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ORIGINAL PAPER

Physiological analysis reveals relatively higher salt tolerance in roots of *Ilex integra* than in those of *Ilex purpurea*

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Abstract Determining the responses of candidate plants to salt stress is a prerequisite for selecting and breeding suitable plants with high salt tolerance to grow in coastal mudflat areas with high salinity. Here, 2-year cutting seedlings of *Ilex purpurea* Hassk. (local species) and *I. integra* Thunb. (introduced species) were grown in pots in a glasshouse and irrigated with a Hoagland-NaCl solution at 0, 24, and 48 h. Root samples were collected at 0, 1, 6, 24, and 72 h, and concentration of Na^+ ion; content of proline, soluble carbohydrate, malondialdehyde (MDA), H_2O_2 and ascorbate; and activity of three key antioxidative enzymes were measured. Roots of *I. integra* accumulated relatively less Na^+ and had less membrane lipid peroxidation and H_2O_2 during salt stress, thus indicating a relatively higher salt tolerance than roots of *I. purpurea*. Values for ascorbate content and antioxidant enzymatic activity suggest that the antioxidant ascorbate and antioxidative catalase may play substantial roles for scavenging reactive oxygen species in *I. integra* roots during salt treatment. Thus, *I. integra* is apparently more suitable for growing in local highly saline coastal mudflats.

Keywords Salt tolerance · *Ilex purpurea* · *Ilex integra* · Lipid peroxidation · Na^+ accumulation · Antioxidant capacity

Introduction

Soil salinity causes massive agricultural and horticultural losses worldwide and restricts the distribution of wild species (Zhao et al. 2020). Elucidating the physiological, metabolic, and biochemical responses of plants to salt stress and the mechanisms that contribute to salt tolerance is critical for developing and cultivating salt-tolerant plants and ensuring food and ecological security. Soil salinity inhibits plant growth and development mainly through two phases. The first is osmotic stress, which inhibits plant uptake of water by lowering the external water potential, and the second is ionic toxicity or imbalance, which is primarily associated with excessive accumulation of Na^+ in plant cells (Munns and Tester 2008). Ionic toxicity is generally thought to have a relatively later onset than osmotic stress and to operate at a timescale of days, weeks or even months (Munns 2002). Prolonged salt stress may result in arrested shoot growth and finally plant death. Among plant organs, roots are usually exposed to salt or to drying soil directly, so their capacity to tolerate osmotic stress imposed by salinity and take up or exclude ions is closely associated with plant performance in salt environments. Theoretically, increasing root tolerance to both the osmotic and ionic components of salinity stress may synergistically improve plant productivity under salt stress (Munns and Tester 2008).

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To maintain a low intracellular osmotic potential under high salinity, plants have evolved physiological or metabolic strategies to accumulate osmolytes such as proline, glycine betaine, soluble sugars, polyamines, and proteins from the late embryogenesis abundant (LEA) superfamily (Verslues et al. 2006). Proline is known to play a dominant role in osmotic adjustment under salt stress (Mansour and Ali 2017) and may act to stabilize proteins and membrane structures or scavenge reactive oxygen species (ROS) to attenuate oxidative stress under high salinity (Ashraf and Foolad 2007; Ben Rejeb et al. 2014). However, a high level of proline accumulation is not always correlated with osmotolerance as demonstrated by an Arabidopsis mutant that accumulates higher proline levels and is hypersensitive to salt (Liu and Zhu 1997). A genome-wide association analysis revealed that only proline accumulation in roots, not in leaves, was positively correlated with salinity tolerance in *Medicago truncatula* (Kang et al. 2019), indicating that different organs with proline accumulation may have differential role in plant salt tolerance.

As aerobic organisms, plants inevitably form reactive oxygen species (ROS), predominantly as H_2O_2 when stressed by high salinity (Pang and Wang 2008). ROS at low levels may act as signal molecules, but they can cause oxidative damage to membrane lipids, proteins, nucleic acids and pigments when over-produced (Noctor and Foyer 1998; Zhao et al. 2020), as occurs during salt stress (Gupta and Huang 2014). Although plants have well-developed enzymatic and non-enzymatic antioxidative systems to control ROS levels (Gupta and Huang 2014), the balance between producing and quenching ROS may be destroyed when scavenging systems are disrupted by salt stress. Excess production or accumulation of ROS, alone or in combination with other metabolites, can cause lipid peroxidation, protein modification, DNA strand breakage and cell death (Biswas and Mano 2015; Locato and De Gara 2018). Plant performance under salinity stress can be improved by increasing either the plant's capacity to scavenge ROS, enzymatically or non-enzymatically. For example, overexpression of certain antioxidant enzyme genes improved the growth of salt-stressed plants (Diaz-Vivancos et al. 2013; Eltayeb et al. 2007; Prashanth et al. 2008), and plants that synthesize more glutathione had greater salinity tolerance (Roxas et al. 1997; Ruiz and Blumwald 2002).

The coastal mudflats in China total 2.114×10^6 ha of intermittent strips along 32,000 km of coastline (Yang and Wang 2015). They are regarded as important alternative land sources for agriculture, but in reality the high salinity and low fertility are not suitable for most crops (Bai et al. 2018; Hniličková et al. 2019). To ameliorate these poor conditions, numerous engineering, physical, chemical, agronomic and biological strategies have been developed (Wan et al. 2017). The agronomic and biological ameliorations aim to increase soil organic ingredients and/or improve salt resistance of selected plants/crops. Such biological strategies are thought to be the most economic and sustainable because halophytes not only absorb soil salts and improve physicochemical properties of soils, but also promote habitats for microbes and animals as saline agriculture and forest become established in coastal mudflats (Yang and Wang 2015). However, finding suitable halophytes or plants with salt tolerance for the different local environments of coastal mudflats all over world is a challenge.

Selecting or breeding salt-tolerant species that can be grown on the coastal mudflat areas is essential for primary utilization of that kind of land with high salinity. However, because salt tolerance is a comprehensive response, a successful strategy to improve salt tolerance depends largely on targeting the key salt tolerance mechanisms for a specific plant species (Roy et al. 2014). Salt tolerance of annual crops can be assessed by comparing biomass production by the plant growing in saline conditions to that when grown in control conditions over a certain time. However, for woody plants, usually with a relatively slower growth rate, other ways than biomass comparison, e.g., simple and quick physiological variables may be employed to compare salt tolerance (Baraldi et al. 2019).

The search for suitable plants to grow in a local coastal mudflat can reasonably start with testing endemic or introduced plants for their salt tolerance and assessing their potential to add high value. We chose two *Ilex* species, which are evergreen, woody plants, and confer more ecological advantages than annual plants in mudflat amelioration. *Ilex purpurea* Hassk. is a widely distributed endemic in Yangzi River valley and valued for landscaping for its bright red fruits (Shi et al. 2019). It also has high resistance to wind and can tolerate wet soils but not extended flooding. *I. integra* Thunb., distributed in the coastal regions of Zhejiang and Fujian provinces in southeastern China and southern areas of Japan and South Korea (Xu et al. 2007), is speculated to have high salt tolerance and is being considered as a suitable import for the coastal mudflat in Jiangsu Province in eastern China, which totals 0.6×10^6 ha, about one fourth of the total coastal mudflat area in China (Wan et al. 2017). Because little is known about the physiological responses of the two species to salt stress or differences in salt tolerance, in the present study, we grew 2-year old cutting seedlings of *I. purpurea* and *I. integra* in a glasshouse and irrigated the soil with a Hoagland-NaCl solution. After different durations of the salt treatment, the roots were sampled to measure concentrations of Na^+, contents of proline, soluble carbohydrate, malondialdehyde (MDA), H_2O_2 and ascorbate, and activities of three antioxidative enzymes.

Materials and methods

Plant materials and salt treatment

Two-year plants of *I. purpurea* and *I. integra*, started from cuttings and about 50–60 cm tall growing in a local nursery field, were dug up and the soil washed from the roots with tap water. Plants were then transplanted into a mixture of garden soil and perlite (1 : 1 v/v) in 1-L pots (1 plant per pot) and grown in a glasshouse at the Baima Experimental Station (31°6′ N, 119°18′ E) of Nanjing Forestry University. Half-strength Hoagland solution (Hoagland and Arnon 1950) was applied to the top of the soil in the pots (500 mL per pot) every 4 d for 2 weeks; the soil surface did not dry out during this time. The soil in each pot was then irrigated with 500 mL of salt solution (half-strength Hoagland solution containing 250 mM NaCl) (between 9:00 to 10:00 am) at time 0 (first treatment), 24 and 48 h to make sure that the soil was totally saturated with the salt solution; any excess solution drained through the holes in the bottom of the pots. A total of 75 plants were included in salt treatment for each species.

Sample harvesting

Nine plants were selected randomly at time 0, 1, 6, 24 and 72 h, then separated randomly into three groups as three biological replicates. The root systems of plants were carefully dug out, quickly washed with tap water to remove any soil and separated into two parts. One part was frozen in liquid N, then stored at –80 °C until further ground to fine powder using a freezer mill. The second part was dried at 105 °C for 3 h, then at 65 °C for 48 h, then ground to a fine powder using an electric mill.

Measurements

Sodium ion concentration

The dry powder sample (0.3 g) was digested with 10 mL acid mixture of 15.6 N HNO_3 and 11.65 N $HClO_4$ (4 : 1 v/v) at room temperature for 48 h. The digestion volume was then condensed to 2 mL, diluted in a 50 mL volumetric flask with 48 mL deionized water, then filtered through a 0.45-μm membrane filter. The concentration of sodium ion was determined with a flame atomic absorption spectrometer (AA900T, Perkin Elmer, USA) and expressed in mg g^{-1} dry mass as mean of 3 sample replicates.

Malondialdehyde

The level of membrane lipid peroxidation in samples was estimated by measuring malondialdehyde (MDA) content. About 0.3 g of the frozen-root powder was homogenized in 1 mL of 80% (v/v) cold ethanol on ice and then centrifuged at 16,000 × *g* for 20 min at 4 °C. The supernatant (0.6 mL) was mixed with 0.6 mL of 20% (w/v) trichloroacetic acid containing 0.65% (w/v) thiobarbituric acid. The mixture was heated at 95 °C for 30 min, then cooled quickly in an ice bath and centrifuged at 10,000 × *g* for 10 min. The absorbance of the supernatant was measured at 532 nm and the amount of MDA in the supernatant was calculated as described previously (Campo et al. 2014).

Soluble sugar and proline

The frozen-root powder was homogenized with 80% (v/v) ethanol at room temperature. After centrifugation at 16,000 × g for 10 min, the supernatant was decanted to a clean tube. The pellet was then subjected to the extraction process two more times, and the supernatants bulked. Total soluble sugar in the supernatant was determined using the anthrone-sulfuric acid method (McCready et al. 1950). Proline was also determined spectrophotometrically using the ninhydrin method (Bates et al. 1973).

H_2O_2

Based on the modified method (Ferguson et al. 1983), we homogenized the frozen-root powder (about fresh mass 1.0 g) in 3 mL cold acetone, centrifuged the samples at 5000 × *g* for 10 min at 4 °C, and added 0.2 mL of the supernatant to 0.8 mL cold acetone, which was then mixed with 0.1 mL of 20% $TiCl_4$-HCl solution and 0.2 mL strong ammonia hydroxide, and centrifuged at 3000 × *g* for 10 min. The resultant peroxide-Ti compound was washed with acetone 3–5 times, dissolved in 3 mL of 2 M H_2SO_4, and the absorbance measured at 410 nm. The content of H_2O_2 (μmol g^{-1} fresh mass) was determined using a standard curve.

Ascorbate

Ascorbate contents were determined according to a previous method (Kampfenkel et al. 1995) with minor modifications. About 0.3 g of frozen-root powder was homogenized in ice-cold mortar with 0.6 mL cold solution of 6% (w/v) trichloroacetic acid (TCA). The content of reduced ascorbate (ASA) in TCA extract was determined by its capacity to reduce ferric ion to ferrous ion and was measured based on the characteristic absorbance at 525 nm of a complex formed by ferrous ion and α-α′-bipyridl. The total ascorbate pool (ASA-pool) was measured after adding excess dithiothreitol to TCA extracts to reduce the oxidized ascorbate (dehydroascorbate, DHA) to ASA (Gillespie and Ainsworth 2007).

Catalase, peroxidase dismutase and superoxide dismutatase activity

About 0.2 g of frozen-root powder was homogenized in 2.5 mL cold extraction buffer (50 mM sodium phosphate buffer pH 7.0, containing 1% m/v polyvinylpyrrolidone), then centrifuged at 15,000 × *g* for 20 min at 4 °C. The supernatant was then used to assay catalase (CAT; EC 1.11.1.6) and guaiacol peroxidase (POD; EC 1.11.1.7) activity. The same process was conducted for extracting superoxide dismutase (SOD; EC 1.15.1.1) except that the extraction buffer was 50 mM phosphate buffer (pH 7.8) containing 2% (m/v) polyvinylpyrrolidone, 0.3% (v/v) Triton X-100, and 0.1 mM EDTA. The soluble protein concentration of enzyme solutions was determined using the Bradford method with bovine serum albumin as the standard (Bradford 1976).

Activities of CAT and POD were measured according to a previous report (Liu and Huang 2000). One unit of activity was defined as an absorbance change of 0.01 per min at 240 nm for CAT and 470 nm for POD, respectively. The activity of SOD was determined by measuring the inhibition of the photoreduction of nitro blue tetrazolium (NBT) (Cavalcanti et al. 2004); one unit of SOD activity was defined as the amount of enzyme that inhibits 50% of NBT photoreduction. Absorbance of the solutions described was measured using a UV-1900 UV–Visible Spectrophotometer (Shimadzu Corp., Kyoto, Japan).

Statistical analyses

Means were subjected to analysis of variance according to the model for completely randomized design using SPSS version 25.0 (IBM, Armonk, NY, USA). Differences between means ± SD of two species at a certain sampling time were evaluated by Student's *t*-test at 0.05 probability level. Differences among means ± SD at various sampling times for a certain species were first estimated by one-way variance analysis (ANOVA) and then evaluated by Duncan's multiple range test at 0.05 probability level.

Results

Variations of Na^+ concentrations in the two *Ilex* species

Before the salt treatment started, the Na^+ concentration in *I. integra* roots was 1.30 mg g^{-1} DW (dry weight), 2.67-fold higher than in *I. purpurea roots* (0.48 mg g^{-1}) (Fig. 1). With increasing duration of the salt treatment, the Na^+ concentrations in *I. integra* roots gradually increased from 1.43 mg g^{-1} after 1 h of treatment to 2.01 mg g^{-1} after 24 h. After 72 h salt treatment, the Na^+ concentration in *I. integra* roots was 1.76-fold higher than before the treatment.

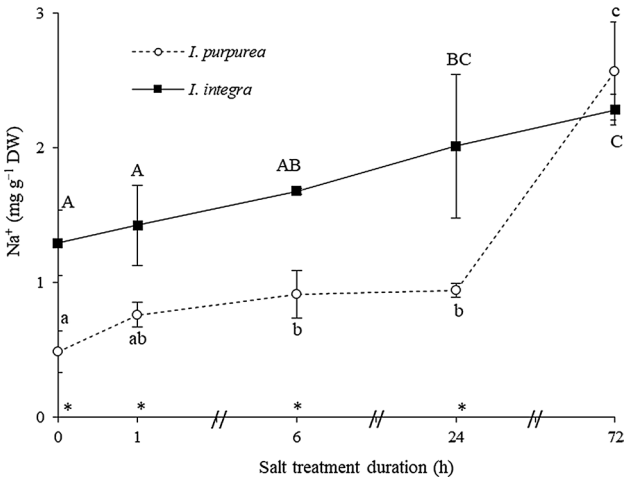

Fig. 1 Mean (±SD) Na^+ concentration in roots of two *Ilex* species after different durations of salt treatment. An asterisk by the *x*-axis indicates a significant difference in concentration between the two species at that sampling time based on Student's *t*-test ($p < 0.05$). A significant difference among concentrations at the sampling times is indicated by different capital letters for *I. integra* and by different lowercase letters for *I. purpurea* based on an ANOVA and Duncan's multiple range test ($p < 0.05$) ($n = 3$)

However, although Na^+ concentration in *I. purpurea* roots only increased slightly from 0.76 mg g^{-1} after 1 h of treatment to 0.94 mg g^{-1} after 24 h, the concentration increased sharply after 72 h to 2.57 mg g^{-1}, 5.31-fold higher than before the treatment, and was thus similar to the level in the roots of *I. integra*.

Malondialdehyde (MDA) concentrations

Malondialdehyde (MDA) was present at similar levels in roots of *I. purpurea* and *I. integra* (13.2 and 11.8 µmol g^{-1} FW (fresh weight), respectively) before the salt treatment (Fig. 2). MDA concentrations in roots of both plants increased gradually during salt treatment and peaked at 24 h (28.7 µmol g^{-1} FW for *I. purpurea* and 22.0 µmol g^{-1} FW for *I. integra,* respectively). Notably, the MDA concentration in *I. purpurea* roots during salt treatment was always higher, indicating more membrane damage in *I. purpurea* roots.

Proline and total soluble sugars

The concentration of proline in roots of *I. purpurea* and *I. integra* did not differ significantly before salt treatments (1.63 vs 1.29 µg g^{-1} FW, respectively) and increased similarly during the salt treatment (Fig. 3a). However, the proline concentration was significantly higher in *I. integra* roots after 1, 6, and 24 h; by 72 h, the concentrations no longer differed significantly. For total soluble sugars, the concentration in *I. integra* differed significantly from that in *I. purpurea* roots at

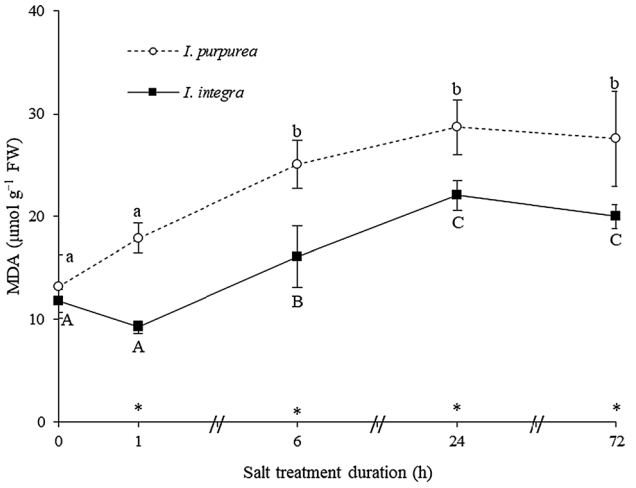

Fig. 2 Mean (±SD) malondialdehyde (MDA) concentrations in roots of two *Ilex* species after different durations of salt treatment. An asterisk by the *x*-axis indicates a significant difference in concentration between the two species at that sampling time based on Student's *t*-test ($p < 0.05$). A significant difference among concentrations at the sampling times is indicated by different capital letters for *I. integra* and by different lowercase letters for *I. purpurea* based on an ANOVA and Duncan's multiple range test ($p < 0.05$) ($n = 3$)

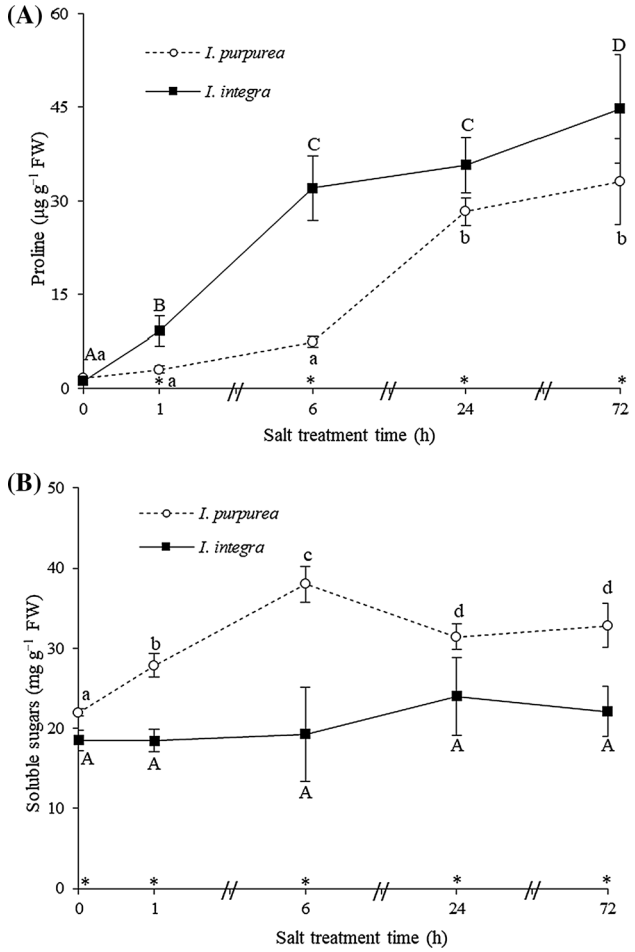

Fig. 3 Concentrations of proline **a** and soluble sugars **b** in roots of two *Ilex* species after different durations of salt treatment. An asterisk by the *x*-axis indicates a significant difference in concentration between the two species at that sampling time based on Student's *t*-test ($p < 0.05$). A significant difference among concentrations for at the sampling times is indicated by different capital letters for *I. integra* and by different lowercase letters for *I. purpurea* based on an ANOVA and Duncan's multiple range test ($p < 0.05$) ($n = 3$)

0 h (2.05 mg g^{-1} FW vs 2.20 mg g^{-1} FW, respectively) and throughout the salt treatment (Fig. 3b), but the level did not differ significantly in its own roots regardless of the duration of the salt exposure. In roots of *I. purpurea*, however, the total sugars increased until 6 h (3.80 mg g^{-1} FW), then declined and leveled off to 3.28 mg g^{-1} FW at 72 h; after the salt treatment began, the levels were always significantly higher than in *I. integra* (Fig. 3b).

H_2O_2 contents

Before the salt treatment, the H_2O_2 content in *I. integra* roots was significantly higher than in *I. purpurea* roots (Fig. 4). In both species, H_2O_2 content increased through 6 h of exposure, then declined through 72 h. Notably, the H_2O_2 content in *I. integra* roots was significantly lower than in *I. purpurea* roots by 72 h, the opposite from 0 h, indicating that more H_2O_2 was accumulated in *I. purpurea* roots after extension of salt treatments.

Ascorbate pool and reduced-ascorbate content

The ascorbate (ASA) pool in roots of both plants was similar from 0 to 24 h of salt exposure (Fig. 5a). Compared to the level at 0 h, the ascorbate pool in *I. purpurea* roots was significantly lower after 1 h of salt treatment, then slightly higher as the duration increased but not significantly higher until after 72 h. The ASA pool in *I. integra* roots did not differ significantly from the 0-h level until 24 and 72 h when it was significantly higher. Although the level in roots of both species did not differ significantly from 0 to 6 h, the ASA pool in *I. integra* roots was significantly higher than in *I. purpurea* roots after 24 and 72 h. Thus, *I. integra* roots may have a relatively higher capacity to increase its ASA pool in response to extended salt exposure. As shown in Fig. 5b, the reduced-ASA content in *I. purpurea* roots decreased gradually from 0.23 μmol g^{-1} at 0 h to 0.09 μmol g^{-1} at 72 h of salt treatment, while reduced-ASA in *I. integra* roots decreased sharply from 0.22 at 0 h to 0.06 μmol g^{-1} after 1 h of salt treatment, then partially recovered to 0.13 μmol g^{-1} at 6 h, then decreased gradually until 72 h of salt treatment, when it did not differ significantly from the lowest level at 1 h.

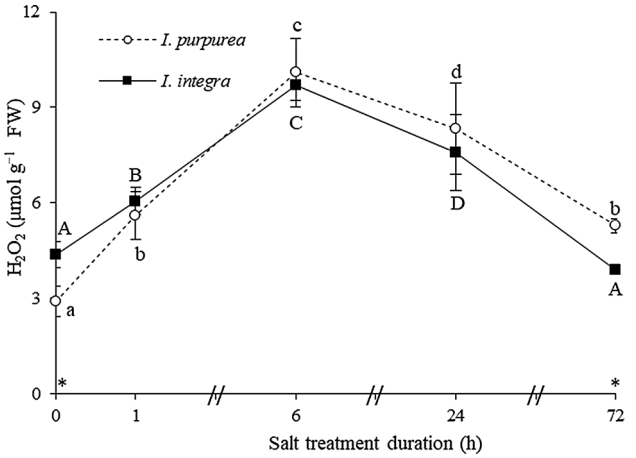

Fig. 4 Mean (±SD) content of H_2O_2 in roots of two *Ilex* species after different durations of salt treatment. An asterisk by the *x*-axis indicates a significant difference in concentration between the two species at that sampling time based on Student's *t*-test ($p<0.05$). A significant difference among concentrations at the sampling times is indicated by different capital letters for *I. integra* and by different lowercase letters for *I. purpurea* based on an ANOVA and Duncan's multiple range test ($p<0.05$)($n=3$)

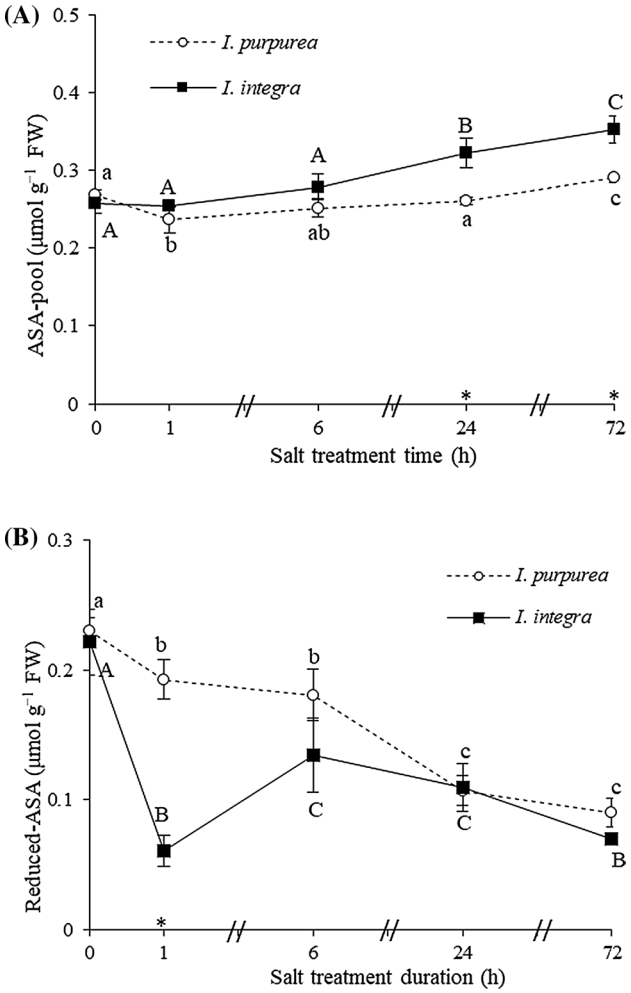

Fig. 5 Mean (±SD) total ascorbate (ASA-pool) and reduced ascorbate (ASA) in roots of two *Ilex* species after different durations of salt treatment. Values are means±SD ($n=3$). An asterisk by the *x*-axis indicates a significant difference in concentration between the two species at that sampling time based on Student's *t*-test ($p<0.05$). A significant difference among concentrations at the sampling times is indicated by different capital letters for *I. integra* and by different lowercase letters for *I. purpurea* based on an ANOVA and Duncan's multiple range test ($p<0.05$) ($n=3$)

Activity of antioxidant enzymes

SOD and POD were significantly higher in *I. purpurea* roots than in *I. integra* roots at all times (Fig. 6a, b). In *I. purpurea* roots, SOD activity was highest (2.40 U mg^{-1} protein) before the salt treatment began, then decreased significantly to 1.33 U mg^{-1} protein and did not then change significantly for the rest of the experiment. In *I. integra* roots, SOD activity increased from 0.03 U mg^{-1} protein at 0 h to maximum values between 6 and 24 h (0.55 U mg^{-1} protein). For *I. integra*, the POD activities were at similar levels in roots before and during salt treatments, possibly indicating that POD is not critical for the response of *I. integra* roots to salinity. However, POD activity in *I. purpurea* roots decreased after 1 h of salt exposure, then increased gradually with the duration of the salt stress. Interestingly, although CAT activity was significantly higher in *I. purpurea* roots than in *I. integra* roots before the salt treatment (Fig. 6c), it decreased in *I. purpurea* roots but increased in *I. integra* roots during the salt exposure. CAT activity was significantly higher in *I. integra* roots, than that in *I. purpurea* roots at 72 h of salt treatment, indicating a higher capacity for scavenging H_2O_2.

Discussion

Before the salt treatment began, roots of *I. integra* had a higher concentration of Na^+ than those of *I. purpurea*, consistent with the natural distribution of *I. integra* in coastal regions with higher salt. During the salt treatments, Na^+ concentrations in the roots of the two *Ilex* species continued to differ. Although the concentration increased gradually in *I. integra* roots with extended salt stress, after 72 h, it was only 1.76-fold higher than before the treatment began. In *I. purpurea*, Na^+ concentration only increased slightly after 24 h of salt exposure, but it sharply increased after 72 h to 5.31-fold higher than at 0 h, thus reaching to a level similar to that in *I. integra*. These results clearly indicated that *I. integra* root responded to exogenous salinity in a controlled way and might encounter mainly osmotic stress during 72 h of salt exposure. However, in *I. purpurea* roots, osmotic stress may persist during 24 h of salt stress because there was no obvious increase in Na^+ concentration, after 72 h

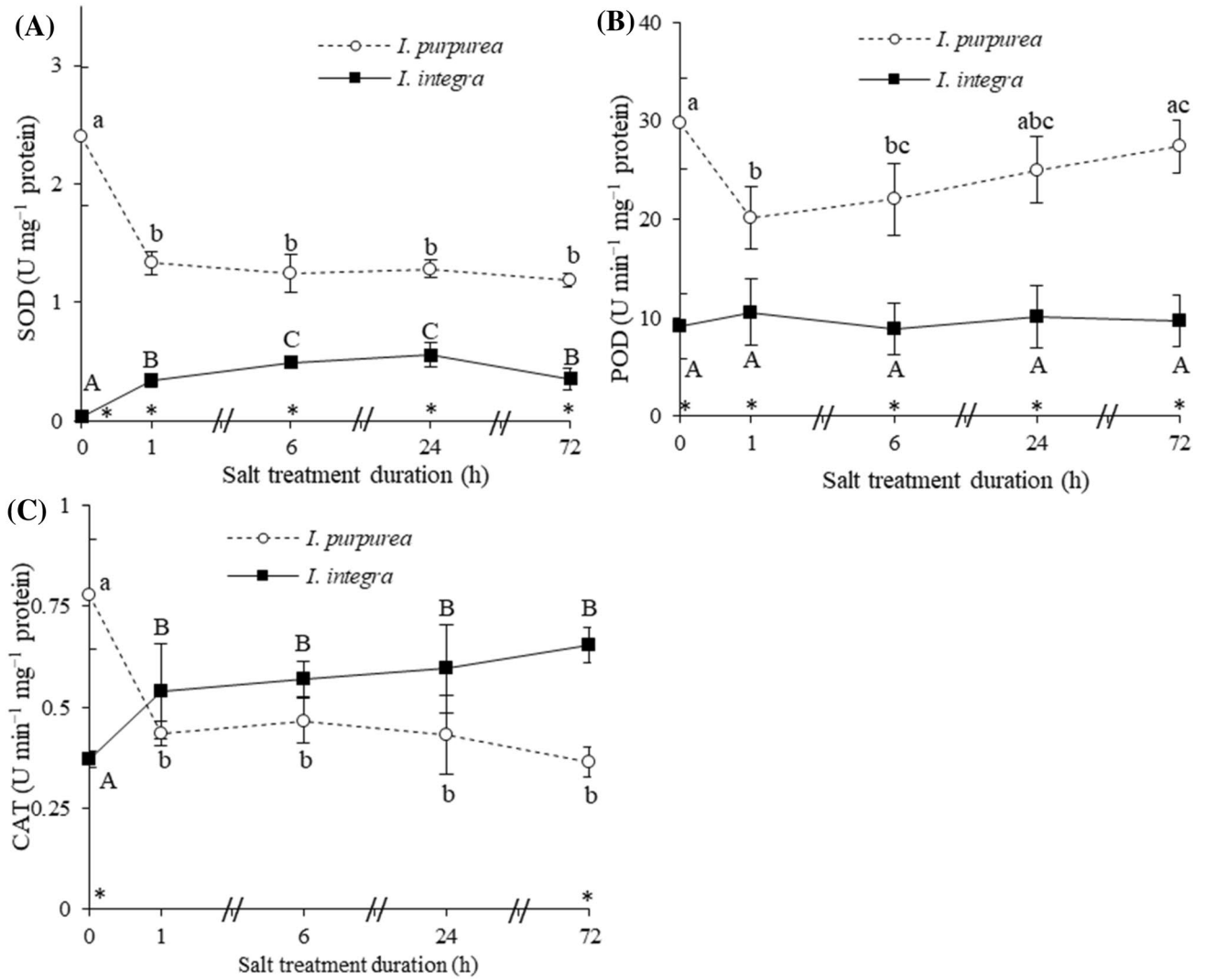

Fig. 6 Activities of SOD **a**, POD **b** and CAT **c** in roots of two *Ilex* species after different durations of salt treatment. An asterisk by the *x*-axis indicates a significant difference in concentration between the two species at that sampling time based on Student's *t*-test ($p < 0.05$). A significant difference among concentrations at the sampling times is indicated by different capital letters for *I. integra* and by different lowercase letters for *I. purpurea* based on an ANOVA and Duncan's multiple range test ($p < 0.05$). ($n = 3$)

Na^+ rapidly accumulated, perhaps due to an ion imbalance or the second phase of the physiological response to salinity (Munns 2002) caused by loss of membrane permeability in the root cells. This result suggests that *I. integra* roots are relatively salt-tolerant and *I. purpurea* root more sensitive to 250 mM NaCl exposures longer than 24 h. Interestingly, *I. integra* roots had more than a twofold higher Na^+ concentration than in *I. purpurea* roots before the salt exposure (Fig. 1), similar to the report for the halophyte seablite (*Suaeda altissima*), which has a higher Na^+ content in roots and leaves than the glycophyte spinach (*Spinacia oleracea*) does, even when they are both grown in a low Na^+ medium (0.5 mM) (Meychik et al. 2013). In tomato, the shoot of salt-resistant genotype PI365967 (*Solanum pimpinellifolium*) contains a higher Na^+ concentration than in the relatively salt-sensitive Moneymaker (*Solanum lycopersicum*) when grown in 1/4 strength Hoagland control solution, but PI365967 has less Na^+ in the shoots than in Moneymaker when they are grown in 1/4 strength Hoagland solution with 200 mM NaCl (Sun et al. 2010). Considering that *I. purpurea* is naturally distributed in the Yangzi River Valley and *I. integra* grows in coastal regions, the higher Na^+ level that is present in *I. integra* without salt exposure compared with its relatively low Na^+ accumulation in roots during salt stress (Fig. 1) may indicate that its roots evolved to adapt to its natural high-salinity habitat, which, together with other physiological mechanisms, may contribute to its higher root salt tolerance.

To maintain a low intracellular osmotic potential under high salinity, plants may synthesize and accumulate various

osmolytes, including soluble sugars, proline, hydroxyproline, glycine betaine, polyamines without disturbing the cellular metabolism (Apse and Blumwald 2002; Mansour and Ali 2017). In this study, although soluble sugars did not increase significantly in *I. integra* roots after salt exposure, they did in *I. purpurea* roots, which had levels significantly higher than in *I. integra* roots during the salt exposure (Fig. 3b), indicating soluble sugar may play a role of osmotic regulation in *I. purpurea* roots. Although initial proline levels did not differ significantly between *I. purpurea* and *I. integra* roots before salt exposure, proline was significantly higher in *I. integra* roots than in *I. purpurea* after 1, 6 and 24 h of salt exposure (Fig. 3a). This result is consistent with previous studies which reported that accumulation of proline is higher in salt-tolerant plant species than in salt-sensitive (Demiral and Türkan 2005; Koca et al. 2007; Kumar and Khare 2016). However, after 72 h of exposure, the proline level in roots of *I. purpurea* no longer differed significantly from that in *I. integra*, perhaps indicating that proline accumulation may only be associated with relatively short-term salt stress.

Maintaining the integrity of cell membrane is a major component of plant tolerance to many environmental stresses including salinity (Mansour et al. 2015). In the present study, the levels of malondialdehyde (MDA), as the product of membrane lipid peroxidation, changed similarly over time in the roots of the two species, but were significantly higher in *I. purpurea* roots than in *I. integra* roots during salt treatment, indicating more membrane lipid peroxidation in *I. purpurea* roots. Interestingly, the level of MDA in *I. purpruea* roots was highest after 24 h of salt treatment and did not coincide with the sharp increase in Na^+ concentration at 72 h, indicating that lipid peroxidation is not alone responsible for the sudden Na^+ influx. Considering that membrane proteins also contribute to plant salt tolerance (Kosová et al. 2013), it would be insightful to compare the proteomics between *I. purpurea* and *I. integra* roots during salt exposure to identify any proteins that may be involved in membrane stabilization and ion homeostasis and contribute to the higher salt-tolerance of *I. integra* (Cheng et al. 2009).

In the present study, the H_2O_2 content was found to be significantly higher in *I. integra* roots than in *I. purpurea* roots before the salt treatment, but after 72 h of salt exposure, the level became significantly lower in *I. integra* roots compared to *I. purpurea* roots, indicating a higher capacity of *I. integra* roots for scavenging H_2O_2 during salt treatments (Fig. 4).

Plant tolerance to high salinity is known to be associated with higher levels of antioxidant activity needed to scavenge ROS (Jabeen et al. 2020), but contradictory results have also been reported (Bose et al. 2013). In present study, the higher H_2O_2 content at 0 h and lower H_2O_2 content after 72 h of salt exposure (Fig. 4) in *I. integra* roots was associated with lower CAT activity at 0 h and a higher CAT activity at 72 h (Fig. 6c), indicating a potential role of CAT in scavenging H_2O_2 during salt stress. In the non-enzymatic ROS scavenging system, ascorbate is thought be the first line of plant defence against oxidative stress (Veljović-Jovanović et al. 2017). Numerous studies on mutant and/or transgenic plants have demonstrated that increased ascorbate levels in certain plants may confer significant abiotic stress tolerance including salinity as reviewed by (Venkatesh and Park 2014). In our study, the total ascorbate pool increased gradually in roots of both species throughout the salt treatment but to a higher level in *I. integra* roots (Fig. 5a). The content of reduced ascorbate in roots of the two species decreased overall from 0 to 72 h of salt exposure with no significant difference between two plants at any time except 1 h. At 1 h of salt treatment, a sharp decreasing of reduced ascorbate was found in *I. integra* roots, possibly indicating a quicker consumption of reduced ascorbate for efficient ROS scavenging at the beginning stage of salt treatment.

Conclusion

I. integra roots apparently are relatively more salt tolerant than *I. purpurea* roots, and the non-enzymatic antioxidant ascorbate and the antioxidative enzyme catalase seem to play substantial roles in scavenging ROS in *I. integra* roots during salt exposure.

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