Acta Physiol Plant (2016) 38:119
DOI 10.1007/s11738-016-2137-3

ORIGINAL ARTICLE

Effect of salinity and waterlogging on growth, anatomical and antioxidative responses in *Mentha aquatica* L.

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Received: 5 August 2015 / Revised: 31 March 2016 / Accepted: 8 April 2016 / Published online: 22 April 2016

Abstract Salinity and waterlogging are two stresses which in nature often occur simultaneously. In this work, effects of combined waterlogging and salinity stresses are studied on the anatomical alteration, changes of enzymatic antioxidant system and lipid peroxidation in *Mentha aquatica* L. plants. Seedlings were cultured in half-strength Hoagland medium 50 days after sowing, and were treated under combination of three waterlogging levels (well drained, moderately drained and waterlogging) and NaCl (0, 50, 100, 150 mM) for 30 days. Moderately drained and waterlogging conditions induced differently aerenchyma formation in roots of *M. aquatica* salt-treated and untreated plants. Moreover, stele diameter and endodermis layer were also affected by salt stress and waterlogging. Salt stress significantly decreased growth, relative water content (RWC), protein level, catalase (CAT) and polyphenol oxidase (PPO) activities, and increased proline content, MDA content, H_2O_2 level and activities of superoxide dismutase (SOD), peroxidase (POX), and ascorbate peroxidase (APX). Waterlogging in salt-untreated plants increased significantly growth parameters, RWC, protein content, antioxidant enzyme activity, and decreased proline content, H_2O_2 and MDA levels. In salt-treated plant, waterlogging caused strong induction of antioxidant enzymes activities especially at severe stress condition. These results suggest *M. aquatica* is a waterlogging tolerant plant due to significant increase of antioxidant activity, membrane stability and growth under water stress. High antioxidant capacity under waterlogging can be a protective strategy against oxidative damage, and help to salt stress alleviation.

Keywords Waterlogging · Salt stress · *Mentha aquatica* · Antioxidant activity · Growth

Abbreviations

ROS	Reactive oxygen species
TCA	Trichloroacetic acid
TBA	Thiobarbituric acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Introduction

Salinity stress limits growth and production of major crops by ionic stress, osmotic imbalance and high production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide and hydroxyl radical (Aghaleh et al. 2009; Khedr et al. 2003). The ROS substances are harmful to cellular proteins and lipids (Golldack et al. 2014; Miller et al. 2010). To counteract ROS, plants can up regulate their antioxidative enzymes and non-enzymatic antioxidants (Ashraf 2009; Kachout et al. 2013).

Communicated by U Feller.

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The general effects of salinity (Merati et al. 2014; Khorasaninejad et al. 2010) and waterlogging (Lenssen et al. 2000; Gibbs and Greenway 2003; Bansal and Srivastava 2012) on many plant species are well documented, but the mixed effects of waterlogging and salinity are not fully understood. Waterlogging becomes an additional constraint to plant growth in saline areas, besides the osmotic and the Na^+ or Cl^- toxic effects, the soil becomes oxygen deficient (hypoxic) (Barrett-Lennard and Shabala 2013). The main effects of hypoxic conditions on plants are the reduction of ATP formation, the lower growth and survival of roots, the lower uptake of nutrients, and the reduction in the capacity of plants to exclude toxic ions like Na^+ and Cl^- from leaves (Kozlowski 1997; Barrett-Lennard 2003; Barrett-Lennard and Shabala 2013). Oxygen deficiency due to waterlogging causes the closure of stomata, reduction in CO_2 concentration of leaves, and therefore a decline in photosynthesis (Folzer et al. 2006). Oxygen demands of plants can be decreased by mechanisms such as low respiration rate, improving antioxidant capacity and aerenchyma development (Noctor and Foyer 1998; Evans 2004).

Mentha aquatica L. as an aromatic perennial and medicinal plant belongs to the Lamiaceae, and grows on wet ground, near of rivers and lakes. It is a source of essential oils; so, it is used in food as flavor, traditional medicine and pharmaceutical industries (Hajian et al. 2011). In spite of some works on the effects of salinity and waterlogging on *Mentha* species, there is rare information available regarding the mixed effects of waterlogging and salt stress. Lenssen et al. 2000 showed that flooding reduced total dry weight, and induced shoot length and adventitious roots in *M. aquatica* plants. Dry weight, root length, essential oil percent and proline content decreased in *M. piperita* under salt stress (Khorasaninejad et al. 2010; Roodbari et al. 2013). Lipid peroxidation, electrolyte leakage and antioxidant enzyme activity markedly increased in *M. pulegium* under salt stress (Karray-Bouraoui et al. 2010). Song et al. (2011) showed that the adventitious root increased in *Suaeda salsa* under combined waterlogging and salinity. The fermentation enzyme activity, net transport of Na^+ to the shoots and antioxidant activity increased in *Suaeda maritime* under combined waterlogging and salinity (Wetson and flowers 2010; Alhdad et al. 2013). We did not find any data on the mixed effects of salt stress and waterlogging in *M. aquatica*. The hypothesis of the present study is that salt tolerance is higher in *M. aquatica* plants flooded in saline water against to those under well-drained conditions by adaptive responses including aerenchyma formation and strong induction of antioxidative enzymes.

Materials and methods

Plant materials and stress treatments

Mentha aquatica L. seeds were collected from Noshahr (Mazandaran Province, Iran) in summer 2013. Seeds were germinated in Tref pit and perlite with a ratio of 1–2 in a green-house with 16/8 h (light/dark period) per 24 h and 25/18 °C (day/night temperature) and 40–45 % humidity. The 50-day-old seedlings (3–4 cm) were transplanted into the plastic pots (14 × 12 cm) containing perlite. Pots were put in another container containing 1/2 Hoagland solution. Seedlings were treated with various levels of waterlogging and NaCl (0, 50, 100, 150 mM). Waterlogging treatment was applied using Hoagland solution 5 cm above the pot level, the moderately drained condition contained Hoagland solution half level of pot, and in well-drained condition, the pots contained nutrient at the bottom (Fig. 1). After 30 days of waterlogging and salinity treatments, plants with four replicates were collected for the analyses.

Growth parameters

Fresh weight (FW) and dry weight (DW) of leaves and roots were determined on six individual plants. For determination of DW, samples were dried for 72 h at 40 °C.

RWC estimation in leaves was performed based on Wheatherley (1950).

$$\text{RWC}\ (\%) = (\text{FW} - \text{DW})/(\text{SW} - \text{DW}) \times 100$$

Saturated weight (SW) of fresh leaves were defined by immersing samples in ultra pure water for 24 h in the dark at 4 °C, and DW determination of leaves were dried in oven for 72 h at 40 °C until constant weight was reached.

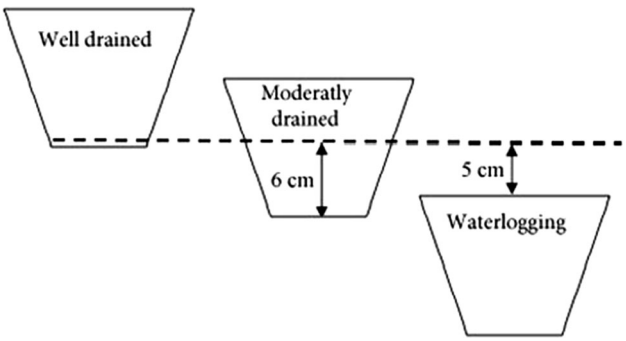

Fig. 1 Depiction of three waterlogging levels. The *dot line* represents the water level. The base of the pots for well drained were equal to the water level, moderately drained treatments were placed so the water level was half below of pot level, waterlogging were placed so the water level was above pot level

Anatomical study

Roots were fixed in formalin–alcohol–glacial acetic acid (90:5:5 % v/v) for 24 h. Sections of root were cut manually using razor 1 cm below the root–shoot junction. The sections were cleared with sodium hypochlorite, dehydrated and stained with Methyl Green and Bismarck Brown colors (Noorbakhsh et al. 2008). Sections were observed by microscope (BX51 Olympus, Tokyo, Japan) and images were taken with a digital camera (DP12 Olympus). The measurement of stele diameter was carried out on the photographs using Microstructure Measurement Software.

Lipid peroxidation and H_2O_2 level

Lipid peroxidation was quantified by measuring malondialdehyde (MDA) content (Heath and Packer 1968). Leaf and root fresh tissues (0.5 g) were homogenized with 2 ml of trichloroacetic acid (TCA 0.1 % w/v) and centrifuged at 13,000×*g* for 30 min. Two ml of thiobarbituric acid (TBA 0.5 %) in 20 % TCA was added to 1 ml aliquot of the supernatant. The mixture was taken into a hot water bath at 95 °C for 30 min and immediately cooled in an ice bath. Absorbance of the supernatant was recorded at 532 and 600 nm after centrifugation at 10,000×*g* for 15 min. The MDA concentration was calculated using the extinction coefficient of 155 mM^{-1}.

Hydrogen peroxide level was determined based on Velikova et al. (2000). Leaf and root fresh tissues (1 g) were homogenized by 5 ml of 0.1 % (w/v) TCA into an ice bath. The mixture was centrifuged at 12,000*g* for 15 min at 4 °C, and then, 0.5 ml of the extract mixed to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. Absorbance of the supernatants was recorded at 390 nm and H_2O_2 contents were measured using a standard curve.

Proline content

Free proline was measured according to Bates et al. (1973). The dried leaves (0.5 g) were homogenized for 5 min in 5 ml of 3 % aqueous sulphosalicylic acid and centrifuged at 12,000×*g* for 15 min at 4 °C. One ml of extract added to 1 ml de-ionized water, 2 ml acid ninhydrin and 2 ml glacial acetic acid and the mixture was heated in water bath at 100 °C for 1 h. Then, 2 ml toluene was added to the reaction mixture and the toluene phase absorbance was recorded at 520 nm.

Protein extraction

For total protein determination, 0.8 g fresh leaves were homogenized at 4 °C in 1 M Tris–HCl (pH 6.8) using a mortar. The homogenates were centrifuged twice at 4 °C for 30 min at 13,000*g* using a Heraeus 400R microfuge. The supernatants were kept at −70 °C until protein determination and enzyme assay. Protein concentration was determined according to Bradford (1976) method, using bovine serum albumin (BSA) as standard. The absorbance was measured at 595 nm by UV–visible spectrophotometer (UV-160, Shimadzu, Tokyo, Japan).

Superoxide dismutase (SOD) activity

Total SOD activity was determined by monitoring inhibition of photochemical reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 560 nm based on Giannopolitis and Ries (1977). The reaction mixture composed of 50 mM sodium phosphate buffer (pH 7.5), 13 mM L-methionine, 75 µM NBT, 0.1 mM EDTA, 75 µM riboflavin and 30 µl enzyme extract. The reaction mixtures were illuminated for 16 min and the absorbances were recorded at 560 nm against the non-illuminated blank. One unit of SOD activity was specified as the amount of enzyme which caused 50 % inhibition in NBT reaction.

Peroxidase (POD) activity

Peroxidase activities were quantified based on Abeles and Biles (1991). The reaction mixture composed of 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 30 µl enzyme extract. Absorbance of the reaction solution was measured at 530 nm. The POD activity was specified as 1 µM of benzidine oxidized per min per mg protein [Unit mg^{-1} (protein)].

Polyphenol oxidase (PPO) activity

Polyphenol oxidase activity was measured based on Raymond et al. (1993). Reaction mixture composed of 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.8), 0.2 ml of 20 mM pyrogallol, and 30 µl of enzyme extract. The absorbance was determined at 430 nm. The PPO activity was quantified as 1 µM of pyrogallol oxidized per min per mg protein [Unit mg^{-1}(protein)].

Ascorbate peroxidase (APX) activity

APX activity was estimated by monitoring of absorbance decrease at 290 nm (Jebara et al. 2005). Reaction mixture was composed of 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM of H_2O_2, 0.5 mM of ascorbate, and 10 µl enzyme extract. The reaction was started by adding of H_2O_2, and ascorbate oxidation was calculated at 290 nm for 1 min. Enzyme activity was measured using the molar

extinction coefficient for ascorbate (2.8 mM^{-1} cm^{-1}), and the results were expressed as 1 μM of ascorbate oxidized per min per mg protein [Unit mg^{-1} (protein)] at 25 ± 2 °C.

Statistical analyses

The experiments were done in randomized complete block design. The data were analyzed using either one or two-way analysis of variance (ANOVA) by SPSS (version 18). The mean differences were compared using the lowest standard deviations (LSD) test. Each data was the mean of four replicates ($n = 4$) in each group and P values ≤ 0.05 are considered to be significant.

Results

Growth parameters and RWC content

Growth parameters of salt-treated plants were followed by measuring leaf and root FW and DW and RWC on three waterlogging levels. Under salinity stress, growth parameters decreased significantly in *M. aquatica* in a concentration-dependent manner (Fig. 2). Waterlogging significantly affects growth parameters ($P \leq 0.05$). Waterlogging in control and 50 mM NaCl increased markedly growth parameters, while these parameters slightly increased at severe salt stress comparing to well drained. At 150 mM NaCl, moderately drained and waterlogging treated plants showed an 22.7 and 15.2 % increase of leaf DW as compared to well drained, respectively. Waterlogging also increased root DW under salt stress. A 30.76 and 17.94 % increase of root DW was observed at 150 mM NaCl in moderately drained and waterlogging-treated plants (Table 1).

RWC content decreased significantly ($P \leq 0.05$) under different salinity treatments (Table 1), and lower RWC content was observed at higher level of NaCl (150 mM). Waterlogging in salt-treated and -untreated plants also increased significantly RWC content in *M. aquatica* leaves. At 150 mM NaCl, waterlogging increased RWC content (41.7 %) as compared to well-drained condition.

Anatomical response

Cross section of *M. aquatica* roots at 0 and 100 mM NaCl treated plants under three waterlogging levels were presented in Fig. 3. In salt-untreated plants, aerenchyma formation was induced under both moderately drained and waterlogging condition, but strongly induced under moderately drained as compared to waterlogging. Under salt stress, aerenchyma formation was prominent, and roots contained more metaxylem in waterlogging as compared to moderately drained condition. Also, roots developed an additional layer of endodermis at comparable level of waterlogging (Fig. 3b, f), and possessed wider stele especially at moderately drained level. Salt stress increased stele diameter (28.6 %) at moderately drained comparing to control plants (Fig. 4). Also, the number of xylem vessels increased and their sized decreased in *M. aquatica* under salt stress (Fig. 3e, f, g).

Lipid peroxidation and H_2O_2 content

The H_2O_2 level in *M. aquatica* leaves and roots increased significantly under salt stress especially at severe stress condition ($P \leq 0.05$) (Fig. 5a). In salt-untreated plants, the highest and lowest H_2O_2 content were determined under well-drained and waterlogging-treated plants, respectively. Under salt stress, waterlogging decreased H_2O_2 level as compared to well drained. At 150 mM NaCl, waterlogging decreased H_2O_2 level in *M. aquatic* leaves (13.3 %) and roots (12.04 %) as compared to well drained.

Lipid peroxidation was quantified as MDA content. Under salt stress, MDA content increased significantly in *M. aquatica* leaf and root tissues, and the content in leaves was higher than that of roots ($P \leq 0.05$) (Fig. 5b). In salt-untreated plants, the lowest and highest MDA contents were determined in waterlogging and well drained treated plants, respectively. Combined effect of waterlogging and salinity decreased significantly MDA content, and a 25.3 and 13.8 % decrease of MDA were observed at 150 mM NaCl as compared to well drained for leaves and roots, respectively ($P \leq 0.05$).

Proline content

Proline content of *M. aquatic* leaves significantly increased up to 100 mM NaCl and then decreased slightly at 150 mM NaCl ($P \leq 0.05$) (Fig. 6). In salt-untreated plants, the highest and lowest proline contents were measured in well drained and waterlogging, respectively, and a 33.3 % decrease was observed in waterlogging as compared to well drained treatment. Combined effect of waterlogging and salinity decreased significantly proline content in *M. aquatic* leaves comparing to well-drained condition ($P \leq 0.05$). Waterlogging cause to decrease of 57.14 and 21.97 % proline content at 100 and 150 mM NaCl comparing to well-drained treatment, respectively.

Soluble protein content

Salinity increased markedly protein content up to 100 mM NaCl, and then significantly decreased at 150 mM NaCl ($P \leq 0.05$) (Fig. 7a). In salt-untreated plants, waterlogging

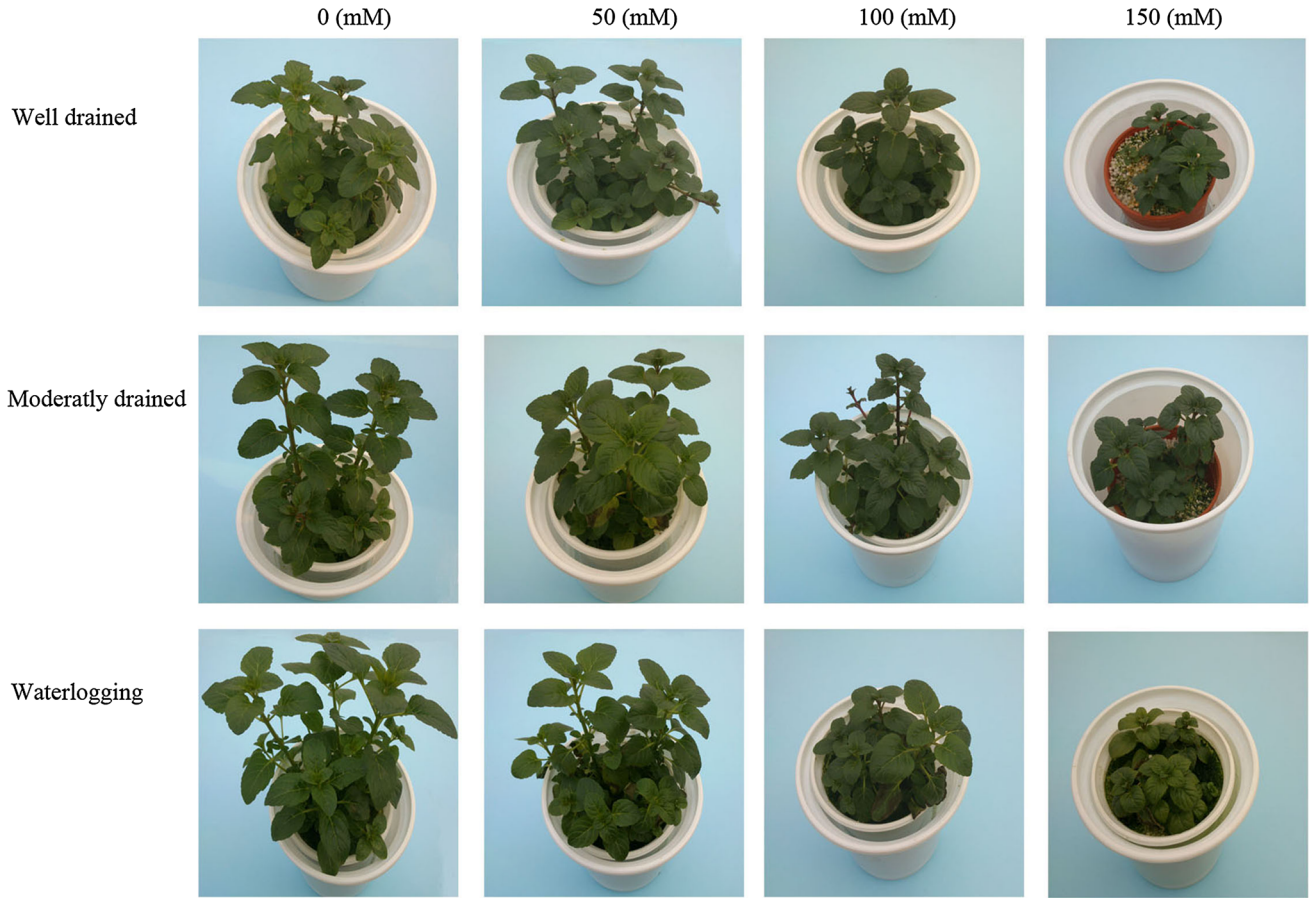

Fig. 2 Different growth responses of *M. aquatica* plants under three waterlogging levels (well drained, moderately drained and waterlogging) and salinity (0, 50, 100, 150 mM NaCl) after 30 days

showed the highest protein content, and a 31.5 % increase was observed in waterlogging-treated plants as compared to well drained. Mixed effect of waterlogging and salinity increased protein content comparing to well drained plants. At severe salt stress condition, waterlogging increased protein content (24.2 %) as compared to well drained.

Antioxidant enzymes activities

SOD activity increased significantly under salt stress comparing to control plants. Effect of waterlogging was different and depended on irrigation level ($P \leq 0.05$). Waterlogging showed the highest SOD activity in salt-treated and untreated plants comparing to well and moderately drained (Fig. 7b). Mixed effect of waterlogging and salinity caused strong induction of SOD activity and showed higher SOD activity than that of other waterlogging levels. At 150 mM NaCl, waterlogging increased an 41.55 and 45.01 % SOD activity as compared to moderately and well drained, respectively.

POX activity increased considerably with the increase of salinity level especially at 150 mM NaCl ($P \leq 0.05$). In salt-untreated plants, the highest POX activity was determined under waterlogging (Fig. 7c). Combined effect of waterlogging and salinity cause to strong induction of POX activity comparing to control plants, and the highest POX activity was determined in the plants treated to waterlogging at 150 mM NaCl. Waterlogging caused an 66.35 and 70.52 % increase in POX activity at 100 and 150 mM NaCl comparing to well drained, respectively ($P \leq 0.05$).

Salinity decreased significantly PPO activity in *M. aquatica* leaves ($P \leq 0.05$). In salt-untreated plants, waterlogging and moderately drained showed an 25.31 and 65.46 % increase of PPO activity as compared to well drained (Fig. 7d). Combined effect of waterlogging and salinity increased significantly MDA content, and an 77.07 and 178.03 % increase of PPO activity were observed at 100 and 150 mM NaCl comparing to well drained, respectively ($P \leq 0.05$).

CAT activity increased with the increase of NaCl concentration, and decreased at severe salt stress. The highest CAT activity was detected at 100 mM NaCl comparing to control (Fig. 7e). In salt-untreated plants, waterlogging showed the highest CAT activity, and a 93.38 % increase

Table 1 Effect of three waterlogging levels and salinity (0, 50, 100, 150 mM NaCl) on leaf FW and DW, root FW and DW and RWC content of *M. aquatica* plants after 30 days

Parameters	Water levels	Salt stress			
		0	50	100	150
Leaf FW (g plant^{-1})	Well drained	2.13 ± 0.135 d	2.09 ± 0.154 d	1.54 ± 0.221 ef	1.04 ± 0.143 f
	Moderately drained	3.97 ± 0.165 b	3.62 ± 0.124 c	1.94 ± 0.101 d	1.12 ± 0.093 f
	Waterlogging	4.54 ± 0.266 a	4.08 ± 0.223 b	1.77 ± 0.131 e	1.07 ± 0.138 f
Leaf DW (g plant^{-1})	Well drained	0.37 ± 0.025 c	0.31 ± 0.014 d	0.14 ± 0.021 ef	0.079 ± 0.004 g
	Moderately drained	0.42 ± 0.023 b	0.44 ± 0.018 b	0.18 ± 0.013 e	0.097 ± 0.006 g
	Waterlogging	0.51 ± 0.031 a	0.49 ± 0.027 a	0.15 ± 0.025 ef	0.091 ± 0.005 g
Root FW (g plant^{-1})	Well drained	1.76 ± 0.29 a	1.15 ± 0.43 d	0.67 ± 0.36 ef	0.43 ± 0.031 f
	Moderately drained	1.92 ± 0.22 b	1.73 ± 0.38 b	0.84 ± 0.24 e	0.53 ± 0.026 f
	Waterlogging	2.19 ± 0.42 c	1.98 ± 0.40 a	0.63 ± 0.25 ef	0.49 ± 0.015 f
Root DW (g plant^{-1})	Well drained	0.11 ± 0.021 cd	0.083 ± 0.031 de	0.052 ± 0.092 f	0.039 ± 0.007 f
	Moderately drained	0.16 ± 0.023 b	0.101 ± 0.052 cd	0.068 ± 0.064 ef	0.051 ± 0.006 f
	Waterlogging	0.19 ± 0.042 a	0.126 ± 0.051 c	0.059 ± 0.052 ef	0.046 ± 0.005 f
RWC (%)	Well drained	72.55 ± 2.32 d	70.25 ± 2.43 d	60.68 ± 3.62 f	52.71 ± 3.14 g
	Moderately drained	78.21 ± 2.02 b	74.13 ± 1.03 c	68.43 ± 1.24 de	65.75 ± 1.76 e
	Waterlogging	83.21 ± 2.13 a	81.90 ± 1.22 a	73.69 ± 1.42 c	74.70 ± 2.21 c

Values are given as mean ± SE ($n = 4$) in each group. Different letters indicate significant differences at $P \leq 0.05$ (LSD)

of CAT activity was detected in waterlogging treated plants comparing to well drained. Mixed effect of waterlogging and salinity significantly increased about 112.16 and 93.52 % of CAT activity at 100 and 150 mM NaCl comparing to well drained, respectively ($P \leq 0.05$).

APX activity increased markedly under salt stress especially at severe stress condition. In salt-untreated plants, moderately drained and waterlogging treated plants showed the lowest and highest APX activity, respectively, and an 64.8 % increase of APX activity was observed in waterlogging as compared to moderately drained ($P \leq 0.05$) (Fig. 5f). Combined effect of waterlogging and salinity increased APX activity in *M. aquatica* leaves. At 150 mM NaCl, waterlogging showed a 40.6 and 99.5 % increases of APX activity as compared to moderately and well drained, respectively.

Discussion

Salt stress adaptation in plants is complex and affected by internal constitutive salt tolerance mechanisms and external environmental factors. In this research, salinity decreased growth parameters and RWC content in *M. aquatica* plants (Table 1). Similar decrease in growth including plant height, fresh and dry weight has been previously observed in *M. piperita* (*Khorasaninejad* et al. 2010) and *M. pulegium* (Merati et al. 2014) under salinity. Decrease of plant growth can be related with a reduction in photosynthetic activity under salinity (Munns and Tester 2008). In the present study, cross section of roots showed an additional layer of endodermis and reduction in xylem vessels size especially at moderately drained level under salt stress. According to Walker et al. (1984) salinity induced suberization of the endodermis and hypodermis in citrus genotypes roots and Casparian strip developed near to the root apex than that of non-treated roots. Increased suberization and decreased xylem size can cause the restriction of ions movement through the vasculature to xylem elements in tissues which could lead to growth reduction (Kozlowski 1997). Waterlogging increased growth parameters in salt-treated and untreated plants. The metaxylem density, stele diameter and RWC content also increased in moderately drained and waterlogging conditions (Table 1; Fig. 3). Development of metaxylem in root may contribute to more convenient water and sap flow in the xylem (Kozlowski 1997). It seems that metaxylem development and increase of stele diameter and vascular number are the adaptive mechanisms to maintain better water flow and higher RWC in *M. aquatica.* Combined effect of waterlogging and salinity caused higher induction of aerenchyma than that of waterlogging. The aerenchyma formation in plant organs is due to cortex degeneration and cell death. Ethylene as a plant hormone leads to the death and disintegration of cells in the root cortex (Taiz and Zeiger 2010). Other chemicals such as NO could also

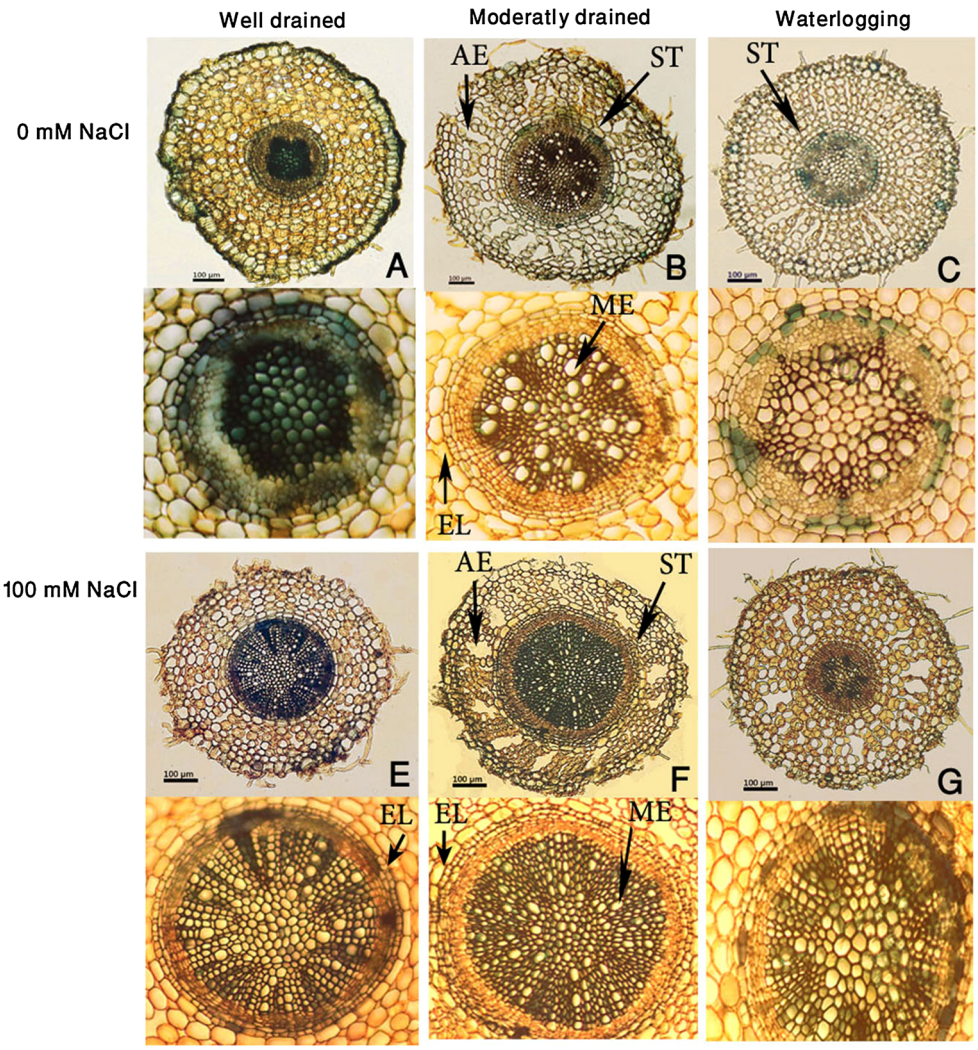

Fig. 3 Cross section of *M. aquatica* root (1 cm below shoot–root junction) in three waterlogging levels (well drained, moderately drained and waterlogging), and salinity (0 and 100 mM NaCl). *AE* aeranchyma, *ST* stele, *EL* endodermis layer, *ME* metaxylem

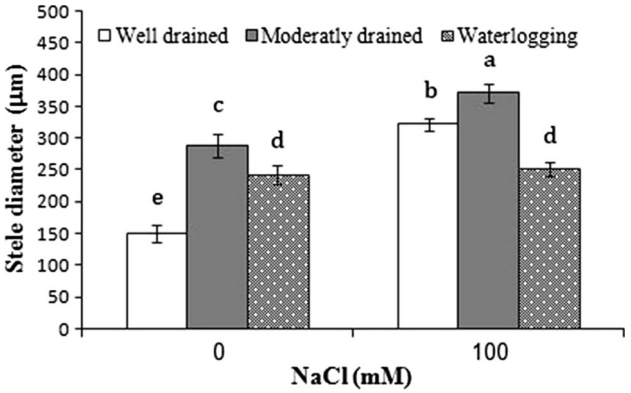

Fig. 4 Stele diameter of *M. aquatica* root (1 cm below shoot–root junction) in three waterlogging levels (well drained, moderately drained and waterlogging), and salinity (0 and 100 mM NaCl). Values are given as mean ± SE ($n = 5$) in each group. *Different letters* indicate significant differences at $P \leq 0.05$ (LSD)

contribute such phenomena (Evans 2004; Jackson and Armstrong 1999). The spaces of these cells formerly occupied provide the gas-filled voids that facilitate movement of oxygen (Taiz and Zeiger 2010).

Peroxidation of membrane lipids of higher plants is the result of free radical oxidative damage at the endo-membranes (Demiral and Türkan 2004). A significant increase in the MDA and H_2O_2 contents was observed under salinity especially at 150 mM NaCl after 30 days (Fig. 5). A similar result has been previously reported in *M. pulegium* under osmotic stress (Hassanpour et al. 2012; Karray-Bouraoui et al. 2010). CAT is the most efficient enzyme in scavenging toxic H_2O_2 in plant cells (Dewir et al. 2006). Increased H_2O_2 concentration especially at severe salt stress condition could be due to the decrease in CAT activity in this research. Waterlogging in control and salt-treated plants decreased significantly MDA content and

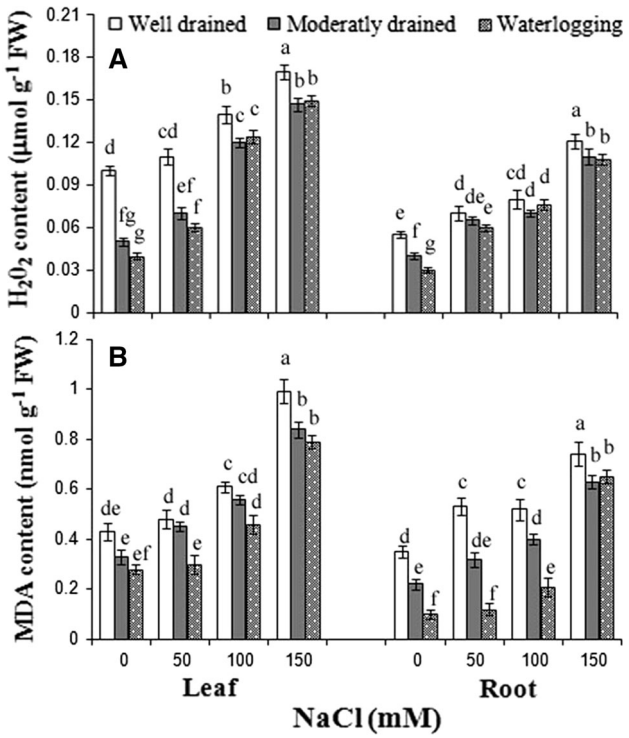

Fig. 5 The H_2O_2 (**a**) and MDA contents (**b**) of *M. aquatica* leaves and roots under three waterlogging levels (well drained, moderately drained and waterlogging) and salinity (0, 50, 100, 150 mM NaCl) after 30 days. Values are given as mean ± SE ($n = 4$) in each group. *Different letters* indicate significant differences at $P \leq 0.05$ (LSD)

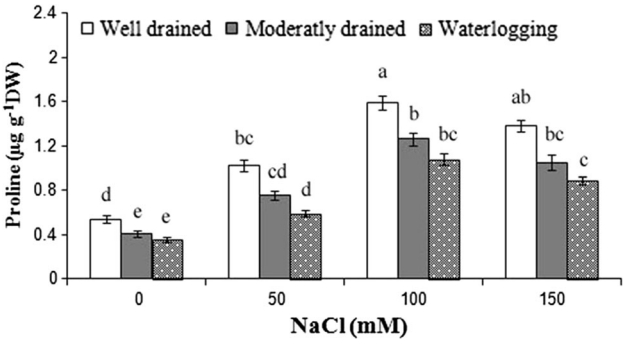

Fig. 6 Proline contents of *M. aquatica* leaves under three waterlogging levels (well drained, moderately drained and waterlogging) and salinity (0, 50, 100, 150 mM NaCl) after 30 days. Values are given as mean ± SE ($n = 4$) in each group. *Different letters* indicate significant differences at $P \leq 0.05$ (LSD)

H_2O_2 level in both organs as compared to well drained (Fig. 5). Cell membrane stability can be regarded as a good index for stress tolerance in plants (Premachandra et al. 1992). Oxygen deprivation and solute leakage disintegrated cell membrane under waterlogging in pea plants (Rawyler et al. 2002). Simova-Stoilova et al. (2012) showed that lipid hydroperoxides content increased markedly in the sensitive *Trifolium* genotype (*Trifolium pretense* L.) along with waterlogging prolongation. Moreover, the content of MDA in tolerant genotype (*Trifolium repens* L.) was lower than the sensitive genotype. It seems that high membrane stability and reduction of H_2O_2 level under waterlogging may help to salt stress alleviation in *M. aquatica*.

Proline content increased significantly under salinity, and the highest proline content was observed in well drained as compared to other water-drained levels. Proline as an osmolyte may play a significant role in maintaining leaf osmotic potential and turgor under osmotic stress (Sadiqov et al. 2002). Proline could also protect protein configurations during dehydration. A positive relation between proline accumulation and osmotic stress tolerance has been reported too (Aly and Latif 2011). In this research, mixed effect of waterlogging and salinity decreased proline content in control and salt-treated plants (Fig. 6). Exogenous proline increased the protein content in *Pancratium maritimum* L. under salinity (Khedr et al. 2003). It seems that the decrease of proline content may relate with the formation of new proteins for oxidative stress tolerance in *M. aquatica.*

The total protein content decreased at 150 mM NaCl in *M. aquatica* leaves (Fig. 7). Some studies including Merati et al. (2014) in *M. pulegium* and Aghaleh et al. (2009) in *Salicornia persica* and *europaea* reported protein reduction under severe salt stress. The decrease in protein content under osmotic stress could be the result of decline in protein synthesis, acceleration of protein degradation and amino acid recycling (Levitt 1980). Mixed effect of waterlogging and salinity strongly induced total protein content in *M. aquatica* leaves. Increased protein contents by waterlogging may be associated with the proteins and enzymes synthesis involved in the physiological changes and stress response proteins called anaerobic proteins (Gibbs and Greenway 2003). A set of about 20 anaerobic proteins under low oxygen treatment was detected by two-dimensional electrophoresis (Sachs et al. 1980), and many of these induced proteins belong to the glycolytic and fermentation pathways (Dolferus et al. 2003).

The antioxidant defense mechanisms protect plants against oxidative stress damages. One of these mechanisms involves antioxidative enzymes including SOD, CAT, APX, POD and etc. (Ashraf 2009; Kachout et al. 2013). In this study, SOD, POX and APX activities were markedly induced under salt stress (Fig. 7). Similar results were previously identified in *M. pulegium* (Merati et al. 2014), and *Cicer arietinum* (Mafakheri et al. 2011) and *Cakile maritima* (Ksouri et al. 2007) under salt stress. SOD converts superoxide radical to molecular oxygen and H_2O_2 (Scandalios 1993), and POX and APX enzymes are responsible for removal of H_2O_2 from chloroplasts and cytosol (Dicko et al. 2006). Induced antioxidant enzyme activity could correlate with increase of plant protection

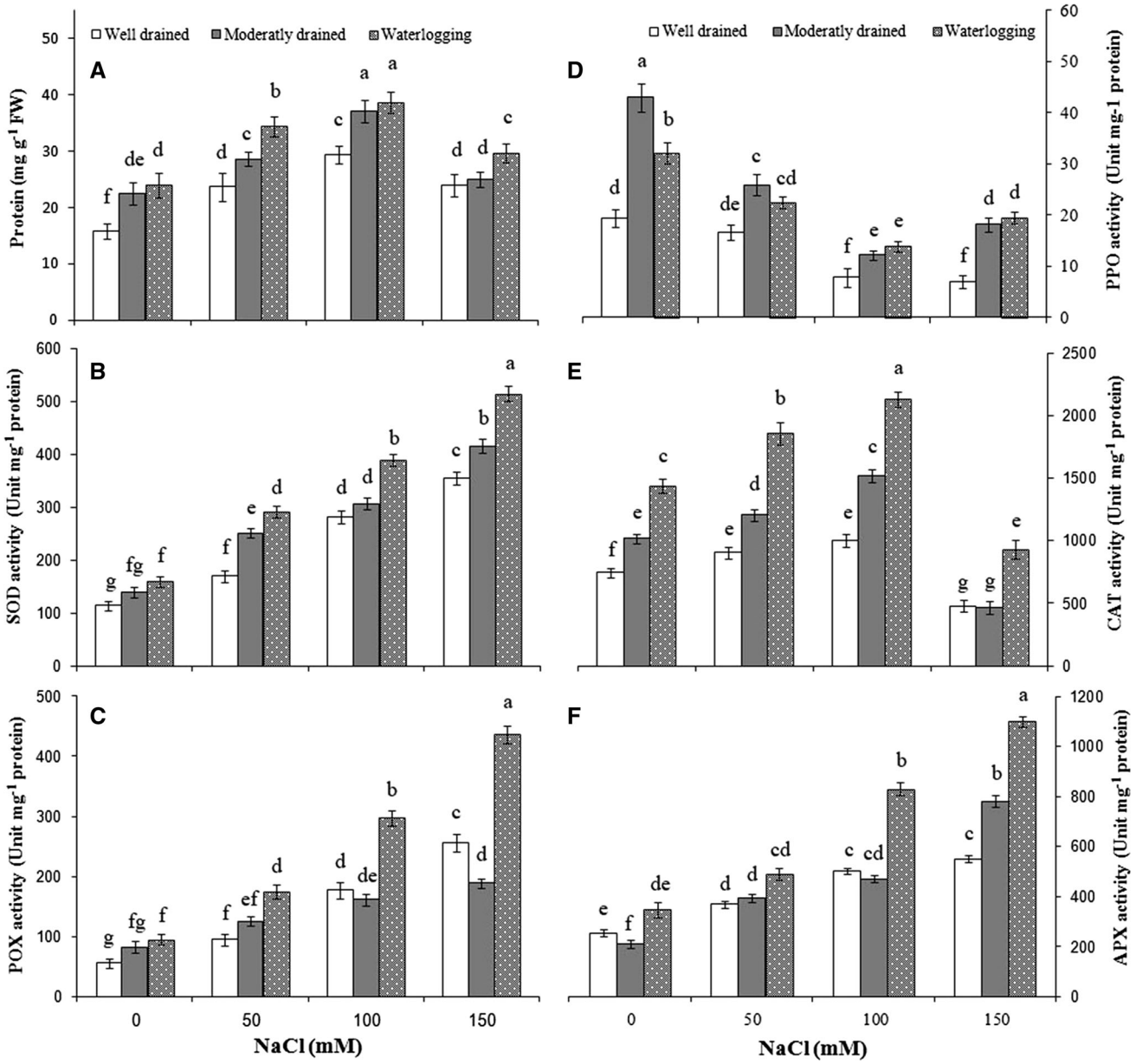

Fig. 7 Protein content (**a**) and SOD (**b**), POX (**c**), PPO (**d**), (CAT (**e**) and APX (**f**) activities of *M. aquatica* leaves under three waterlogging levels (well drained, moderately drained and waterlogging) and salinity (0, 50, 100, 150 mM NaCl) after 30 days. Values are given as mean ± SE ($n = 4$) in each group. *Different letters* indicate significant differences at $P \leq 0.05$ (LSD)

from damage (Asada 1999). Combined waterlogging and salinity caused to more increase of these enzymes activities especially at severe salinity condition ($P \leq 0.05$) (Fig. 7). Salinity and hypoxia could cause excessive ROS and induce antioxidative mechanisms (Blokhina et al. 2003). Alhdad et al. 2013 showed that combination of waterlogging and salinity induced higher DPPH and superoxide anion scavenging capacity in *Suaeda maritime*. Sairam et al. (2009) showed that waterlogging for 24 h induced significantly Cu/Zn-SOD gene expression in waterlogging tolerant genotype of *Cajanus cajan* L. (ICPL 84023). It seems that higher activities of ROS scavenging enzymes under waterlogging could effectively alleviate the oxidative damage under salinity condition.

Polyphenolics synthesis and accumulation in plants are generally induced in response to various kinds of stresses (Ksouri et al. 2007; Giorgi et al. 2009), and PPO is the main responsible enzyme for phenolics oxidation. PPO activity decreased significantly under salinity in *M. aquatica* leaves (Fig. 7d). Merati et al. (2014) showed PPO activity increased in *M. pulegium* under salt stress, but in *Phaseolus Mungo,* PPO and CAT activities decreased

under salt stress (Dash and Panda 2001). Decreased PPO activity in this study may express that PPO did not have a function in reduction of the phenol content and protection of *M. aquatica* plant under salt stress. Waterlogging and moderately drained treatments increased markedly PPO activity in control and salt treated plants (Fig. 7d). There is a little study about the role of PPO activity under waterlogging resistance. Bansal and Srivastava (2012) showed that waterlogging induced PPO activity in *Cajanus cajan* L. plants. Increased polyphenol oxidase activity under waterlogging may be responsible for oxidation and degradation of phenolics produced during oxidative stress. The present study indicates the conclusive role of PPO in waterlogging resistance in *M. aquatica*, but the mechanism remains to be elucidated.

CAT activity significantly increased up to 100 mM NaCl and then decreased at 150 mM to the level lower than the control (Fig. 7e). Similar results were observed in *Chrysanthemum (*Hossain et al. 2009) and *M. pulegium* (Merati et al. 2014) under salt stress. Combined effect of waterlogging and salinity induced strongly CAT and APX activities as compared to other water-drained levels (Fig. 7). Increased CAT activity under waterlogging have been previously observed in barley (Yordanova et al. 2004) and pigeonpea (Kumutha et al. 2009). It seems that waterlogging by more enhancing of antioxidant enzyme activity can decrease the negative effect of salt stress in *M. aquatica*.

In summary, *M. aquatica* plants should be considered as a waterlogging tolerant species since waterlogging after 30 days increased dry weight, RWC, protein content, antioxidant activity and decreased H_2O_2 level, lipid peroxidation and proline content as compared to well drained condition. Anatomical study showed aerenchyma formation and stele diameter increased in *M. aquatica* roots under waterlogging as compared to well drained. Salinity decreased significantly growth parameters, RWC, protein content. The decrease of *M. aquatica* growth under salinity, especially at 150 mM NaCl could be related to sensitivity of *M. aquatica* to salinity. Combined effect of waterlogging and salinity caused partial alleviation of salt stress by strong induction of antioxidant enzyme activity, promotion of root aerenchyma formation, decrease in metaxylem size and increase in endodermis layers. However, future studies on NO content and hormonal responses will be required to gaining complementary information on the effect of waterlogging on salinity alleviation in *M. aquatica* plants.

Author contribution statement Bahareh Sadat Haddadi contributed to the bench experiments. Dr. Vahid Niknam and Dr. Halimeh Hassanpour have designed and supervised the project, and organized the manuscript. All authors read and approved the manuscript.

Acknowledgments We thank Dr. Farideh Attar and Dr. Yasaman Salmaki from University of Tehran for helping us during anatomical studies.

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