Bio-priming mitigates detrimental effects of salinity on maize improving antioxidant defense and preserving photosynthetic efficiency

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Bio-priming enhances salinity tolerance in maize seedlings.

Panuccio M.R., [1] Chaabani S[2]., Roula R[2]., and Muscolo A[1]*

[1] Agriculture Department, Mediterranea University, Feo di Vito-89122 Reggio Calabria Italy

[2] High Agronomic Institute of chott Mariem University of Sousse

*Corresponding author: Professor Adele Muscolo

Agriculture Department, Mediterranea University,

Feo di Vito-89122 Reggio Calabria Italy

Email: amuscolo@unirc.it

Tel: 003909661694364

ABSTRACT

Salinity is an abiotic stress which seriously affects crop production over the world, particularly in arid and semi-arid regions, with harmful effects on germination, growth and yield. Maize (Zea mays L.), cultivated in a wide spectrum of soil and climatic conditions, is the third most important cereal crop after rice and wheat, moderately sensitive to salt stress. A saline level more than 0.25 M NaCl damages maize plants, causing severe wilting. In this study, the effects of hydro-priming (distilled water) and bio-priming (*Rosmarinus officinalis L.* and *Artemisia L.* leaf extracts) on seed germination and seedling growth of maize, under 100 mM NaCl salinity were investigated. The factorial experiments were carried out in greenhouse under controlled condition (25°C in 12/12 day/night) based on a completely randomized design with three replicates. Results showed that both hydro- and bio-priming increased germination percentage and germination indexes in maize seeds. Rosmarinus extract was the most effective in inducing salt resistance in 30 days old seedlings, with beneficial effects in the strengthening of the antioxidant system and in the maintenance of a higher photosynthetic efficiency under salt stress condition.

Keywords: Antioxidants; Artemisia; Priming; Rosmarinus, Salinity, Zea mays.

1. Introduction

Salinity is one of the major abiotic stresses that negatively affects crop productivity. More than 800 million hectares of land worldwide are affected by either salinity (397 million hectares) or sodicity (434 million hectares) with a decline, by more than 50 percent, in average yield of the major crop plants (FAO, 2011; Munns and Tester, 2008). Salt stress occurs in areas where soils are naturally high in salts and precipitations are low and/or where irrigation, hydraulic lifting of salty underground water, or invasion of sea water in coastal areas bring salt to the surface soil. NaCl is the predominant salt causing salinization worldwide (Munns and Tester, 2008). The most widely accepted effects of salinity are decrease in germination percentage, germination rate and in growth and metabolism of seedlings by creating an external osmotic potential that prevents water uptake, or by causing specific ion toxicity and ion imbalance. Salinity affects also cellular metabolism including photosynthesis and synthesis of compatible solutes called “osmolytes” like proline, sugars (Amirjani, 2011) and proteins (Sen and Alikamanogli, 2011). Maize (Zea mays L.) is the third most important cereal crop after rice and wheat and it is grown under a wide spectrum of soil and climatic conditions. It is an important C4 plant from the Poaceae family, moderately sensitive to salt stress (Farooq et al., 2015). A saline level containing more than 0.25 M NaCl may damage maize plants and stunt growth causing severe wilting (Menezes-Benavente et al., 2004). For this reason, the aim of this work was to find a method that could alert and/or attenuate the negative impact of salts increasing the tolerance to salinity in maize plants. Seed priming is an easy, low cost and low risk technique recently used to overcome the salinity problem in agricultural lands (Ibrahim, 2016; Chen and Arora, 2013) It is a pre-sowing treatment and the most important priming treatments include halo-priming (soaking seeds in inorganic salt solutions), solid matrix priming (treatment of seeds with solid matrices), osmo-priming (soaking seeds in

solutions of different organic osmotic) and bio-priming (using a priming mixture integrated with bioactive molecules or beneficial microorganisms) (Nouman et al., 2014). All the above listed priming are able to 1) stimulate metabolic processes involved in the early phases of germination, producing high germination rate and great germination percentage 2) induce uniformity and faster emergence of seedlings from primed seeds, bring vigorous growth in adverse conditions (Imran et al., 2013).

Maher et al. (2013) and Tzortzakis (2009) indicated also that seed priming in fenugreek, endive and chicory increased final germination percentage, germination speed and radicle length over the non-primed treatments in saline conditions. Generally, farmers facing with saline problems, cannot reclaim soil, or use expensive plant hormones, antioxidants or nutrients for seed priming (Basra et al., 2011; Imran et al., 2013). So, there is the need to explore new plant growth enhancers, naturals and environmentally friendly which should be reliable and economically sustainable under prevailing salinity conditions. This study was planned to investigate the potential of *Rosmarinus officinalis L*. and *Artemisia L*. aqueous extracts as seed bio-priming agents. These species, mostly found in arid and semi-arid areas, are widely distributed in Mediterranean countries, and represent new sources of natural antioxidant and antimicrobial agents. Thus, we hypothesized that seed priming with these natural extracts may alleviate salt stress in germinating maize seeds and improve seedling establishment by modulating antioxidants, photosynthetic pigments, ionic homeostasis and photosynthesis.

2. Materials and methods

2.1 Extract preparation and chemical characterization

Rosmarinus (*Rosmarinus officinalis* L.) and Artemisia (*Artemisia erba alba* L.) fresh leaves were collected between February and March 2016 from the region of Chouachi-HadjebAyoun (Tunisia 35° 23′ North 9° 32′ Est), identified according to the flora of Tunisia (Pottier-Alapetite, 1981), were

dried and grounded . Aqueous extracts were prepared by soaking the dried leaves, overnight in distilled water (1:10 w/v). The suspensions were filtered with Whatman's paper. Extracts have been analyzed by HPLC analysis with a Knauer (Berlin, Germany) apparatus interfaced to a DAD detector (model 2600). HPLC-DAD technique is commonly used to detect antioxidants in many matrix (Giuffrè, 2013). For separation a binary gradient was prepared: (A) bi-deionized water and (B) acetonitrile, both were acidified at pH 3 by with formic acid. The applied gradient was: 0-20 min, 95% A and 5% B; 20-50 min, the eluent B increased from 5 to 40%; 50-60 min, eluent B increased from 40% to 95%; 60-65 min, the eluent B decreased from 95% to 5%; 65-70 min 95% A and 5% B in isocratic. The analysis was performed with a constant flow rate of 1 ml/min. A Knauer C18 Eurosphere II separation column (Berlin, Germany) was used (250 mm length x 4.6 mm internal diameter x 5 μm particle size). All standard components (purity ≥ 97%) were purchased from Sigma Co. (St. Louis, MO). All solvents and reagents (HPLC grade) were purchased from Panreac (Barcelona, Spain). The identification of unknown components in the extracts were performed by comparison the retention times of the detected compounds with those of appropriate standards.

2.2 Seed priming

For each treatment, 30 health seeds were surface sterilized with 5% sodium hypochlorite for 5 min and then rinsed with sterile bi-distilled water. Aqueous extracts of Rosmarinus (RP) and Artemisia (AP) were used for seed priming (Durak et al., 2016) . Maize seeds were soaked in extracts ratio 1:5 (w/v) for 24 hours in darkness at room temperature. The Hydro-primed (HP), bio-primed (RP, AP) and the un-primed (CNP) seeds were dried back to their original moisture contents at room temperature and were used as control. The experiments were performed in triplicate.

2.3 Assessment of seed germination and morphological, physiological and biochemical responses of seedlings

After priming, seed germination tests were carried out. 10 seeds for each treatment (priming and control) were placed in plastic pots (26 cm diameter × 27 cm height) filled with sand and equilibrated with water (control) or NaCl 100 mM. 70% of field capacity was maintained with distilled water. Each treatment was replicated three times. Experiments were carried out in climatic chamber at 25°C in a 12/12-h photoperiod for 30 d. Seeds were considered germinated when a visible coleoptile protrusion was observed. The germinated seeds were counted daily and the germination percentage was calculated at the 7 days. Germination index (GI), mean germination time (MGT), time to reach 50% germination (T_{50})and total germination percentage TG were calculated as follow:

$GI=\sum(G_t/T_t)$; $MGT=\sum(G_t \times T_t)/\sum G_t$ and $T50= t_i + [(N/2-n_i)\ (t_j - t_i)\ ]/(n_j-n_i)$.

Gt is the number of germinated seeds on day t, T_t is the time corresponding to G_t in days, N is the final number of germination. ni and nj are the cumulative number of seeds germinated by counts adjacent at times when $ni<N/2<nj$ (Zhang et al., 2007).

Root length and shoot height were measured manually with a ruler and dry weights were determined after drying at 80 °C for 24 h.

2.4 Photosynthetic pigments in seedlings

Fresh leaves, 0.05g for each treatment, were mixed with 2.5 ml of pure ethanol and incubated for 24 h at 4°C in the dark. After, the samples were centrifuged for 10 minutes at 7000 rpm. For chlorophyll and carotenoid analysis, the absorbance of supernatants was recorded at 649 nm, 665 nm, and 470 nm and their concentrations (mg/g fresh weight) were calculated using Lichtenthaler's equations. (Lichtenthaler,

1987). Anthocyanins were extracted incubating 20 mg of fresh leaf in 0.5 ml of methanol: HCl (99:1). After 24 h incubation at 4°C, samples were centrifuged at 6000 g for 10 min at 4°C. (Panuccio et al., 2016). The absorbance was read at 530 and 657 nm and anthocyanin content (μg anthocyanin /g fresh weight) was calculated according to the following equation: [A530nm – (0,025*A657nm)*ml of extract] /g fresh weight

2.5 Chlorophyll fluorescence imaging

Photosynthetic efficiency of primed and un-primed seedlings in absence and in presence of salinity was evaluated by using an Imaging PAM Fluorometer (Walz, Effeltrich, Germany). The chlorophyll fluorescence parameters detected were: Maximum quantum yield of PSII photochemistry (Fv/Fm); Effective quantum yield of PSII photochemistry (Y(II)); Quantum yield of regulated energy dissipation at PSII (Y(NPQ); Quantum yield of non-regulated energy dissipation at PSII (Y(NO); Non photochemical quenching coefficient (NPQ) and Electron transport rate (ETR).

2.6 Antioxidant enzyme assay

Fresh leaves, 0.5 g, were extracted (1:3 w:v) using a chilled mortar (4°C) in a 0.1 M phosphate buffer solution (pH 7.0), containing 100 mg polyvinylpolypyrrolidone (PVPP) and 0.1 mM Na_2-EDTA. The homogenates were centrifuged at 14000 g at 4°C for 15 min and the supernatants were used. All enzyme activities were measured at 25 °C by a UV–visible light spectrophotometer (UV-1800 CE, Shimadzu, Japan).

Catalase (CAT, EC 1.11.1.6) activity was assessed, evaluating the disappearance of H_2O_2 at 240 nm. The extinction coefficient (ε) = 0.036 $mM^{-1}cm^{-1}$ was used (Beaumont et al.,1990).

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was evaluated monitoring the decrease in absorbance at 290 nm for the oxidation of ascorbate (Nakano and

178 Asada,1981). Enzyme activity was quantified using the molar extinction coefficient for
179 ascorbate (2.8 $mM^{-1}cm^{-1}$).
180 Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by recording the
181 decrease in absorbance of formazan produced from NBT, at 560 nm (Gupta et al.,
182 1993). The reaction mixture (3mL) contained 0.1 ml of 200 mM methionine, 0.1 ml of
183 2.25 mM nitro-blue tetrazolium, 0.1 ml of 3 mM EDTA, 1.5 ml of 100 mM potassium
184 phosphate buffer, 1 ml of distilled water and 0.05 ml of enzyme extract. The reaction
185 was started by adding 0.1 mL of riboflavin (60 µM) and placing the tubes below a light
186 source of two 15 W florescent lamps for 15 min. After, tubes were covered with
187 aluminum paper and the light was switched off. Tubes without enzyme extract were
188 used as control and they developed maximum colour. A non-irradiated complete
189 reaction mixture was the blank. One unit of enzyme activity corresponded to the
190 quantity of enzyme reducing the absorbance (560) nm of samples of 50%, compared to
191 tubes lacking enzymes.
192 All enzymatic activities were expressed as enzyme units (U)/mg fresh weight. One unit
193 of enzyme was the amount of enzyme necessary to decompose 1 mmol of substrate/ min
194 at 25°C.
195

196 *2.7 Total phenols and reduced glutathione analysis*

197 Total phenol content was determined with the Folin–Ciocalteu reagent according to the
198 method of Julkenen-Titto (1985). The reduced glutathione (GSH) was assayed
199 according the method of Jollow et al. (1974). Fresh leaves were homogenized in 3%
200 CCl_3COOH at 4 °C and centrifuged at 1000g for 10 min at 4 °C. The absorbance was
201 measured at 412 nm and related to a calibration curve of GSH solutions.

202 *2.8 Measurement of root morphology*

Seedlings were harvested 30 days after treatments and root weight was measured. Roots were analyzed by using the Epson Expression/STD 1600 scanner and personal computer (Intel Pentium III/500 CPU, 128 MB RAM), Regent Instrument, Inc. Root morphology was detected with the Win-RHIZO image analysis system (Regent Instruments, Quebec, Canada) (Panuccio et al., 2014).

2.9 Ion Analysis

Cations (Na^+, K^+, Ca^{2+}, Mg^{2+}) and anion (Cl_2) were extracted from leaves and analysed by ion chromatography (DIONEX ICS-1100). For anions, 0.500 g of dried material was extracted using 50 ml of anion solution (Na_2CO_3 / $NaHCO_3$ 3.5 mM) under stirring for 20 minutes, then the homogenate was filtered and the chromatographic analysis was carried out. For cations, 1 g of dry material was ashed at 550 ° C for 5-6 hours in a porcelain capsule. The ash was then mineralized for 30 minutes at 100 ° C using 1M HCl solution. Finally, the obtained solution was filtered and analyzed by ion chromatograph, eluent (Methansulfonic acid 20 mM).

2.10 Statistical analysis

The experiments were in randomized complete block design with three replicates. Statistical analyses were performed using one-way ANOVA and mean comparisons were made using Tukey's test ($p<0.05$). All data were analysed using SYSTAT 13.0 software (SPSS Inc.) The germination percentage data were previously subjected to arcsine transformation but were reported in tables as untransformed values.

3. Results

3.1 Composition of Artemisia and Rosmarinus leaf extracts

HPLC analysis showed qualitative and quantitative differences in the composition of the leaf acqueous extracts of the two officinal plants (Table 1). Rosmarinus extract

contained rosmarinic acid (63%) rutin (21%) carnosinic acid (6%) and neochlorogenic acid (2.6%) that were absent in the artemisia extract. In contrast, Artemisia extract contained narirutin (8%), naringin (13%) and protochatecuic acid (2%) that were absent in rosmarinus leaf extract. Chlorogenic (52%) and syringic (6%) acids were present in greater quantity in Artemisia than in rosmarinus leaf extract.

3.2 Seed germination

Bio and hydro-priming, in absence of salinity (0 mM NaCl), increased significantly ($p > 0.05$) total germination percentage (TG %) and germination index (GI) in comparison to untreated seeds (CNP) (Table 2), and decreased MGT and T_{50}. RP and HP were the treatments with the greatest positive effects on maize TG % and GI (Table 2). Under 100 mM NaCl, RP and AP increased the germination (TG %) and GI of about 60-70% compared to CNP, and the T_{50} and MGT values were lower. No significant differences in T_{50} and MGT were observed between bio and hydro-priming treatments (Table 2). Analysis of Variance showed that GI, T_{50} and TG% were mainly affected by priming and salinity, while MGT only by type of priming treatment. The interaction between salinity and priming didn't affect significantly all the germination indices.

Table 1

Chemical composition of leaf aqueous extracts of *Rosmarinus officinalis L*. and *Artemisia L.* Values are the means of three replicates ± Standard Deviation (SD)

Rosmarinus extract		
	mg/l	SD
Gallic Acid	3.864	0.018
Neoclorogenic Ac.	4.092	0.097
Clorogenic Acid	1.961	0.062
Siringic Acid	1.969	0.061
Rutin	32.623	0.060
Rosmarinic Acid	99.116	0.004
Apigenin	4.089	0.050
Carnosol	1.047	0.052
Carnosic Acid	9.183	0.052

Artemisia extract		
	mg/l	SD
Gallic Acid	1.655	0.005
Protocatechic Acid	3.093	0.028
Clorogenic Acid	32.395	0.134
Siringic Acid	6.506	0.108
Narirutin	5.043	0.060
Naringin	7.838	0.035
3,4-Di-O-caffeoylquinic ac.	1.907	0.013
Luteolin	0.518	0.003
Apigenin	2.175	0.010
Kaempferol	0.384	0.019

Table 2.

Effect of NaCl (100 mM) and seed priming on germination indexes of maize. Control no Priming (CNP) Hydro-Priming (HP) Rosmarinus Priming (RP) Artemisia Priming (AP). Values are the means of three experiments ± SE. Different letters indicate significant differences ($P \leq 0.05$) among different plant treatments at the same salt concentration. ANOVA *** P≤ 0.001; **P≤ 0.01; *P≤ 0.05 (ANOVA and mean comparison with Tukey's test).

		MGT			ANOVA summary	
NaCl	**CNP**	**HP**	**RP**	**AP**	Salt	0.02
0	4.07 ±0.19 a	2.96 ±0.26 b	3.07 ±0.06 b	3.14 ±0.06 b	Priming	16.22***
100	3.81 ±0.12 a	3.23 ±0.07 b	3.10 ±0.06 b	3.16 ±0.05 b	Salt x Prim.	1.09
		GI				
NaCl	**CNP**	**HP**	**RP**	**AP**	Salt	5.97*
0	6.17 ±1.31 c	10.91 ±1.7 b	13.6 ±0.97 a	12.04±0.3ab	Priming	29.99***
100	5.03 ±1.16 c	9.38 ±1.41 b	12.18 ±2.3 a	10.52±1.1ab	Salt x Prim.	0.026
		T_{50}				
NaCl	**CNP**	**HP**	**RP**	**AP**	Salt	10.86**
0	4.96 ±0.80 a	3.11 ±0.76 b	2.21 ±0.34 c	3.09 ±0.16 b	Priming	27.43***
100	5.25 ±0.43 a	3.88 ±0.33 b	3.21 ±0.32 b	3.60 ±0.17 b	Salt x Prim.	0.66
		TG %				
NaCl	**CNP**	**HP**	**RP**	**AP**	Salt	5.26*
0	60.1±3c	76.7±5b	96.7±5a	90.3±5a	Priming	22.60***
100	46.7±5d	73.3±1c	90.1±2a	82.3±2b	Salt x Prim.	0.35

Table 3.

Shoot and root length of 30 days old maize seedlings derived from no-primed (CNP), hydro-primed (HP), Rosmarinus (RP) and Artemisia (AP) bio-primed seeds and grown in the absence (0) or in the presence of 100 mM NaCl. Values are the means of three experiments ± SE. Different letters indicate significant differences ($P \leq 0.05$) among different plant treatments at the same salt concentration. * indicates significant differences ($P \leq 0.05$) between different salt concentrations of the same priming treatment.

	Shoot Length	
	0 mM NaCl	**100 mM NaCl**
CNP	39.7±2.5 b*	30.0±2.6 c
HP	48.3±3.1 a	47.5±1.3 a
RP	48.0±2.3 a	48.0±2.3 a
AP	50.3±1.5 a*	41.0±1.0 b
	Root Length	
	0 mM NaCl	**100 mM NaCl**
CNP	31.3±2.5c*	25.1±1.5c
HP	47.5±2.3b*	30.2±1.0b
RP	57.3±1.5 a*	45.3±1.3a
AP	57.0± 2.0a*	47.5±2.0a

3.3 Seedling growth

Hydro- and bio-priming increased shoot and root length both in presence and in absence of salinity (Table 3). Bio-priming stimulated better their growth in comparison to HP and control. No significant differences between the two bio-priming treatments (RP and AP) were observed both in absence and in presence of salinity. In absence of salinity, total root length was increased mainly by HP, instead in presence of 100 mM NaCl, the greatest elongation was observed in presence of RP and AP (Table 4). Root volume increased in primed seedlings, in presence and in absence of salinity, and the greatest enhancement was observed with RP treatments.

Table 4

Root parameters of 30 days old maize seedlings derived from no-primed (CNP), hydro-primed (HP), Rosmarinus (RP) and Artemisia (AP) bio-primed seeds and grown in the absence (0) or in the presence of 100 mM NaCl. Values are the means of three replicates ± SE. Different letters indicate significant differences ($P \leq 0.05$) among different plant treatments at the same salt concentration. ANOVA *** P≤ 0.001; **P≤ 0.01; *P≤ 0.05 (ANOVA and mean comparison with Tukey's test).

Total root length (cm)					ANOVA summary	
NaCl	**CNP**	**HP**	**RP**	**AP**	Salt	5753.5***
0	5454.7 ±25.6b	8113.6 ±11.0 a	4947.0 ±22.0c	5728.7 ±15.1b	Priming	3059.1***
100	2598.6 ±11.1d	3750.9 ±13.5c	5077.1 ±43.02a	4950.7 ±15.2b	Salt x Prim.	2651.1***
Root volume (cm^3)						
NaCl	**CNP**	**HP**	**RP**	**AP**	Salt	1561.3***
0	1.83 ±0.01 d	3.15 ±0.004 c	4.04 ±0.01 a	3.25 ±0.01 b	Priming	1341.8***
100	2.05 ±0.02c	2.39 ±0.03b	2.99 ±0.01a	2.07 ±0.05c	Salt x Prim.	328.9***
Surface root area (cm^2)						
NaCl	**CNP**	**HP**	**RP**	**AP**	Salt	193.2***
0	519.6 ±7.7d	1792.9 ±7.0a	591.0 ±7.0c	1658.9 ±13.4b	Priming	488.9***
100	1588.9 ±45.5a	1060.7 ±19.0d	1382.1 ±58.5b	1133.9 ±23.1c	Salt x Prim.	1870.2***
Root DW (g $plant^{-1}$)						
NaCl	**CNP**	**HP**	**RP**	**AP**	Salt	721.4***
0	0.08 ±0.01 d	0.11 ±0.001 c	0.15 ±0.003 a	0.14 ±0.001 b	Priming	238.4***
100	0.04 ±0.01 c	0.10 ±0.002 a	0.10 ±0.004 a	0.08 ±0.003 b	Salt x Prim.	62.0***

Root surface area increased in absenceof salinity with HP and AP priming in respect to untreated, conversely, under salinity, CNP roots showed the highest surface area. Root dry weight (DW) was significantly the highest when seedlings were pretreated with Rosmarinus extract (Table 4). ANOVA analysis underlined that Total root length, was the parameters most affected by salinity, priming and their interaction (F-ratios) (Table 4). Salinity more than priming treatments influenced DW and root volume; conversely, the interaction of the two variables mainly affected root surface area (Table 4). Under salinity, the specific length (SRL) and the fineness (RF) of roots in the RP and AP primed seedlings increased significantly (Fig. 1). These results indicated longer roots per unit of root mass respect to control and HP seedlings in which a reduction of SRL and RF was instead evident (Fig.1). The interaction between treatment and salinity had a significant effect on both SRL and RF values (F-ratios). The ratio between root mass and volume (RTD) decreased at 100mM NaCl, except for the root of the HP primed seedlings. The salinity mainly affected RTD (F-ratio) and this can be explained by the remarkable reduction of the radical dry weights caused by salt (Fig. 1).

Table 5

Phenols and reduced glutathione (GSH) in leaves of 30 days old maize seedlings derived from no- primed (CNP), hydro-primed (HP), Rosmarinus (RP) and Artemisia (AP) bio-primed seeds and grown at 0 and 100 mM NaCl. Values are the means of three replicates experiments ± SE. Different letters in the same row denote significant differences among treatments ($P \leq 0.05$). ANOVA *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$ (ANOVA and mean comparison with Tukey's test).

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		Phenols (µg GAE/g D.W.)			ANOVA summary	
NaCl	**CNP**	**HP**	**RP**	**AP**	Salt	50.57***
0	24.78 ± 2.61a	22.23 ± 1.0a	23.53 ± 1.16a	22.12 ± 0.77a	Priming	5.63**
100	8.32 ± 0.64b	19.97 ± 1.34a	20.11 ± 0.04a	21.63 ± 0.07a	Salt x Prim.	22.88***
		GSH (µmoles GSH/g F.W.)				
NaCl	**CNP**	**HP**	**RP**	**AP**	Salt	20419***
0	9.54 ± 0.03b	13.29 ± 0.1a	13.33 ± 0.04a	9.18 ± 0.04b	Priming	11484***
100	11.31 ± 0.07d	17.44 ± 0.03b	24.15 ± 0.03a	13.14 ± 0.03c	Salt x Prim.	2930***

Figure1

Specific root length (SRL = root length/root DW), root tissue density ((RTD = root DW/root volume), root fineness (RF= root length/root volume) of 30 days old maize seedlings, derived from no-primed (CNP), hydro-primed (HP), Rosmarinus (RP) and Artemisia (AP) bio-primed seeds and grown at 0 and 100 mM NaCl. Values are the means of three replicates experiments ± SE. Bars with different letters are statistically different at ($P \leq 0.05$). ANOVA *** P≤ 0.001; **P≤ 0.01; *P≤ 0.05 (ANOVA and mean comparison with Tukey's test).

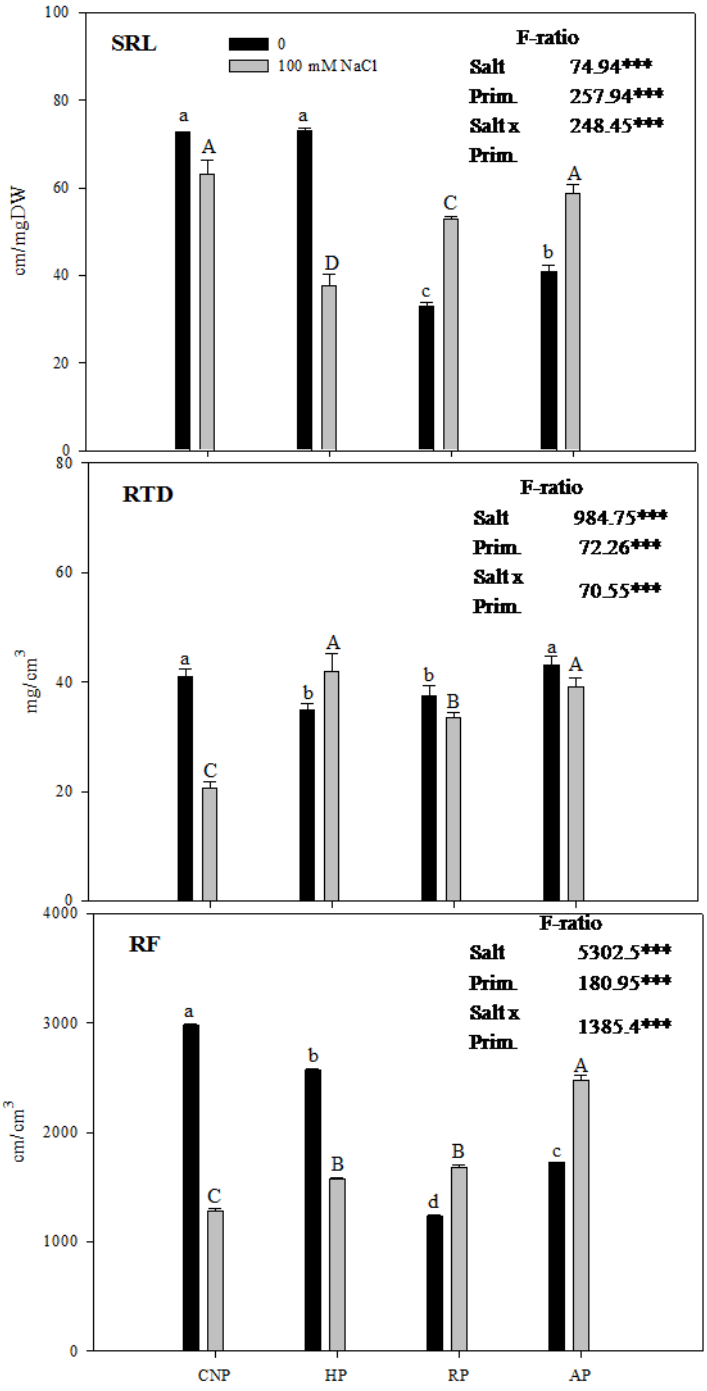
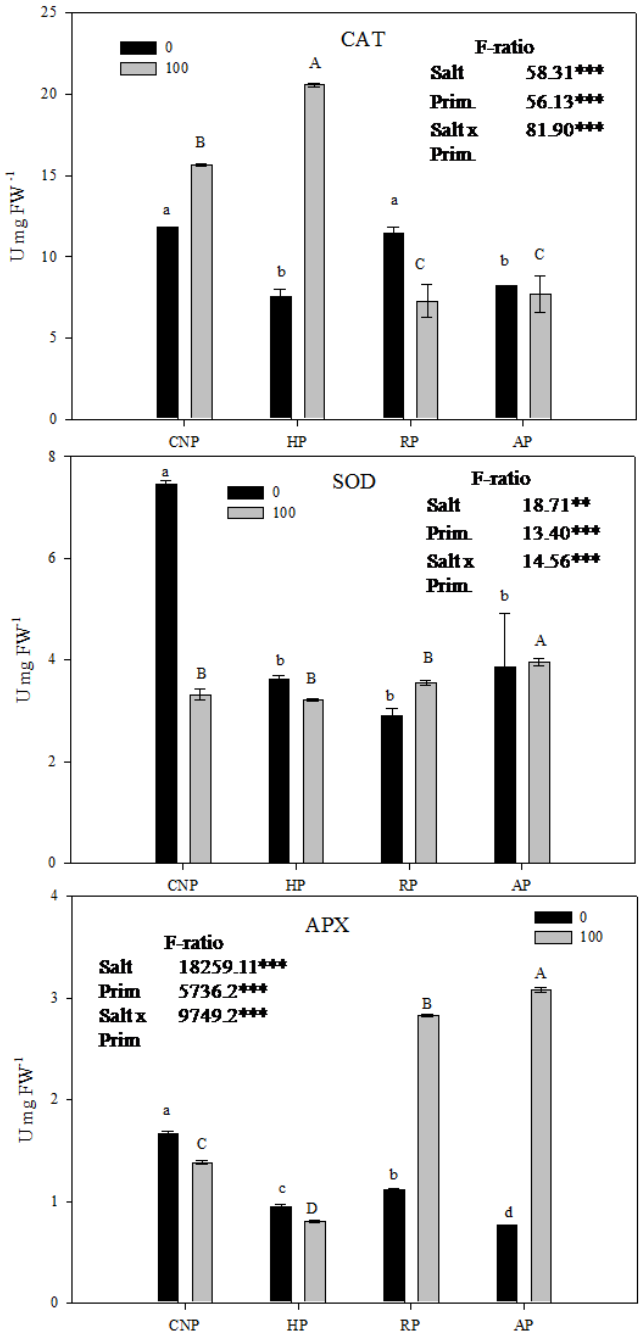


Figure 2

Antioxidant enzymatic activities in leaves of 30 days old maize seedlings: Catalase (CAT), Superoxide dismutase (SOD), Ascorbate peroxidase (APX). Seedlings were derived from no-primed (CNP), hydro-primed (HP), Rosmarinus (RP) and Artemisia (AP) bio-primed seeds and grown at 0 and 100 mM NaCl. Values are the means of three replicates experiments ± SE. Bars with different letters are statistically different at ($P \leq 0.05$). ANOVA *** P≤ 0.001; **P≤ 0.01; *P≤ 0.05 (ANOVA and mean comparison withTukey's test).



3.4 Enzyme activities, phenols and antioxidants

Antioxidant enzyme activities of seedlings resulted differently influenced by salt and by priming treatment (Fig.2). In untreated leaves (CNP), CAT activity significantly increased (+ 25%) under salinity, while SOD and APX activities lowered (Fig. 2). The same trend was shown for HP treated seedlings. In AP primed seedlings, SOD and CAT activities were not significantly influenced by 100mM NaCl, and the highest APX activity was detected (Fig. 2). The salinity in RP primed seedlings caused remarkable increases in SOD and APX activities, while CAT activity was reduced. F-ratios values evidenced that salt and priming, individually and in combination, significantly affected the antioxidant enzymatic system of maize leaves (Fig. 2). CAT activity was mainly influenced by the interaction of salt and treatment, whereas the salinity induced the major changes in SOD and APX activities. In absence of salinity, in all seedlings, regardless of the treatments, the phenol content was similar (Table 5). The salinity caused significant reductions in phenols only in leaves of untreated seedlings. The amount of reduced glutathione (GSH) increased at 100mM NaCl in all samples and the highest concentrations were detected in leaves of HP and RP primed seedlings. The salinity induced the most significant effects both on phenols and GSH contents (F-ratios).

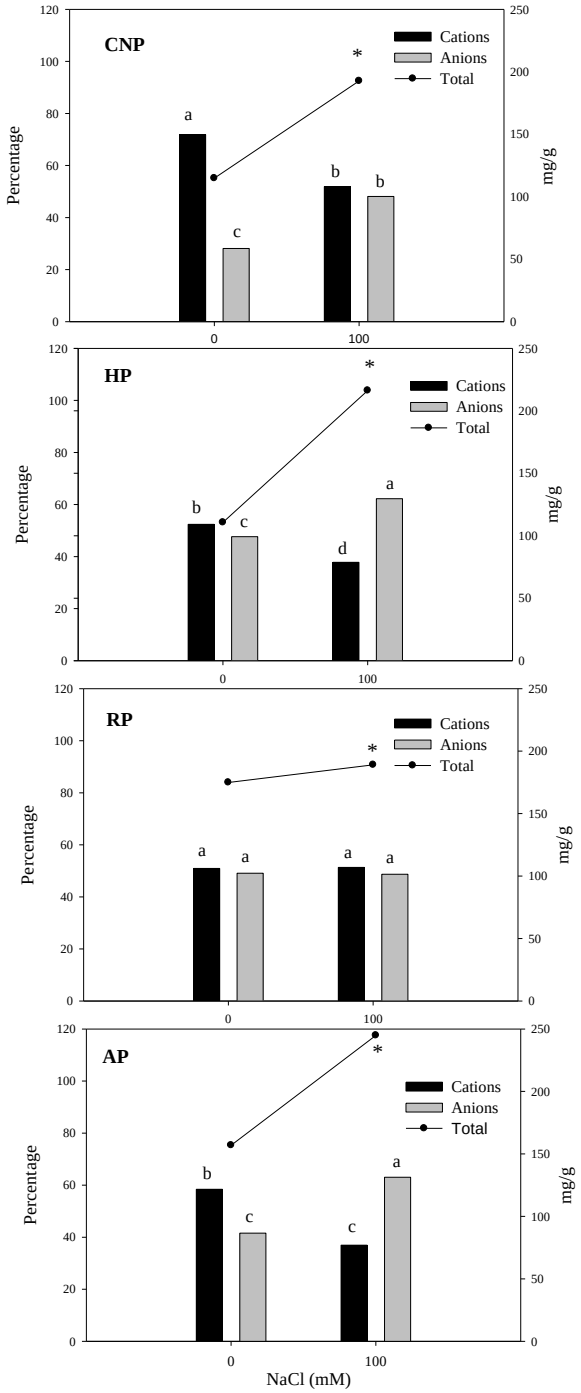
3.5 Ion accumulation

Under salinity, total ion content increased significantly (+ 40%) in leaves of un-primed seedlings, with a remarkable decrease in cation and a simultaneous increase in anion percentage (Fig. 3). Under salinity HP and AP primed seedlings had the highest content of total ions and the major anion percentages compared to all other treatments (Fig. 3).

In RP primed seedlings, only a slight increase in the total ion content (+8%) was detected, without causing any significant change in the percentages of anions and cations (Fig. 3).

Figure 3

Total ion content and anion and cation percentages in leaves of 30 days old maize seedlings derived from no- primed (CNP), hydro-primed (HP), Rosmarinus (RP) and Artemisia (AP) bio-primed seeds and grown at 0 and 100 mM NaCl. Values are the means of three replicates experiments ± SE. Bars with different letters are statistically



different at ($P \leq 0.05$).

Figure 4.

Na^+ K^+ and Cl^- content in roots and shoots of in leaves of 30 days old maize seedlings derived from no-primed (CNP), hydro-primed (HP), Rosmarinus (RP) and Artemisia (AP) bio-primed seeds and grown at 0 and 100 mM NaCl. Values are the means of three replicates experiments ± SE. Bars with different letters are statistically different at ($P \leq 0.05$).

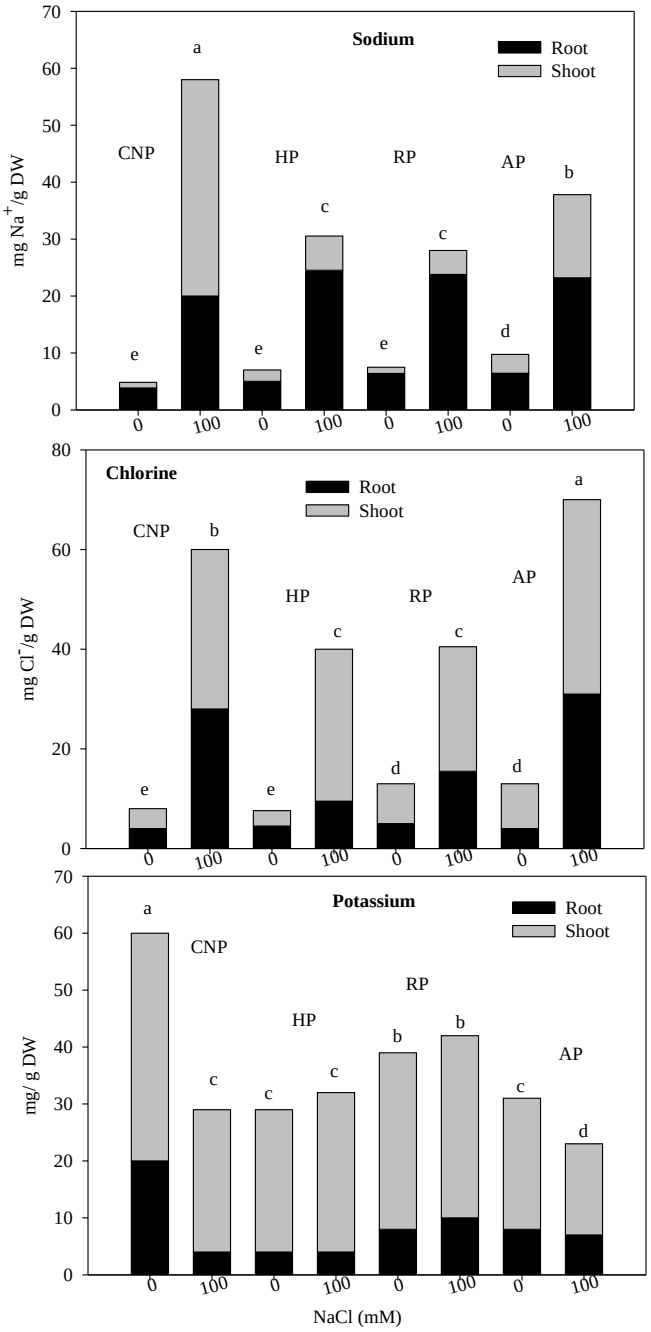


Table 6

Cation content against sodium and chloride, in leaves of 30 days old maize seedlings derived from no-primed (CNP), hydro-primed (HP), Rosmarinus (RP) and Artemisia (AP) bio-primed seeds and grown at 0 and 100 mM NaCl. Values are the means of three experiments ± SE. Different letters in the same column denote significant differences among treatments ($P \leq 0.05$).

		K^+/Na^+	Ca^{2+}/Na^+	Mg^{2+}/Na^+	K^+/Cl^-	Ca^{2+}/Cl^-	Mg^{2+}/Cl^-	Na^+/Cl^-
CNP	**0**	8.75±0.7 a	1.37±0.02 a	1.32±0.02 a	2.64±0.04 a	0.41±0.02 b	0.40±0.02 a	0.32±0.01 d
	100	0.49±0.05 e	0.14±0.01 e	0.09±0.01 e	0.47±0.02 e	0.14±0.01 d	0.10±0.1 1 d	0.97±0.03 a
HP	**0**	2.09±0.10 b	0.73±0.05 b	0.74±0.03 b	1.19±0.05 b	0.39±0.02 b	0.42±0.02 a	0.57±0.02 c
	100	1.06±0.11 d	0.37±0.02 d	0.44±0.04 d	0.61±0.03 d	0.22±0.02 c	0.25±0.02 b	0.56±0.02 c
RP	**0**	1.64±0.05 c	0.66±0.04 b	0.55±0.04 c	1.05±0.03 c	0.42±0.03 b	0.37±0.03 a	0.64±0.03 b
	100	1.51±0.05 c	0.57±0.03 b	0.39±0.03 d	1.03±0.01 c	0.39±0.03 b	0.27±0.03 b	0.69±0.04 b
AP	**0**	1.02±0.06 d	0.49±0.02 c	0.44±0.03 d	1.03±0.04 c	0.49±0.02 a	0.44±0.02 a	1.01±0.03 a
	100	0.14±0.02 e	0.42±0.01 d	0.39±0.02 d	0.21±0.02 f	0.14±0.02 d	0.14±0.02 c	0.34±0.01 d

Table 7.

Photosynthetic pigments in leaves of 30 days old maize seedlings derived from no-primed (CNP), hydro-primed (HP), Rosmarinus (RP) and Artemisia (AP) bio-primed seeds and grown at 0 and 100 mM NaCl. Values are the means of three replicates experiments ± SE. Different letters in the same row denote significant differences among treatments ($P \leq 0.05$).

	CNP	**HP**	**RP**	**AP**
Total Chlorophy (mg/g F.W.)				
0	10.93 ± 0.11d	13.30 ± 0.08c	17.92 ± 0.13a	16.65 ± 0.18b
100	19.81 ± 0.02c	20.75 ± 0.04c	27.64 ± 0.07a	22.61 ± 0.10b
Chlorophyll a (mg/g F.W.)				
0	7.92 ± 0.08d	9.56 ± 0.06c	11.76 ± 0.09a	13.40 ± 0,09a
100	11.42 ± 0.01c	11.67 ± 0.02c	14.89 ± 0.03b	16.04 ± 0.07a
Chlorophyll b (mg/g F.W.)				
0	3.03 ± 0.03d	3.74 ± 0.02b	6.16 ± 0.04a	3.25 ± 0.04c
100	8.39 ± 0.01c	9.09 ± 0.02b	12.75 ± 0.04a	6.57 ± 0.03d
Carotenoids (mg/g F.W.)				
0	2.12 ± 0,02c	2.49 ± 0.02b	3.30 ± 0.02a	1.10 ± 0.01d
100	0.63 ± 0.03c	0.69 ± 0.02c	1.00 ± 0.01b	4.63 ± 0.02a
Anthocyanins (µg/g F.W.)				
0	7.08 ± 0.07c	7.45 ± 0.05b	6.44 ± 0.04d	15.08 ± 0.16a
100	12.52 ± 0.13a	10.27 ± 0.02c	11.56 ± 0.03b	12.40 ± 0.05a

Table 8

Effects of salinity and priming treatments on chlorophyll fluorescence parameters in leaves of 30 days old maize seedlings derived from no priming (CNP), hydro-priming (HP), Rosmarinus (RP) and Artemisia (AP) bio-priming seeds and grown at 0 and 100 mM NaCl. Values are the means of three replicates experiments ± SE. Different letters in the same row denote significant differences among treatments ($P \leq 0.05$).

	CNP	**HP**	**RP**	**AP**
Fv/Fm				
0	0.54 ± 0.02c	0.66 ± 0.02a	0.62 ± 0.02b	0.59 ± 0 .01b
100	0.43 ± 0.02c	0.51 ± 0.01b	0.54 ± 0.01b	0.58 ± 0.02a
Y(II)				
0	0.35 ± 0.01c	0.40 ± 0.01b	0.43 ± 0.01a	0.45 ± 0.01a
100	0.19 ± 0.02c	0.21 ± 0.02c	0.30 ± 0.01b	0.35 ± 0.01a
Y (NPQ)				
0	0.29 ± 0.02a	0.27 ± 0.01a	0.20 ± 0.02b	0.23 ± 0.01b
100	0.33 ± 0.03b	0.41 ± 0.03a	0.23 ± 0.01c	0.25 ± 0.01c
Y(NO)				
0	0.23 ± 0.01a	0.21 ± 0.01a	0.14 ± 0.01b	0.14 ± 0.03b
100	0.30 ± 0.03a	0.20 ± 0.02b	0.13 ± 0.02c	0.14 ± 0.02c
NPQ				
0	0.43 ± 0.02a	0.33 ± 0.03b	0.35 ± 0.02b	0.40 ± 0.01a
100	0.58 ± 0.02a	0.50 ± 0.03b	0.39 ± 0.01c	0.39 ± 0.03c
ETR				
0	35.02± 0.02b	46.50± 1.02a	45.25± 0.21a	45.04± 0.31a
100	19.02± 0.23d	30.56± 0.14c	33.76± 0.30b	40.99± 1.21a

405 The salt condition significantly increased the Na^+ and Cl^- concentrations in all seedlings
406 (Fig. 4). Na^+ was generally higher in roots of primed seedling. Conversely, CNP had the
407 highest sodium and chloride content, mainly accumulated in shoots (Fig. 4). In CNP and
408 AP primed seedlings a significant decrease in K^+ content was observed. Under salinity,
409 HP and RP primed seedlings showed a similar trend regarding content, uptake and
410 distribution of Na^+ and Cl^- ions. In RP seedlings, at 100mMNaCl, the highest K^+
411 content was detected, mainly accumulated in shoots. (Fig. 4). In shoot of CNP plants
412 grown in absence of salinity, the K^+/Na^+, Ca^{2+}/Na^+, Mg^{2+}/Na^+ ratios were the highest
413 (Table 6). Conversely, under salinity the above mentioned ratios were significantly
414 lower in comparison with all primed plants. In AP seedlings, due to the highest Cl^- ion
415 accumulation, at 100mM NaCl, the lowest ratios of cations against chloride were
416 detected .

417 *3.6 Chlorophyll, carotenoid and anthocyanin content*

418 Total chlorophyll content increased in all seedlings under salinity (Table 7). At 100 mM
419 NaCl, the highest amount of total chlorophyll was detected in RP leaves. Salinity caused
420 a decrease in carotenoids and a simultaneous increase in anthocyanin content in all
421 samples, except for AP seedlings which showed an opposite behaviour (Table 7)

422 Bio-primed seedlings had the higher Y(II), ETR, and Fv/Fm values in respect to un-
423 primed seedlings both in presence and in absence of salinity (Tab.8). In CNP and HP
424 primed seedlings, a decline in effective quantum yield of photochemical energy
425 conversion in PSII, Y(II), was accompanied by a significant increase in the quantum
426 yield of regulated energy dissipation in PSII (YNPQ). The quantum yield of non-
427 regulated energy dissipation at PSII, Y(NO), significantly increased under salinity only
428 in CNP leaves, differently a weak decrease in primed seedlings was observed. Salinity
429 caused an increase in non-photochemical quenching (NPQ) of all samples to prevent
430 photoinhibition in leaves.

4. Discussion

Salinity represents nowadays a serious problem for the Mediterranean countries, and in particular for North African regions where changing climatic conditions are further exacerbating water scarcity and soil salinity. It is known that soil salinity, with high percentages of chloride and sodium, affects plant growth by modifying their morphological, anatomical biochemical and physiological traits (Muscolo et al., 2015; Panuccio et al., 2003) in particular way at seed and seedling stage (Ibrahim, 2016). Our results showed that the detrimental effects of salinity on germination were less severe when maize seeds were pretreated with RP and AP extracts rather than water or untreated. This ameliorative effect on germination under salinity could be related to the phytochemical content of these two extracts. Rosmarinic extract contained great amount of rosmarinic acid that is known to have numerous beneficial and protective effects at cellular levels with antioxidant potential, and it is considered as an effective scavenger compound against Na^+ toxicity, combating cellular damage under stress (Adomako-Bonsu et al., 2017). Conversely, the effectiveness of AP extract could be prevalently related to its content of chlorogenic and syringic acids, referred as potent cellular antioxidants with scavenging properties (Xu et al., 2017). Generally, salinity causes oxidative stress in plants, by disruption of the balance between ROS production and elimination, leading to an increase in antioxidant compounds such as phenols, reduced glutathione, anthocyanins, carotenoids, and with a shift from primary to secondary metabolism (Munns & Tester, 2008). In maize, the negative effects of salt stress depend on the stress degree and plant growth stages (Imran et al., 2013). Our results showed, under salinity, a significant increase in reduced glutathione (GSH) content of all seedlings, confirming its essential role in keeping ROS under control. GSH can be involved as an antioxidant in direct reactions with free radicals, or in cooperation with ascorbate in the ascorbate-glutathione cycle which plays a central role in integration of

redox signals. According to the most significant increase in GSH content, APX activity increased in AP and RP primed seedlings under salinity. Among antioxidant enzymes, CAT activity is generally low under normal growth conditions and it increases only at relatively high H_2O_2 concentrations or under stress conditions, to support APX, SOD, and other enzymes primarily involved in ROS homeostasis (Papalia et al., 2017). At 100mM NaCl, the lowest activities of CAT in RP and AP primed seedlings suggested that priming treatment was able to contrast the salt sensitivity of maize seedlings. In numerous cases, the beneficial impact of priming on plant growth is more evident under non-optimal than under optimal conditions because of an increase in stress resistance (Ibraim, 2016). As reported by some authors, several components of the ROS-mediated signaling pathways are accumulated and activated during the first hydration phase and the final degree of stress resistance of seedlings can be linked to the persistence, even after germination, of all the antioxidant mechanisms activated in seed (Paparella et al., 2015). Then the priming treatment with AP and RP extracts, containing molecules with recognized antioxidant properties, may have contributed to amplify and strengthen the antioxidant response leading to an increase in salt resistance of maize seedlings.

Maize seedling grown in presence of salinity, showed an accumulation of sodium and chloride. The ability of primed seedlings to limit Na^+ transport into shoot, compared to untreated plants, was important for the maintenance of growth rates and protection of the metabolic process from the toxic effect of Na^+. This results perfectly agree with findings reported by Jamil et al. (2012) in rice, by Akram et al. (2011) in sunflower, by Perveen et al. (2012) in wheat, demonstrating that the plant growth reduction under saline condition was dependent mainly by the accumulation of sodium at leaf level. The increase of sodium content caused a decrease in K^+/Na^+ ratios of all seedlings. In maize plants, salt toxicity is mainly due to antagonistic effect of Na^+ on K^+ uptake and to a strong interference of sodium and chloride ions with other essential mineral elements,

leading to severe nutritional imbalances (Farooq et al., 2015). In HP and RP primed seedling exposed to salt stress, Na^+ and Cl^- concentrations were significantly lower compared to all other samples, and potassium content did not changed. These results suggest that HP and RP primed plants were able to avoid an excessive sodium entry and maintain an efficient K^+ uptake as adaptive strategy under salt stress. Increased sodium accumulation also disturbs calcium nutrition (Shahzad et al., 2012). The Ca^{2+} content, at 100mM NaCl, was significantly higher in primed seedlings, and the maintenance of adequate levels of Ca^{2+}, under stress condition, is important by considering the fundamental role of this ion in stabilizing the cell wall and membrane and also as a signal in induction of the antioxidant enzymes. As reported by many authors, the decrease in growth observed in many plants subjected to salinity stress is often associated with a decline in their photosynthetic capacity (Akram et al., 2011). This decrease aggravates the amount of excess excitation energy which should be appropriately dissipated to avoid that uncontrolled production of ROS and photosynthetic apparatus damage occur. The synthesis of anthocyanins is induced in many plants for their protective role against light and other stress conditions. Eryılmaz (2006) demonstrated an increase in anthocyanins in response to salt stress in tomato and cabbage seedlings, evidencing a positive correlation between anthocyanins and NaCl. Our results, confirmed the involvement of anthocyanins in maize response to salinity and the highest content was in leaves of CNP seedlings, indicating an higher stress condition respect to that of primed plants. Salt stress decreases photosynthetic performance in plants, leading to a decline of CO_2 fixation and an enhancement of the oxigenase activity of RUBPco (Kangasjärvi et al., 2012). Our results evidenced that priming treatments, with RP and AP extracts, were able to preserve photosynthetic apparatus and photosynthetic efficiency of maize seedlings against salt stress. Salinity lowered the potential photochemical activity of PSII, expressed by Fv/Fm ratios in

maize, as reported by Han et al. (2010), Akram et al. (2011). The lowest Fv/Fm values were detected in CNP and HP primed seedlings, indicating that the conversion efficiency of primary light energy and the potential activity of PSII were mainly affected. In addition, in CNP plants, the significant increase in Y(NO), the index of non regulated energy dissipation at PSII, confirmed a damage to photosynthetic apparatus due to the stress condition. Photodamage steps are mediated by reactive oxygen species and the main target are the photosynthetic reaction centers, primarily of photosystem II (PSII), and likely also PSI (Ruban et al., 2012). The general NPQ increase in all samples may reflect heat dissipation of light energy in the antenna system. NPQ, often referred to as "feedback de-excitation", is considered the most important short-term reversible photoprotective process in higher plants (Lambrev et al., 2012).

The priming treatment influenced also the growth and morphology of root apparatus. The root system possesses a certain phenotypic plasticity and under stress condition this variability represents a major survival strategy allowing plants to concentrate their resources where nutrients and water are more easily available (Panuccio et al., 2014). Salinity reduced total root length in CNP and HP primed seedlings indicating a decrease in carbon skeleton supply from the shoot. This reduced root growth and elongation was also confirmed by the lowest values of SRL and RF, structural root traits associated with the nutrient acquisition capacities of plants, that respond rapidly to stresses and environmental changes. A different behavior was observed in root system of AP and RP primed seedlings, where the greatest SRL and RF ratios suggested that the plants maximized the effectiveness of roots in water and nutrient uptake (Fitter and Stickland, 1991). Both the extracts are effective in inducing salt resistance in maize, however RP treated seedlings showed a better response to salinity and in AP treated seedlings some stress signals were detected. The root and shoot grew less, the content of anthocyanins and carotenoids were higher while total chlorophyll was lower than rosmarinus treated

ones. In AP primed seedlings, under salinity, was also observed a significant increase in total ion content and a different ion compartmentalization of cations and anions between root and shoot, leading to ion imbalance with consequent possible toxicity.

5. Conclusion

In short, although bio-primed maize seedlings didn't maintain equal growth under salt stress in comparison with the same plants grown under normal conditions, they showed a better growth performance under salinity as compared to untreated and hydro-primed seedlings. Beneficial effects of bio-priming treatment are mainly expressed by the improvement of nutritional and nutraceutical qualities due to the increasing of antioxidant compounds with relevant beneficial effect on human health. The use of natural extracts as bio-priming agents could be attractive because it reduces the risk of negative environmental impact in respect to the use of chemical agents, representing an up and coming ecofriendly technique to overcome agricultural problems in degraded land. The results obtained evidence a new potential application of these two species already widely used in the pharmaceutical and cosmetic field. Bio-priming represents a non-expensive and a value added practice that greatly can increase yield of salt sensitive crops in saline lands, improving the economic returns to farmers in Tunisia

Conflicts of interest

The authors have declared that no competing interests exist.

Author contributions

All authors discussed the results and commented on the manuscript.
Muscolo Adele designed the project.
Panuccio Maria Rosaria analyzed the data.
Chaabani Saber, Roula Rabia worked in the laboratory
Muscolo Adele and Panuccio Maria Rosaria wrote the manuscript.

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