Journal of Plant Interactions

ISSN: 1742-9145 (Print) 1742-9153 (Online) Journal homepage: https://www.tandfonline.com/loi/tjpi20

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To cite this article: Mohammad Javad Merati, Halimeh Hassanpour, Vahid Niknam & Masoud Mirmasoumi (2014) Exogenous application of penconazole regulates plant growth and antioxidative responses in salt-stressed *Mentha pulegium* L., Journal of Plant Interactions, 9:1, 791-801, DOI: 10.1080/17429145.2014.948084

To link to this article: https://doi.org/10.1080/17429145.2014.948084

Published online: 24 Dec 2014.

Journal of Plant Interactions, 2014
Vol. 9, No. 1, 791–801, http://dx.doi.org/10.1080/17429145.2014.948084

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RESEARCH ARTICLE

Exogenous application of penconazole regulates plant growth and antioxidative responses in salt-stressed *Mentha pulegium* L.

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(*Received 27 February 2014; accepted 21 July 2014*)

The mechanism of growth amelioration in salt-stressed pennyroyal (*Mentha pulegium* L.) was investigated by exogenous application of penconazole (PEN). Seven weeks after sowing, seedlings were treated with increasing NaCl concentrations (0, 25, 50, and 75 mM) with or without PEN (15 mg l^{-1}) and were harvested randomly at different times. Results showed that some growth parameters and the relative water content (RWC) decreased under salt stress, while lipid peroxidation, H_2O_2 content, activities of superoxide dismutase (SOD; EC 1.15.1.1), peroxidase (POX; EC 1.11.1.7), polyphenol oxidase (PPO; EC 1.10.3.1), catalase (CAT; EC 1.11.1.6), and ascorbate peroxidase (APX; EC 1.11.1.1) remarkably increased. Exogenous application of PEN increased some growth parameters, RWC, antioxidant enzyme activities, and H_2O_2 content, but the effects of PEN were more significant under salt stress conditions. PEN treatment also decreased lipid peroxidation. These results suggest that PEN-induced tolerance to salt stress in *M. pulegium* plants may be related to regulation of antioxidative responses and H_2O_2 level.

Keywords: antioxidant enzymes; *Mentha pulegium*; penconazole; salinity stress

Introduction

Salinity is an important environmental stress that in plants imposes both ionic toxicity and osmotic stress, leading to the reduction of plant growth and crop production (Munns et al. 2006; Bano et al. 2014). Pennyroyal (*Mentha pulegium* L.) is an aromatic and medicinal plants belonging to the Lamiaceae family present in the humid regions of Iran. It is widely used in traditional medicine, food processing, perfumery, and pharmaceutical products because of the high quality of its essential oil (Shirazi et al. 2004, Omran et al. 2012; Soares et al. 2012). Like most of the cultivated plants, growth and yield of this plant can be affected under salt stress.

One of the plant responses to environment stresses is the rapid and increased generation of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radicals (OH^-), which can damage membrane lipids, proteins, and nucleic acids (Liang et al. 2003; Patade et al. 2011). Different plants develop several protection mechanisms to eliminate ROS and prevent oxidative damage. Among of them, enzymatic antioxidants such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), peroxidase (POX; EC 1.11.1.7), and ascorbate peroxidase (APX; EC 1.11.1.1) provide the first line of defense against ROS (Demiral & Turkan 2004; Jebara et al. 2005).

Manipulation of crop production with chemicals is one of the most important advancement achieved in agriculture. The medicinal plant yield under stress can be increased by application of plant growth regulators (PGRs). Penconazole (PEN) [1-(2,4-dichloro-β-propylphenethyl)-1*H*-1,2,4-triazole] is a triazolic fungicide which has PGR properties (Fletcher et al. 2000). Triazole compounds induce a variety of morphological and biochemical responses in plants including stimulation of root growth, reduction in free-radical damage, and increase in antioxidant potential (Jaleel, Gopi, Manivannan & Panneerselvam 2007; Manivannan et al. 2007). These qualities make them ideal to increase resistance to salt stress in medicinal plants. Our previous work showed that PEN minimizes the negative effects of drought stress with lower membrane damage and could be used for partial amelioration of drought stress in *M. pulegium* plants (Hassanpour et al. 2012). The effect of salinity on antioxidative responses of *M. pulegium* plants was previously studied by Oueslati et al. (2010). The aim of the present study was to investigate the effect of salt stress on some physiological and biochemical parameters and to assess the possibility of improving salt tolerance of *M. pulegium* by application of PEN.

Materials and methods

Plant material and treatments

Seeds of *M. pulegium* were collected during summer 2012 from Chalous (Province of Mazandaran, Iran). Seeds were sown in Tref peat in a greenhouse with a 16 h light/8 h dark photoperiod at 25/18°C day/night temperatures and 50% air humidity. Plastic pots were filled with perlite and seedlings were thinned to five per pot 7 weeks after sowing. Plants were irrigated with complete Hoagland solution. Pots were divided in eight groups and treated with different salinity concentration

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and the PEN treatment. Seedlings were treated with 0, 25, 50, and 75 mM NaCl with or without PEN (15 mg l^{-1}). PEN was applied uniformly to the plants as a fine spray using an atomizer once a week and the optimum concentration was determined according to Hassanpour et al. (2012). Seedlings were uprooted during four harvest times (10, 20, 30, and 40 days after treatments) and four plants per treatment were used for analyses in all the experiments.

Growth parameters

Plants were evaluated after four interval harvests of salt stress in terms of fresh weight (FW) and dry weight (DW). Relative water content (RWC) of leaves was estimated according to Wheatherley (1973). Saturated weight (SW) of the plant was determined by keeping them in de-ionized water at 4°C in the dark for 24 h, and DW was obtained after oven drying at 45°C for 72 h, the time point at which a constant weight was reached.

$$\mathrm{RWC}(\%) = \left(\frac{\mathrm{FW} - \mathrm{DW}}{\mathrm{SW} - \mathrm{DW}}\right) \times 100$$

Lipid peroxidation and H_2O_2 content

Lipid peroxidation was determined by measuring malondialdehyde (MDA) content (Heath & Packer 1968). Plant material (0.25 g) from leaf, shoot, and root samples was homogenized with 2.5 ml of 0.1% trichloroacetic acid (TCA) and the homogenate was centrifuged at 10,000×*g* for 10 min. For every 1 ml of the aliquot of the supernatant, 4 ml of 20% TCA containing 0.5% thiobarbituric acid (TBA) was supplemented. The mixture was heated at 95°C for 30 min and quickly chilled in an ice bath. Afterwards, the mixture was centrifuged at 10,000*g* for 15 min and the absorbance of the supernatant was evaluated at 532 and 600 nm. The concentration of MDA was estimated by using an extinction coefficient of 155 mM^{-1}.

Hydrogen peroxide levels were assayed according to Velikova et al. (2000). Plant materials (0.5 g) were homogenized with 5 ml of 0.1% (w/v) TCA in an ice bath. The homogenate was centrifuged at 12,000×*g* for 15 min, and 0.5 ml of the extract added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. Absorbance of the supernatant was estimated at 390 nm, and H_2O_2 content was calculated using a standard curve.

Extraction and estimation of antioxidant enzymes

For determination of enzyme activities, leaves, shoots, and roots of plants were homogenized at 4°C with a mortar in1 M Tris-HCl (pH 6.8) at 4°C with a mortar in 1 M Tris-HCl (pH 6.8) and 2% (w/v) polyvinyl polypyrrolidone to avoid phenol oxidative effects. The homogenate was centrifuged in a refrigerated centrifuge at 13,000×*g* for 30 min, and supernatant was utilized for total protein content assessment and enzyme assays (Bradford 1976).

Table 1. Results of two-way analysis of variance (ANOVA) of PEN, NaCl, harvest time (Day) and their interaction (PEN × NaCl × Day) for growth parameters, RWC, H_2O_2 content, MDA level and SOD, POD, PPO, CAT, and APX activities in *M. pulegium*.

	Independent variable			
Dependent variable	PEN	Salt	Day	PEN × Salt × Day
Shoot FW	0.008^{ns}	2.729***	1.676***	0.007^{ns}
Root FW	0.865***	0.799***	1.345***	0.046***
Shoot DW	0.002^{ns}	0.18**	0.30***	0.001^{ns}
Root DW	0.003***	0.005***	0.015***	0.001**
RWC	1133.27***	1439.35***	553.62***	9.274*
Leaf SOD activities	1.101***	4.204***	14.212***	0.389^{ns}
Shoot SOD activities	2.82***	17.71***	36.27***	0.051^{ns}
Root SOD activities	18.56***	65.67***	64.66***	0.566***
Leaf POX activities	1.59***	11.92***	7.89***	0.355^{ns}
Shoot POX activities	65.245***	198.77***	80.27***	2.118***
Root POX activities	30.117***	162.389***	60.469***	0.147***
Leaf PPO activities	80.061***	567.396***	326.349***	0.296^{ns}
Shoot PPO activities	30.642***	169.019***	26.954***	0.183^{ns}
Root PPO activities	78.784***	1.089***	254.138***	0.903^{ns}
Leaf CAT activities	1678.60***	7590.04***	4750.17***	17.93***
Shoot CAT activities	515.18***	4801.24***	4306.37***	3.28^{ns}
Root CAT activities	83.08***	597.98***	340.99***	1.64**
Leaf APX activities	9939.6***	31759.9***	13568.4***	302.6***
Shoot APX activities	1615.4***	6842.78***	2184.64***	17.69^{ns}
Root APX activities	36.01.405***	40117.98***	17482.17***	20.99*
Leaf H_2O_2 content	0.020***	0.013***	0.010***	0.008^{ns}
Root H_2O_2 content	0.13*	0.109***	0.097***	0.009^{ns}
Leaf MDA content	44.22***	97.23***	141.99***	1.22**
Root MDA content	2.49***	8.53***	8.704***	0.165**

Note: Mean squares are indicated at 5% level.
ns, not significant.
*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

SOD activity was estimated by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT) at 560 nm, as regarded by Giannopolitis and Ries (1977) in a reaction mixture (1.5 mL) containing of 50 mM sodium phosphate buffer (pH 7.5), 0.1 µM EDTA, 13 mM L-methionine, 75 µM NBT, 75 µM riboflavin, and 30 µL enzyme extract. Riboflavin was added last and tubes were shaken and illuminated for 15 min, and absorbance of the reaction mixture was recorded at 560 nm against the non-illuminated blank. One unit of SOD activity was determined as the amount of enzyme that inhibits 50% NBT photoreduction.

POX activity was measured according to Abeles and Biles (1991). The reaction mixture consisted 4 ml of 0.2 M acetate buffer (pH 4.8), 0.4 ml H_2O_2 (3%), 0.2 ml of 20 mM benzidine, and 0.03 ml enzyme extract. Alteration in absorbance of the reaction solution was measured at 530 nm. The POD activity was determined as 1 µM of benzidine oxidized per min per mg protein [unit mg^{-1} (protein)].

PPO activity was determined at 40°C by the method of Raymond et al. (1993) based on the oxidization of pyrogallol. The reaction mixture contained 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.8), 0.2 ml pyrogallol 20 mM, and 0.03 ml enzyme extract. The increase in absorbance was recorded at 430 nm. The PPO activity was determined as µM of pyrogallol oxidized per min per mg protein [unit mg^{-1} (protein)].

APX activity was measured according to Jebara et al. (2005). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM hydrogen peroxide, and 10 µl of enzyme extract in a total volume of 1 ml. The concentration of oxidized ascorbate was determined by the decrease in absorbance at 290 nm. The concentration of oxidized ascorbate was calculated by using extinction coefficient ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$). One unit of APX was defined as 1 µM oxidized ascorbate per min per mg protein [unit mg^{-1} (protein)].

Total CAT activities were estimated according to Aebi (1984), which measures the initial rate of disappearance of H_2O_2 at 240 nm. The reaction mixture consisted 0.625 ml of 50 mM sodium phosphate buffer (pH 7.0) with 75 µl H_2O_2 (3%) and 5 µl enzyme extract. The enzyme activity was defined as 1 µM of H_2O_2 oxidized per min per mg protein [unit mg^{-1} (protein)].

Statistical analysis

The experiments were carried out in completely randomized block design. Data were analyzed by one-way

Table 2. Effects of NaCl and PEN treatments during four interval harvest times (10, 20, 30, and 40 days) on growth parameters and RWC content in *M. pulegium*.

Treatments		Parameters				
Day and NaCl	Penconazole	Shoot FW (g)	Root FW (g)	Shoot DW (g)	Root DW (g)	RWC (%)
10 (Control)	−PEN	0.75 ± 0.021 ef	0.55 ± 0.016 f	0.083 ± 0.007 e	0.053 ± 0.004 f	82.98 ± 0.70 ab
10 (25 mM)		0.73 ± 0.025 ef	0.60 ± 0.032 f	0.083 ± 0.007 e	0.057 ± 0.006 f	80.57 ± 0.64 ab
10 (50 mM)		0.67 ± 0.044 ef	0.50 ± 0.012 fg	0.067 ± 0.005 f	0.058 ± 0.007 f	77.23 ± 1.41 bc
10 (75 mM)		0.46 ± 0.029 g	0.37 ± 0.023 g	0.056 ± 0.004 f	0.042 ± 0.004 g	73.31 ± 1.28 c
20 (Control)		1.19 ± 0.030 cd	0.73 ± 0.038 e	0.121 ± 0.008 e	0.066 ± 0.008 ef	83.60 ± 0.73 ab
20 (25 mM)		1.11 ± 0.032 d	0.82 ± 0.042 e	0.105 ± 0.007 ef	0.076 ± 0.005 e	78.88 ± 1.35 cd
20 (50 mM)		0.88 ± 0.043 e	0.63 ± 0.019 f	0.081 ± 0.008 ef	0.080 ± 0.005 e	72.31 ± 1.32 de
20 (75 mM)		0.55 ± 0.031 f	0.44 ± 0.009 g	0.066 ± 0.009 f	0.049 ± 0.004 g	66.85 ± 0.92 d
30 (Control)		1.50 ± 0.041 bc	1.02 ± 0.044 cd	0.163 ± 0.008 bc	0.080 ± 0.006 de	82.27 ± 1.06 ab
30 (25 mM)		1.35 ± 0.043 c	1.09 ± 0.035 cd	0.150 ± 0.008 c	0.094 ± 0.005 d	75.13 ± 1.64 c
30 (50 mM)		1.01 ± 0.049 de	0.69 ± 0.032 ef	0.116 ± 0.005 d	0.107 ± 0.006 c	60.58 ± 0.57 d
30 (75 mM)		0.61 ± 0.051 f	0.50 ± 0.023 fg	0.072 ± 0.008 ef	0.057 ± 0.004 f	51.39 ± 1.54 ef
40 (Control)		1.90 ± 0.112 a	1.27 ± 0.06 b	0.213 ± 0.012 a	0.106 ± 0.006 c	83.26 ± 1.28 ab
40 (25 mM)		1.51 ± 0.097 bc	1.33 ± 0.022 a	0.180 ± 0.007 bc	0.120 ± 0.007 b	70.19 ± 1.42 ef
40 (50 mM)		1.10 ± 0.029 d	0.80 ± 0.036 e	0.137 ± 0.007 cd	0.125 ± 0.008 b	56.64 ± 1.14 de
40 (75 mM)		0.64 ± 0.037 ef	0.53 ± 0.017 fg	0.073 ± 0.009 ef	0.069 ± 0.006 ef	42.22 ± 1.49 fg
10 (Control)	+PEN	0.73 ± 0.02 ef	0.59 ± 0.014 f	0.079 ± 0.007 ef	0.058 ± 0.005 f	83.36 ± 1.33 ab
10 (25 mM)		0.71 ± 0.029 ef	0.75 ± 0.04 f	0.070 ± 0.006 ef	0.063 ± 0.004 ef	82.05 ± 1.43 ab
10 (50 mM)		0.57 ± 0.012 f	0.60 ± 0.032 fg	0.060 ± 0.013 f	0.067 ± 0.007 ef	81.9 ± 1.26 ab
10 (75 mM)		0.41 ± 0.017 g	0.48 ± 0.037 g	0.059 ± 0.005 f	0.053 ± 0.006 f	79.78 ± 1.24 b
20 (Control)		1.00 ± 0.052 de	0.83 ± 0.038 cd	0.086 ± 0.007 e	0.077 ± 0.004 e	85.53 ± 0.66 a
20 (25 mM)		0.91 ± 0.054 e	1.01 ± 0.038 de	0.071 ± 0.007 ef	0.093 ± 0.007 d	83.21 ± 0.44 ab
20 (50 mM)		0.77 ± 0.072 ef	0.77 ± 0.042 e	0.076 ± 0.006 ef	0.092 ± 0.004 d	79.18 ± 0.93 b
20 (75 mM)		0.55 ± 0.034 f	0.63 ± 0.017 ef	0.074 ± 0.006 ef	0.063 ± 0.007 ef	74.9 ± 1.08 bc
30 (Control)		1.26 ± 0.046 cd	1.16 ± 0.032 bc	0.145 ± 0.008 cd	0.080 ± 0.006 de	84.48 ± 1.62 a
30 (25 mM)		1.09 ± 0.058 d	1.25 ± 0.055 b	0.140 ± 0.008 cd	0.102 ± 0.006 c	81.86 ± 1.87 ab
30 (50 mM)		0.86 ± 0.056 e	0.83 ± 0.03 de	0.118 ± 0.007 d	0.127 ± 0.006 b	72.09 ± 1.16 c
30 (75 mM)		0.58 ± 0.035 f	0.74 ± 0.025 e	0.084 ± 0.007 e	0.079 ± 0.005 de	62.16 ± 0.85 d
40 (Control)		1.68 ± 0.054 b	1.39 ± 0.052 ab	0.186 ± 0.008 ab	0.117 ± 0.006 c	86.81 ± 1.18 a
40 (25 mM)		1.30 ± 0.05 c	1.43 ± 0.058 a	0.164 ± 0.013 bc	0.130 ± 0.009 b	79.3 ± 1.28 b
40 (50 mM)		0.95 ± 0.066 de	0.99 ± 0.032 cd	0.143 ± 0.007 cd	0.155 ± 0.009 a	71.14 ± 1.14 c
40 (75 mM)		0.54 ± 0.038 f	0.72 ± 0.023 e	0.092 ± 0.009 e	0.102 ± 0.007 c	54.98 ± 1.18 e

Note: Means ± SE based on four replicates are presented. Different letters indicate significant differences at $P \leq 0.05$ (LSD).

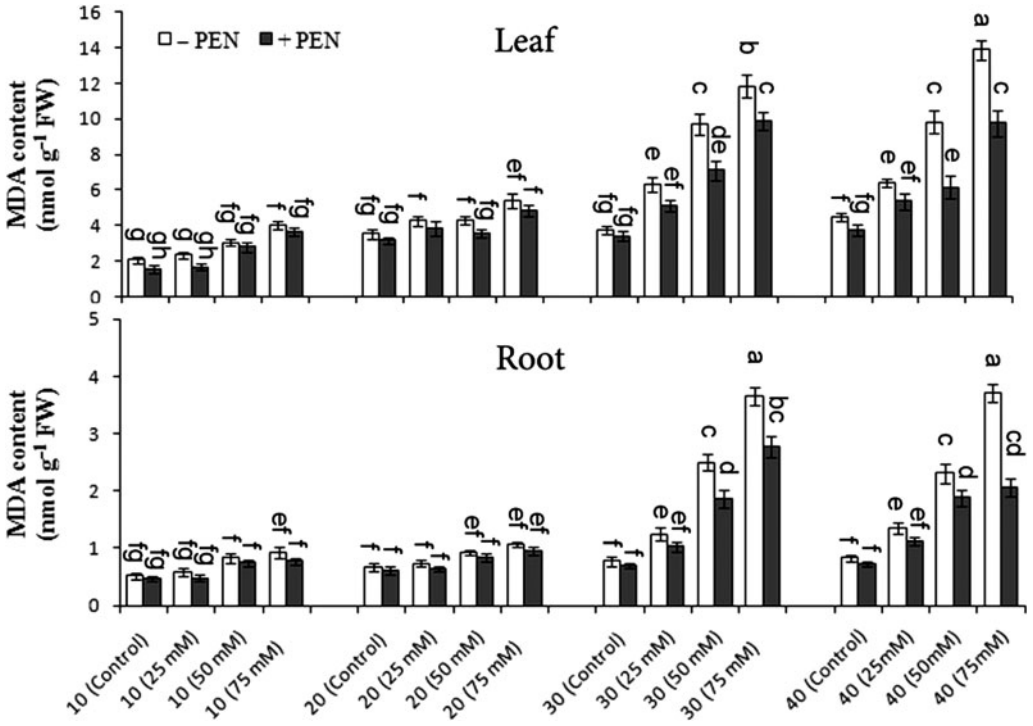

Figure 1. The effect of NaCl (0, 25, 50, and 75 mM) and penconazole (−PEN and +PEN) treatments on lipid peroxidation (MDA) content of *M. pulegium* leaves and roots during four harvest times (10, 20, 30, and 40 days). Vertical bars indicate mean ± SE of four replicates. Different letters indicate significant differences at $P \leq 0.05$ (LSD).

analysis of variance (ANOVA) using SPSS (version 19). The mean differences were compared by the lowest standard deviations (LSD) test. Each data point was the mean of four replicates (n = 4) in each group and P values ≤0.05 were considered as significant.

Results

Root and shoot growth was followed by measuring FW and DW on four interval harvest times. Under salt stress, growth parameters significantly decreased in *M. pulegium* with the increasing salinity concentration.

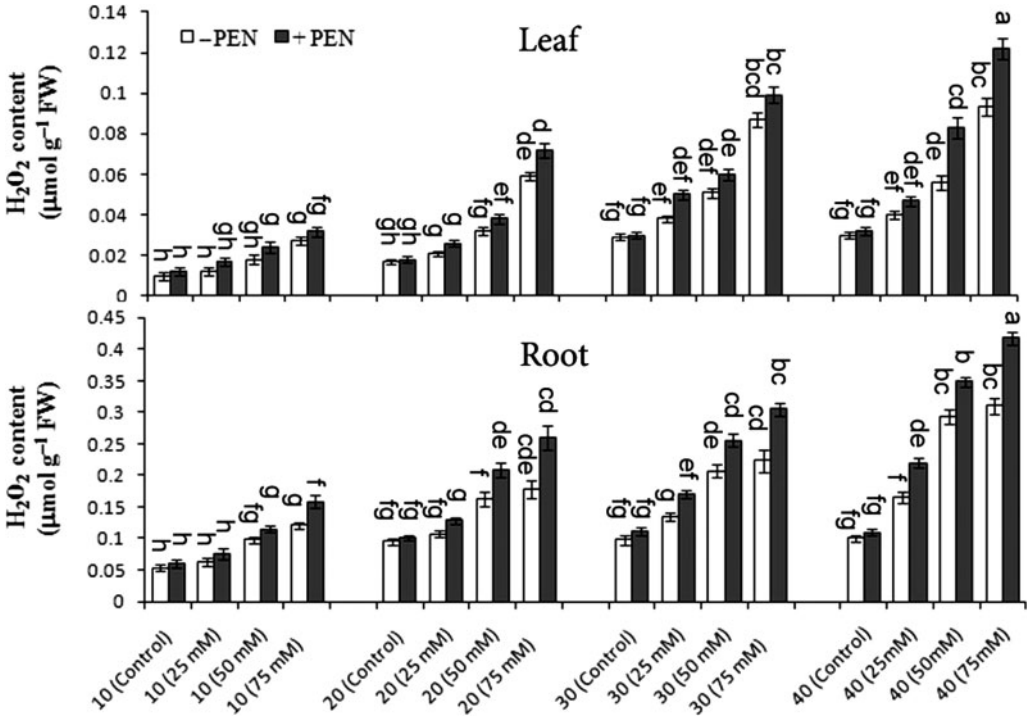

Figure 2. The effect of NaCl (0, 25, 50, and 75 mM) and penconazole (−PEN and +PEN) treatments on H_2O_2 content of *M. pulegium* leaves and roots during four harvest times (10, 20, 30, and 40 days). Vertical bars indicate mean ± SE of four replicates. Different letters indicate significant differences at $P \leq 0.05$ (LSD).

There was a significant effect of harvest time on growth parameters (Tables 1 and 2). PEN treatment in salt-stressed and unstressed plants decreased the negative effect of salt stress on growth parameters, especially at 75 mM NaCl. PEN treatment significantly increased root growth (FW and DW) especially on long-term expose to salinity as compared to plants without PEN ($P \leq 0.05$). At 50 and 75 mM NaCl with PEN, root FW increased 23.8% and 35.8%, and root DW increased 24% and 47.8% on day 40 as compared to without PEN, respectively (Table 2). It was found that PEN treatment had more effect on stress tolerance at severe stress conditions.

The RWC content significantly decreased under different salinity conditions (Table 1). At 50 and 75 mM NaCl, RWC content decreased at all harvest times and the lower RWC content was observed with increasing harvest days. PEN treatment in salt-stressed and unstressed plants significantly increased RWC content, while the effects of PEN were more pronounced in salt-stressed plants. At 50 and 75 mM NaCl with PEN, RWC increased 18.9% and 20.9% on day 30, and 25.6% and 30.2% on day 40 with higher value than without PEN (Table 2). There was a significant interaction among salinity, PEN, and harvest time for RWC content (Table 1).

MDA content significantly increased under different salinity especially at 50 and 75 mM NaCl as compared to control, and the increase level was higher on day 40 than the other harvest days (Figure 1). PEN treatment in salt-stressed and unstressed plants significantly decreased MDA content in both organs, and the effect of PEN was more pronounced in PEN-treated plants. At 75 mM NaCl and control with PEN, MDA content decreased 29.7% and 16.5% in leaves, and 44.3% and 12.04% in roots on day 40 as compared to without PEN, respectively. There was a significant interaction among salinity, PEN, and harvest time for MDA content in both the organs (Table 1).

For determination of ROS scavenging capacity, H_2O_2 levels of leaf and root organs were estimated under stress conditions. The H_2O_2 level was higher in roots than leaves, and significantly increased at 50 and 75 mM NaCl as compared to controls especially on day 40 (Figure 1). There was a significant effect of harvest time on H_2O_2 levels (Table 1). Under salt stress, H_2O_2

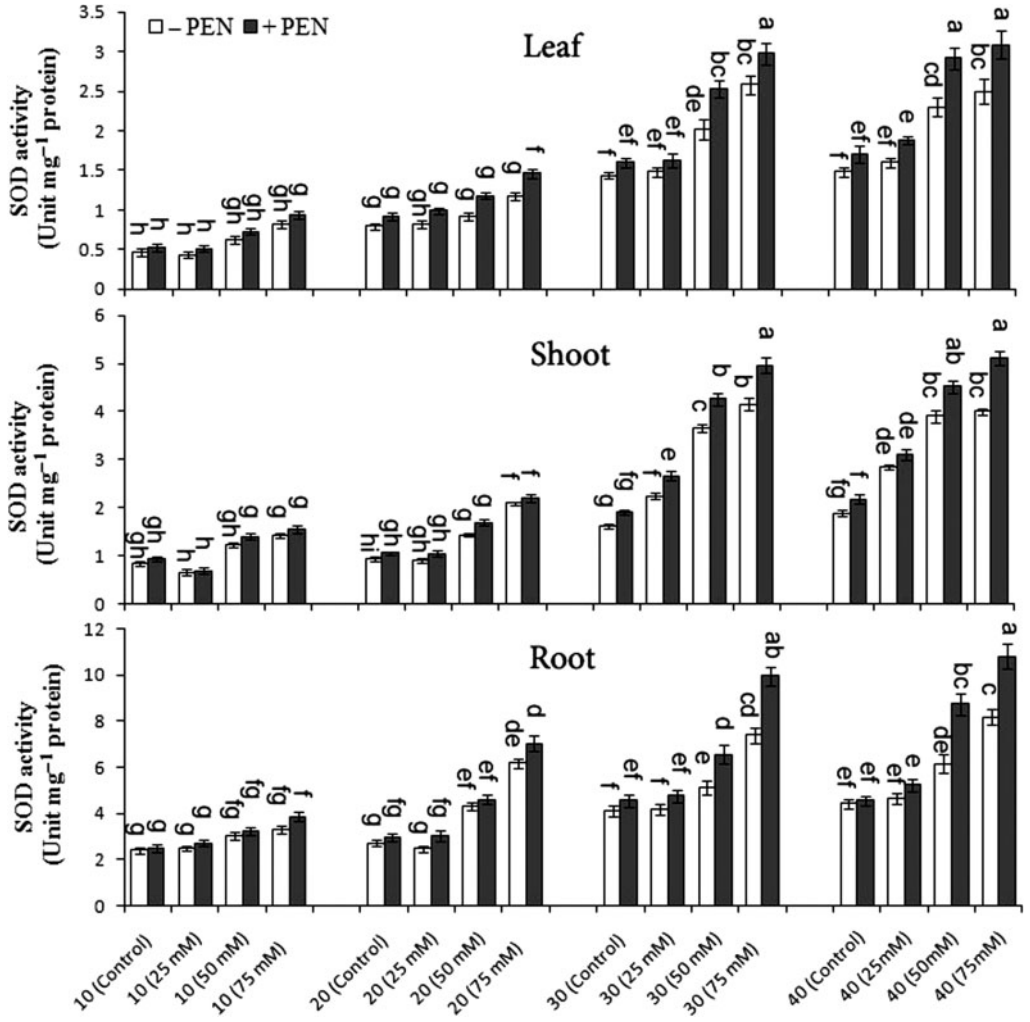

Figure 3. The effect of NaCl (0, 25, 50, and 75 mM) and penconazole (−PEN and +PEN) treatments on SOD activities of *M. pulegium* leaves, shoots, and roots during four harvest times (10, 20, 30, and 40 days). Vertical bars indicate mean ± SE of four replicates. Different letters indicate significant differences at $P \leq 0.05$ (LSD).

level significantly increased especially on day 40 at 75 mM NaCl in *M. pulegium*. PEN treatment to salt-stressed and unstressed plants increased the H_2O_2 level in leaves and roots organs, while the effect of PEN was only significant in leaves. At 75 mM NaCl and control with PEN, H_2O_2 level increased 31.2% and 6.7% in leaves and 34.8% and 8.9% in roots on day 40 with higher values than those obtained without PEN, respectively (Figure 2). There was no significant interaction among salinity, PEN, and harvest time for H_2O_2 level in both organs of *M. pulegium* (Table 1).

The differences in antioxidant enzyme (SOD, POX, APX, CAT, and PPO) activities are depicted in Figures 3–7. During the experimental period, PEN treatment caused more induction of these enzyme activities in all plant organs. Under different NaCl stress, SOD activity was significantly increased in all organs, especially in roots and activities increased with increasing harvest times. PEN treatment to salt-stressed plants caused a higher induction of SOD activity when compared to unstressed plants. At 75 mM NaCl with PEN, SOD activity increased 23.2% in leaves, 27.1% in shoots, and 34.64% in roots on day 40 as compared to without PEN (Figure 3). There was significant interaction among salinity, PEN, and harvest time for SOD activity in roots and none significant in shoots (Table 1).

POX activity in *M. pulegium* significantly increased under salinity conditions (Table 1). The highest POX activity was observed at 50 and 75 mM NaCl on day 30, as compared to controls and then decreased slightly on day 40. POX activity was higher in shoots than in roots and was induced by the PEN treatment (Figure 4). At 50 and 75 mM NaCl with PEN, POX activity increased 18.9% and 22.3.9% in leaves, 24.1% and 31.5% in shoots, and 24.9% and 21.1% in roots on day 30 with higher values than those obtained without PEN, respectively. In contrast to leaves, there was a significant interaction among salinity, PEN, and harvest time for SOD activity in shoots and roots.

PPO activity significantly increased in all plant organs under salinity, PEN treatment, and harvest time, but there was no significant interaction between all of them (Table 1). PPO activity was higher in roots than

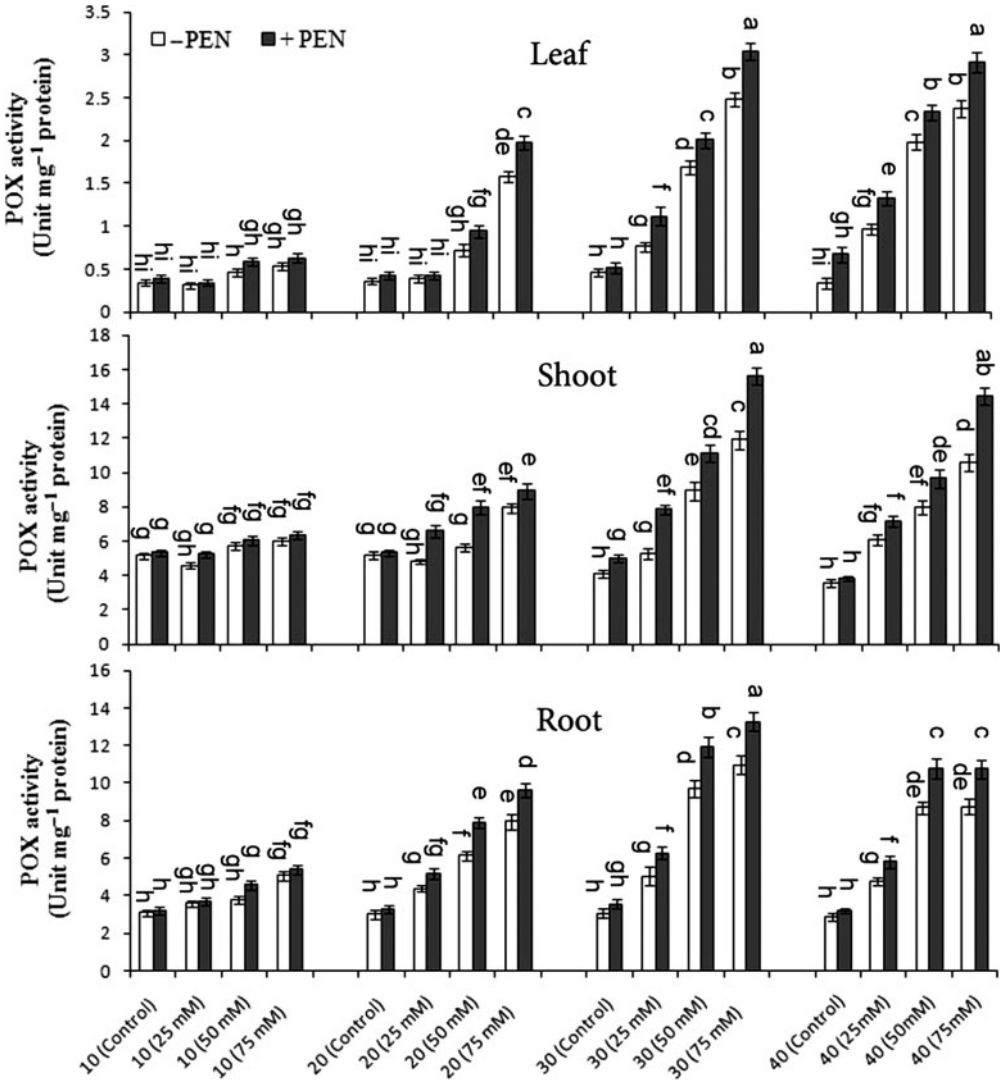

Figure 4. The effect of NaCl (0, 25, 50, and 75 mM) and penconazole (−PEN and +PEN) treatments on POX activities of *M. pulegium* leaves, shoots, and roots during four harvest times (10, 20, 30, and 40 days). Vertical bars indicate mean ± SE of four replicates. Different letters indicate significant differences at $P \leq 0.05$ (LSD).

in leaves and shoots, and more induced by the PEN treatment. The highest PPO activity was observed on day 30 as compared to other harvest times. Under salt stress, PPO activity increased from control to 50 mM NaCl and then decreased at higher NaCl concentration (75 mM NaCl) in all organs (Figure 5). PEN treatment in salt-stressed and unstressed plants increased PPO activity in all organs and the increasing level was higher in salt-stressed plants. At 50 mM NaCl with PEN, PPO activity increased 15.67% in leaves, 10.9% in shoots, and 21.3% in roots on day 30 as compared to without PEN.

CAT activity significantly increased under different NaCl stress as compared to controls and was pronounced with PEN. The highest CAT activity was observed in leaves with respect to other organs, especially on day 30, and then decreased on day 40 (Figure 6). At 75 mM NaCl with PEN, CAT activity increased 30.5% in leaves, 22.02% in shoots, and 22.2% in roots on day 30 as compared to without PEN, respectively. There was a significant interaction among salinity, PEN, and harvest time for CAT activity in leaves and roots, but it was not significant in shoots (Table 1).

APX activity significantly increased under different salinity concentrations when compared to controls and was induced with PEN treatment. The highest APX activity in *M. pulegium* was observed in roots than the other organs and remarkably increased on day 40 as compared to other harvest days (Figure 7). At 75 mM NaCl with PEN, APX activity increased 32.8% in leaves, 12.8% in shoots, and 17.8% in roots as compared to without PEN on day 40, respectively. There was a significant interaction among salinity, PEN, and harvest time for APX activity in leaves and roots, but it was not significant in shoots (Table 1).

Discussion

Plant adaptations to salt stress are complex and affected by internal constitutive salt tolerance mechanisms and external environmental factors. In our experiment, growth parameters and RWC content were remarkably inhibited under different salinity conditions in *M. pulegium*, especially on long-term NaCl exposure. Osmotic stress and ion toxicity are caused by salt stress, and can decrease cell chemical activity and turgor (Serrano et al. 1999).

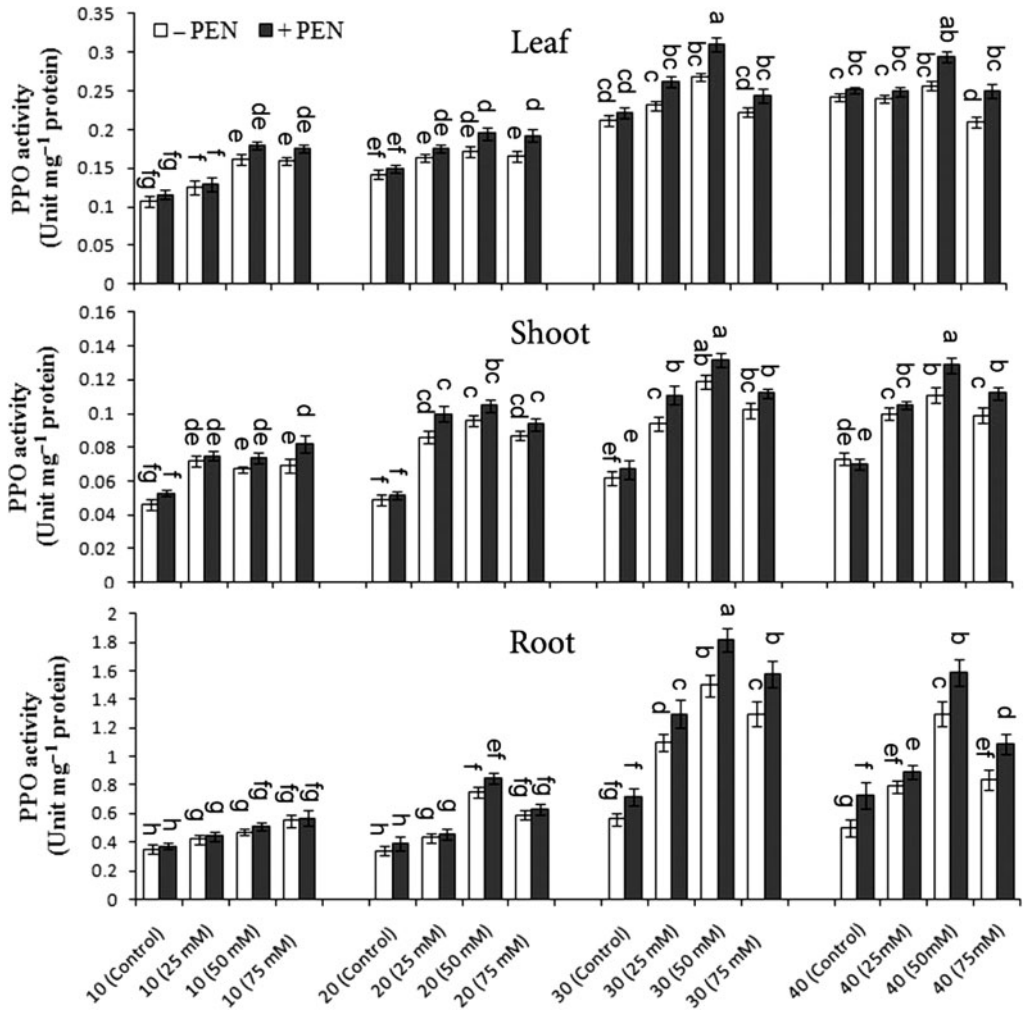

Figure 5. The effect of NaCl (0, 25, 50, and 75 mM) and penconazole (−PEN and +PEN) treatments on PPO activities of *M. pulegium* leaves, shoots, and roots during four harvest times (10, 20, 30, and 40 days). Vertical bars indicate mean ± SE of four replicates. Different letters indicate significant differences at $P \leq 0.05$ (LSD).

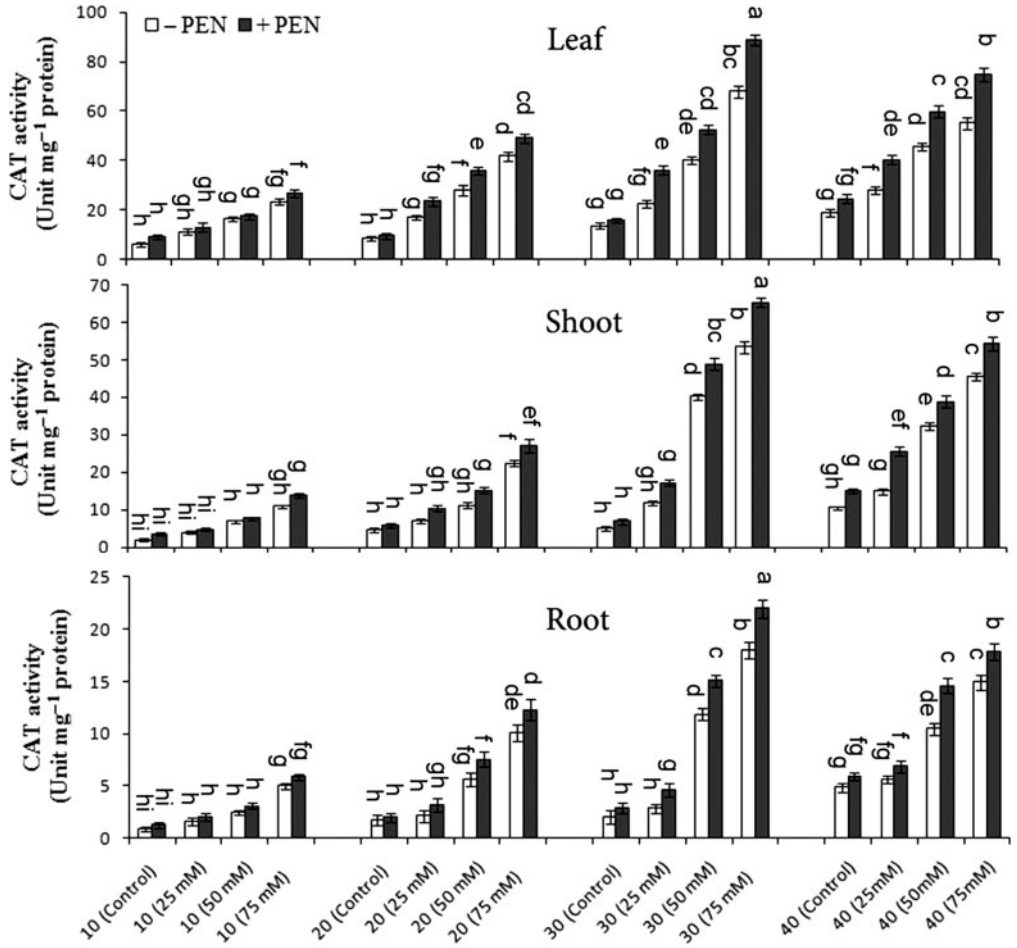

Figure 6. The effect of NaCl (0, 25, 50, and 75 mM) and penconazole (−PEN and +PEN) treatments on CAT activities of *M. pulegium* leaves, shoots, and roots during four harvest times (10, 20, 30, and 40 days). Vertical bars indicate mean ± SE of four replicates. Different letters indicate significant differences at $P \leq 0.05$ (LSD).

Since cell growth depends on turgor pressure which enlarges the cell walls, lack of turgor entails danger for cell survival. In this study, salt stress caused a growth inhibition and RWC reduction in *M. pulegium* plants (Table 2). Reduction of growth and water status have been previously observed in different studies (Jaleel, Gopi, Manivannan & Panneerselvam 2007; Shaheen et al. 2013). Indeed, in order to increase stress tolerance, *M. pulegium* plants with reduction growth could conduct energy to increase antioxidant defense system. Application of PEN ameliorated the adverse effects of salt stress by increasing root growth and RWC content in *M. pulegium* (Table 2). Similar results were reported in wheat (Hajihashemi et al. 2007) and *Catharanthus roseus* (Jaleel et al. 2008) with other triazoles. The increase of growth induced by PEN has been associated with the ability of triazoles to increase the endogenous cytokinin content (Grossman 1990) and cell division. Triazoles also induce the accumulation of osmolytes (Jaleel, Gopi, Manivannan & Panneerselvam 2007), which may function as a mechanism that maximizes the water potential gradient between soil and plant (Gomathinayagam et al. 2007). Hassanpour et al. (2013) demonstrated that PEN induced the proline accumulation in drought-stressed *M. pulegium* plants.

Peroxidation of lipid membranes of higher plants reflects free radical-induced oxidative damage at the cellular level under oxidative stress conditions (Demiral & Turkan 2004). A significant increase in the MDA and H_2O_2 levels was observed under salinity stress especially at 75 mM NaCl on day 40 (Figures 1 and 2). PEN treatment in salt-stressed and unstressed plants caused an increase of H_2O_2 level and a decrease of MDA content. Our results are in agreement with the findings of Hassanpour et al. (2012) and Berova et al. (2002), who found that triazole treatment reduced MDA content in pennyroyal and wheat, respectively. It seems that triazoles are able to induce stress-like symptoms for stimulation of antioxidative system. This might be the reason for the general increase of H_2O_2 content in *M. pulegium* (Figure 2). The increase in H_2O_2 was also reported previously in triazole-stressed *C. roseous* (Jaleel, Gopi & Panneerselvam 2007). Our study showed that H_2O_2 levels were higher in roots than those of leaves, in contrast to MDA. (Figures 1 and 2). Plants have evolved a complex antioxidant system to protect cellular membranes and organelles from damaging effects of ROS, which is composed of low-molecular mass antioxidants (glutathione, ascorbate, and carotenoids) as well as ROS-scavenging enzymes (Foyer et al. 1991; Salah et al. 2011;

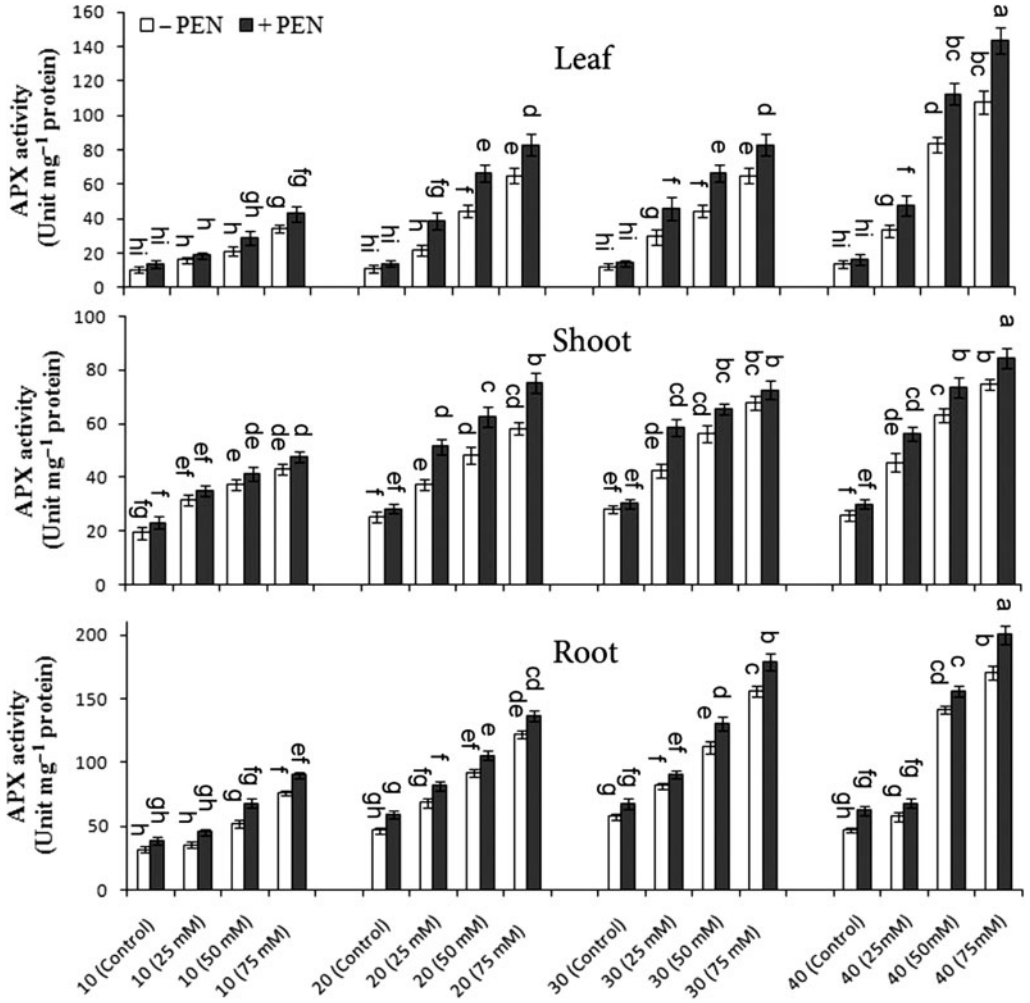

Figure 7. The effect of NaCl (0, 25, 50, and 75 mM) and penconazole (−PEN and +PEN) treatments on APX activities of *M. pulegium* leaves, shoots, and roots during four harvest times (10, 20, 30, and 40 days). Vertical bars indicate mean ± SE of four replicates. Different letters indicate significant differences at $P \leq 0.05$ (LSD).

Bano et al. 2014). It seems that the high accumulation of H_2O_2 content in roots is able to induce antioxidant system and protect root cells.

Plants respond to abiotic stress by increasing their antioxidant capacity to support them to restore the normal cellular equilibrium between production and scavenging of ROS. SOD catalyzes the disproportion of superoxide radical to molecular oxygen and H_2O_2 (Scandalios 1993) and hence decreases the risk of hydroxyl radical formation from superoxide via the Haber–Weiss type reaction. SOD activity in all the organs of *M. pulegium* significantly increased under salinity condition (Figure 3). Increased levels of SOD activity in roots reflect higher dismutating capacity as compared to shoots and leaves (Figure 3). Similar results previously have been reported in *M. pulegium* under drought stress (Hassanpour et al. 2012) and in *Zea mays* under salt stress (Kaya et al. 2013). Salt stress induced NO and H_2O_2 productions in wheat (*Triticum aestivum* L.) leaves, and NO is known to affect O_2^- generation enzymes (Haihua et al. 2002). SOD activity remarkably increased on day 40 especially at 75 mM NaCl with PEN treatment. Similar findings were presented in higher plants such as paclobutrazol-treated salt-stressed *C. roseus* (Jaleel, Gopi, Manivannan & Panneerselvam 2007), propiconazole-treated *Vigna unguiculata*, and PEN-treated *M. pulegium* under drought stress (Manivannan et al. 2007; Hassanpour et al. 2012).

POXs are involved in removal of H_2O_2 from chloroplasts and cytosol, and many different physiological functions including the oxidation of toxic compounds, the biosynthesis of cell walls (lignin and suberin), growth and developmental processes, etc. (Jbir et al. 2001; Dicko et al. 2006). Activity of POX has been reported to increase under salt stress and relatively higher activity has been reported in sensitive rice cultivars than in tolerant ones (Demiral & Turkan 2004). Likewise, in this study, a decrease was found in POX activity of *M. pulegium* plants after 40 days exposure to different NaCl concentrations than that of day 30 (Figure 4). In a similar way, Dani et al. (2005) found decreased polypeptide levels of cell wall-associated peroxidases measured by 2D electrophoresis in the apoplastic fluid from leaves of tobacco plants under NaCl stress. Plants increase lignin synthesis during NaCl stress to prevent water loss through the cell walls (Garcia et al. 1997). The slight decrease of POX activity in *M. pulegium* after

day 40 in salt-stressed and unstressed plants can prevent or reduce lignification processes, help to uptake water through the cell walls, or induce the activity of other antioxidant enzyme activities such as APX and CAT on day 40. PEN treatment significantly increased POX activity in salt-stressed and unstressed *M. pulegium*, while the effect of PEN was higher in salt-stressed plants.

PPO is the major enzyme responsible for oxidation of phenolic compounds. PPO activity considerably increased under salt stress in all organs of *M. pulegium* especially at 50 mM NaCl and then decreased slightly at 75 mM NaCl (Figure 5). Increased PPO activity may reduce the phenol content thereby protecting the content of IAA (Fletcher et al. 2000), and this can enhance cell and cell wall growth. This result is consistent with the findings in *V. unguiculata* (Manivannan et al. 2007) under drought stress and propiconazole treatment. It seems that the inducing of root growth by PEN may be related to change of growth regulator levels and cell growth in the PEN-treated plants.

CAT and APX are two of the most important enzymes in removing toxic H_2O_2 from plant cells (Dewir et al. 2006). APX and CAT activities significantly increased under different NaCl-treated groups and more induced by PEN treatment (Figures 6 and 7). The highest APX activity was observed in PEN-treated plants on day 40 (Figure 7) and CAT activity was observed on day 30 in *M. pulegium* (Figure 6). H_2O_2 is an integral component of cell signaling cascades and an indispensable second messenger in biotic and abiotic stress situations (Mittler 2002; Pastori & Foyer 2002). It seems that CAT and APX regulations serve to limit excessive H_2O_2 accumulation, and allow to occurring essential signaling functions.

In conclusion, salt stress caused a reduction in growth parameters, RWC, and an increase in MDA content, H_2O_2 level, and antioxidant enzyme activities of *M. pulegium*. Highest SOD and APX enzyme activities were observed on long-term stress conditions (day 40), whereas POX, PPO, and CAT activities were observed on day 30. There was a significant difference among different organs in responses to salinity and PEN treatments. PEN treatment in salt-stressed plants decreased the negative effect of salinity on growth, RWC, membrane damage, and induced H_2O_2 level and antioxidative defense responses. From these results, it can be concluded that the application of PEN alone or in combination with salt stress ameliorated salinity effects in *M. pulegium* plants by improving antioxidant systems and regulation of H_2O_2 level for signaling functions. Further work on the cellular signaling and non-enzymatic antioxidant compounds is required to gain more information about how PEN treatment ameliorates salinity effects in *M. pulegium* plants.

Acknowledgment

The authors wish to thank Tehran University for providing research facilities.

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