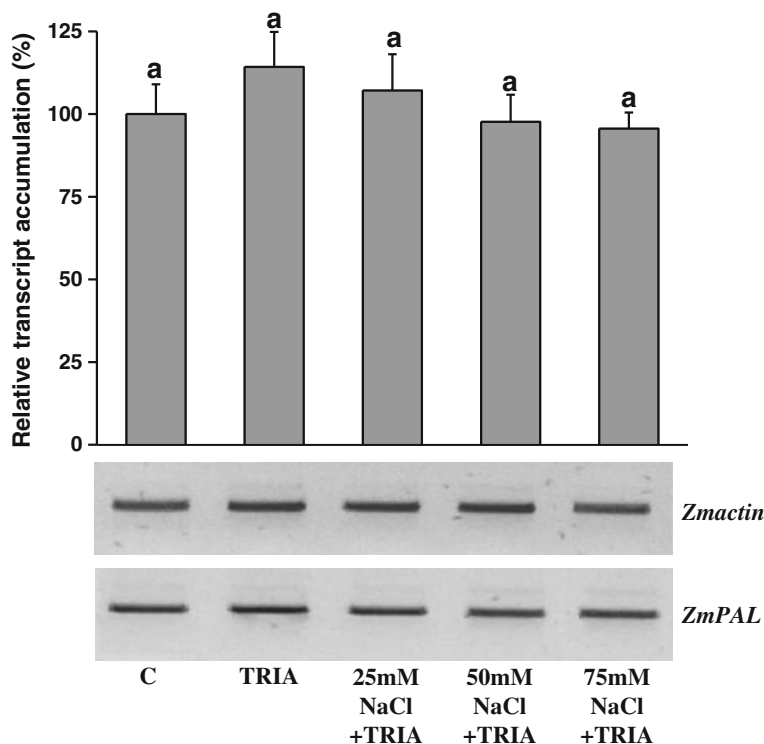
however, comprises conventional breeding methods that are often time consuming, difficult and rely on genetic variability (Javid et al. 2011). Alternatively, the exogenous application of plant growth regulators, as well the proper management of biostimulants that contain some of them, can be exploited to help plants to overcome salt stress.



**Fig. 1** Relative transcript accumulation of the gene encoding phenylalanine ammonia-lyase (*ZmPAL*) in leaves of *Z. mays* plants grown for 12 days in a complete culture solution and/or NaCl and treated for 2 days with 1 mg L<sup>-1</sup> EM or 11.2  $\mu\text{M}$  TRIA. The constitutive *Zmact* (actin) gene was used as internal control. The accumulation of the gene transcript was first normalized relative to

the *Zmact* transcript, and then expressed as relative to the control (=100 %). Analyses were repeated at least four times for each cDNA obtained from two different RNA extractions. Different letters indicate significant differences between treatments ( $p < 0.05$ ) according to Student-Newman-Keuls test

**Fig. 2** Relative transcript accumulation of the gene encoding phenylalanine ammonia-lyase (*ZmPAL*) in leaves of *Z. mays* plants grown for 12 days in a complete culture solution and/or NaCl and treated for 2 days with 11.2  $\mu$ M TRIA. The constitutive *Zmact* (actin) gene was used as internal control. The accumulation of the gene transcript was first normalized relative to the *Zmact* transcript, and then expressed relative to the control (=100 %). Analyses were repeated at least four times for each cDNA obtained from two different RNA extractions



On this account, the present study has investigated the capacity of a protein hydrolysate-based biostimulant derived from alfalfa plants to stimulate short-term growth in the presence of NaCl in maize plants. EM significantly stimulated the growth of NaCl-treated plants likely because of its content in plant growth regulators, such as IAA and TRIA. Exogenous IAA has been shown to improve the root and shoot growth of wheat seedlings under saline condition (Egamberdieva 2009), and TRIA ameliorated salt-stress induced damages when exogenously supplied to wheat and radish plants (Çavuşoğlu et al. 2008; Perveen et al. 2011). Compared to other biostimulant substances (Schiavon et al. 2010; Trevisan et al. 2010), the amount of IAA in EM was lower, but adequate to explain the auxin-like activity of EM previously reported by Schiavon et al. (2008). The TRIA concentration in EM was consistent with exogenous TRIA concentrations reported by the current literature, which are effective in improving plant growth (Kilic et al. 2010; Naeem et al. 2009; Çavuşoğlu et al. 2008; Khan et al. 2009). Since the effects of pure TRIA on plant weight were similar to those induced by EM, it is presumable that TRIA was the active compound in EM mainly responsible for the plant biomass increase.

According to Knowles and Ries (1981), the positive effect exerted by TRIA and EM on plant growth occurred through stimulation of nitrogen assimilation. Increased uptake of nitrogen could account for enhanced photosynthesis and improved translocation of photosynthates and other metabolites to the sinks that contribute to the greater yield of plants supplied with EM. Furthermore, the values of protein content, GS and GOGAT activities were even higher in salt-stressed plants after EM treatment in most cases. These results are consistent with previous studies that showed the capacity of TRIA to up-regulate genes involved in the photosynthesis process (Chen et al. 2002), and to stimulate leaf photochemical efficiency (Cavani et al. 2006), nitrate reductase activity and protein synthesis (Muthuchelian et al. 1994).

The SPAD index, which is indicative of chlorophyll content, decreased in salt-treated plants. This reduction could be considered a part of the senescence response occurring under salt stress (Hörtensteiner 2006). On this account, the enhancement of SPAD index by EM in salt-treated plants appeared to be a consequence of the stimulating effect of EM.

Salinity increased the activity of CAT, GPX and APX, which are considered crucial enzymes in regulating intracellular  $H_2O_2$  levels in plants. The increase of

antioxidant enzyme activity indicates both protection against damage and control of redox signalling, and supports the evidence that the antioxidative defence system plays a pivotal role in overall plant salt tolerance (de Azevedo Neto et al. 2005; Abogadallah 2010). The increase of antioxidant enzyme activity was probably due to the higher content of proline measured in plants grown under NaCl stress. Indeed, in several studies proline has been reported to accumulate in response to salinity, and induce the activity of antioxidant enzymes (Khedr et al. 2003; Hoque et al. 2007).

The addition of EM to NaCl-treated plants decreased the activity of antioxidant enzymes in leaves. This result could be surprising, as the toxic effects generated in plants under salt stress are often reduced by the activation of antioxidant enzymes implied in ROS scavenging (Asma et al. 2010; Łukawska-Kuźma K et al. 2012). A possible explanation is that ROS, despite their toxicity at high concentration, may also play a pivotal role in stress signalling mechanisms by acting as second messengers, as suggested by an increasing number of studies (Boudsocq and Laurière 2005; Droillard et al. 2004).

The maintenance of physiologically relevant concentrations of proline in maize positively contributed to plant growth under salinity stress. High levels of proline are known to function as very efficient regulators of plasma membrane  $K^+$  ion channels (Shabala et al. 2005; Cuin and Shabala 2005) and thus, are directly involved in the regulation of the intracellular  $K^+/Na^+$  ratio, a key trait for salinity tolerance in plants (Flowers and Colmer 2008; Maathuis and Amtmann 1999; Shabala and Cuin 2008). Consistently with the increase of proline in leaves, the content of  $K^+$  in salt-treated plants was maintained by EM or TRIA at the same level than in control plants or even stimulated. As reported by Mangano et al. (2008), the increased resistance to high NaCl concentrations could be explained by enhanced  $K^+$  and/or by reduced  $Na^+$  accumulation in cells. Furthermore, increased  $K^+$  accumulation may lead to membrane depolarization which reduces the driving force for the transport of toxic cations (Mangano et al. 2008).

The  $K^+/Na^+$  ratio was remarkably high in plants supplied with NaCl in the absence of EM or TRIA, and the decrease of growth was observed. Growth restraint may represent a strategy adopted by plants to survive under adverse environmental conditions, as it may enable the reallocation of assimilates to support mechanisms that promote survival of plants to adversity. Moreover,

smaller plants may be less susceptible to stress because they possess less surface area (Achard et al. 2006).

The increased content of phenols and flavonoids in maize plants grown under salt stress indicated that these compounds are important antioxidants implicated in the tolerance response to salinity. However, the enhancement of flavonoids on one hand, and the decrease of phenols on the other hand in NaCl-treated plants after EM application, suggested that EM stimulated the flavonoid pathway to the detriment of that involved in phenol biosynthesis.

The increase of flavonoid content was consistent with the variation in activity of a key enzyme (PAL) involved in phenylpropanoid biosynthesis. It has been reported that the gene encoding this protein is responsive to a number of abiotic and biotic stresses in many plant species (Huang et al. 2010) and can be induced by biostimulants (Schiavon et al. 2010; Ertani et al. 2011). Our results prove that PAL is responsive to salt-stress and its activity and gene expression are both up-regulated by EM under salinity. However, the up-regulation of the *ZmPal* gene by EM in salt-treated plants could not be ascribed to the content in TRIA of the biostimulant. Rather, IAA in EM might be responsible for the increased transcription of *ZmPal*, in agreement with a previous study that showed induction of PAL gene expression by auxins contained in humic substances (Schiavon et al. 2010).

The stimulation of the phenylpropanoid pathway by EM could be due to the improvement of N assimilation, although an alternative metabolic route that couples the proline biosynthesis to the pentose phosphate pathway has been proposed (Shetty and McCue 2003; Shetty and Wahlqvist 2004). According to this hypothesis, the increase of proline under stress would be partially responsible for the triggering of the phenolic compound build-up.

In conclusion, our results indicate that the use of plant hydrolysates as biostimulant products may represent a promising tool for the amelioration of the adverse effects of salinity in maize. The positive effects of EM on plant growth and metabolism might be ascribed not only to the formulation of the product (small peptides and amino acids), but also to the content of important plant growth regulators. It is noteworthy, however, that the effects of EM have been evaluated in the short-period. As previously discussed, growth restraint may represent a strategy of plants to cope with stress conditions (Achard et al. 2006). On

this account, the effects of EM on plant growth need to be investigated also in long-term experiments to assay if the initial stimulation of growth exerts a negative long-term effect.

**Acknowledgments** This research was funded by ILSA S.p.A.

## References

- Abogadallah GM (2010) Antioxidative defense under salt stress. *Plant Signal Behav* 4:369–374
- Achard P, Cheng H, De Grauwe L, Decat J, Schoutteten H, Moritz T, Van Der Straeten D, Peng J, Harberd NP (2006) Integration of plant responses to environmentally activated phytohormonal signals. *Science* 311:91–94
- Agastian P, Kingsley SJ, Vivekanandan M (2000) Effect of salinity on photosynthesis and biochemical characteristics in mulberry genotypes. *Photosynthetica* 38:287–290
- Ahmed A, Mukherjee S, Deobagkar M, Naik T, Nandi D (2010) Rapid burst of H<sub>2</sub>O<sub>2</sub> by plant growth regulators increases intracellular Ca<sup>2+</sup> amounts and modulates CD<sup>4+</sup> T cell activation. *Int Immunopharmacol* 11:1397–1405
- Arnaldos TL, Ferrer MA, Garcia AAC, Munoz R (2001) Changes in peroxidase activity and isoperoxidase pattern during strawberry (*Fragaria x ananassa*) callus development. *J Plant Physiol* 159:429–435
- Arshi A, Abdin MZ, Iqbal M (2002) Growth and metabolism of senna as affected by salt stress. *Biol Plant* 45:295–298
- Avila C, Rotella JR, Canovas FM, De Castro IN, Valpuesta V (1987) Different characteristics of the two glutamate synthetases in green leaves of *Lycopersicon esculentum*. *Plant Physiol* 85:1036–1039
- Blokhina O, Virolainen E, Fagerstedt KV (2003) Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Ann Bot* 91:179–194
- Boudsocq M, Laurière C (2005) Osmotic signaling in plants. Multiple pathways mediated by emerging kinase families. *Plant Physiol* 138:1185–1194
- Bradford MM (1984) A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Brownell PF, Crossland CJ (1974) Growth responses to sodium by *Bryophyllum tubiflorum* under conditions inducing crasulacean acid metabolism. *Plant Physiol* 54:416–417
- Canovas FM, Canton FR, Gallardo F, Garcia-Gutierrez A, de Vicente A (1991) Accumulation of glutamine synthetase during early development of maritime pine (*Pinus pinaster*) seedlings. *Planta* 185:372–378
- Carpici EB, Celik N, Bayram G (2009) Effects of salt stress on germination of some maize (*Zea mays* L.) cultivars. *Afr J Biotechnol* 19:4918–4922
- Carpici EB, Celik N, Bayram G, Asikb BB (2010) The effects of salt stress on the growth, biochemical parameter and mineral element content of some maize (*Zea mays* L.) cultivars. *Afr J Biotechnol* 41:6937–6942
- Cavani L, ter Halle A, Richard C, Ciavatta C (2006) Photosensitizing properties of protein hydrolysates-based fertilizers. *J Agric Food Chem* 54:9160–9167
- Çavuşoğlu K, Kılıç S, Kabak K (2008) Effects of some plant growth regulators on stem anatomy of radish seedlings grown under saline (NaCl) conditions. *Plant Soil Environ* 54:428–433
- Chance B, Maehly AC (1955) Assay of catalases and peroxidases. *Methods Enzymol* 2:764–775
- Chen SK, Subler S, Edwards CA (2002) Effects of agricultural biostimulants on soil microbial activity and nitrogen dynamics. *Appl Soil Ecol* 19:249–259
- Chen SK, Edwards CA, Subler S (2003) The influence of two agricultural biostimulants on nitrogen transformations, microbial activity, and plant growth in soil microcosms. *Soil Biol Biochem* 35:9–19
- Cuin TA, Shabala S (2005) Exogenously supplied compatible solutes rapidly ameliorate NaCl-induced potassium efflux from barley roots. *Plant Cell Physiol* 46:1924–1933
- De Azevedo Neto AD, Prisco JT, Enéas-Filho J, Medeiros JV, Gomes-Filho E (2005) Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. *Environ Exp Bot* 56:87–94
- de Vasconcelos ACF, Zhang XZ, Ervin EH, Kiehl JD (2009) Enzymatic antioxidant responses to biostimulants in maize and soybean subject to drought. *Sci Agric* 66:395–402
- Droillard MJ, Boudsocq M, Barbier-Brygoo H, Laurière C (2004) Involvement of MPK4 in osmotic stress response pathways in cell suspensions and plantlets of *Arabidopsis thaliana*: activation by hypoosmolarity and negative role in hyperosmolarity tolerance. *FEBS Lett* 574:42–48
- Egamberdieva D (2009) Alleviation of salt stress by plant growth regulators and IAA producing bacteria in wheat. *Acta Physiol Plant* 31:861–864
- Ertani A, Cavani L, Pizzeghello D, Brandellero E, Altissimo A, Ciavatta C, Nardi S (2009) Biostimulant activities of two protein hydrolysates on the growth and nitrogen metabolism in maize seedlings. *J Plant Nutr Soil Sci* 172:237–244
- Ertani A, Schiavon M, Altissimo A, Franceschi C, Nardi S (2011) Phenol-containing organic substances stimulate phenylpropanoid metabolism in *Zea mays* L. *J Plant Nutr Soil Sci* 3:496–503
- Flores P, Botella MA, Martinez V, Cedra A (2000) Ionic and osmotic effects on nitrate reductase activity in tomato seedlings. *J Plant Physiol* 156:552–557
- Flowers TJ, Colmer TD (2008) Salinity tolerance in halophytes. *New Phytol* 179:945–963
- Gao S, Ouyang C, Wang S, Xu Y, Tang L, Chen F (2008) Effects of salt stress on growth, antioxidant enzyme and phenylalanine ammonia-lyase activities in *Jatropha curcas* L. seedlings. *Plant Soil Environ* 54:374–381
- Garcia-Sanchez MJ, Jaime MP, Ramos A, Sanders D, Fernandez JA (2000) Sodium-dependent nitrate transport at the plasma membrane of leaf cells of the marine higher plant *Zostera marina* L. *Plant Physiol* 122:879–883
- Giridara KS, Matta RA, Sudhakar C (2003) NaCl effects on proline metabolism in two high yielding genotypes of mulberry (*Morus alba* L.) with contrasting salt tolerance. *Plant Sci* 165:1245–1251
- Greenway H, Munns R (1980) Mechanism of salt tolerance in non halophytes. *Annu Rev Plant Physiol* 31:149–190

- Hernandez JA, Campillo A, Jimenez A, Alacon JJ, Sevilla F (1999) Response of antioxidant systems and leaf water relations to NaCl stress in pea plants. *New Phytol* 141:241–251
- Hoque MA, Banu MNA, Okuma E, Murata Y (2007) Exogenous proline and glycinebetaine increase NaCl-induced a scorbate-glutathione cycle enzyme activities, and proline improves salt tolerance more than glycinebetaine in tobacco Bright Yellow-2 suspension-cultured cells. *J Plant Physiol* 11:1457–1468
- Hörtensteiner S (2006) Chlorophyll degradation during senescence. *Annu Rev Plant Biol* 57:55–77
- Huang JL, Gu M, Lai ZB, Fan BF, Shi K, Zhou YH, Yu JQ, Chen ZX (2010) Functional analysis of the arabidopsis PAL gene family in plant growth, development, and response to environmental stress. *Plant Physiol* 153:1526–1538
- Javid MG, Sorooshzadeh A, Moradi F, Sanavy AMM, Allahdadi J (2011) The role of phytohormones in alleviating salt stress in crop plants. *AJCS* 6:726–734
- Kauffman GL III, Kneivel DP, Watschke TL (2007) Effects of a biostimulant on the heat tolerance associated with photosynthetic capacity, membrane thermostability, and polyphenol production of perennial ryegrass. *Crop Sci* 47:261–267
- Khan W, Rayirath UP, Subramanian S, Jithesh MN, Hodges DM, Critchley AT, Craigie JS, Norrie J, Prithiviraj B (2009) Seaweed extracts as biostimulants of plant growth and development. *Plant Growth Regul* 28:386–399
- Khedr AHA, Abbas MA, Wahid AAA, Quick WP, Abogadallah GM (2003) Proline induces the expression of salt-stress-responsive proteins and may improve the adaptation of *Pancreaticum maritimum* L. to salt-stress. *J Exp Bot* 54:2553–2562
- Kilic NK, Duygu E, Donmez G (2010) Triacontanol hormone stimulates population, growth and brilliant blue red dye removal by common duckweed from culture media. *J Hazard Mater* 182:525–530
- Knowles NR, Ries SK (1981) Rapid growth and apparent total nitrogen increase in rice and corn plants following application of triacontanol. *Plant Physiol* 68:1279–1284
- Kumaravelu G, Livingstone VD, Ramanujam MP (2000) Triacontanol-induced changes in the growth, photosynthetic pigments, cell metabolites, flowering and yield of green gram. *Biol Plant* 43:287–290
- Lukawska-Kuźma K, Podgórska A, Rychter AM (2011) Plasma membrane-generated ROS and their possible contribution to leaf cell growth of cucumber (*Cucumis sativus*) MSC16 mitochondrial mutant 34:721–730
- Lukawska-Kuźma K, Podgórska A, Rychter AM (2012) Plasma membrane-generated ROS and their possible contribution to leaf cell growth of cucumber (*Cucumis sativus*) MSC16 mitochondrial mutant. *Acta Physiol Plant* 34:721–730
- Lutts S, Majerus V, Kinet JM (1999) NaCl effects on proline metabolism in rice (*Oriza sativa*) seedlings. *Physiol Plant* 105:450–458
- Maathuis FJM, Amtmann A (1999) K<sup>+</sup> nutrition and Na<sup>+</sup> toxicity: the basis of cellular K<sup>+</sup>/Na<sup>+</sup> ratios. *Ann Bot* 84:123–133
- Mangano S, Silberstein S, Santa-Maria GE (2008) Point mutations in the barley HvHAK1 potassium transporter lead to improved K<sup>+</sup>-nutrition and enhanced resistance to salt stress. *FEBS Lett* 582:3922–3928
- Marschner H (1995) Mineral nutrition of higher plants. Academic, London, p 674
- Meenakshi S, Manica GD, Tamil MS, Arumugam M, Balasubramanian T (2009) Total flavonoid in vitro antioxidant activity of two seaweeds of Rameshwaram Coast. *Glob J Pharmacol* 3:59–62
- Mori T, Sakurai M, Sakuta M (2001) Effects of conditioned medium on activities of PAL, CHS, DAHP synthase (DS-Co and DS-Mn) and anthocyanin production in suspension cultures of *Fragaria ananassa*. *Plant Sci* 160:355–360
- Muthuchelian K, Murugan G, Harigovindan R, Nedunchezian N, Kulandaivelu G (1994) Effect of triacontanol in flooded *Erythrina variegata* seedlings to changes in (CO<sub>2</sub>)-C-14 fixation and ribulose-1,5 Bisphosphate carboxylase photosystem, and nitrate reductase activities. *Photosynthetica* 30:407–413
- Naeem M, Khan MMA, Moinuddin, Siddiqui MH (2009) Triacantanol stimulates nitrogen-fixation, enzyme activities, photosynthesis, crop productivity and quality of hyacinth bean (*Lablab purpureus* L.). *Sci Hortic* 121:389–396
- Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol* 22:867–880
- Nardi S, Pizzeghello D, Gessa C, Ferrarese L, Trainotti L, Casadoro G (2000) A low molecular weight humic fraction on nitrate uptake and protein synthesis in maize seedlings. *Soil Biol Biochem* 32:415–419
- Ochoa-Alfaro AE, Silva-Ortega CO, Becerra-Flora A, Flores-Rivas J, Jimenez-Bremont JF (2008) Effect of salt stress, proline, and polyamines on seed germination of *Opuntia streptacantha*. *J Prof Assoc Cact* 10:56–70
- Ohnishi JI, Flugge UI, Heldt HW, Kanai R (1990) Involvement of Na<sup>+</sup> in active uptake of pyruvate in mesophyll chloroplasts of some C4 plants. *Plant Physiol* 94:950–959
- Osswald WF, Kraus R, Hippeli S, Benz B, Volpert R, Elstner EF (1992) Comparison of the enzymatic activities of dehydroascorbic acid reductase, glutathione reductase, catalase, peroxidase and superoxide dismutase of healthy and damaged spruce needles (*Picea abies* L.). *J Plant Physiol* 139:742–748
- Ouyang B, Yang T, Li HX, Zhang L, Zhang YY, Zhang JH, Fei ZJ, Ye ZB (2007) Identification of early salt stress response genes in tomato root by suppression subtractive hybridization and microarray analysis. *J Exp Bot* 58:507–520
- Ozden M, Demirel U, Kahraman A (2009) Effects of proline on antioxidant system in leaves of grapevine (*Vitis vinifera* L.) exposed to oxidative stress by H<sub>2</sub>O<sub>2</sub>. *Sci Hortic* 119:163–168
- Parida AK, Das AB, Mitra B (2004) Effects of salt on growth, ion accumulation, photosynthesis and leaf anatomy of the mangrove, *Bruguiera parviflora*. *Trees-Struct Funct* 18:167–174
- Perveen S, Shahbaz M, Ashraf M (2011) Modulation in activities of antioxidant enzymes in salt stressed and non-stressed wheat (*Triticum aestivum* L.) plants raised from seed treated with triacontanol Pak. *J. Bot* 43:2463–2468
- Ries SK, Violet W (1977) Growth responses of rice seedlings to triacontanol in light and dark. *Planta* 135:77–82
- Schiavon M, Ertani E, Nardi S (2008) Effects of an Alfa-Alfa protein hydrolysate on the gene expression and activity of enzymes of TCA cycle and N metabolism in *Zea mays* L. *J Agric Food Chem* 172:237–244
- Schiavon M, Pizzeghello D, Muscolo A, Vaccaro S, Francioso O, Nardi S (2010) High molecular size humic substances

- enhance phenylpropanoid metabolism in maize (*Zea mays* L.). *J Chem Ecol* 36:662–669
- Shabala S, Cuin TA (2008) Potassium transport and plant salt tolerance. *Physiol Plant* 133:651–669
- Shabala S, Mackay A (2011) Plant responses to drought and salinity stress: developments in a post-genomic era Editor Turkan I. In book series. *Advances in botanical research* 57:151–199
- Shabala L, Cuin TA, Newman I, Shabala S (2005) Salinity-induced ion flux patterns from the excised roots of *Arabidopsis* sos mutants. *Planta* 222:1041–1050
- Shetty K, McCue P (2003) Phenolic antioxidant biosynthesis in plants for functional food application: integration of systems biology and biotechnological approaches. *Food Biotechnol* 2:67–97
- Shetty K, Wahlqvist M (2004) A model for the role of the proline-linked pentose-phosphate pathway in phenolic phytochemical biosynthesis and mechanism of action for human health and environmental applications. *Asia Pac J Clin Nutr* 1:1–24
- Soussi M, Lluch C, Ocana A (1998) Effects of salt stress on growth, photosynthesis and nitrogen fixation in chick-pea (*Cicer arietinum* L.). *J Exp Bot* 49:1329–1337
- Trevisan S, Pizzeghello D, Ruperti B, Francioso O, Sassi A, Palme K, Quaggiotti S, Nardi S (2010) Humic substances induce lateral root formation and expression of the early auxin-responsive IAA19 gene and DR5 synthetic element in *Arabidopsis*. *Plant Biol* 12:604–614
- Viégas RA, Silveira JAG, Junior ARL, Queiroz JE, Fausto MJM (2001) Effects of NaCl salinity on growth and inorganic solute accumulation in young cashew plants. *Braz J Agric Environ Eng* 5:216–222
- Zhao FY, Liu T, Xu ZJ (2010) Modified responses of root growth and reactive oxygen species-scavenging system to combined salt and heat stress in transgenic rice. *Russ J Plant Physiol* 57:518–525