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Polyphenol-enriched spelt husk extracts improve growth and stress-related biochemical parameters under moderate salt stress in maize plants

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Abstract

Biostimulants improve yield, quality, and stress acclimation in crops. In this work, we tested the possibility of using phenolics-rich extracts from spelt (*Triticum dicoccum* L.) husks to attenuate the effects of salt stress (100-200 mM NaCl) in maize. Two methanolic extracts were prepared from the soluble-conjugated (SC), and the insoluble-bound (IB) phenolic acid fractions of the spelt husk, and their effects were investigated on several stress-associated biochemical parameters, such as proline, lipid peroxidation, H_2O_2, GSH levels, and ion content. Results show that SC and IB fractions of husk extracts behaved very differently, no doubt due to their greatly divergent chemical composition, as revealed by both GC-MS and HPLC analyses. The efficacy of treatments in mitigating salt stress was also dose- and timing-dependent. IB, even at the lower concentration tested, was able to recover the performance of stressed plants in terms of growth, photosynthetic pigments content, and levels of salt stress markers. Recovery of shoot growth to control levels and reduction of stress-induced proline accumulation occurred regardless of whether plants were pre-treated or post-treated with IB, whereas only pre-treatment with the higher dose of IB was effective in mitigating oxidative stress. Although in some cases SC and even methanol alone exerted some positive effects, they could also be deleterious whereas IB never was. Overall, results indicate that a polyphenol-containing extract obtained from spelt by-products can behave as biostimulant in maize plants and can mitigate their response to salt stress, by acting on different biochemical targets.

Keywords: agricultural by-products, biostimulant, oxidative stress, phenolic acids, salt stress, *Triticum dicoccum*, *Zea mays*

Introduction

Salinity is one of the major abiotic stresses that affects plant growth and biomass production as well as crop yield. More than 800 million hectares worldwide are currently affected by salt and with global climate change, drought and salinity are expected to increase even further affecting more than 50% of all arable lands by the year 2050 (Zhu 2002; Wang et al. 2003). High concentrations of salt decrease water potential, thereby restricting water and nutrient uptake by roots (Ashraf 2004); they are also responsible for nutritional and hormonal imbalance as well as oxidative stress (Basile et al. 2011), and they reduce chlorophyll content and RuBisCO activity (Rady 2013; Kahrizi et al. 2012) leading to impaired plant growth and productivity.

The application of biostimulants has been proposed as an innovative agronomic practice, not only to ensure optimal nutrient uptake, crop yield, and quality (Chen et al. 2003; Schiavon et al, 2008, 2010;

Ertani et al. 2009; Khan et al. 2009), but also to mitigate the detrimental effects of environmental stresses, including high salinity (Feitosa de Vasconcelos et al, 2009; Povero et al. 2016; Desoky et al. 2018). The nature and activity of biostimulants is heterogeneous, since they include humic acids, protein hydrolysates, extracts from seaweeds and higher plant species, as well as beneficial microbes, such as plant growth-promoting bacteria, and mycorrhizal fungi (Ertani et al, 2013, 2016; du Jardin 2015). For this reason, their chemical composition is also highly variable, consisting of a broad range of bioactive compounds, characterized by a huge chemical complexity. An interesting source of biostimulants are agro-industrial residues, such as bark, straw, rice husks, etc. (Ertani et al., 2011a), which often contain bioactive molecules, including antioxidants (Schieber et al. 2001; Balasundram et al. 2005), able to exert a protective effect under a variety of stress conditions.

Phenolic compounds are a very large group of secondary metabolites, ubiquitous in all plant tissues, where they play an important role by providing plants with specific adaptations to changing environmental conditions and by eliciting defense mechanisms (Caretto et al. 2015 and references therein). Indeed, activation of stress acclimation in plants is often accompanied by the synthesis of secondary metabolites (Mazid et al. 2011; Ramakrishna and Ravishankar 2011). In particular, the role of phenolic compounds in mediating stress responses is due to their capacity to scavenge free radicals produced in stress-induced oxidative reactions (Cook and Samman 1996; Halliwell 2008; Sharma et al. 2012), thus protecting cell components from damage (Bulgari et al. 2017). Phenolic compounds, amongst which phenolic acids and flavonoids (El Gharras 2009), are, in fact, reported to possess powerful antioxidant activity *in vitro* (Kähkönen et al. 1999). In recent years, several studies have investigated the application in agriculture of phenol-enriched products derived from food residues, since these secondary metabolites are particularly efficient in stimulating plant growth. In fact, it has been reported that phenolic molecules may exert a gibberellin-like activity (Savy et al. 2017) and also interact with several other plant hormones, including auxins (Ertani et al. 2016). Moreover, they improve plant-water relationships, stomatal function, rate of photosynthesis, and respiration (Ertani et al. 2016).

Maize (*Zea mays* L.), being rich in nutrients, such as starch (72%), protein (10%), fiber (8.5%), and edible oil (4.8%), is one of the major sources of food, sugar, cooking oil, and animal feed (Okoruwa 1997). It is classified as a salt-sensitive crop (Maas & Hoffman 1977). Antioxidant defense mechanisms have been demonstrated to play a fundamental role in mitigating salt stress (Zhu 2001; Sairam and Tyagi 2004) and salt stress tolerance in some maize genotypes was explained by the maintenance and/or increased activity of antioxidant enzymes (De Azevedo Neto et al. 2006). The effects of biostimulants on growth and stress tolerance in maize have been investigated. Maize seedlings treated with plant extracts derived from red grape, blueberry fruits, and hawthorn leaves

showed increased root and leaf biomass, chlorophyll and sugar content, as well as phenolic acids compared to untreated plants (Ertani et al. 2016). Recently, a positive effect against drought stress was also observed in maize treated with an aqueous extract of sorghum leaves (Maqbool and Sadiq 2017).

By-products from spelt (*Triticum dicoccum* L.) processing (husks, glumes and stems) are employed for energy production (electricity or heating) as they present a number of advantages, both in the renewable energy sector and in agriculture and environmental protection (Kiš et al. 2017). Based on the assumption that this by-product may still contain bioactive polyphenols, we assessed the potential of this material as plant biostimulant under stress conditions. To this purpose, a pot trial was conducted to test the performance of methanolic extracts of spelt husks in mitigating the negative impact of salt stress in maize. Since methanol was used as solvent and previous reports demonstrate its important role in plant growth and development (Dorokhov et al. 2018) as well as in mitigating the effects of drought and salt stresses (Mirakori et al. 2009; Wei et al. 2015), the effect of methanol alone was investigated in parallel.

Materials and methods

Spelt husk extract preparation

Two phenolic acid-enriched extracts were prepared from husks of *Triticum dicoccum* L. (kindly provided by Terra Bio Soc. Coop, Schieti di Urbino, PU, Italy) according to the protocol described in Antognoni et al. (2017). Husk samples (1.5 g) were extracted with 30 mL acetone/methanol/water mixture (7:7:6, v/v/v) in an ultrasound bath at 30 °C for 30 min. The homogenate was centrifuged at 1,500 x *g* for 20 min and the pellet was re-extracted once with the same procedure. Pooled supernatants and pellets were used to prepare the soluble-conjugated (SC) and the insoluble-bound (IB) phenolic acid fractions, respectively. An aliquot (8 mL) of supernatant was mixed with 2 mL 10 M NaOH and hydrolyzed under nitrogen flow and constant stirring for 1 h. The solution was then acidified to pH 2 with 12 M HCl and extracted three times with an equal volume of a diethyl ether/ethyl acetate (1/1, v/v) mixture. The organic extracts were merged, brought to dryness in a rotary evaporator, and re-dissolved with 2 mL of methanol. A 0.5 g aliquot of the pellet was mixed with 40 mL of 2 M NaOH and hydrolyzed under nitrogen flow and constant stirring for 1 h. The sample was then centrifuged for 20 min at 1.500 x *g*; the supernatant was acidified to pH 2 and then subjected to the extraction procedure already described for the SC fraction.

Gas chromatography-mass spectrometry (GC-MS) analysis of extracts

An aliquot (*ca.* 1 mg) of each dry extract was derivatized with 200 µL of bis-(trimethyl) trifluoroacetamide (BSTFA) including 1% trimethylchlorosilane (TMCS) (Sigma-Aldrich, Milan, Italy) and 200 µL of pyridine (Sigma-Aldrich, Milan, Italy) for 2 h at room temperature (RT). The silylating reagent was removed under a nitrogen stream and the sample re-dissolved in 1 mL of *n*-heptane. The GC-MS analysis of the trimethylsilyl derivatives was carried out using a Trace GC Ultra coupled to an ion-trap mass spectrometer detector Polaris Q and equipped with a split/splitless injector (Thermo Scientific, Italy). The column was a Supelco SLB-5ms, 30 x 0.25 mm, 0.1 µm film thickness (Supelco, Sigma-Aldrich, Milan, Italy). Column oven temperature was programmed as follows: 80 °C held for 2 min, up to 280 °C at 10 °C min^{-1}, held for 10 min, up to 300 °C at 10 °C min^{-1} and held for 10 min. Helium was used as carrier gas at a flow rate of 1 mL min^{-1}. The injector, transfer line, and ion source were held at 280, 280 and 200 °C, respectively. Split ratio was 1:10 and injection volume 1 µL. The mass spectra were recorded under electron ionization (EI) conditions at 70 eV electron energy with a mass range from *m/z* 50 to 650. Relative percentage amount of each identified compound was expressed as percent peak area relative to total ion current GC-MS peak area without using the correction factor.

HPLC analysis of extracts

Twenty µL of each extract were injected into an HPLC system (Jasco, Tokyo, Japan; PU-4180 pump, MD-4015 PDA detector, AS-4050 autosampler). The stationary phase was an Agilent (Santa Clara, CA, USA) Zorbax Eclipse Plus C18 reversed-phase column (100 mm x 3 mm I.D., particle size 3.5 µm).The chromatographic method for the analysis of phenolic acids was adapted from Mattila et al. (2005). Gradient elution was carried out with a mixture of acidic phosphate buffer and acetonitrile flowing at 0.7 mL min^{-1}. The signals at 254, 280, and 329 nm were used for analyte quantitation. Identification and quantification were performed based on standard compounds (gallic, *p*-hydroxybenzoic, syringic, ferulic, *p*-coumaric, cinnamic, and caffeic acids). The recovery values in spiked samples ranged from 78.8 to 92.2% (RSD $< 9.8\%$, n = 6).

The sum of all individual phenolic acid concentrations was calculated and used to express the total phenolics acid index (TPAI) for each extract.

Plant material

Seeds of *Zea mays* L. (var. FAO 700, kindly provided by Società Italiana Sementi, San Lazzaro di Savena, Bologna, Italy) were washed under running tap water for 30 min and then placed on wet filter paper in Petri dishes. They were germinated for two days in the dark at 25 °C. Seedlings were then transferred to plastic pots (12 x 8 x 7 cm) with drainage holes and containing Perlite soaked in

Hoagland's nutrient solution (Hoagland and Arnon 1950). The pots were placed on trays to which half-strength Hoagland's solution was added when necessary in order to keep the Perlite fully wet. Plants were grown in a growth chamber with a photoperiod of 16/8 h day/night (400 µmol m^{-2} s^{-1} flux density supplied by fluorescent TL/D Aquarelle Philips lamps) at a temperature of 24 ± 2 °C. During the day, an additional irradiation (180 m^{-2} s^{-1} flux density from Lumatek HPS Grow lamps) was supplied for 5 h.

Salt treatment and husk extract application

After 8-10 days, the pots (four per treatment and each containing five plants) were transferred to trays containing Hoagland's solution added with either 0 (control), 50, 100, or 200 mM NaCl. The solution in the trays was changed every two days to maintain the same salt concentration. Plants were harvested at 5, 12, and 19 days after the start of salt treatment.

Husk extracts (HEs) were added to the nutrient solution at two different concentrations, 0.1 or 1.0 mL L^{-1}, in the trays before (pre-treatment) or after (post-treatment) the addition of NaCl as follows:

a) pre-treatment (PRE): IB, SC or methanol (solvent control) were added for two days before the beginning of salt treatment. Plants were then transferred to trays containing Hoagland's solution added with 0, 100 or 200 mM NaCl.
b) post-treatment (POST): IB, SC or methanol (solvent control) were added two days after the start of salt treatment; plants were grown in the presence of extract or methanol plus NaCl until sampling.

Plants were harvested at 12 (100 mM NaCl) or 8 days (200 mM NaCl) from the start of salt treatment. Shoots and roots were weighed separately and then ground to a powder in liquid nitrogen; some samples were freeze-dried, while others were left frozen and kept at -80 °C until use. For dry weight (DW) determination, shoots and roots were dried in an oven at 80 °C for two days.

Photosynthetic pigment determination

Freeze-dried shoot samples (50 mg) were extracted in a chilled mortar with 80% (v/v) cold acetone (1:5 ratio) in dim light. The homogenate was centrifuged at 10,000 x *g* for 10 min at 4 °C and the extraction repeated once. The supernatants were pooled and absorbance determined spectrophotometrically (V-530 Jasco, Jasco Corporation, Tokyo, Japan) at 663 nm (chlorophyll *a*), 647 nm (chlorophyll *b*), and 470 nm (carotenoids and xanthophylls). Pigment concentrations were estimated based on specific absorbance coefficients (Lichtenthaler 1987) and are expressed as mg g^{-1} DW.

Lipid peroxidation assay

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) production by the thiobarbituric acid (TBA) reaction method (Velikova et al. 2000). Absorbance of the supernatant was read at 532 nm. After subtracting the value for non-specific absorption at 600 nm, the concentration of the MDA-TBA complex was calculated from the extinction coefficient 155 mM^{-1} cm^{-1}.

Hydrogen peroxide determination

Hydrogen peroxide (H_2O_2) levels were determined according to Velikova et al. (2000). Frozen shoot and root tissue (500 mg) was extracted on an ice-bath with 5 mL 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 12,000 x *g* for 15 min. Then, an aliquot (0.5 mL) of the supernatant was added to a mixture of 0.5 mL potassium phosphate buffer (10 mM, pH 7.0) and 1 mL 1 M KI. After 10 min at RT, the absorbance of the samples was spectrophotometrically determined at 390 nm. The concentration of H_2O_2 was calculated from a standard curve.

Proline determination

Shoot and root proline concentrations were estimated following the method of Bates et al. (1973) with slight modifications. About 20 mg of freeze-dried plant material was crushed in 1.2 mL 3% sulphosalicylic acid and the homogenate centrifuged at 16,000 x *g* at RT for 20 min. A 0.5 mL aliquot of the supernatant was made up to 1 mL with water and to this 1 mL glacial acetic acid and 1 mL ninhydrin reagent [2.5% ninhydrin in glacial acetic acid-distilled water-85% orthophosphoric acid (6:3:1)] were added. The reaction mixtures were kept in a water bath at 90 °C for 1 h to develop the colour. Test tubes were then cooled in an ice-bath, and 3 mL toluene added to separate the chromophore. Absorbance of the toluene phase was read in a spectrophotometer at 546 nm, and proline concentration calculated by comparing sample absorbance with the standard proline curve.

Glutathione (GSH) measurement

GSH was determined as previously described by Brundu et al. (2016). Approximately 20-25 mg of freeze-dried shoots or roots were transferred to microcentrifuge tubes containing 200 μL of precipitating solution (1.67 g glacial meta-phosphoric acid, 0.2 g disodium EDTA, and 30 g NaCl in 100 mL). Samples were homogenized on ice with a mortar and pestle, kept on ice for 10 min and then centrifuged at 12,000 × *g* for 10 min at 4 °C. Fifteen μL 0.3 M Na_2HPO_4 were added to 60 μL of extract followed immediately by 45 μL 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) prepared as follows: 20 mg DTNB in 100 mL 1% (w/v) sodium citrate solution. The mixture was stirred for 1

min at RT, then left at RT for another 5 min and finally used for GSH determination by Reverse Phase-HPLC (Jasco LG-980-02, Jasco Europe S.R.L., Cremella, LC, Italy).

Na^+ and K^+ content determination

Freeze-dried samples were mineralized (MDS 2000, CEM, Italy) with a mixture of concentrated HNO_3/H_2O_2 (8+2 mL). All chemicals used in sample treatment were suprapure grade (HNO_3 65%, H_2O_2 30%, Merck Suprapur, Darmstadt, Germany). Na^+ and K^+ concentrations were measured by inductively coupled plasma-optical emission spectrometry using a Perkin Elmer Optima 8000 ICP-OES Spectrometer (PerkinElmer Inc., Shelton, CT, USA). Ultrapure water (Milli-Q system, Millipore Corporation, USA) was used for all solutions. Standard solutions were prepared from stock solutions of ultrapure grade metals (PerkinElmer Pure Plus, 100 mg L^{-1}). The error of metal determinations, based on variation in replicate analyses (n=2) on the same samples, was 10% or lower.

Results

Phytochemical characterization of HEs

The phytochemical composition of both IB and SC was investigated through HPLC-DAD and GC-MS analyses. As shown in Table 1, five phenolic acids were detected by HPLC-DAD in both extracts, i.e., two simple phenolics (*p*-hydroxybenzoic, and syringic acids) and three hydroxycinnamic acids (ferulic, *p*-coumaric, and caffeic). The most abundant were ferulic and *p*-coumaric acids, which together represented *ca*. 75% and 97% of total phenolic acids in SC and IB, respectively. In absolute terms, IB contained about 25-fold higher ferulic and *p*-coumaric acids compared to SC and more than six-fold higher caffeic acid; syringic acid was about two-fold higher in SC than IB (Table 1). In both extracts, *p*-hydroxybenzoic acid was detected at a similar concentration (Table 1). Total phenolic acid index was 17.71 μg mL^{-1} for SC and 713.82 μg mL^{-1} for IB (Table 1).

Phenolic acid	SC		IB	
	μg mL^{-1}	μg g DW^{-1}	μg mL^{-1}	μg g DW^{-1}
p-OH benzoic acid	1.72 ± 0.7	16.15 ± 6.6	2.77 ± 0.4	13.23 ±1.9
Syringic acid	1.63 ± 0.7	15.36 ± 6.6	1.84 ± 0.3	8.79 ± 1.4
p-Coumaric acid	7.22 ± 0.9	67.74 ± 8.4	343.37 ±12.1	1635.10 ± 57.6
Caffeic acid	0.38 ± 0.05	3.61 ± 0.5	5.11 ± 0.2	24.34 ± 0.9
Ferulic acid	6.76 ± 0.4	63.45 ± 3.8	360.73 ± 19.4	1717.76 ± 92.4
Total phenolic acid index	17.71 ±1.33	166.31 ± 13.1	713.82±12.11	3399.22 ±108.9

Table 1. HPLC-DAD analysis of phenolic acid composition of spelt husk extracts. Data are the means ± S.E. of three independent extractions.

In addition, GC-MS analysis of IB revealed the presence of vanillin (0.7%), vanillic acid (1.6%) and a relatively higher percentage of *m*-coumaric (10.5%) and isoferulic acids (8.4%) (Table 2). GC-MS analysis of SC showed the presence of malic (14.2%), palmitic (27.6%), linoleic (16.8%), oleic (25.6%), and stearic (3.2%) acids accompanied by lesser amounts of 2-hydroxyglutaric acid (0.2%), *m*-anisic acid methyl ester (0.7%), campesterol (0.2%), stigmasterol (0.3%), and β-sitosterol (0.4%) (Table 2). Fatty acids, absent in IB, represented about 73% of all compounds identified in SC. Among phenolic acids, ferulic and *p*-coumaric acids were the major constituents in both extracts representing *ca.* 75% and 98% in SC and IB, respectively, in agreement with the results of the HPLC-DAD analysis. Vanillin and isoferulic acid were absent in SC.

	SC		IB	
Compound	**Mean (%)[a]**	**RSD[b] (%)**	**Mean (%)[a]**	**RSD[b] (%)**
Malic acid	14.2 ± 0.4	2.8	-	-
Vanillin	-	-	0.7 ± 0.01	3.2
2-Hydroxyglutaric acid	0.2 ± 0.01	2.0	-	-
p-Hydroxybenzoic acid	0.5 ± 0.01	3.6	0.2 ± 0.01	3.8
m-Anisic acid methyl ester	0.7 ± 0.01	1.2	-	-

Vanillic acid	1.8 ± 0.01	1.8	1.6 ± 0.1	4.4
m-Coumaric acid	0.7 ± 0.01	0.4	10.5 ± 0.1	0.7
Syringic acid	1.4 ± 0.1	4.0	0.4 ± 0.01	6.4
Isoferulic acid	-	-	8.4 ± 0.2	2.0
p-Coumaric acid	3.3 ± 0.1	1.7	35.2 ± 0.4	1.0
Palmitic acid	27.6 ± 1.2	4.2	-	-
Ferulic acid	3.0 ± 0.01	1.0	42.1 ± 0.1	0.3
Caffeic acid	0.1 ± 0.01	4.6	0.9 ± 0.1	5.9
Linoleic acid	16.8 ± 1.1	6.5	-	-
Oleic acid	25.6 ± 0.4	1.5	-	-
Stearic acid	3.2 ± 0.1	1.9	-	-
Campesterol	0.2 ± 0.01	1.6	-	-
Stigmasterol	0.3 ± 0.01	2.1	-	-
β-Sitosterol	0.4 ± 0.01	1.3	-	-

Table 2. GC-MS analysis of spelt husk extracts.

[a]Percent peak area relative to total ion current GC-MS peak area ± SD (n = 3).

[b]Relative standard deviation.

Effects of salt treatment on maize plants

In order to characterize the response to salinity of the maize genotype used in this study, a time-course analysis of growth and stress-related biochemical parameters was performed with three different concentrations of NaCl. Shoot fresh weight (FW) and dry weight (DW) were negatively affected by 50, 100 and 200 mM NaCl starting on day 12, with the strongest inhibition in FW (58% relative to controls) at the highest concentration and longest exposure time (19 days; Table 3). Root FW was less affected by all three salt concentrations, with a maximum decline (*ca*. 30-40%) with 200 mM NaCl after 12 and 19 days; root DW was not significantly affected (Table 3).

		Shoots				Roots			
Time (days)	NaCl (mM)	FW (g $plant^{-1}$)	DW (g $plant^{-1}$)	RFW	RDW	FW (g $plant^{-1}$)	DW (g $plant^{-1}$)	RFW	RDW
5	0	0.689 ± 0.208^{a}	0.055 ± 0.017^{a}	100	100	0.572 ± 0.154^{a}	0.03 ± 0.017^{a}	100	100
	50	0.945 ± 0.227^{a}	0.072 ± 0.017^{a}	137.2	130.9	0.655 ± 0.029^{a}	0.038 ± 0.017^{a}	114.5	115.2
	100	0.905 ± 0.216^{a}	0.076 ± 0.016^{a}	131.3	138.2	0.709 ± 0.089^{a}	0.036 ± 0.016^{a}	124.0	109.1
	200	0.907 ± 0.282^{a}	0.083 ± 0.026^{a}	131.6	150.9	0.628 ± 0.178^{a}	0.044 ± 0.026^{a}	109.8	133.3
12	0	2.620 ± 0.263^{a}	0.206 ± 0.017^{a}	100	100	1.338 ± 0.252^{a}	0.077 ± 0.014^{a}	100	100
	50	1.968 ± 0.078^{b}	0.160 ± 0.022^{b}	75.1	77.7	1.144 ± 0.131^{b}	0.061 ± 0.016^{a}	85.5	79.2
	100	1.923 ± 0.220^{b}	0.158 ± 0.034^{b}	73.4	76.7	1.100 ± 0.137^{b}	0.079 ± 0.014^{a}	82.2	102.3
	200	1.229 ± 0.183^{c}	0.137 ± 0.022^{b}	46.9	66.5	0.823 ± 0.126^{c}	0.065 ± 0.006^{a}	61.5	84.4
19	0	4.350 ± 0.39^{a}	0.350 ± 0.03^{a}	100	100	1.596 ± 0.25^{a}	0.100 ± 0.02^{a}	100	100
	50	4.220 ± 1.10^{a}	0.366 ± 0.06^{a}	97.0	105.7	1.560 ± 0.34^{a}	0.099 ± 0.05^{a}	97.7	99.0
	100	2.910 ± 0.55^{b}	0.281 ± 0.06^{ab}	66.9	80.0	1.400 ± 0.30^{ab}	0.097 ± 0.02^{a}	87.7	97.0
	200	2.820 ± 0.45^{b}	0.221 ± 0.05^{b}	41.8	62.9	1.130 ± 0.07^{b}	0.090 ± 0.01^{a}	70.8	90.0

Table 3. Fresh weight, dry weight, relative fresh weight (RFW), and relative dry weight (RDW) of maize plants after exposure for 5, 12, and 19 days to 0, 50, 100 or 200 mM NaCl. Data are means ± S.D. (n=8). Different letters within a column and for each time point indicate significant differences ($p<0.05$).

At day 5, a small but significant reduction in Chl*a* was observed with 200 mM NaCl, while at days 12 and 19, all pigments showed a significant and dose-dependent reduction (Fig. 1). A similar pattern was observed for total carotenoids content (Fig.1).

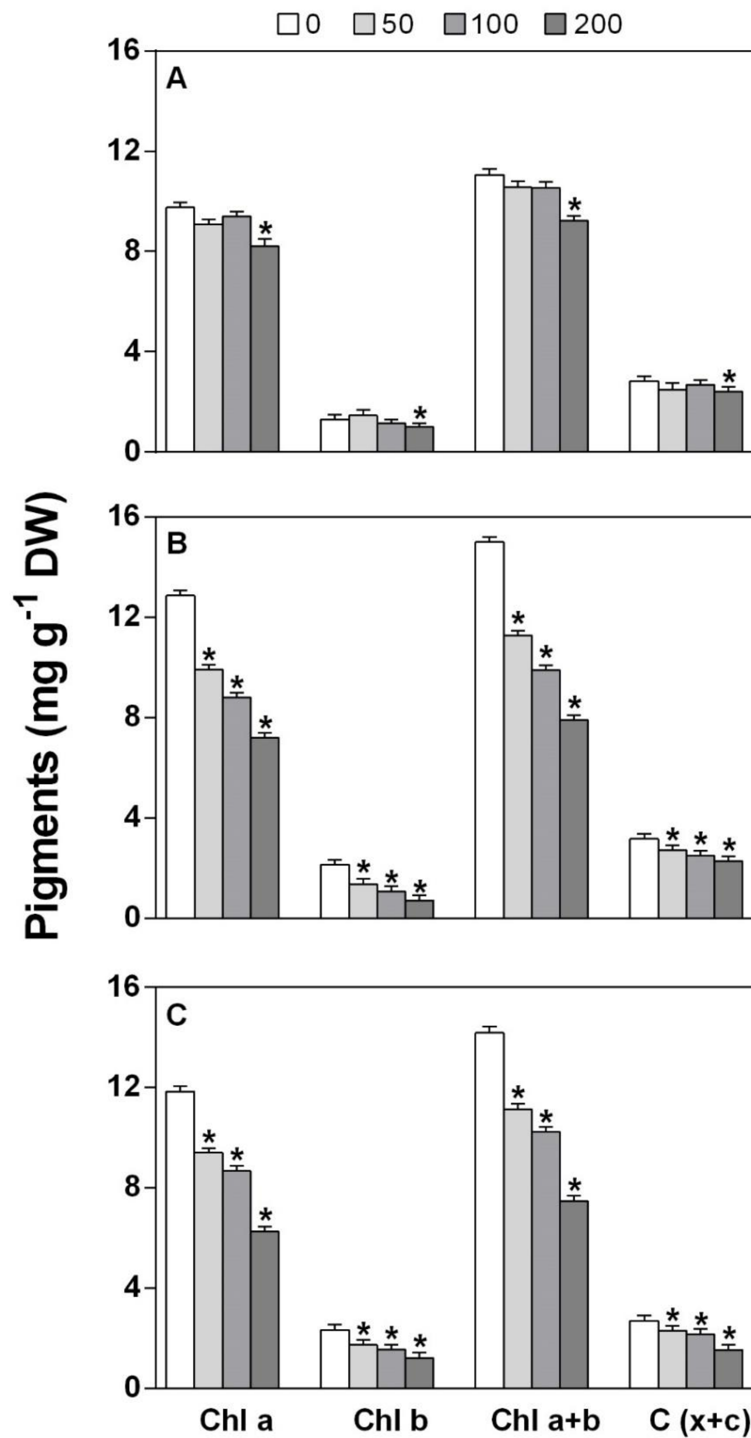


Fig. 1. Concentration of photosynthetic pigments after 5 (A), 12 (B), and 19 (C) days of exposure to 0, 50, 100 or 200 mM NaCl. Data are means ± S.E. (n=3). Asterisks indicate significant differences (P<0.05) relative to controls (0 mM NaCl).

In both shoots and roots of salt-treated plants, a significant increase in proline occurred starting from day 5, but only with 200 mM NaCl. On day 12, the increase was significant relative to controls also with 100 mM NaCl and by day 19 the increase in shoots was *ca.* 6-fold and 26-fold with 100 and 200 mM NaCl, respectively; salt-induced proline accumulation in roots was equally dramatic (Fig. 2).

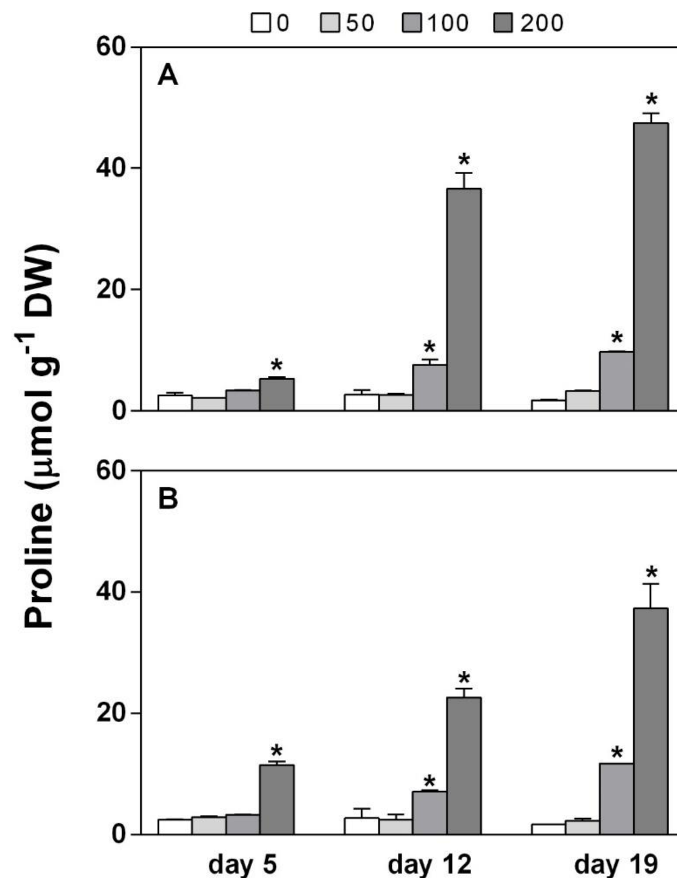


Fig. 2. Proline concentration in shoots (A) and roots (B) after 5, 12, and 19 days of exposure to 0, 50, 100 or 200 mM NaCl. Data are means ± S.E. (n=3). Asterisks indicate significant differences (P<0.05) relative to controls (0 mM NaCl) for each sampling time.

Salt-induced oxidative stress was evaluated by measuring lipid peroxidation (MDA assay) and H_2O_2 production. In shoots, MDA production was significantly enhanced by all NaCl treatments starting from day 5; on day 19, it was *ca*. 40% and 70% higher than in controls with 100 and 200 mM NaCl, respectively (Fig. 3A). In roots, only the highest NaCl concentration significantly enhanced MDA levels, while other treatments had no effect (Fig. 3B). H_2O_2 levels were higher in all NaCl-treated shoots relative to controls at day 5; on day 19, the increase remained significant only for the two higher doses of salt (Fig. 3C). Roots exhibited a similar early response to all salt concentrations, whereas by day 19 only 200 mM NaCl still exerted an effect (Fig. 3D). GSH concentrations in shoots and roots likewise revealed a salt-induced response. At day 5, the lowest concentration of NaCl had

no effect, while at day 12 it caused an increase of shoot GSH content that became significant at day 19; both 100 and 200 mM NaCl significantly enhanced the shoot concentration of GSH as compared with controls at all sampling times (Fig. 3E). In roots, 50 mM NaCl also had no effect, while the most significant increases in GSH levels where measured at 5 and 12 days with 100 and 200 mM NaCl; at day 19, only 200 mM NaCl still exerted a significant effect (Fig. 3F).

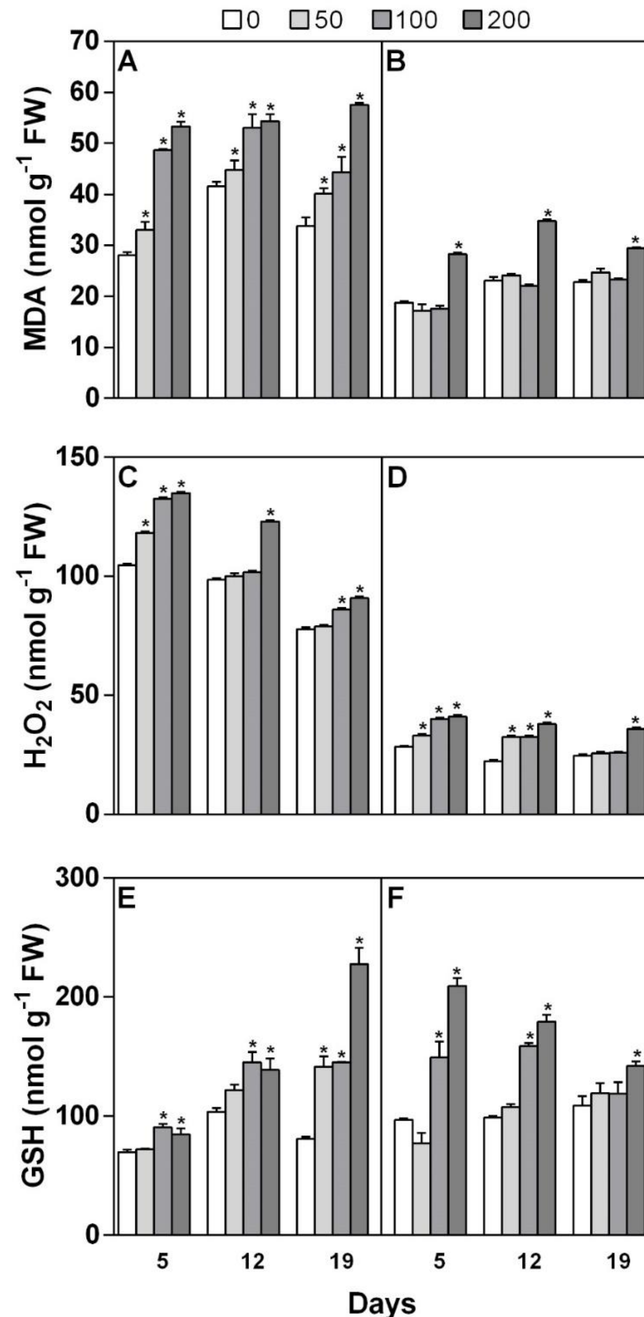


Fig. 3. Malondialdehyde (A, B), H_2O_2 (C, D) and GSH (E, F) concentrations in shoots (A, C, E) and roots (B, D, F) after 5, 12, and 19 days of exposure to 0, 50, 100 or 200 mM NaCl. Data are means ± S.E. (n=3). Asterisks indicate significant differences (*P*<0.05) relative to controls (0 mM NaCl) for each sampling time.

Effects of treatments with 100 mM NaCl and HEs

Pre-treatment with either 0.1 or 1.0 mL L^{-1} IB led to a significant shoot growth recovery, similar for both concentrations, that went *ca*. 22-25% beyond non-saline controls and that was 73.6% (FW) and 77.0% (DW) higher than with NaCl alone (Fig. 4A, C). Conversely, plants pre-treated with SC or methanol exhibited the same reduction in shoot FW and DW as those treated with NaCl alone, regardless of the dose applied. Roots also responded positively to pre-treatment with 1.0 mL L^{-1} IB (PRE-IB 1.0) in terms of FW, but not DW (Fig.4B, D). Improved shoot growth (FW and DW) relative to both control and saline conditions was also registered after post-treatment with IB (POST-IB) irrespective of IB concentration (Fig.4 A, C); a significant (46.2%) growth recovery, but only in terms of DW, was also observed after post-treatment with 1.0 mL L^{-1} SC (POST-SC 1.0) (Fig. 4C). Root growth (FW and DW) in the presence of 100 mM NaCl was likewise improved, even beyond control levels, by POST-IB 1.0 and, as regards DW, also by POST-SC 1.0 (Fig. 4B, D). Methanol had no effect on the growth performance of maize plants.

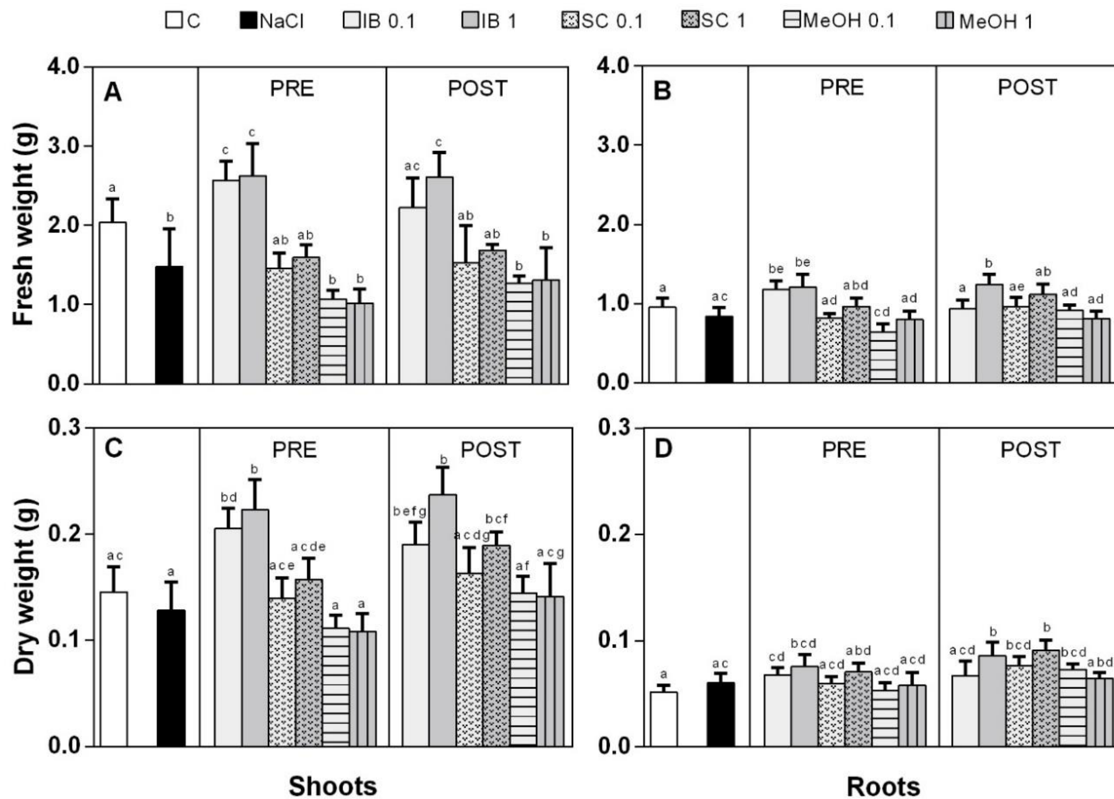


Fig. 4. Shoot (A, C) and root (B, D) fresh and dry weights after 12 days of exposure to 100 mM NaCl in the presence of 0.1 or 1 ml L^{-1} IB, SC, or methanol (MeOH) added two days before (PRE) or two days after (POST) the start of salt treatment. Data are means ± S.E. (n=10). Different letters indicate significant differences (P<0.05).

Under saline conditions, both shoots and roots accumulated Na^+, although the increase, relative to controls, was higher in the former (23-fold) than in the latter (*ca.* 10-fold). By contrast, K^+ levels declined in both organs (25% and 47% in shoots and roots, respectively; Fig. 5 A, B). Na^+ accumulation in shoots was not affected by IB treatments, but it was reduced to the same extent by PRE-SC 1.0 and PRE-MeOH 1.0 (Fig. 5A). Na^+ uptake in roots was most effectively reduced by PRE-MeOH 0.1 and POST-IB 1.0; conversely, it was enhanced by both doses of PRE-IB and POST-SC (Fig. 5B). The decline in shoot K^+ levels in salt-treated *vs* control plants was slightly, but significantly ($p < 0.05$), reverted by PRE-IB 0.1 (FIG 5A). In roots, all treatments with IB as well as PRE-MeOH 1.0 and POST-SC (both doses) increased K^+ levels as compared with salt treatment alone (Fig. 5B). Salt stress caused a strong decline in K^+/Na^+ ratios in both shoots (from *ca.* 45 in controls to 1.5 with NaCl) and roots (from 11.5 to *ca.* 0.7 in control *vs* salt-treated plants). Treatment with HEs or MeOH had no ameliorative effect, except POST-IB 1.0, which doubled the ratio in roots, relative to salt alone, by increasing K^+ without increasing Na^+ levels.

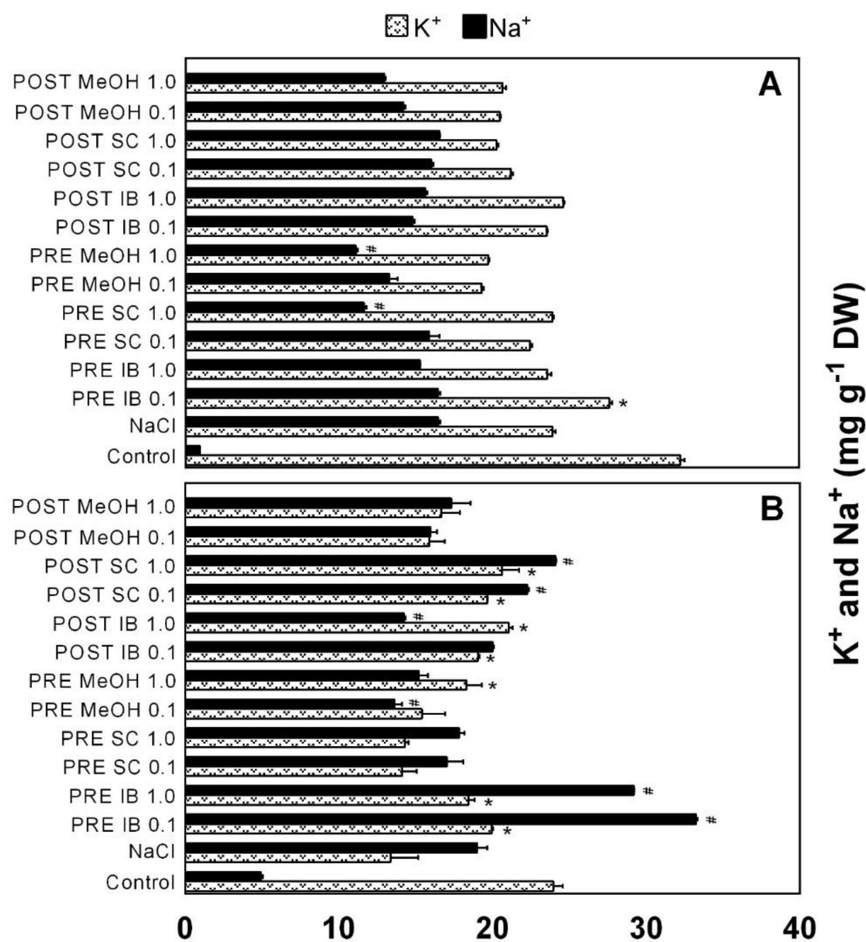


Fig. 5. Shoot (A) and root (B) sodium (Na^+) and potassium (K^+) concentrations after 12 days of exposure to 100 mM NaCl in the presence of 0.1 or 1 ml L^{-1} IB, SC, or methanol (MeOH) added two days before (PRE) or two days after (POST) the start of salt treatment. Data are means ± S.E. (n=3).

The salt-induced decline in Chl*a*, Chl*b* and their sum as well as that in total carotenoids was reverted only by pre-treatment with 0.1 or 1.0 mL L^{-1} IB (Fig. 6A). Post-treatment with IB, however, did not produce the same positive effects (Fig. 6B). Both pre- and post-treatments with SC and MeOH had no effect or even exacerbated the negative response in terms of pigment concentrations (Fig. 6). PRE-IB (both doses) also decreased the Chl*a*/Chl*b* ratio, while other treatments either had no effect or increased it. Finally, the Σcarotenoid/Σchlorophyll ratio ranged from 0.14 to 0.18 and did not change notably in relation to the treatments (data not shown).

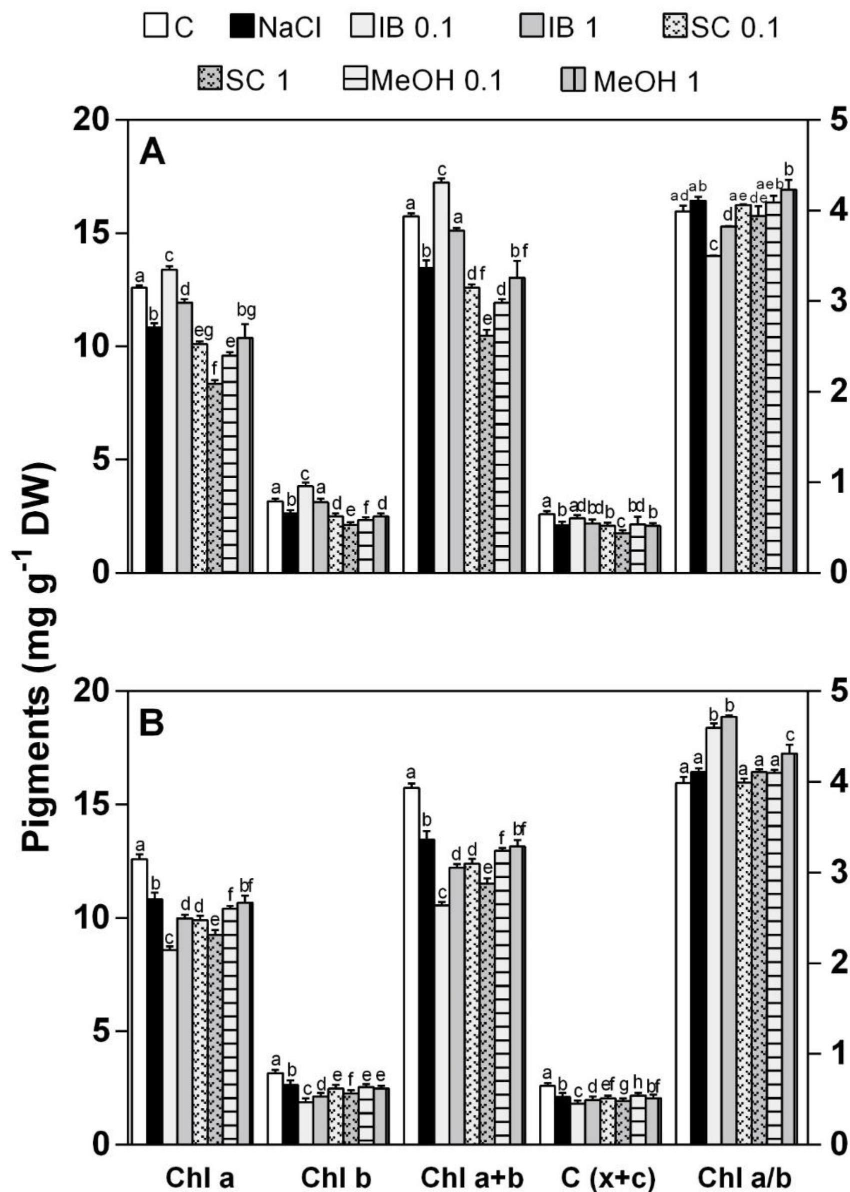


Fig. 6. Concentration of photosynthetic pigments and Chl*a*/Chl*b* ratios after 12 days of exposure to 100 mM NaCl in the presence of 0.1 or 1 ml L^{-1} IB, SC, or methanol (MeOH) added two days before (A) or two days after (B) the start of salt treatment. Data are means ± S.E. (n=3). Different letters indicate significant differences ($P<0.05$).

Upon treatment with IB (both doses), recovery of proline to control levels was observed, without differences between pre- and post-treatment; all other treatments resulted in no change relative to salt alone or even further accumulation (with methanol) of this salt stress-related compound (Fig. 7A, B).

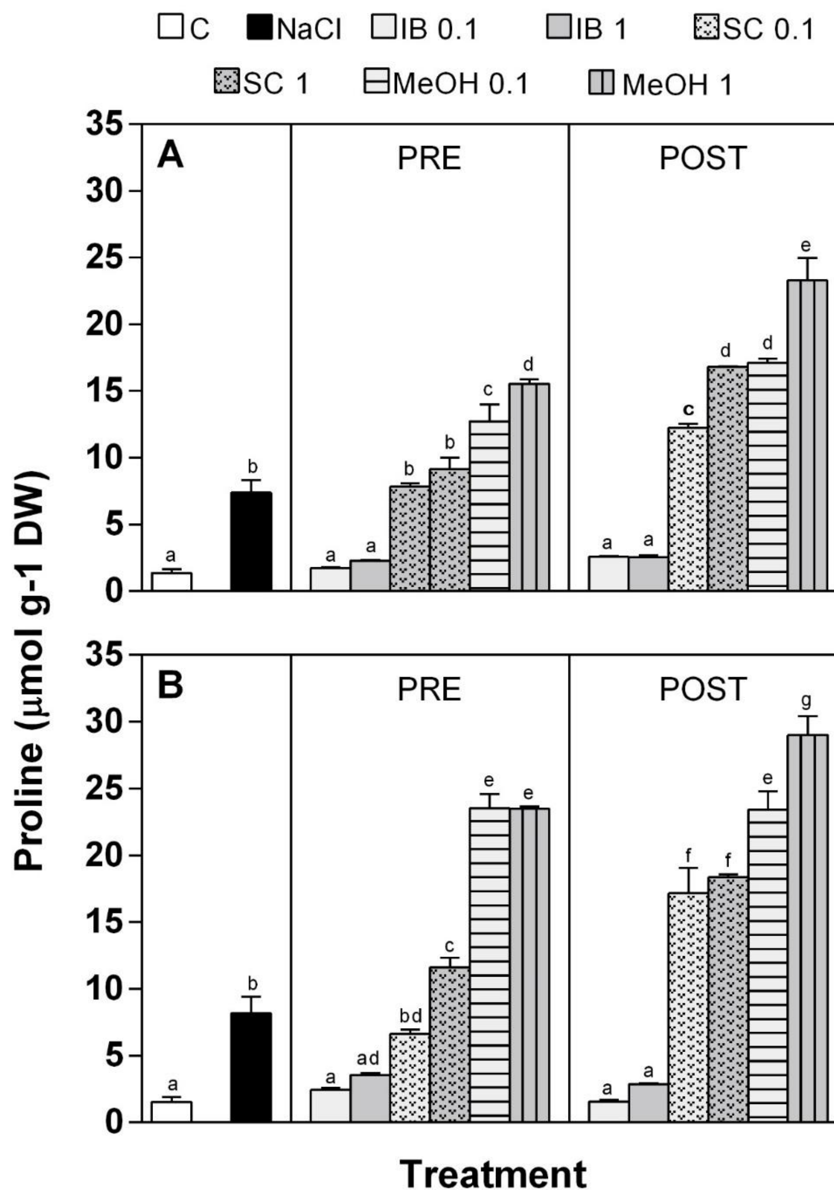


Fig. 7. Proline concentration in shoots (A) and roots (B) after 12 days of exposure to 100 mM NaCl in the presence of 0.1 or 1 ml L^{-1} IB, SC or methanol (MeOH) added two days before (PRE) or two days after (POST) the start of salt treatment. Data are means ± S.E. (n=3). Different letters indicate significant differences (*P*<0.05).

After pre-treatment with either dose of IB, MDA accumulation under saline conditions, indicative of oxidative stress, returned to control values in shoots, although the effect was not significantly different from that of PRE-MeOH 0.1 (Fig. 8A). In roots, all pre-treatments reduced MDA accumulation as compared with NaCl alone, with PRE-IB 0.1 producing the strongest ameliorative effect (Fig. 8B). POST-IB 0.1 also strongly contrasted the salt-induced MDA accumulation in both organs; in shoots, this beneficial effect was significantly stronger than with pre-treatment, while in roots pre-treatment was better than post-treatment. Post-treatment with the lower dose of SC and methanol exerted a similar stress-mitigating effect, but only in roots, so the effect was due to the solvent (Fig. 8A, B).

Salt-induced accumulation of H_2O_2 in shoots and roots was slightly, but significantly, mitigated by all pre-treatments and to the same extent. In post-treatment, however, 0.1 mL L^{-1} IB caused the strongest reduction in shoot H_2O_2 production, while other treatments had no or a negative effect (Fig. 8C, D). In roots, POST-IB 0.1 was again the most effective treatment, while all other post-treatments (except 1.0 mL L^{-1} MeOH) led to only a slight decline in H_2O_2 levels as compared with salt alone (Fig. 8D).

Salt-induced GSH content in shoots was slightly, but significantly, decreased by PRE-IB 1.0, while neither SC nor methanol had any effect. When given after the start and for the entire duration of the salt treatment, none of the extracts contrasted the rise in shoot GSH concentration (Fig. 8E). In roots, the response was quite different. In fact, a significant increase in GSH relative to NaCl was observed, both after pre- and post-treating plants with SC and methanol, while IB had no effect (Fig. 8F).

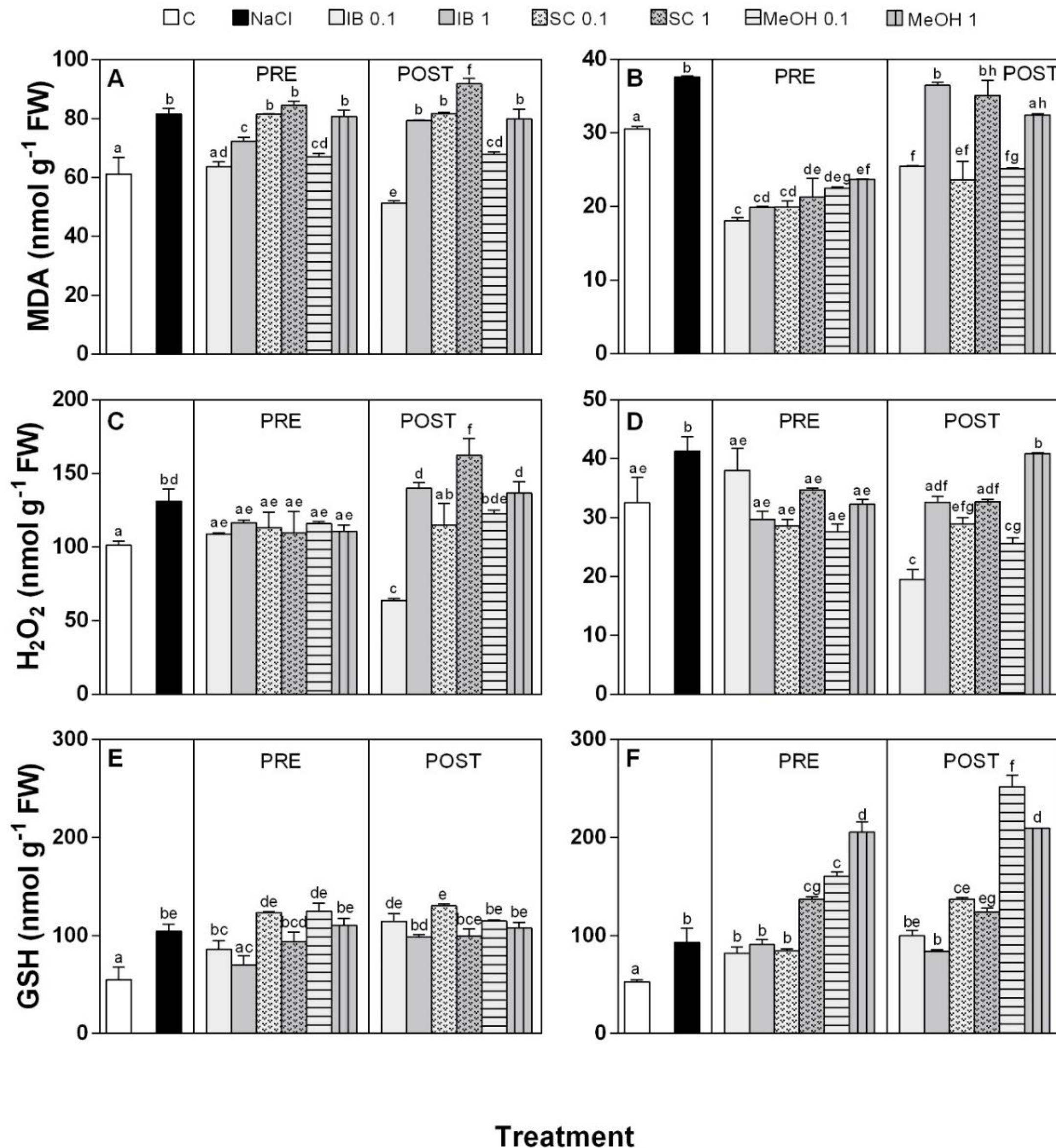


Fig. 8. Malondialdehyde (A, B), H_2O_2 (C, D) and GSH (E, F) concentrations in shoots (A, C, E) and roots (B, D, F) after 12 days of exposure to 100 mM NaCl in the presence of 0.1 or 1 ml L^{-1} IB, SC or methanol (MeOH) added two days before (PRE) or two days after (POST) the start of salt treatment. Data are means ± S.E. (n=3). Different letters indicate significant differences ($P<0.05$).

Effects of treatment with 200 mM NaCl and IB

The efficacy of the extract treatment that gave the best results with 100 mM NaCl (i.e., 1.0 mL L^{-1} IB) was tested with the higher dose of salt (200 mM NaCl) albeit for a shorter duration (8 days). As shown in Table 4, both pre- and post-treatment with IB resulted in a growth stimulation of shoots and roots relative to the salt treatment both in terms of fresh and dry biomass. Pre-treatment with IB improved shoot growth to a greater extent than the post-treatment: *ca*. 53% (FW) and 78% (DW); in

roots, FW and DW increments (relative to salt) were of the order of 50%. Although methanol also stimulated growth relative to salt, the increase was always lower than that of IB, so that net increases were around 30-40%. These positive effects on growth were not, however, accompanied by significant changes in the other parameters. Thus, the decline in photosynthetic pigments and the accumulation of proline, MDA, H_2O_2, and GSH induced by 200 mM NaCl were not mitigated, either by IB or methanol (data not shown).

	Shoot				**Root**			
	Fresh weight variation (%)							
	1 ml l^{-1} IB		**1 ml l^{-1} MeOH**		**1 ml l^{-1} IB**		**1 ml l^{-1} MeOH**	
VS	**pre**	**post**	**pre**	**post**	**pre**	**post**	**pre**	**post**
NaCl	**+ 53.1**	**+ 31.2**	**+ 25.2**	**+ 4.4**	**+ 50.9**	**+ 29.3**	**+ 10.4**	**- 5.7**
	Dry weight variation (%)							
VS	**pre**	**post**	**pre**	**post**	**pre**	**post**	**pre**	**post**
NaCl	**+ 77.8**	**+ 43.4**	**+ 44.4**	**+ 10.1**	**+ 47.5**	**+ 22.0**	**+ 16.9**	**- 5.1**

Table 4. Percent variation in shoot and root FW and DW relative to NaCl after 8 days of exposure to 200 mM NaCl and pre- or post-treatment with 1 mL L^{-1} IB or methanol.

Discussion

Present results indicate that the maize genotype used in this study was moderately sensitive to salt stress. Indeed, reductions in shoot and root growth (except root DW) of 20-33% were observed with 100 mM NaCl. This is in accord with a previous study, aimed at investigating the genetic variability of salt tolerance in maize, in which a reduction in growth from 20 to 80% was already evident at 100 mM NaCl in all tested accessions (Hoque et al., 2015). In the present study, the response to 100 mM salt was characterized by changes in several biochemical parameters, including photosynthetic pigments, proline, oxidative markers such as MDA levels and H_2O_2. Salt stress also caused a nutrient imbalance resulting in a lower K^+/Na^+ ratio. Moreover, all growth, and biochemical changes observed under saline treatments were in general more evident in shoots than in roots, even though the amount of Na^+ accumulated in the two plant portions was similar in absolute terms. The analyses performed in this work also indicate that biochemical salt-induced responses were concentration- and time-dependent. Thus, photosynthetic pigments, proline, MDA, and H_2O_2 levels were, generally speaking, higher with 200 mM NaCl than with the lower doses of salt. In particular, pigments generally declined and proline accumulated in a clear dose-dependent manner. Intensity of the responses also increased with time, except for the oxidative markers (i.e., MDA, H_2O_2, GSH), which instead show a short-term response (day 5) even at the lowest dose of salt. This is in agreement with Ashraf et al. (2018),

who observed a more pronounced accumulation of MDA and H_2O_2 levels at early growth stages compared to later ones in salt-treated maize.

IB and SC were tested for their possible role as biostimulants on maize plants subjected to salt stress. The responses appear to be differentially modulated by the two extracts, and chemical composition may account for these differences. Indeed, only IB, even at the lower concentration tested, was able to recover the performance of stressed plants in terms of growth, photosynthetic pigments content, and levels of salt stress markers, such as proline, lipid peroxidation products, and H_2O_2. Thus, IB can be regarded as a potential biostimulant able to mitigate salt stress. Present results also indicate that the biostimulant activity of IB, in salt-stressed maize plants, strictly depended on timing of treatment. Thus, whereas shoot growth recovery and reduction of proline accumulation occurred when plants were either pre-treated or post-treated with the extract, POST-IB 0.1 was most effective in mitigating oxidative stress. Conversely, the positive effect on photosynthetic pigments, i.e., recovery of Chl*a* and *b* levels, only occurred when IB was applied to plants before salt stress; when applied two days after salt treatment, the stress-alleviating effect was no longer observed. Moreover, the decrease in Chl*a*/*b* ratio induced by PRE-IB relative to control and salt-treated plants may be of particular relevance, since Chl*b* is favoured over Chl*a* in exerting a protective function of the photosystems, due to the lower photo-oxidation rate of the former compared to the latter (de Souza et al., in press). A similar alleviating effect on chlorophyll content in NaCl-stressed bean plants was reported after foliar application Howladar et al. (2014) or seed pre-soaking with *Moringa oleifera* leaf extract (Rady et al. 2013). Ertani et al. (2016) also reported that Chl*a* and Chl*b* were differentially affected by treatment with various plant extracts (blueberry, hawthorn, red grape skin). In *Salicornia*, adaptation to stress was revealed by the maintenance of a high ratio (0.12 to 0.14) of photo-protective pigments (i.e., carotenoids) against light-harvesting chlorophylls (de Souza et al. in press). In our study, neither salt alone nor salt combined with HEs had any effect on this ratio.

Proline accumulation under various types of abiotic stress (Scoccianti et al., 2016), including salinity, is a common response and overproduction of this compatible solute in transgenic plants confers a higher salt stress tolerance (Kishor et al. 1995). In addition to their osmoprotective role, organic osmolytes, such as proline, contribute to contrasting oxidative stress (Szabados and Savouré, 2010). Interestingly, IB, at both doses and times of application, reverted the salt-induced proline increase in shoots and roots to control levels, a strong indication in favor of its stress-mitigating effect**.**

Sodium and chloride are responsible for both osmotic stress and ion-specific toxicity that significantly reduce crop growth and yield. The best characterized mechanisms of tolerance involve limiting Na^+ uptake, excluding Na^+ from leaves, and efficient vacuolar compartmentation of Na^+ (Munns and Tester 2008). Soil salinity also causes ion imbalance by affecting, for example, the uptake of

potassium (K^+), which is an essential macronutrient in plants. Maintaining high K^+/Na^+ ratios is regarded as a major strategy for coping with salinity stress in salt-sensitive (glycophyte) species (e.g., cereals, such as barley) and this is often achieved by K^+ retention rather than Na^+ exclusion (Shabala and Pottosin 2014). The positive effect of moringa leaf extract on shoot K^+ under high salinity was previously reported in wheat (Yasmeen et al., 2013**).** As regards ion homeostasis under salt stress, here we show that none of the treatments improved the K^+/Na^+ ratio in shoots, whereas in roots, post-treatment with the higher dose of IB was able to slightly ameliorate this parameter; the ratio increased due to enhanced K^+ levels and no change in Na^+ levels.

Salt stress is known to result in extensive lipid peroxidation, a parameter that has often been used as indicator of oxidative damage in membranes (Miller et al. 2010). Depending on timing and concentration, both HEs as well as methanol were able to reduce MDA production in salt-stressed roots. In shoots, however, post-treatment with the lower dose of IB was most effective in mitigating salt-induced lipid peroxidation as well as in reducing both shoot and root H_2O_2 concentration. In spite of the dose- and time-dependent ameliorative effect of IB on oxidative stress, growth recovery under saline conditions was observed when the extract was applied at either dose and both in pre- and post-treatment. This suggests that the positive effect of IB on growth was not simply the consequence of reduced oxidative stress, as also confirmed by the results of the experiment conducted using 200 mM NaCl, in which growth recovery was observed, without a substantial change in biochemical parameters.

It is worth noting that partial recovery from salt-induced oxidative stress was also observed with methanol alone, at the lower dose and in both organs. Some authors have investigated the biological functions of solvents used in plant experiments (Savvides et al. 2016), and a clear role of ethanol and methanol in influencing several plant responses has been reported. In *Arabidopsis thaliana* and rice plants, the application of exogenous ethanol enhanced salinity stress tolerance by regulating ROS-related genes and enhancing ROS detoxification (Nguyen et al. 2017); an increase in tolerance to chilling stress has also been reported in rice plants (Kato-Noguchi 2008). As regards methanol, its positive effects on growth and water use efficiency has been documented in various plant species (Behrouzyar et al. 2016), even though they strictly depend on exposure time, tissue morphology and, especially, application method. In tomato, Arabidopsis, and tobacco plants, foliar spraying with methanol enhanced plant growth under normal conditions, while root applications caused severe damage (Rowe et al. 1994; Ramirez et al. 2006). Nevertheless, it has to be underlined that the methanol concentrations (0.01-0.1%, v/v) applied by us to maize roots were much lower than those used in the above-cited studies, and this can explain the, sometimes, ameliorative action or ineffectiveness of this solvent.

In plants, glutathione (G) is an essential component of the cellular antioxidant defense system. It is the substrate of glutathione peroxidase and glutathione-S-transferases, enzymes involved in the removal of ROS, and the ascorbate-G cycle is regarded as the principal means of superoxide and H_2O_2 removal. Reduced/oxidized forms of G (GSH/GSSG) influence the redox status of plant cells. Although increases in GSH levels have been measured in response to chilling, heat shock, and other forms of abiotic stress, the numerous publications on this topic (Tausz et al. 2004 and references therein) indicate that the GSH/GSSG ratio may change one way or another, suggesting that the role of G is particularly complex. In our study, maize plants exposed to 100 mM NaCl exhibited significantly higher GSH levels as compared with non-saline controls, with only a slight reduction in shoot FW/DW and no inhibition of root biomass, suggesting that GSH may have contributed to salt acclimation. The strongest increase in GSH levels, however, occurred with 200 mM NaCl, which significantly depressed plant biomass. Thus, under more severe conditions, although the plant activated this antioxidant response, it was not enough to ensure normal growth. The salt-induced increase in GSH concentrations could be interpreted as an "overcompensation" to keep G in its reduced, active form (Tausz et al. 2004) or an interference with cellular metabolism limiting the conversion of GSH to GSSG. Indeed, De Azevedo Neto et al. (2006) reported that G reductase activity in leaves of salt-stressed maize plants (both salt-tolerant and salt-sensitive) was greater than in control plants. Ruiz and Blumwald (2002) showed that synthesis of cysteine and GSH increased significantly when *Brassica napus* was exposed to salt stress. Similarly, the contents of GSH, MDA, $O_2^{\bullet-}$, and H_2O_2 were significantly increased in bean plants stressed with 200 mM NaCl (Latif and Mohamad 2016). Overall, these results suggest that the processes leading to the accumulation of GSH are salt stress-elicited. The pattern of changes in GSH levels in response to spelt HEs and methanol showed that, in shoots, the higher dose of IB reverted salt-induced GSH accumulation when plants were exposed to 100 mM NaCl. On the other hand, roots responded in a relevant manner only to SC and, especially, methanol, which, instead, caused a further increase in GSH levels. Thus, although GSH is regarded as having a positive (antioxidant) role under stress conditions, our results indicate that GSH increased with increasing stress (100 *vs* 200 mM NaCl), so that the effect of IB can be considered as stress-mitigating, while SC and methanol had the opposite effect.

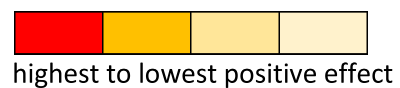
Extract composition plays an outstanding role in determining the efficacy of biostimulants (Bulgari et al., 2015). The phytochemical investigation of the two spelt HEs revealed that their chemical composition was rather different, both in qualitative and quantitative terms. In particular, SC contained, besides phenolic and hydroxycinnamic acids, also organic and fatty acids, which were absent in IB, while the latter had a much higher level of ferulic, *p*-coumaric, and caffeic acids compared to the former. Indeed, most hydroxycinnamic acids in spelt and einkorn (*Triticum*

monococcum L.) grains are localized in cell walls where they are bound to structural components, such as cellulose, lignin, and arabinoxylans (Hidalgo and Brandolini 2014) and this might explain their higher levels in IB compared to SC. The greater amount of hydroxycinnamic acids, especially ferulic and *p*-coumaric acid, in IB could account for its higher efficacy, compared to SC, in counteracting the negative impact of salt stress. Besides the numerous functions of ferulic acid in plant growth and development (e.g., cell wall lignification), a major physiological role is likely to be its potent antioxidant capacity (Graf 1992). Thus, the higher antioxidant potential of IB, revealed by attenuated lipid peroxidation and H_2O_2 accumulation, may be due to its higher ferulic acid content and may have contributed to alleviate the toxicity generated by salt stress. Moreover, the higher fatty acid content of SC may render this extract more hydrophobic, thus less prone to interact with the root surface and be absorbed. Differences in the biostimulatory effects in maize plants of two phenol-containing extracts, deriving from lignin-rich biorefinery wastes, have been recently reported; both extracts were able to act as biostimulants, but at different concentration ranges and through diverse mechanisms, for example, via modification of the plant's hormonal balance (Savy et al. 2017). Other authors also investigated the potential biostimulant effect of polyphenol-enriched fractions derived from plant by-products. Maize plants supplied with two different doses of extracts obtained from dry apple and blueberry residues displayed a significant increase in root and leaf biomass and a higher content in macronutrients and proteins; extracts also exerted a positive impact on secondary metabolism associated with the synthesis of phenolic compounds (Ertani et al. 2011b). Phenolic acids also have allelopathic properties. Allelopathic water extracts (AWE) containing, among other compounds, phenolic acids, can improve tolerance to abiotic stresses when applied to crops, such as wheat (Farooq et al. 2018).

Finally, a growth-stimulating effect of IB was also observed in maize plants grown under non-saline conditions, confirming its potential as biostimulant. This effect could be accounted for by the phytohormone (gibberellin, auxin)-like activity reported for phenols (Ertani et al. 2016; Savy et al. 2017) and their ability to influence endogenous phytohormone levels (Einhellig, 2004).

In conclusion, we show here that polyphenol-containing methanolic extracts prepared from spelt husks have a growth-stimulating and stress-mitigating effect on maize plants by acting on different targets, including accumulation of compatible solutes, photosynthetic pigments, oxidative stress, and ion balance. The plethora of mechanisms activated by the extract can be related to its chemical complexity, as occurs with all biostimulants, with IB generally exerting a more positive action than SC (Fig. 9). Work is in progress to investigate the effect of spelt HEs on plant polyphenol metabolism and regulation of genes involved in polyphenol biosynthesis. Experiments are also underway to test extracts prepared by using more environmentally compatible extraction procedures.

		PRE						POST					
		IB 0.1	IB 1.0	SC 0.1	SC 1.0	M 0.1	M 1.0	IB 0.1	IB 1.0	SC 0.1	SC 1.0	M 0.1	M 1.0
FW	S												
	R												
DW	S												
	R												
Chl*a*	S												
Chl*b*	S												
Chl*a*+*b*	S												
Cc+x	S												
proline	S												
	R												
MDA	S												
	R												
H_2O_2	S												
	R												
GSH	S												
	R												


highest to lowest positive effect

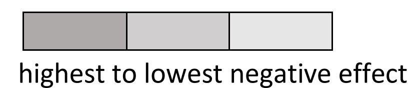

highest to lowest negative effect

Fig. 9 Graphical representation of the effects on growth and metabolic parameters of pre- or post-treatments with IB, SC or methanol in shoots (S) and roots (R) of maize plants grown under saline (100 mM NaCl) conditions. Darker and lighter colour shadings represent relatively higher and lower values, respectively.

Funding

This research was funded by Progetti Valorizzazione DiSB - Università di Urbino 2017/ 2018 to V.S. and by Ricerca Fondamentale Orientata - Università di Bologna to F.A. and S.B.

Declarations of interest: none

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