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Changes in phenolic profile, soluble sugar, proline, and antioxidant enzyme activities of *Polygonum equisetiforme* in response to salinity

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Abstract: This study intended to focus on the effect of different levels of NaCl (0–400 mM, at regular intervals every 100 mM) on the growth, secondary metabolites, and antioxidant enzyme activities of *Polygonum equisetiforme*, a wild plant with medicinal and industrial uses, which often grows in harsh environmental conditions. Our results showed that biomass production significantly decreased with salinity, while the leaf relative water content declined only at high levels of NaCl concentration (300–400 mM). In contrast, these higher doses of salt resulted in a significant increase in malondialdehyde (MDA) content. Both proline and soluble sugar (e.g., fructose, glucose, and sucrose) contents were enhanced under saline conditions. The methanolic extracts of the shoots included 10 flavonoids and 9 phenolic acids. The total phenolic acids (TPA), total flavonoid compounds (TFC), and total phenolic compounds (TPC) increased with salinity, particularly at 300 mM NaCl. An increase in TPA resulted especially from an increase in quinic, gallic, and protocatechuic acids (phenolic compounds), followed by quercetin-3-O-galactoside, catechin, and epicatechin (flavonoid compounds). Superoxide dismutase (SOD) activity increased only at high salinity levels (>200 mM), while glutathione reductase (GR), guaiacol peroxidase (GPX), catalase (CAT), and ascorbate peroxidase (APX) activity increased with salinity level. A positive significant correlation between antioxidant DPPH and TPA, TFC, TPC, CAT, and APX suggests a vital protective role in controlling oxidative stress through the scavenging process. Consequently, our results indicated that *P. equisetiforme* shoots are rich in secondary metabolites, especially phenolic compounds with high potential antioxidant activities. It can be considered a salt-tolerant species able to survive at salinity levels up to 300 mM NaCl.

Key words: *Polygonum equisetiforme*, salt stress, osmolytes, secondary metabolites, antioxidant enzyme activities

1. Introduction

Salinity is known as the most significant serious environmental issue affecting plant production and growth, particularly in arid regions. It usually stimulates declines in stomatal conductance and changes in the photochemical apparatus, which may eventually decrease photosynthetic rates (Boughalleb et al., 2017; Abdellaoui et al., 2017). The inhibitory consequences of salinity also contribute to alterations in metabolic perturbation, water relations, reactive oxygen species (ROS) production, ion toxicity, and tissue damage (Zhu, 2001).

To reduce the negative effects of salt stress, plants initiate different mechanisms, such as enzymatic antioxidants (Parida and Jha, 2010) and nonenzymatic low molecular compounds (Slama et al., 2015), to produce scavenging activity against ROS, which are generated as a response to the oxidative stress that causes subsequent cellular damage, such as lipid peroxidation, protein damage, and membrane destruction (Foyer and Noctor, 2005). Secondary metabolites produced under environmental stresses (e.g., salinity) constitute the majority of plant antioxidants (Dixon and Paiva, 1995). The phenolic compounds are one of the secondary metabolites which are produced in plant tissues to scavenge free radicals and/or to block their production via hydroperoxide decomposition (Agati et al., 2012). Phenolic metabolites display different biological activities, such as antiinflammatory, antiallergic, cardioprotective, vasodilatory, antimicrobial, and antioxidant effects (Balasundram et al., 2006). In addition to the role of secondary metabolites (nonenzymatic antioxidants) in plant protection against oxidative stress, other enzymatic defense systems such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), and glutathione

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reductase (GR) are implicated (Parida and Jha, 2010). Antioxidant enzyme activities in various plant species are correlated with their tolerance to salinity (Abdellaoui et al. 2017; Boughalleb et al., 2017).

Salinity may increase phytochemical contents in plants (Boo et al., 2012), thus increasing the nutritional, aromatic, and medicinal properties which are attributed to a large supply of antioxidant compounds in wild plants such as *Polygonum*. However, an approach of using abiotic stresses to improve the quality of plants must be considered with some caution owing to their potential adverse effects on the growth and productivity of the plants.

Polygonum species characterized by a high proportion of phytochemical compounds are traditionally used in many countries to treat a variety of diseases such as dermatitis, hemorrhoids, insomnia, etc. (Narasimhulu et al., 2014). In Tunisia, *Polygonum equisetiforme* is a widespread plant that is palatable to domestic animals (FAO 1988) and economically profitable for rural populations (Gamoun, 2014). *Polygonum equisetiforme* is also used to treat some illnesses such as sore throat, common colds, and coughs (Khafagi and Dewedar, 2000). Current studies have shown that shoot extracts of Egyptian *P. equisetiforme* are rich in phenolic contents (Hussein et al., 2017); in fact, some flavonoid compounds have important antioxidant activities (El-Toumy et al., 2017).

Although *P. equisetiforme* is a viable species in arid and saline soils, its tolerance level to salt stress is not known. On the other hand, environmental stress applications are known to increase the phenolic content of plants—for example, applying salt stress. Different concentrations of salt were applied to confirm this hypothesis. For this purpose, the effects of salinity on growth, osmolyte accumulation, phenolic content, and antioxidant potential were evaluated, and the salinity tolerance threshold was determined.

2. Materials and methods

2.1. Plant growth conditions

In this experiment, the seeds of *P. equisetiforme* were collected from Djerba in southern Tunisia (33°51′N; 10°54′E) in July 2017. The seed surface was sterilized with a calcium hypochlorite solution before sowing, and subsequently rinsed 5 times with demineralized water. Five seeds were sown in a 5-L plastic pot filled with a mixture of peat and sandy soil (1:2, v/v) and watered with half-strength Hoagland's solution (Hoagland and Arnon, 1950). After 2 weeks, 3 healthy seedlings of similar size were selected from each pot. Seedlings of *P. equisetiforme* were grown under controlled conditions with a relative humidity of 65% during the day and 75% at night, a constant 25 ± 1 °C as an optimum temperature, and a photoperiod of 16 h light with photosynthetic photon flux density (PPFD) of 250 μmol m^{-2} s^{-1} and 8 h darkness regime during the experimental period.

Ninety days after sowing, the seedlings were watered with half-strength Hoagland's nutrient solution (300 mL) and different levels of NaCl (0, 100, 200, 300, and 400 mM). The salt concentration was augmented progressively by 50 mM every 2 days until the salt levels were reached to avoid a shock response. To prevent salt accumulation in the soil, the pots were flushed weekly with 500 mL of water, and nutrient solutions were changed every 2 weeks; any change in treatment conductivity levels was prevented by checking conductivity using a conductivity meter (CDS106, Omega Engineering Inc., Norwalk, CT, USA). All plants in each pot were harvested (roots and shoots separately) after 45 days of salt application, and 4 replicates were performed for all treatments.

2.2. Growth and water parameters

As soon as plants were harvested, the fresh and dry weights of the shoots (SFW and SDW) and roots (RFW and RDW) were measured as g $plant^{-1}$ using a balance with 0.001 g precision. To determinate the turgid weight (TW; g $plant^{-1}$), the shoots were placed in deionized water in darkness for 48 h. A portion of fresh material was kept at −80 °C for further investigations. Other fresh samples were dried in an oven at 70 °C for 48 h to determine SDW and RDW. The leaf relative water content (RWC) was estimated as follows:

$$RWC\ (\%) = ([SFW - SDW] / [TW - SDW]) \times 100.$$

The leaf water potential (Ψ_W) was measured at midday from fully expanded leaves selected from the median shoot part using a Scholander pressure chamber (Model 1000, PMS Instrument Co., Albany, OR, USA).

2.3. Proline content

Proline was extracted and analyzed according to Troll and Lindsley (1955). Briefly, ~200 mg (per replicate of fresh shoots) was ground with a pestle and mortar in 2 mL of 40% methanol; the homogenate was then incubated at 80 °C for 1 h, cooled, and filtered. The colorimetric measurement of proline required a 1:1:1 (v/v/v) of the filtrate, glacial acetic acid, and ninhydrin acid solution (25 mg ninhydrin mL^{-1} in 10.44 M acetic acid and 2.4 M phosphoric acid), and incubation at 95 °C for 1 h. The mixture was put on ice in order to stop the reaction. Afterwards, 4 mL of toluene was added and the absorption of the resultant supernatant was measured at 520 nm using a spectrophotometer (Specord Plus; Analytik Jena, Jena, Germany) with L-proline as a calibration standard.

2.4. Soluble sugar measurement by HPLC

After drying the shoots for 48 h at 70 °C, ~20 mg of crushed dried material per replicate was homogenized with 1 mL of 80% ethanol, and then incubated for 90 min at 70 °C and centrifuged for 10 min at 16,000 rpm. The soluble

sugar content of *P. equisetiforme* shoots was determined using the high-performance liquid chromatography (HPLC) method (Douville et al., 2006). The separation was realized on an XBridge™ amide column (3.5 µm, 4.6 × 150 mm; Waters, Milford, MA, USA). The HPLC grade sugar standard (Sigma–Aldrich, Shanghai, China) was used to calculate the sugar concentration (fructose, sucrose, and glucose).

2.5. Lipid peroxidation

Hernández and Almansa's (2002) method was used to determine the content of malondialdehyde (MDA) in the shoots of *P. equisetiforme*. Briefly, ~500 mg of fresh shoot per replicate was mixed with 5 mL 0.1% (w/v) trichloroacetic acid (TCA) and then centrifuged for 20 min (15,000 rpm, 4 °C). One mL of the collected supernatant was then mixed with 4 mL 0.5% (w/v) thiobarbituric acid (TBA) prepared in 20% (w/v) TCA. The reaction was activated in a water bath set at 90 °C for 30 min, and stopped in an ice bath followed by centrifugation at 10,000 rpm for 5 min. Eventually, the absorption was read at 532 nm and the subtracting nonspecific absorption was also measured at 600 nm. In addition, the MDA content was determined using the extinction coefficient of 155 mM^{-1} cm^{-1}.

2.6. Preparation of methanolic extracts

Dry shoot sample (~300 mg per replicate) was crushed, mixed with 30 mL of methanol (80%) at 40 °C for 24 h, and then centrifuged at 4500 rpm for 20 min. The resultant supernatant was filtered using a 0.2-µm pore size acrodisc syringe filter and kept in the freezer at 20 °C for future use.

2.7. Estimation of DPPH assay

The DPPH (1,1-diphenyl-2-picrylhydrazyl) activity was estimated by Sánchez-Moreno et al.'s (1998) method. To 50 µL of shoot extract, 1.95 mL of DPPH methanolic solution (0.025 g L^{-1}) was added, then vortexed and placed in darkness at room temperature for 30 min. The percentage of radical scavenging activity was determined as follows:

([(Abs control – Abs sample) / Abs control] ×100) at 515 nm; Abs control is the absorption of the control reaction, prepared by substituting the sample under the same operating conditions.

2.8. LC–ESI/MS method

Determination of phenolic content was estimated using LC–ESI/MS analysis. The shoot extracts were analyzed using a Shimadzu UFLC XR system (Kyoto, Japan) with an electrospray ionization source (ESI) operated in negative mode (see details in Mahmoudi et al., 2019).

2.9. Enzyme extractions and assays

Fresh shoot samples (~100 mg per replicate) were homogenized with 100 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA-2Na, 5% (w/v) polyvinylpolypyrrolidone (PVPP), and Triton X-100 (0.1%) fixed at 4 °C. The mixtures were then stirred at 15,000 rpm (4 °C), and supernatants were kept at −80 °C for future use. The protein concentration was estimated using Bradford's (1976) method, with bovine serum albumin (BSA) as a standard. The CAT (EC 1.11.1.6) activity was estimated by measuring the decomposition rate of hydrogen peroxide (H_2O_2) at 240 nm, following the method of Aebi (1984). One unit of CAT activity was defined as the amount of enzyme that catalyzed the decomposition of 1 µmol of H_2O_2 per minute per mg of protein. The activity of APX (EC 1.11.1.11) was investigated through Nakano and Asada's (1981) method. The APX activity was expressed as mmol oxidized ascorbate min^{-1} mg^{-1} protein. The activity of SOD (EC 1.15.1.1) was measured based on the ability of this enzyme to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) into blue formazon. One unit of SOD activity was defined as the amount of enzyme that gave 50% inhibition of NBT reduction as measured at 560 nm. In addition, the activity of GR (EC 1.6.4.2) was estimated according to Schaedle and Bassham's (1977) method, measuring the decrease in absorbance induced by NADPH oxidation at 340 nm. Furthermore, GR activity was calculated as nmol NADPH oxidized min^{-1} mg^{-1} protein (for further details, see Abdellaoui et al., 2017).

2.10. Statistical analysis

The experimental data were statistically analyzed using SPSS v. 16.0 software (SPSS Inc., Chicago, IL, USA), and Duncan's post-hoc test ($\alpha = 0.05\%$) was applied to compare the treatments for each studied parameter. Correlations analyses were conducted using the regression program in SPSS 16.0.

3. Results

3.1. Biomass production and water parameters

The production of biomass (e.g., SDW and RDW) linearly decreased with increasing salinity (Figure 1a). The amount of decrease in the SDW was almost 16.0%, 33.6%, 51.2%, and 62.4% at 100, 200, 300, and 400 mM NaCl compared to the control, respectively. Similarly, RDW was significantly reduced by 8.0%, 16.2%, 31.2%, and 47.4% at 100, 200, 300, and 400 mM NaCl compared with the control, respectively (Figure 1a). RDW was less affected by salinity than SDW. The shoot/root ratio remained almost unchanged until 100 mM NaCl, and then significantly decreased compared to that of the control (Figure 1a). Although there was no significant variation between treatments in terms of RWC until 200 mM NaCl (<11.9% compared with the control), it significantly decreased by 14.6% and 23.5% at 300 and 400 mM NaCl compared to the control, respectively (Figure 1b). The rate of decrease in RWC was 0.041% per mM increase of NaCl concentration. Similarly, the Ψ_W of salt-treated plants was reduced by 0.0084% per mM increase of NaCl, which was about 4.4-fold lower in 400 mM NaCl when compared to untreated plants (Figure 1c).

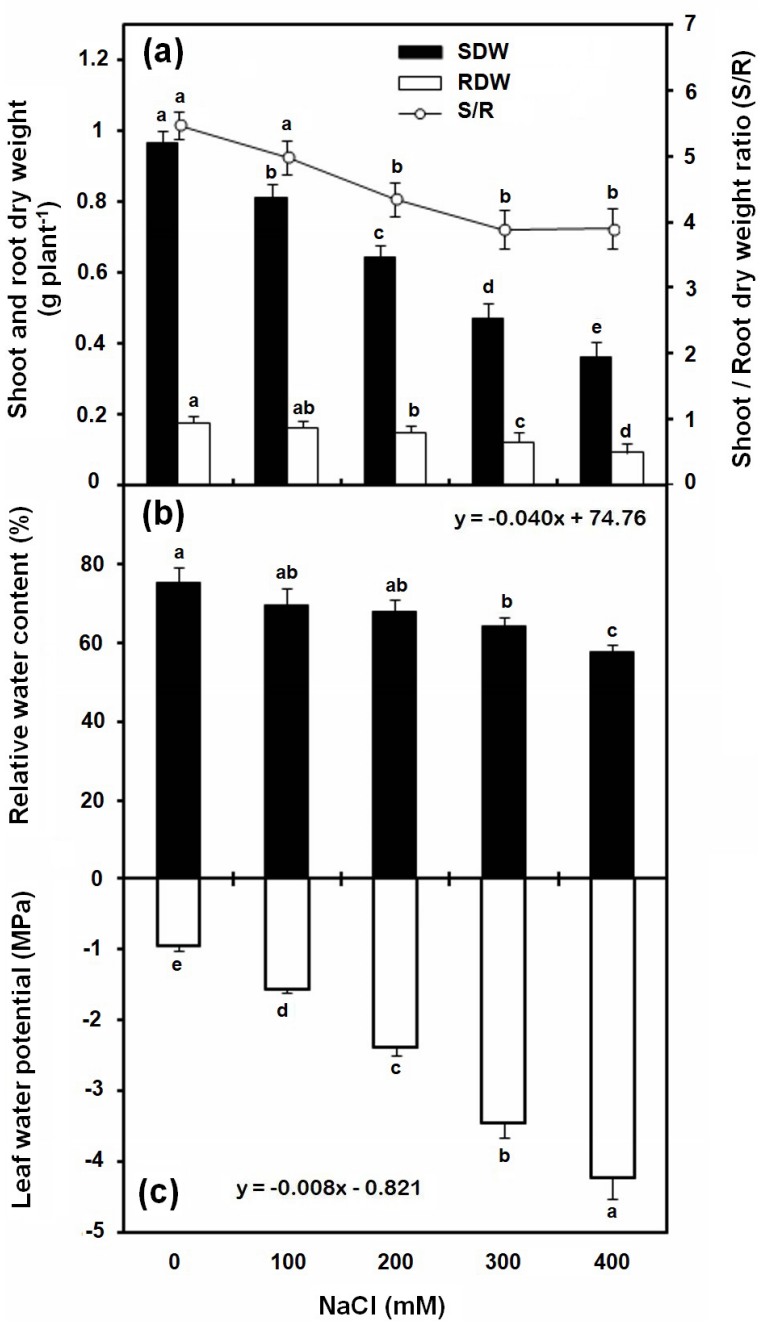

Figure 1. Effects of salt stress on (a) shoot dry weight (SDW, g plant^{-1}), root dry weight (RDW, g plant^{-1}), and shoot/root dry weight ratio (S/R), (b) leaf relative water content (RWC, %), and (c) leaf water potential (Ψw, MPa) in 3-month-old plants of *P. equisetiforme*. Values are means of 4 replicates ± SE. Bars with different letters are significantly different at $P < 0.05$ level according to Duncan's multiple range test.

3.2. Osmolyte, MDA content, and antioxidant capacity

The proline content measured in *P. equisetiforme* leaves remained unchanged under 0–100 mM of NaCl (6.2–8.5 mg g^{-1} SFW, as average 0 and 100 mM), then increased by 1.9-, 3.6-, and 5.6-fold under 200, 300, and 400 mM NaCl, respectively, compared to untreated plants (Figure 2). There was a significant increase in the content of fructose, glucose, and sucrose as a result of an increase in NaCl concentration; the patterns were similar for all of them (Figure 2). The lowest contents of fructose, glucose, and

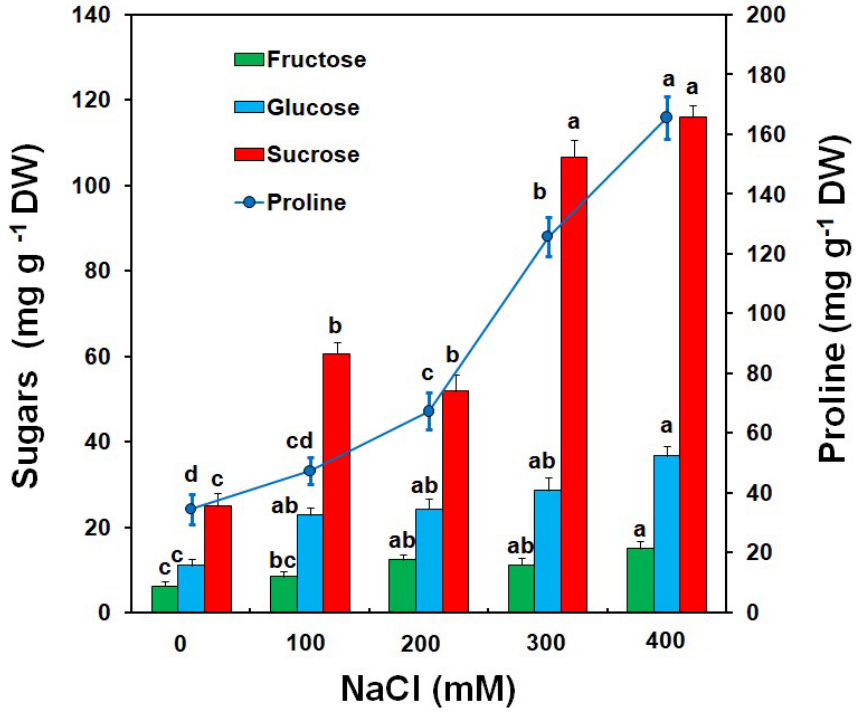

Figure 2. Effects of salt stress on proline and soluble sugar (i.e., fructose, glucose, and sucrose) contents in leaves of the 3-month-old plants of *P. equisetiforme* seedlings. Values are means of 4 replicates ± SE. Bars with different letters are significantly different at $P < 0.05$ level according to Duncan's multiple range test.

sucrose were found in the plants subjected to 0 mM NaCl (6.1, 11.0, and 24.8 mg g^{-1} SFW, respectively). However, the content of fructose (35.8%, 99.3%, 79.9%, and 141.9%), of glucose (106.5%, 119.4%, 159.2%, and 232.6%) and of sucrose (143.9%, 108.7%, 328.6%, and 366.5%) increased significantly at 100, 200, 300, and 400 mM NaCl compared to the control plants, respectively (Figure 2). No significant differences were observed among all NaCl treatments in terms of fructose content when NaCl concentration level was >200 mM. The MDA content was not influenced by increasing NaCl concentration from control to moderate salinity (0–200 mM NaCl; 4.06 to 5.50 µmol g^{-1} SFW as average), while the higher levels of salinity (300–400 mM) caused a significant increase in its value (1.8- to 2.5-fold, respectively, compared to the control) (Figure 3). The antioxidant capacity of *P. equisetiforme* evaluated by DPPH radical scavenging assay was increased by 6.1%, 20.8%, 21.0%, and 7.1% at 100, 200, 300, and 400 mM NaCl compared to the control (Figure 3).

3.3. Phenolic acids and flavonoids contents

The LC–ESI/MS analysis identified 10 flavonoids and 9 phenolic acids; the latter were the major compounds in the methanolic extract of *P. equisetiforme* shoots (Table 1). For control plants, quinic acid (1055 ± 81.2 µg g^{-1} DW) and gallic acid (452 ± 52.3 µg g^{-1} DW) were the major compounds, followed by p-coumaric acid (58.0 ± 7.0 µg g^{-1} DW) and protocatechuic acid (56.5 ± 7.1 µg g^{-1} DW). Regarding flavonoids, quercetin-3-O-galactoside (158 ± 9.3 µg g^{-1} DW) showed the highest content, followed by epicatechin (61.4 ± 5.4 µg g^{-1} DW) and catechin (54.0 ± 6.3 µg g^{-1} DW) in *P. equisetiforme* shoots under control conditions. The averages of total phenolic compounds, total phenolic acids, and total flavonoid contents increased with increases in NaCl concentration, and the maximum values were registered at 300 mM NaCl. In fact, the quinic acid content showed a gradual increasing pattern with increasing salinity by 46.6%, 67.1%, 144.6%, and 91.3% at 100, 200, 300, and 400 mM NaCl, respectively, compared to the control. The contents of gallic acid and protocatechuic acid remained steady at 100 mM NaCl compared to the control. However, increasing NaCl concentration in the medium culture resulted in increasing gallic acid content by 52.6%, 97.8%, and 74.4%, and protocatechuic acid content by 67.7%, 84.8%, and 110.3%, at 200, 300, and 400 mM respectively, compared to the control (Table 1). However, the trans-cinnamic acid content increased by 84.4% and 124.0% at 300 and 400 mM respectively compared to the control plants. Of the phenolic acids detected, the lowest accumulation was that of caffeic acid; however, it increased significantly from 39.8% to 100.7%

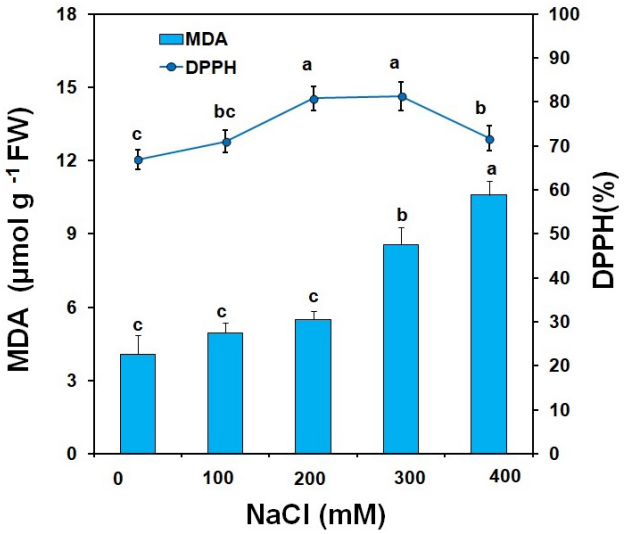

Figure 3. Effects of salt stress on MDA content (µmol g^{-1} SFW) and DPPH activity (%) in the shoots of 3-month-old plants of *P. equisetiforme* seedlings. Values are means of 4 replicates ± SE. Bars with different letters are significantly different at $P < 0.05$ level according to Duncan's multiple range test.

at 200 and 400 mM NaCl respectively compared to the control (Table 1). The contents of the flavonoid compounds catechin (+), epicatechin, quercetin-3-O-galactoside, and quercetin were remarkably enhanced as salinity increased; i.e., at 100–400 mM NaCl, the amount of these flavonoids increased by 2.5- to 5.0-fold, 2.3- to 4.1-fold, 1.3- to 2.6-fold, and 1.7- to 2.5-fold, respectively, in comparison with the control plants (Table 1). Under low and moderate salinity levels (100–200 mM NaCl), cirsiliol, quercetin-3-O-rhamonoside, and naringenin content showed no significant differences with the control, while they increased significantly at higher salinity levels (300–400 mM NaCl), by 43.3%, 81.0%, and 84.6%, at 400 mM NaCl, respectively, from those found in the control. However, the results showed that the content of some compounds such as 4-O-caffeoylquinic acid, syringic acid, p-coumaric acid, transferulic acid, naringin, luteolin, and cirsilineol were not statistically changed between the untreated and NaCl-stressed plants (Table 1).

3.4. Antioxidant enzymes activities

Results of the enzymes' antioxidant activities (SOD, CAT, APX, GPX, and GR) are presented in Table 2. The SOD activity was not changed significantly under low to moderate salt stress (100–200 mM NaCl); however, it increased by approximately 1.6- and 2.1-fold respectively at 300 and 400 mM NaCl in comparison with the control. CAT activity increased significantly with salinity level. The highest value was recorded at 200 mM (2.4-fold compared to the control). Similarly, APX exhibited the same trend as CAT, with maximum activity at 300 mM, and then decreased at 400 mM, but remained higher than control plants. GPX activity was significantly improved with increasing NaCl; it increased by 2.6- to 8.2-fold respectively at 100 and 400 mM compared to the control. At 100 mM NaCl, no change was registered in the GR activity, whereas it increased by 1.6-, 2.9-, and 4.2-fold at 200, 300, and 400 mM NaCl respectively compared to the control (Table 2).

3.5. Correlation analysis

A correlation analysis was performed between MDA and DPPH scavenging activity on the one hand vs. the secondary metabolites and enzymes antioxidant activities on the other hand for describing the relationship between these variables (Table 3). The results indicated that MDA content was positively correlated ($P < 0.05$) with proline (0.968**), fructose (0.825**), glucose (0.857**), sucrose (0.845**), TPC (0.742**), TFC (0.701**), TPA (0.699**), and the activities of GR (0.977**), SOD (0.912**), and APX (0.712**). In addition, significant positive correlations were observed among DPPH and TPA (0.696**), TFC (0.797**), and TPC (0.722**), and the activities of CAT (0.900**) and APX (0.671**) (Table 3).

4. Discussion

Salt stress can lead to different physiological and biochemical modifications, resulting in many changes in both the structure and function of cells (Takemura et al., 2000). The present work found that salinity had a negative effect on the SDW and RDW of *P. equisetiforme*. A reduction in plant growth might be due to the osmotic effect induced by salinity leading to a decrease in turgor pressure, as well as several metabolic changes (Munns and Tester, 2008). Salinity also had a negative effect on RWC, which showed a gradual decrease when the plant was exposed to salinity exceeding 200 mM NaCl, leading to water deficit and loss of turgor. Similarly, RWC was also decreased in many glycophyte species cultivated under saline conditions (Kaya et al., 2007; Pulavarty et al., 2015). Furthermore, the Ψ_w of *P. equisetiforme* was significantly decreased with increasing salinity. In the cell cytoplasm, reduced water potential can be interpreted as an adaptive mechanism to allow the cells which are subjected to lower water to have the potential to improve their ability to maintain turgor pressure (Boughalleb et al., 2017).

The oxidative stress induced by salinity can lead to an excess of ROS production resulting in lipid peroxidation (Farmer and Mueller, 2013), which can be estimated by the MDA content (Dhindsa et al., 1981). The present study indicated a gradual increase of *P. equisetiforme* MDA content under salt stress. This increase was significant at 300 and 400 mM, suggesting that severe stress-induced lipid peroxidation resulted in deterioration of the integrity of the membrane. However, no significant change of the

Table 1. Salinity (0, 100, 200, 300, and 400 mM NaCl) effect on phenolic acids and flavonoids compounds (μg g^{-1} DW) identified in the shoots of the 3-month-old plants of *P. equisetiforme*.

No.	RT (min)	Compound name	Chemical class	Treatments				
				Control	100 mM	200 mM	300 mM	400 mM
1	2.06	Quinic acid	Phenolic acid	1055 ± 81.2c	1548 ± 113b	1764 ± 127b	2372 ± 159a	2119 ± 132a
2	4.05	Gallic acid	Phenolic acid	452 ± 52.3b	506 ± 48.1b	691 ± 72.3a	894 ± 91.5a	723 ± 87.4a
3	7.03	Protocatchuic acid	Phenolic acid	56.5 ± 7.1c	75.8 ± 9.0bc	94.8 ± 8.7ab	104.5 ± 8.2ab	118.9 ± 10.6a
4	12.32	4-O-caffeoylquinic acid	Phenolic acid	0.39 ± 0.06a	0.31 ± 0.05a	0.24 ± 0.04a	0.37 ± 0.03a	0.31 ± 0.05a
5	14.77	Caffeic acid	Phenolic acid	2.7 ± 0.12c	2.8 ± 1.90c	3.9 ± 0.25b	4.2 ± 0.31b	5.6 ± 4.50a
6	16.45	Syringic acid	Phenolic acid	19.8 ± 1.3a	18.1 ± 1.5a	19.0 ± 1.7a	20.7 ± 2.3a	18.3 ± 2.4a
7	21.15	p-coumaric acid	Phenolic acid	58.0 ± 7.0a	47.2 ± 6.8a	45.3 ± 7.5a	57.8 ± 6.4a	46.1 ± 5.3a
8	23.40	Trans ferulic acid	Phenolic acid	8.4 ± 0.7a	8.3 ± 0.9a	8.2 ± 0.5a	9.0 ± 0.7a	9.4 ± 0.6a
9	32.23	Trans cinnamic acid	Phenolic acid	12.1 ± 0.9c	13.0 ± 1.1c	13.8 ± 0.8c	22.4 ± 1.7b	27.3 ± 2.1a
10	11.43	Catechin (+)	Flavonoid	54.0 ± 6.3d	138.1 ± 9.9c	165.8 ± 8.4c	274.4 ± 19.1a	237.3 ± 13.3b
11	24.51	Quercetin-3-o-galactoside	Flavonoid	158 ± 9.3b	211 ± 14.9b	419 ± 18.2a	376 ± 25.9a	393 ± 24.2a
12	26.97	Quercetin-3-o-rhamonoside	Flavonoid	8.7 ± 0.6bc	6.5 ± 0.3c	8.5 ± 0.7bc	9.3 ± 0.8b	11.8 ± 0.9a
13	26.05	Naringin	Flavonoid	1.2 ± 0.2a	1.2 ± 0.3a	1.7 ± 0.2a	1.4 ± 0.1a	1.6 ± 0.2a
14	16.87	Epicatechin	Flavonoid	61.4 ± 5.4c	141 ± 8.9b	254 ± 11.9a	199 ± 12.2b	187 ± 13.5b
15	32.70	Quercetin	Flavonoid	5.5 ± 0.4c	10.7 ± 0.5b	9.7 ± 0.4b	12.8 ± 0.8ab	14.0 ± 1.2a
16	34.18	Naringenin	Flavonoid	1.3 ± 0.1b	1.6 ± 0.1b	1.7 ± 0.2b	2.4 ± 0.3a	2.5 ± 0.2a
17	35.59	Luteolin	Flavonoid	2.4 ± 0.1a	2.2 ± 0.1a	2.6 ± 0.2a	2.1 ± 0.2a	2.0 ± 0.1a
18	35.59	Cirsiliol	Flavonoid	12.1 ± 0.4c	10.5 ± 0.5c	12.2 ± 0.7c	14.4 ± 1.7b	17.4 ± 1.5a
19	38.88	Cirsilineol	Flavonoid	0.61 ± 0.04a	0.38 ± 0.05a	0.36 ± 0.07a	0.43 ± 0.05a	0.5 ± 0.07a
Phenol acids average				1665 ± 96.8e	2220 ± 108d	2640 ± 136c	3485 ± 119a	3069 ± 125b
Flavonoids average				306 ± 28.2c	524 ± 23b	877 ± 26.9a	893 ± 33.4a	867 ± 35.7a
Total phenols average				1972 ± 103d	2745 ± 134c	3517 ± 156b	4379 ± 215a	3936 ± 191b

Data are means values ± standard deviation (n = 3). The different lowercase letters (a–e) in the same row indicate significantly different values at $P < 0.05$ as described by Duncan's multiple range test. RT is the retention time.

Table 2. Effects of salt stress (0, 100, 200, 300, and 400 mM NaCl) on superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), and gluthation reductase (GR) activities in the shoots of the 3-month-old plants of *P. equisetiforme*.

Parameters	Treatments				
	Control	100 mM	200 mM	300 mM	400 mM
SOD (units mg^{-1} protein)	1.39 ± 0.08c	1.62 ± 0.08c	1.76 ± 0.09c	2.23 ± 0.12b	2.98 ± 0.15a
CAT (units mg^{-1} protein)	10.3 ± 0.36e	14.7 ± 0.41d	24.9 ± 0.57a	21.3 ± 0.62b	19.1 ± 0.52c
APX (units mg^{-1} protein)	23.1 ± 0.74e	30.4 ± 0.82d	43.3 ± 0.95c	77.4 ± 1.50a	54.8 ± 1.42b
GR (units mg^{-1} protein)	8.8 ± 0.29d	10.3 ± 0.27d	14.1 ± 0.33c	26.0 ± 0.52b	37.4 ± 0.83a
GPX (units mg^{-1} protein)	0.27 ± 0.01e	0.75 ± 0.03d	1.20 ± 0.05c	2.23 ± 0.07b	2.31 ± 0.08a

Data are means values ± standard deviation (n = 4). The different lowercase letters (a–e) in the same row indicate significantly different values at $P < 0.05$ as described by Duncan's multiple range test.

Table 3. Correlation coefficients (*r*) between malondialdehyde (MDA) content and secondary metabolites (proline fructose, glucose, sucrose, and phenolic compounds) and enzymes antioxidant activities, and between DPPH scavenging activity and secondary metabolite and enzyme antioxidant activities. APX, ascorbate peroxidase; CAT, catalase; GPX, guaiacol peroxidase; GR, glutathione reductase; SOD, superoxide dismutase; TFC, total flavonoid compounds; TPA, total phenolic acids; TPC, total phenolic compounds.

Correlation	Proline	Fructose	Glucose	Sucrose	TPA	TFC	TPC	SOD	CAT	GPX	APX	GR
MDA	0.968**	0.825**	0.857**	0.845**	0.699**	0.701**	0.742**	0.912**	0.421	0.902**	0.712**	0.977**
DPPH	0.202	0.476	0.338	0.312	0.696**	0.797**	0.722**	0.161	0.900**	0.510	0.671**	0.222

Significant coefficients at $P < 0.05$ with ** and * significant at 0.01 and 0.05 probability levels, respectively.

MDA content at low to moderate stress (100–200 mM NaCl) was observed. Our results corroborate with findings showing that an unchanged lipid peroxidation level seems to be a characteristic of tolerant plants coping with salinity (Ashraf and Harris, 2004). In order to minimize the oxidative damage caused by ROS generated under salt stress, the plants exhibit both enzymatic and nonenzymatic antioxidant defense systems (Foyer and Noctor, 2005).

The proline content of *P. equisetiforme* was significantly enhanced at moderate to severe salt stress (200–400 mM NaCl). As suggested by Munns and Tester (2008), high levels of metabolites such as proline are an important adaptive response toward salt tolerance by contributing to cellular osmotic adjustment and cellular and physiological homeostasis maintenance in plants. Moreover, a positive relationship between MDA and proline accumulation ($r = 0.968^{**}$; Table 3) was recorded, suggesting that this osmolyte participates effectively in scavenging the ROS generated, thereby protecting the cells from oxidative damage (Matysik et al., 2002). In addition, the soluble sugar content (i.e., fructose, glucose, and sucrose) of *P. equisetiforme* shoots was increased under salt stress. Similarly, Gil-Quintana et al. (2013) showed that sucrose, glucose, and fructose are the most common soluble sugars accumulated in plants subjected to abiotic stress. The accumulation of sucrose and glucose compared to the proline content suggests their main role in the process of osmotic adjustment in *P. equisetiforme* under salt stress, which is in agreement with results on *Astragalus gombiformis* (Boughalleb et al., 2017) and *Stipa lagascae* (Abdellaoui et al., 2017). In view of the positive and highly significant correlation between MDA and the accumulation of soluble sugars (i.e., fructose, glucose, and sucrose) (Table 3), they could act as scavengers of free radicals and provide membrane protection, which is similar to the results of Singh et al. (2015). Moreover, soluble sugars are also involved in the oxidative pentose phosphate pathway that allows the reduction of $NADP^+$ to NADPH, which is essential for scavenging of ROS (Hu et al., 2012). Indeed, the reduced NADPH is involved in continuously replenishing the GSH pools as a cofactor of glutathione reductase (Rescigno and Perham, 1994), and additionally in keeping catalase in its active form (Hillar and Nicholls, 1992).

In our study, the major compounds found in the extract of *P. equisetiforme* shoots are quinic and gallic acid (phenolic acids) followed by quercetin-3-O-galactoside, epicatechin, and catechin (flavonoids). These results confirm those on other populations of *P. equisetiforme* (Mahmoudi et al., 2019). As a result of increasing the level of salinity, some compounds including quinic acid, catechin, epicatechin, quercetin-3-O-galactoside, and quercetin were remarkably increased. However, cirsiliol, quercetin-3-O-rhamonoside, and naringenin increased only at high salinity levels (300–400 mM NaCl). Likewise, Caliskan et al. (2017) reported that chlorogenic acid, rutin, hyperoside, isoquercetin, quercitrin, and quercetin contents of *Hypericum pruinatum* plantlets increased significantly under higher saline conditions. Minh et al. (2016) also found that the content of *p*-coumaric acid, protocatechuic acid, and ferulic acid were higher in rice (*Oryza sativa*) treated with NaCl. Among the 19 phenolic compounds identified in *P. equisetiforme* shoots, 7 compounds (4-O-caffeoylquinic acid, syringic acid, p-coumaric acid, trans-ferulic acid, naringin, luteolin, and cirsilineol) were not affected by NaCl treatments (Table 1). Our results showed that the highest accumulation of phenolic compounds was found in *P. equisetiforme* plants subjected to 300 mM NaCl. This richness in secondary metabolites could be useful in the medicinal, pharmaceutical, and cosmetic industries.

The antioxidant activity, expressed by the ability to neutralize DPPH radical, showed significant values at 200 and 300 mM NaCl. This could be directly linked to the increase in phenolic acid and flavonoid contents (i.e., quinic acid, gallic acid, quercetin-3-O-galactoside, epicatechin, and catechin) confirmed by the high and positive correlation among DPPH, TPC, and TFC (Table 3), which corroborate with findings showing that accumulation of phenolic acids and flavonoids could be considered an adaptative strategy for overcoming oxidative

damage (Upadhyay et al., 2010; Vallverdú-Queralt et al., 2013). At 400 mM NaCl, the reduced levels of DPPH suggest a failure in the ROS scavenging system, resulting in the imbalance between the produced antioxidants and ROS, which explains the chlorosis and/or necrosis of almost 20% of the leaves (data not shown).

In addition to the vital role of metabolites in scavenging ROS, several antioxidant enzymes are associated with the antioxidant defense system which helps plants to better overcome abiotic stresses (Apel and Hirt, 2004). In our study, SOD activity increased at higher salinity levels (300–400 mM NaCl), while it remained constant at low and moderate doses of NaCl (≤200 mM), similar to results obtained in *Astragalus gombiformis* (Boughalleb et al., 2017) and *Stipa lagascae* (Abdellaoui et al., 2017). This increase in SOD activity generates high levels of H_2O_2, which acts as a precursor for other ROS (Bose et al., 2014). Likewise, CAT activity increased at all levels of salinity; higher values were recorded at 200 and 300 mM NaCl. In agreement with our findings, CAT catalyzes the degradation or reduction of H_2O_2 to H_2O and O_2, consequently completing the detoxification process initiated by SOD (Chelikani et al., 2004; Gill and Tuteja, 2010).

Among the most important enzymes in the ascorbate–glutathione cycle, APX and GR catalyze the reduction of H_2O_2 into O_2 and H_2O coupled to ascorbate oxidation and the reduction of oxidized glutathione disulfide (GSSG) to glutathione (GSH) (Saed-Moucheshi et al., 2014). In *P. equisetiforme* shoots, the APX activity showed a notable increase at all salinity levels, with higher activity at 300 mM NaCl. APX uses ascorbate as the electron donor to detoxify the H_2O_2 into O_2 and H_2O in chloroplast, cytosol, mitochondria, and peroxisomes (Caverzan et al., 2012). Similarly, the GR activity demonstrated the same behavior; however, it remained steady under low salinity levels. In agreement with previous reports, activities of APX and GR increased with increasing salt stress (Benzarti et al., 2012). The increase in GR activity ensures the modulation of the glutathione redox state, which increases the rate of NADPH oxidation to minimize the formation of ROS (Muscolo et al., 2013). Similar to the results of previous studies indicating an increase in GPX activity induced by salt stress in rice (Khan and Panda, 2008) and in wheatgrass (Sheikh-Mohamadi et al., 2017), *P. equisetiforme* shoots showed an increase of GPX activity under salinity, with the highest values at 300 mM and 400 mM NaCl. GPX decomposes H_2O_2 to produce H_2O and O_2 (Mehlhorn et al., 1996). Our results showed that the enzymatic antioxidant system is very effective even at a 300 mM dose of NaCl, beyond which the DPPH content and the activities of CAT and APX in *P. equisetiform* were decreased.

In conclusion, *P. equisetiforme* is a highly common wild plant growing in drought and salt-affected areas which is widely used in traditional and contemporary medicinal applications. Our study showed that *P. equisetiforme* is a valuable source of polyphenols including gallic acid, quinic acid, and protocatchuic acid, with catechin, epicatechin, and quercetin-3-O-galactoside as major compounds. Salinity progressively increased the phenol content with a maximum of 4379 μg g^{-1} DW at 300 mM NaCl. Similarly, the enzymatic antioxidant activity (CAT, SOD, APX, GR, and GPX) gradually increased with the increase of salt stress. The maximum activity of CAT was recorded at 200 mM, while that of APX was at 300 mM NaCl. However, SOD, GR, and GPX increased their activity even at 400 mM NaCl. Additionally, our results showed that the tolerance threshold of *P. equisetiforme* was 300 mM NaCl, corresponding to a biomass reduction of 50%, which suggests that *P. equisetiforme* could be considered a salt-tolerant species. Finally, it is recommended that detailed field experiments with various salinity regimes should be carried out to confirm these conclusions.

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