Plant Physiology and Biochemistry 112 (2017) 326–334

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Saline stress enhanced accumulation of leaf phenolics in honeysuckle (*Lonicera japonica* Thunb.) without induction of oxidative stress

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ARTICLE INFO

Article history:
Received 7 November 2016
Received in revised form
28 December 2016
Accepted 21 January 2017
Available online 22 January 2017

Keywords:
Antioxidant enzyme
Ascorbate
Phenylalanine ammonia-lyase
Phenolics
Photosynthetic electron transport

ABSTRACT

Honeysuckle (*Lonicera japonica* Thunb.) is a traditional medicinal plant in Chinese, and chlorogenic acid and luteolosid are its specific bioactive phenolic compounds. This study was to investigate leaf antioxidant responses in honeysuckle to saline stress with emphasis on phenolics through hydroponic experiments and field trials. NaCl stress did not stimulate antioxidant system including superoxide dismutase, ascorbate peroxidase, catalase and ascorbate, and had no significant effect on lipid peroxidation in the leaves. Consistently, no inhibition on photochemical capacity of photosystems suggested that reactive oxygen species (ROS) was maintained at a normal level under NaCl stress. However, leaf phenolic synthesis was activated by NaCl stress, indicated by elevated genes transcription and activity of phenylalanine ammonia-lyase and increased phenolics concentration. Specifically, leaf chlorogenic acid concentration was increased by 67.43% and 48.86% after 15 days of 150 and 300 mM NaCl stress, and the increase of luteolosid concentration was 54.26% and 39.74%. The accumulated phenolics hardly helped detoxify ROS *in vivo* in absence of oxidative stress, but the elevated phenolic synthesis might restrict ROS generation by consuming reduction equivalents. As with NaCl stress, soil salinity also increased concentrations of leaf phenolics including chlorogenic acid and luteolosid without exacerbated lipid peroxidation. In conclusion, leaf phenolics accumulation is a mechanism for the acclimation to saline stress probably by preventing oxidative stress in honeysuckle; leaf medicinal quality of honeysuckle can be improved by saline stress due to the accumulation of bioactive phenolic compounds.

1. Introduction

Salt stress causes ion homeostasis disruption in plant cells and results in oxidative damage with excess generation of reactive oxygen species (ROS) (Gill and Tuteja, 2010; Munns and Tester, 2008; Zhu, 2016). In photosynthetic organisms, the major ROS generation sites are located at the reaction centers of photosystem I and II (PSI and PSII) in chloroplast (Asada, 2006). In particular, superoxide anion ($O_2^{\bullet -}$) tends to be generated through linear photosynthetic electron transport due to nicotinamide adenine dinucleotide phosphate (NADPH) accumulation, when CO_2 assimilation is inhibited under abiotic stresses (Takahashi and Murata, 2008). Subsequently, $O_2^{\bullet -}$ is transformed to hydrogen peroxide (H_2O_2) through dismutation reaction, and finally, the most dangerous ROS, hydroxyl radical, is generated through Fenton reaction (Gill and Tuteja, 2010).

Plants have evolved a suite of antioxidant system to protect against oxidative damage by scavenging ROS. The antioxidant system consists of: (1) metabolites, the most abundant being ascorbate (AsA); (2) ROS scavenging enzymes, such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Gill and Tuteja, 2010). SOD catalyzes the dismutation of $O_2^{\bullet -}$ to H_2O_2, which is subsequently scavenged by APX and CAT, and AsA can directly scavenge ROS or act as reducing substrate for APX to detoxify H_2O_2 (Gill and Tuteja, 2010). Thus, they work together to

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbate; CAT, catalase; ETR, electron transport rate; Fv/Fm, the maximal photochemical capacity of PSII; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; NADPH, nicotinamide adenine dinucleotide phosphate; $O_2^{\bullet}$, superoxide anion; PAL, phenylalanine ammonia-lyase; Pn, Photosynthetic rate; PSI, photosystem I; PSII, photosystem II; ROS, reactive oxygen species; SOD, superoxide dismutase; $\Delta MR/MR_0$, the maximal photochemical capacity of PSI.

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http://dx.doi.org/10.1016/j.plaphy.2017.01.020
0981-9428/

limit the generation of hydroxyl radical by controlling the levels of $O_2^{-\bullet}$ and H_2O_2. Under saline stress, plant antioxidant system is often stimulated to defense oxidative injury, and antioxidant capacity has been used as a criterion for the comparison of salt tolerance among crop cultivars (Ashrafi et al., 2015; Kiani-Pouya, 2015; Zhang et al., 2014). Phenolic compounds which are secondary metabolites have high ROS scavenging capacity, and accumulation of phenolics is commonly considered as a positive self-protection way to strengthen ROS scavenging ability in plants upon salt-induced oxidative stress (Abrol et al., 2012; Colla et al., 2013; Falleh et al., 2012; Ksouri et al., 2007; Shao et al., 2015; Zhao et al., 2015). Nevertheless, antioxidant activity of phenolic compounds is mainly proved by *in vitro* free radical scavenging experiments, and their roles in mitigating oxidative stress *in vivo* are still open to debate (Hernandez et al., 2009; Hernandez and Van Breusegem, 2010). Phenolic compounds are mainly conserved in vacuoles, and can scavenge ROS diffused from chloroplasts under severe oxidative stress (Ferreres et al., 2011; Niggeweg et al., 2004). Thus, as a traditional viewpoint, phenolic compounds are defined as secondary antioxidants, and play an assisting role in detoxifying ROS, when the primary antioxidant system is depressed under severe stress (Fini et al., 2011). However, this traditional viewpoint has been challenged recently, because moderate saline stress is more liable to induce accumulation of phenolics in plants without severe oxidative stress and depression of antioxidant system (Abrol et al., 2012; Karray-Bouraoui et al., 2010; Ksouri et al., 2007; Shao et al., 2015; Zhao et al., 2015). Therefore, the relation between antioxidant system and phenolic metabolites in resisting oxidative stress is still not clear.

Honeysuckle (*Lonicera japonica* Thunb.) is a twining semi-evergreen vine native to East Asia, and chlorogenic acid and luteolosid are its specific bioactive phenolic compounds. In Chinese, honeysuckle is a traditional medicinal plant, and has been widely used in healthcare, food and cosmetics industries. At present, studies attach importance to salt-induced changes in concentrations of bioactive phenolic compounds in medicinal plants (Colla et al., 2013; Karray-Bouraoui et al., 2010; Shao et al., 2015; Zhao et al., 2015). However, antioxidant mechanisms of phenolics *in vivo* are often overlooked, and the relation between accumulation of phenolics and antioxidant system induction under saline stress is less concerned. In addition, salt-induced accumulation of phenolics in plants is majorly demonstrated by hydroponic experiments and unusually evidenced by field trials. Especially, Bautista et al. (2016) reported that plant phenolics concentration was weakly correlated with soil salinization in natural habitat. Thus, salt-induced accumulation of phenolics in plants lacks ecological proof and practical value.

In recent years, we identified a salt tolerant honeysuckle cultivar and demonstrated its phytoremediation effect on saline soil (Yan et al., 2015, 2016). In this study, we aimed to explore leaf antioxidant responses in honeysuckle under saline stress with emphasis on phenolics through hydroponic experiments and field trials. We hypothesized that the accumulation of leaf phenolics could be improved in honeysuckle to protect against oxidative damage under saline stress by assisting antioxidant system to scavenge ROS.

2. Materials and methods

2.1. Hydroponic experiments

Bare-rooted honeysuckle plants were planted in plastic pots filled with quartz sand. The plants were watered with Hoagland solution (pH 5.7) and placed in a climatic chamber (Qiushi, China). The photon flux density, day/night temperature and humidity were controlled at 200 μmol m^{-2} s^{-1} (12 h per day from 07:00 to 19:00), 25/18 °C and 65% in the chamber. After 60 days, healthy and uniform plants were selected for salt treatments. NaCl was added to nutrient solution incrementally by daily 50 mM steps to final concentrations of 150 and 300 mM. Nutrient solution without adding NaCl was used for the cultivation of control plants. After 0, 7 and 15 days of salt stress, the newly expanded leaves were sampled from four replicate plants in each treatment for the measurements of physiological parameters.

2.2. Field trials

The experiment site was established in Dongying Halophyte Arboretum, Dongying Academy of Agricultural Sciences, Shandong province, China (37°24′N, 118°39′E and 8.8 m above sea level). This area has a warm temperate continental monsoon climate. The annual average temperature and precipitation at this site are 12.8 °C and 555.9 mm, respectively.

Bare-rooted honeysuckle plants were planted in non-saline area in the arboretum in November, 2013. Four replicate plots (3 m × 4 m) were constructed in non-saline and saline areas. The initial soil nutrients, salinity and pH were reported in our recent study (Yan et al., 2016). The average electronic conductance and sodium adsorption ratio were 486 μs cm^{-1} and 9.51, respectively, in non-saline plots and 910 μs cm^{-1} and 16.43, respectively, in saline plots. To avoid border effects, an isolation belt of 0.5 m was left around the plots. The plots were ploughed, and compound fertilization was applied at 750 kg ha^{-1}. In April, 2014, 45 plants were transplanted to each plot; plant and row spacing was 0.5 m and 0.75 m. The newly expanded leaves were sampled from three randomly selected plants in each plot in growth seasons of 2014 and 2015.

2.3. Measurements of photosynthetic parameters

Photosynthetic rate (Pn) and electron transport rate (ETR) were detected by using an open photosynthetic system (LI-6400XTR, Li-Cor, Lincoln, NE, USA) equipped with a fluorescence leaf chamber (6400-40 LCF, Li-Cor). Temperature, CO_2 concentration and actinic light intensity were, respectively, set at 25 °C, 400 μmol mol^{-1} and 1000 μmol m^{-2} s^{-1} in the leaf cuvette. Pn was recorded, when it reached a steady level. Steady-state fluorescence yield was also recorded, and then a saturating actinic light pulse of 8000 μmol m^{-2} s^{-1} for 0.7 s was used to produce maximum fluorescence yield by temporarily inhibiting PSII photochemistry for measuring actual photochemical efficiency of PSII (ΦPSII). ETR were calculated as ΦPSII × PPFD × 0.84 × 0.5 (Maxwell and Johnson, 2000).

A multifunctional plant efficiency analyzer (MPEA, Hansatech, UK) was used to measure the maximal photochemical capacity of PSI ($\Delta MR/MR_0$) and PSII (Fv/Fm) (Yan et al., 2013a). The leaves were kept in the dark for 30 min before the measurements. Thereafter, the leaves were orderly illuminated with 1 s red light (627 nm, 5000 μmol photons m^{-2} s^{-1}), 10 s far red light (735 nm, 200 μmol photons m^{-2} s^{-1}) and 2 s red light (627 nm, 5000 μmol photons m^{-2} s^{-1}). Chlorophyll fluorescence and modulated 820 nm reflection were simultaneously recorded during the illumination. Monitoring modulated reflection change near 820 nm is a very convenient way to follow redox state of PSI. The relative value of maximal difference of 820 nm reflection during the last 2 s red illumination was used to indicate $\Delta MR/MR_0$ (Schansker et al., 2003). Chlorophyll fluorescence transients were quantified to calculate Fv/Fm (Kalaji et al., 2014).

2.4. Measurements of antioxidant enzymes activities, AsA content and redox state and malondialdehyde (MDA) content

The methods of extraction and measurement have been illuminated in detail in our previous study (Yan et al., 2010). SOD activity (U g^{-1} FW) was determined by the inhibition on nitro blue tetrazolium reduction. APX (μmol AsA min^{-1} g^{-1} FW) and CAT (mmol H_2O_2 min^{-1} g^{-1} FW) activities were, respectively, estimated by AsA oxidation rate and H_2O_2 decomposing rate. AsA content (mg g^{-1} FW) was detected by using a high performance liquid chromatograph (Thermo, USA) with a hypersil C18 column (5.0 μm particles size, 4.6 × 150 mm). Dithiothreitol was used to completely reduce the oxidized AsA for detecting total AsA content and calculating AsA redox state. MDA content was reckoned by the reaction with thiobarbituric acid.

2.5. Measurements of phenolic concentration and DPPH scavenging capacity

Leaves were dried at 40 °C to constant weight, and ground to pass through 0.25 mm sieve for measuring phenolics concentration. Dry leaf powder (0.1 g) was homogenized in 10 ml 60% (V/V) methanol, and ultrasonic extraction was carried out at 40 °C for 40 min. The mixture was centrifuged for 10 min at 10000×*g*. The supernatant (0.1 ml) was mixed with 0.15 ml Folin–Ciocalteau reagent, 0.15 ml of 10% (W/V) Na_2CO_3 and 4.6 ml of distilled water. After 80 min incubation at room temperature in the dark, the absorbance at 760 nm was measured. Total phenolics content was determined by using a standard curve plotted with known concentrations of gallic acid (Colla et al., 2013). Flavonoid content was measured by using differential spectrophotometry method with $ZrOCl_2 \cdot 8H_2O$ for avoiding the disturbance of chlorogenic acid (Zhou et al., 2007). The supernatant (0.2 ml) was mixed with 1.5 ml $ZrOCl_2 \cdot 8H_2O$ (0.2%) and 3.3 ml methanol, and another 0.2 ml supernatant was mixed with 4.8 ml methanol. Then, the mixtures were incubated at room temperature in the dark for 1 h, and the differences of absorbance at 410 nm were determined. Flavonoid content was calculated by using a standard curve plotted with known concentrations of lutin.

Chlorogenic acid and luteoloside concentrations were assayed according to Zhang et al. (2007) with some modification. The assay was performed in a high performance liquid chromatography system (Thermo, USA) with a hypersil C18 column (5.0 μm particles size, 4.6 mm × 150 mm). The supernatant was filtered through a 0.45 μm membrane filter before injecting into the column; the injection volume was 10 μl. The mobile phase consisted of 0.3% (v/v) formic acid aqueous solution (A) and acetonitrile (B), and a gradient elution program was applied as follows: at 0 min, the volume ratio between A and B was 92/8, and the ratio was changed to 83/17 from 0 to 12 min, subsequently to 80/20 from 16 to 21 min, and finally returned to 92/8 at 22 min through a linear gradient. The flow rate was 1.0 ml min^{-1}, and the column temperature was 38 °C. Chlorogenic acid and luteoloside in samples were identified by comparing their retention times in UV spectra with those of the standards (Fig. 1). Chlorogenic acid and luteoloside were, respectively, detected at 330 nm and 360 nm, and their concentrations were determined by using a standard curve plotted with known concentrations of the standards.

The diluted supernatant (0.3 ml) and pure methanol (0.3 ml) were, respectively, mixed with 60 μM DPPH methanol solution (3 ml). The mixture was shacked vigorously and incubated for 30 min in the dark at room temperature, and then the absorbance at 517 nm was assayed (Khan et al., 2013). DPPH scavenging rate (%) was calculated as $(A_{blank}-A_{sample})/A_{blank} \times 100$, where A_{sample} and A_{blank} were the absorbance of mixtures in the presence and absence of plant extract, respectively.

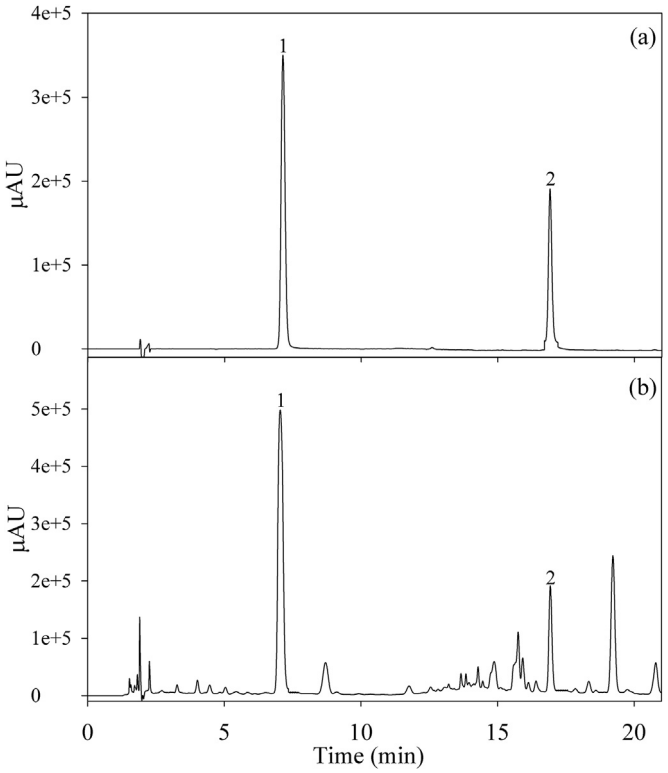

Fig. 1. High performance liquid chromatogram of chlorogenic acid (peak 1) and luteolosid (peak 2) of standards (a) and a leaf sample (b).

2.6. Measurement of phenylalanine ammonia-lyase (PAL) activity

Plant tissues (0.2 g) were ground under liquid nitrogen, and homogenized in 5 ml of 50 mM borate buffer (pH 8.8) containing 20 mM β-mercaptoethanol, 5% (v/w) PVP, 1 mM $EDTANa_2$ and 0.1% (v/w) Triton X-100. The homogenate was centrifuged at 10000 × *g* and 4 °C for 20 min, and the supernatant was collected. For determining PAL activity, 0.2 ml supernatant was mixed with 2.8 ml 0.02 M L-phenylalanine (dissolved in 50 mM borate buffer at pH 8.8), and incubated for 1 h at 30 °C. The increase in OD_{290nm} due to the formation of cinnamate acid was measured, and PAL activity was expressed as the change in OD_{290nm} h^{-1} mg^{-1} protein (Lister et al., 1996). Protein was estimated by coomassie brilliant blue staining with bovine serum albumin as a standard.

2.7. Real time quantitative PCR analysis

For real time expression analysis, the PCR mix contained 1 ml of diluted cDNA (10 ng), 10 ml of SYBR Green PCR Master Mix (Applied Biosystems, USA) and 200 nM of specific primers in a final volume of 20 μl. Actin (*ACT*) (GenBank Accession No. GQ241342) was used as an internal reference gene to calculate relative transcript levels. The primers for *ACT, PAL1, PAL2 and PAL3* are listed in Table 1. All PCRs were performed using a Fast Real Time PCR System (Applied Biosystems, USA) under the following conditions: 2 min at 95 °C, 40 cycles of 15 s at 95 °C, and 60 s at 60 °C in optical 96-well reaction plates. The specificity of amplicons was verified by melting curve analysis. Three technical replicates were analyzed for each gene.

Table 1
Primers for real time quantitative PCR. PAL indicates the gene of phenylalanine ammonia-lyase. PAL gene family consists of *PAL1*, *PAL2* and *PAL3*. Actin (*ACT*) is an internal reference gene.

Gene	Sense primer	Antisense primer
ACT	CCAGTATTGTAGGTAGACCAAGAC	TCAATGGGGTATTTCAAGGTAAGG
PAL1	GCCAATCCAGTCACTAACC	CGTAAATTCTCCTCCAAATGC
PAL2	GCTCGCCCTTGTTAATGG	GTGGTGCTTCAACTTATGC
PAL3	TGAACGCTGGAATCTTTGG	GGTGATGTTGTGGTTGAGG

2.8. Statistical analysis

One-way ANOVAs were carried out by using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) for all sets of data. The values presented are the means of samples collected from four replicate plants in hydroponic experiments and four replicate plots in field trials. The comparisons of means were determined using a least significant difference test, and the differences were considered significant at $P < 0.05$.

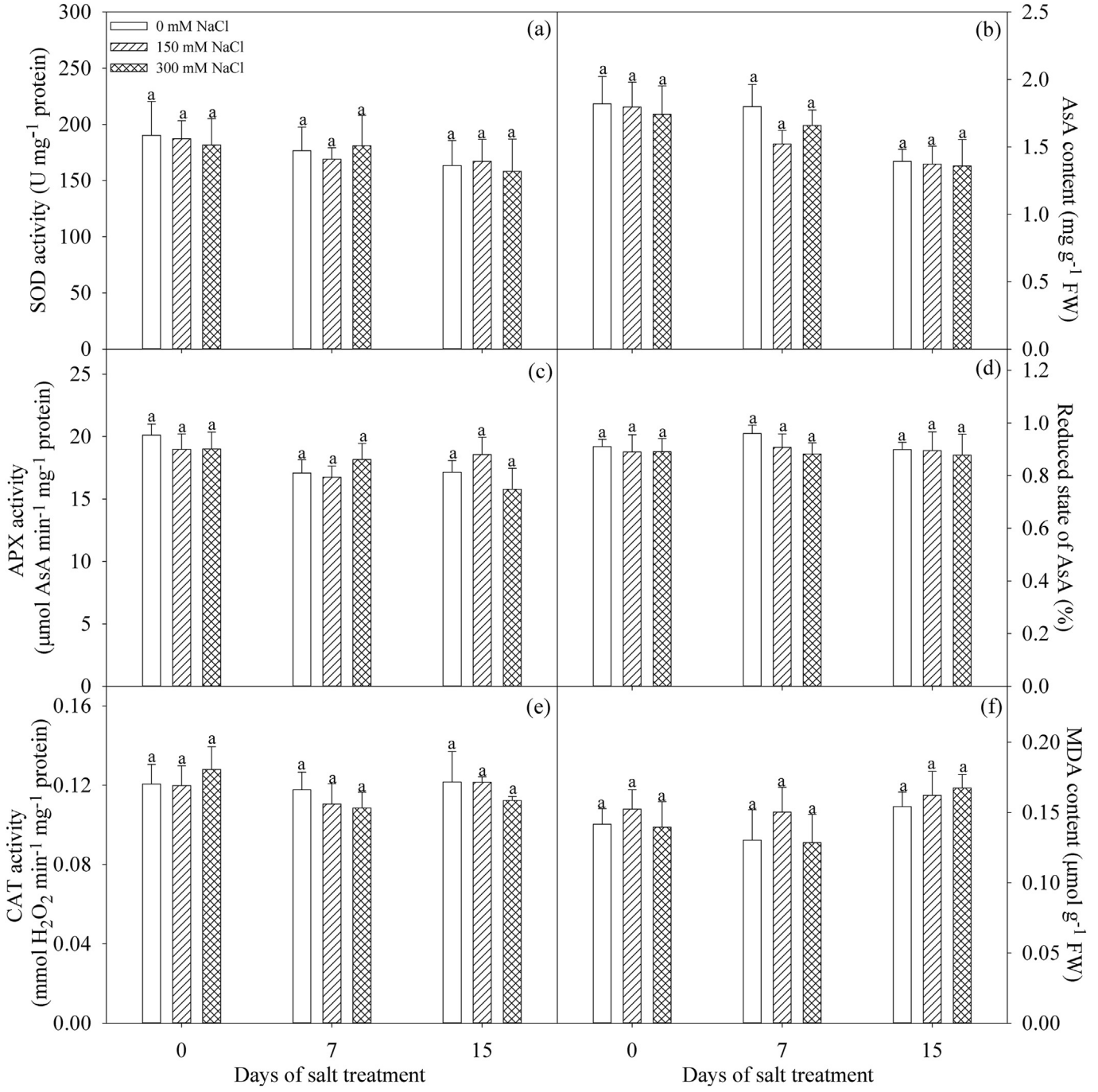

Fig. 2. Effects of saline stress on superoxide dismutase (SOD, a), ascorbate peroxidase (APX, c) and catalase (CAT, e) activities, ascorbate (AsA) content (b) and redox state (d), and malondialdehyde (MDA) content (f) in the leaves of honeysuckle. FW indicates dry and fresh weight. Data in the figure indicate the means of four replicates (±SD). Different letters on error bars indicate salt-induced significant differences at $P < 0.05$.

3. Results

3.1. Antioxidant system and MDA content under NaCl stress in hydroponic experiments

After 7 and 15 days of NaCl stress, SOD, APX and CAT activities in the leaves were not significantly affected, as were MDA content and AsA content and redox state (Fig. 2). Thus, antioxidant system was not stimulated by NaCl stress.

3.2. Photosynthetic rate and chlorophyll fluorescence parameters under NaCl stress in hydroponic experiments

Pn was significantly decreased by 14.56% and 32.45% after 7 days of 150 and 300 mM NaCl stress, and the decrease was up to 29.93% and 60.22% at day 15 (Fig. 3a). ETR was significantly decreased by 17.28% and 27.53% after 7 days of 150 and 300 mM NaCl stress, and the decrease reached 21.30% and 50.02% at day 15 (Fig. 3b). Fv/Fm and $\Delta MR/MR_0$ were not markedly affected after 7 and 15 days of NaCl stress (Fig. 3c and d).

3.3. Phenolics concentration, PAL activity and DPPH scavenging rate under NaCl stress in hydroponic experiments

Significant increases in concentrations of leaf total phenolics and flavonoid were noted after 7 days of NaCl stress, and the increases became greater when NaCl stress was prolonged to 15 days (Fig. 4a and b). Consistently, leaf chlorogenic acid and luteoloside concentrations were also significantly increased after 7 days of NaCl stress, and at day 15, the increase of chlorogenic acid concentration was up to 67.43% and 48.86%, respectively, under 150 and 300 mM NaCl stress, while the increase of luteoloside concentration was 54.26% and 39.74%, respectively (Fig. 4c and d). Remarkable increases in PAL activity and DPPH scavenging rate were observed after 7 and 15 days of NaCl stress (Fig. 4e and f). The increase of phenolics concentration was greater under moderate stress with 150 mM NaCl compared with severe stress with 300 mM NaCl (Fig. 4a–d).

3.4. Gene transcription under NaCl stress in hydroponic experiments

PAL gene family consists of *PAL1*, *PAL2* and *PAL3*, and their transcription was significantly elevated by 150 and 300 mM NaCl at day 7 and 15 (Fig. 5).

3.5. Phenolics concentration in plants in saline plots in field trials

Concentrations of total phenolics, flavonoid, chlorogenic acid and luteoloside in the leaves were maintained at a higher level in

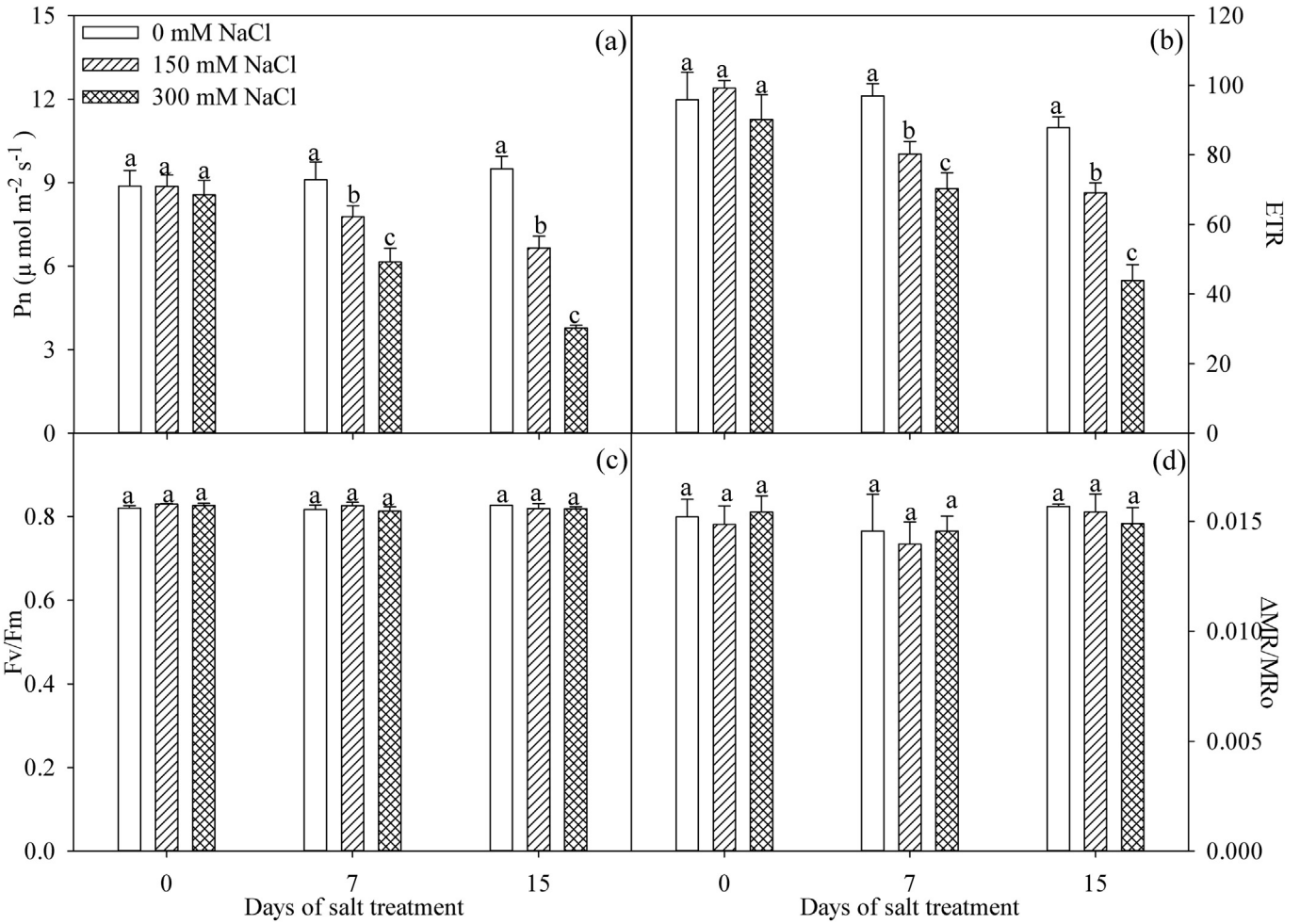

Fig. 3. Effects of saline stress on photosynthetic rate (Pn, a), electron transport rate (ETR, b), the maximal photochemical capacity of PSI ($\Delta MR/MR_0$, c) and PSII (Fv/Fm, d) in the leaves of honeysuckle. Data in the figure indicate the means of four replicates (±SD). Different letters on error bars indicate salt-induced significant differences at $P < 0.05$.

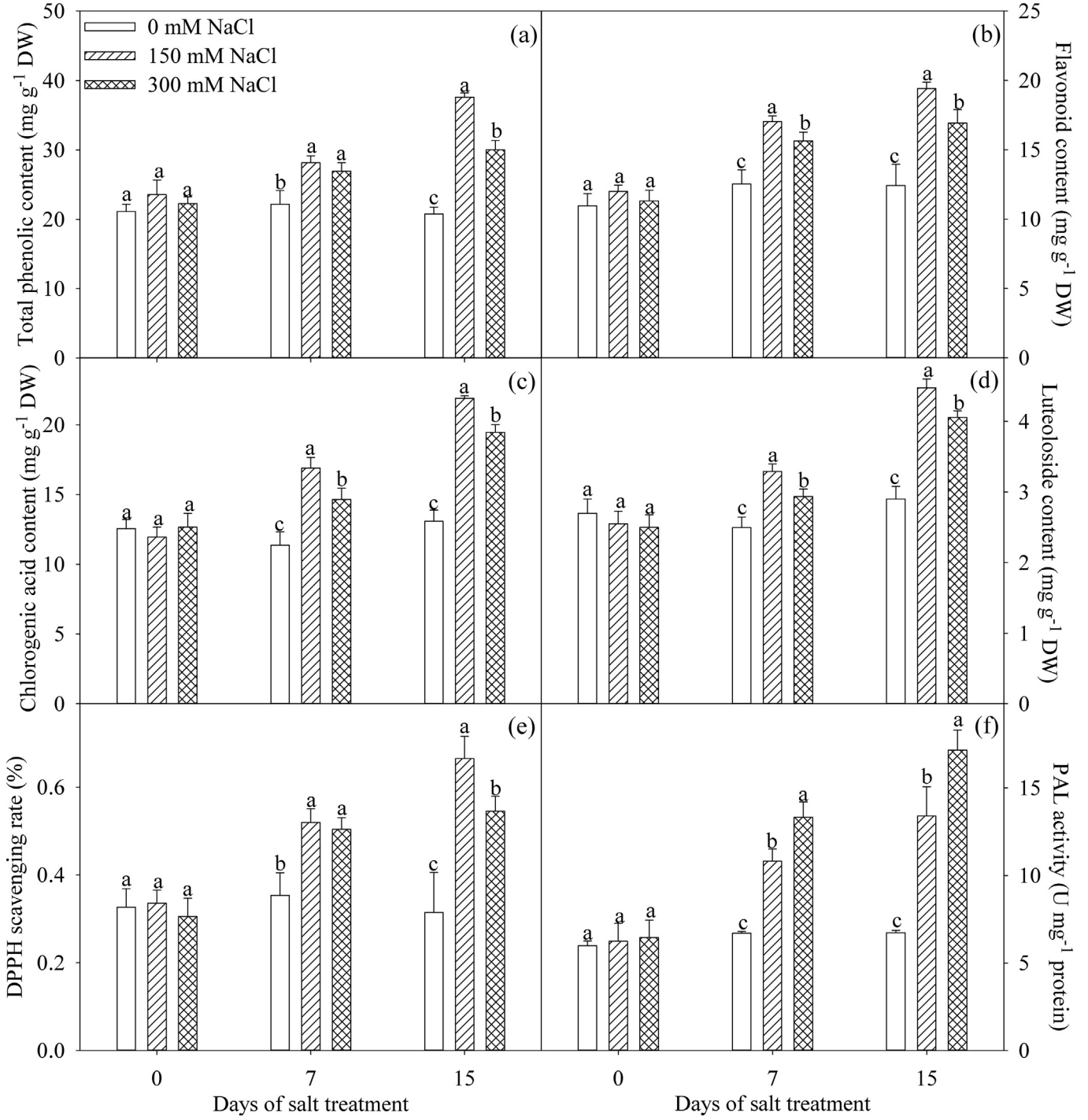

Fig. 4. Effects of saline stress on contents of total phenolics (a), flavonoid (b), chlorogenic acid (c) and luteolosid (d), DPPH scavenging rate (e) and phenylalanine ammonia-lyase (PAL) activity (f) in the leaves of honeysuckle. DW indicates dry weight. Data in the figure indicate the means of four replicates (±SD). Different letters on error bars indicate salt-induced significant differences at $P < 0.05$.

plants in saline plots compared with non-saline plots in growth seasons of 2014 and 2015 (Fig. 6a–h). The differences of their concentrations between the plants from saline and non-saline plots were insignificant in some months (Fig. 6a–h). No significant changes in MDA content were observed in the leaves of plants exposed to soil salinity (Fig. 6i and j).

4. Discussion

ROS are able to bring about lipid peroxidation by initiating a chain reaction on polyunsaturated fatty acids, and MDA content representing the extent of lipid peroxidation is a classic parameter to reflect oxidative injury (Gill and Tuteja, 2010; Yan et al., 2013b).

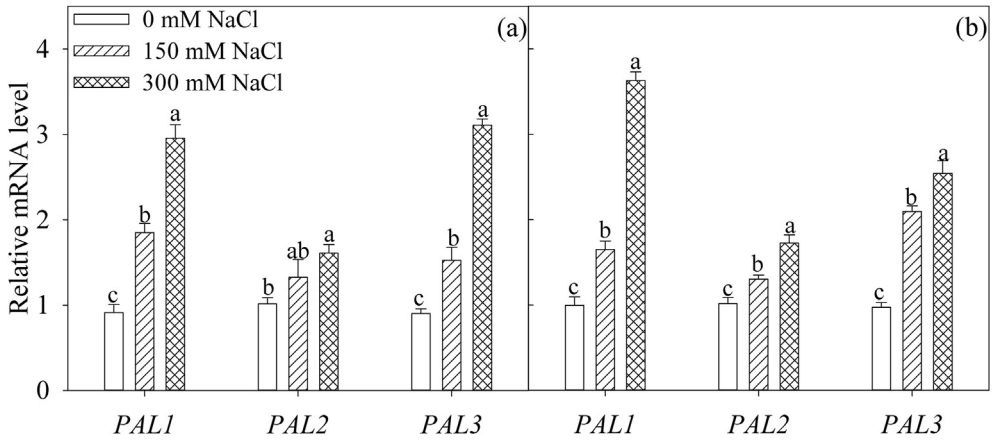

Fig. 5. Phenylalanine ammonia-lyase (PAL) genes transcription in the leaves of honeysuckle under saline stress for 7 (a) and 15 (b) days. PAL gene family consists of *PAL*1, *PAL*2 and *PAL*3. Data in the figure indicate the means of four replicates (±SD). Different letters on error bars indicate salt-induced significant differences at $P < 0.05$.

In this study, insignificant changes of MDA content suggested that oxidative injury did not occur in the leaves of honeysuckle under NaCl stress in the hydroponic experiments (Fig. 2f). In parallel with aggravated lipid peroxidation, oxidative stress commonly stimulates antioxidant system in plant tissues, and no induction on antioxidant system confirmed that oxidative stress was prevented without elevation of ROS production in the leaves of honeysuckle under NaCl stress in the hydroponic experiments (Fig. 2a–e). As widely known, photoinhibition of photosystems results from oxidization of reaction center proteins by ROS (Sonoike, 2011; Takahashi and Murata, 2008), and particularly, Oukarroum et al. (2015) demonstrated that PSI and PSII photochemical capacity was negatively correlated with ROS production. Thus, no significant changes in $\Delta MR/MR_0$ and Fv/Fm further proved that ROS production was controlled at a normal level for preventing oxidative injury in the leaves of honeysuckle under NaCl stress in the hydroponic experiments (Fig. 3c and d). However, the inhibited CO_2 assimilation under NaCl stress could lead to more ROS production through linear photosynthetic electron transport by inducing NADPH accumulation (Fig. 3a). Correspondingly, ROS production was suppressed by virtue of declined ETR under NaCl stress in the hydroponic experiments (Fig. 3b). The declined ETR was a positive response, which probably resulted from elevated dissipation of excess excitation energy by photoprotective mechanisms such as xanthophyll cycle (Brestic et al., 2015, 2016; Jahns and Holzwarth, 2012). Therefore, oxidative damage was prevented by limiting ROS production in the leaves of honeysuckle under NaCl stress in the hydroponic experiments.

According to traditional viewpoint, accumulation of phenolics should not appear in the leaves of honeysuckle under NaCl stress in light of no obvious oxidative stress. On the contrary, NaCl stress significantly increased phenolics concentration, and elevated genes transcription and activity of PAL in the leaves of honeysuckle in the hydroponic experiments, which was a key enzyme in the pathway of phenolic synthesis (Figs. 4 and 5). In spite of elevated DPPH scavenging rate *in vitro* (Fig. 4e), the accumulated phenolics rarely had the chance to scavenge ROS *in vivo* in absence of oxidative stress. Thus, it is surprising that much carbon was consumed to synthesize phenolics upon the decreased CO_2 assimilation. A quite quantity of NADPH is required for phenolic synthesis (Grace and Logan, 2000), and Hernandez and Van Breusegem (2010) suggested that the protective role of flavonoid as energy escape valve during abiotic stresses was largely underrated in comparison to their antioxidant function. Therefore, similar to photoprotective mechanisms, phenolic synthesis helped inhibit ROS production by alleviating NADPH accumulation in the leaves of honeysuckle under NaCl stress. It is worth to note that increased leaf phenolics concentration without oxidative injury and antioxidant system induction under NaCl stress is an uncommon finding. The reason may be that NaCl treatments with low light in the hydroponic experiments were not beneficial to ROS production. Similarly, this uncommon finding was also reported in other medicinal plant, *Swertia chirata*, under mild NaCl stress, and the stimulation of antioxidant enzymes in this plant did not appear until salt-induced oxidative stress became greater (Abrol et al., 2012). Therefore, we suppose that antioxidant system induction stems from the increase of ROS generation, but phenolic biosynthesis is induced by NADPH accumulation.

NaCl stress improved leaf medicinal quality of honeysuckle by promoting accumulation of chlorogenic acid and luteolosid in the hydroponic experiments (Fig. 4c and d). Similar to other medicinal plants (Abrol et al., 2012; Colla et al., 2013; Ksouri et al., 2007; Shao et al., 2015), moderate saline stress was more beneficial to the accumulation of bioactive compounds in the leaves of honeysuckle (Fig. 4c and d), as severe stress greatly reduced carbon supply for phenolic synthesis by inhibiting CO_2 assimilation. In line with NaCl stress in the hydroponic experiments, soil salinity also enhanced concentrations of phenolic compounds in the leaves of honeysuckle without aggravated lipid peroxidation in the field trials (Fig. 6). Thus, leaf phenolics accumulation was also an ecological mechanism in honeysuckle for the adaption to saline soil. The inconsistent result of Bautista et al. (2016) may derive from species difference and specific climatic conditions. However, we agree with them that environmental factors such as light, temperature and precipitation influence plant phenolic synthesis. Therefore, leaf phenolics concentration showed seasonal changes in the field trials (Fig. 6a–h); the insignificant difference of leaf phenolics concentration between plants in saline and non-saline plots in some months might derive from dilution effects of those environmental factors (Fig. 6a–h). Importantly, soil salinity improved leaf medicinal quality in honeysuckle by promoting accumulation of chlorogenic acid and luteolosid in the field trials (Fig. 6e–h). In combination with its phytoremediation effect on saline soil (Yan et al., 2016), honeysuckle is a promising material for planting in saline land.

In agreement with the hypothesis, leaf phenolics accumulation was improved in honeysuckle under saline stress, however, the accumulated leaf phenolics did not directly assisted antioxidant system to scavenge ROS. In addition, saline stress is beneficial to the accumulation of leaf bioactive compounds in honeysuckle.

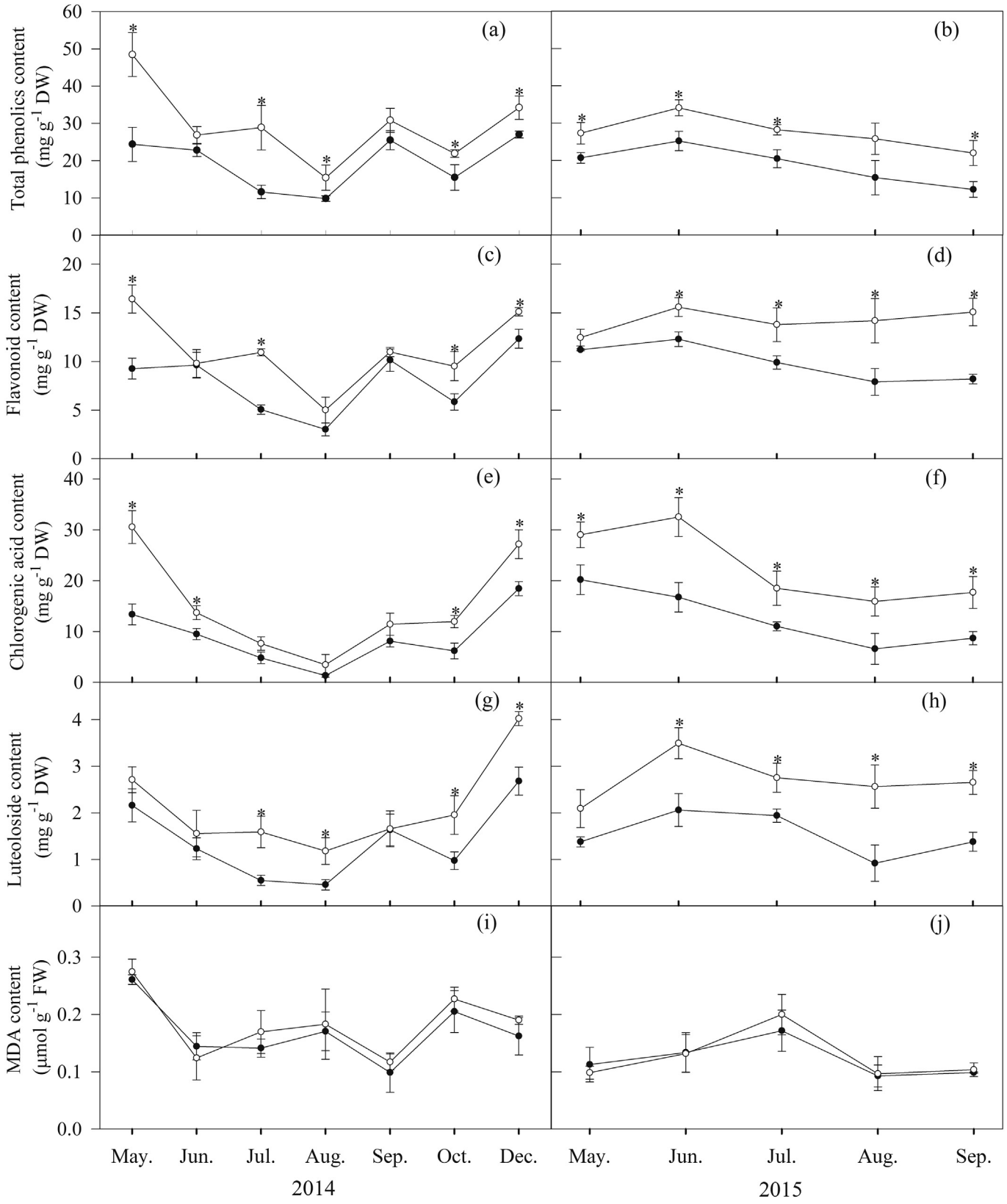

Fig. 6. Seasonal changes in contents of total phenolics (a, b), flavonoid (c, d), chlorogenic acid (e, f), luteolosid (g, h) and malondialdehyde (MDA) (i, j) in the leaves of honeysuckle in non-saline (closed symbol) and saline (open symbol) plots. DW and FW indicate dry and fresh weight, respectively. Data in the figure indicate the means of four replicate plots (±SD). Significant differences induced by salinity are indicated by asterisks: $^{*}P < 0.05$.

Authors contribution

KY designed the experiments, performed data analysis, and wrote the manuscript. SZ participated in experimental design and reviewed the manuscript. LB participated in conducting the experiments. XC assisted in the construction of experimental plots.

Acknowledgements

This research was jointly supported by the National Natural Science Foundation of China (41201292), the Yantai Science and Technology Planning Project (2015ZH069) and the Opening Foundation of the State Key Lab of Crop Biology, Shandong Agricultural University (2016KF07).

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