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EXOGENOUS APPLICATION OF PROLINE ALLEVIATES SALT-INDUCED TOXICITY IN SAINFOIN SEEDLINGS

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ABSTRACT

To clarify the roles of exogenously applied proline in mitigating Na^+ toxicity in sainfoin (*Onobrychisviciaefolia* Scop.), in this study, the changes of physiological and biochemical parameters were investigated in 15-day-old seedlings subjected with 0, 25 and 100 mmol/L NaCl with or without 2.5mmol/L proline for 7d. The results showed that both low salt (25mmol/L) and high salt (100mmol/L) significantly reduced fresh weight, dry weight, and water content (exception for low salt) in plants compared with the control. High salt also significantly increased concentration of malondialdehyde (MDA) and relative membrane permeability (RMP) in shoots. Our results showed that the addition of 2.5mmol/L proline remarkably mitigated Na^+ toxicity in plants under both low- and high-salt stress. The mitigation of toxicity may be caused by regulating Na^+/K^+ ratio and increasing accumulation of proline. This study may provide physiological insights into understanding the mechanisms of salinity tolerance in plants treated with exogenous proline.

Keywords: legume forages, cell membrane stability, ions homeostasis, salinity tolerance, K^+ uptake.

INTRODUCTION

Salt stress is one of the most serious environmental factors that adversely affects crop growth and productivity throughout the world (Zhang and Shi, 2013; Flowers and Colmer, 2015). It has been estimated that more than 20% of all cultivated land around the world contains level of salt high enough to cause salinity stress on crop plants (Munns and Tester, 2008). Salinity induces osmotic stress from low soil water potential, ion toxicity from accumulation of Na^+ and Cl^-, and oxidative stress from reactive oxygen species such as hydrogen peroxide (H_2O_2), hydroxylradical (OH^-), and superoxide (O_2) (Nounjan and Theerakulpisut, 2012). Many crop plants including legume forages are susceptible and cannot survive under conditions of high salinity or can survive only with reduced yields (Zhang and Shi,2013).

Sainfoin (*Onobrychis viciaefolia* Scop.) is an important perennial legume forage with higher crude protein contents which makes its productivity fundamental for beef cattle, sheep meat and dairy production industries (Carbonero *et al.*,2011) and it is planted in the arid and semi-arid areas of northern China. Furthermore, the high ability of nitrogen fixation of sainfoin makes it adaptive to a wide range of soil and climatic conditions (Re *et al*., 2014). Although sainfoin is considered as a relatively salt tolerant species compared with other legume forages, it is sensitive to salt stress in seedling stage. To alleviate the deleterious effects of salinity, a lot of management and curative practices have been adopted. One of those is to apply exogenous proline under salt conditions (Agami, 2014).

Proline plays a critical role as compatible cytoplasmic solute against salinity stress, and acts as protectant of membrane, enzymes and other proteins (Ashraf and Foolad, 2007). It has been shown that salt-tolerant plants can accumulate more proline under salinity stress (Bavei *et al*., 2011). It was well documented that exogenous application of proline had beneficial effects on plants exposed to salinity conditions (Ahmed *et al*., 2010; Abdelhamid *et al*., 2013; Wutipraditkul *et al*., 2015). Furthermore, previous studies suggested that exogenously applied proline protects enzymes, scavenges free radicals, and prevents oxidation under salinity stress (Wutipraditkul *et al*.,2015). However, whether exogenous proline alleviates salt-induced toxicity in sainfoin seedlings is still unclear.

In the present study, to clarify the roles of proline in the salt tolerant mechanism, we investigated the effects of exogenously applied proline on plants growth, Na^+,K^+ and proline concentrations in sainfoin seedlings under salinity conditions.

MATERIALS AND METHODS

Plant materials, cultivation conditions and treatments: The seeds of sainfoin (*O.viciaefolia* Scop., cv."Gansu") were kindly provided by Professor Yanzhong Li, from Lanzhou University, China, in mid August, 2014. Seeds were germinated on filter paper in Petri dishes wetted with distilled water at 25° C in the dark. After 3d of germination, uniform seedlings were carefully transferred to vermiculite irrigated with the modified Hoagl and nutrient solution (Wu *et al*., 2013). The seedlings were grown in the same chamber, where

the environmental conditions were as follows: temperature 20°C at night and 25°C at day, photon flux density 560µmolm^{-2}s^{-1} ,photo period 8/16 h for night / day cycle, and relative humidity 65% (Wu *et al*. ,2013). 15-day- old seedlings were treated with control (C, neither salt nor proline), proline (P, 2.5mmol/L), low salt (LS,25mmmol/LNaCl), high salt (HS,100mmol/LNaCl), low salt plus proline (LS+P,25mmol/L NaCl plus 2.5mmol/Lproline) and high salt plus proline (HS+P,100mmol/L NaCl plus 2.5mmol/L proline) for 7d, respectively. The treatment solution was renewed every 3d.

Determination of growth and water content: At the end of treatments, plant roots were washed twice for a total of 8 min in ice-cold water containing 20 mmol/L $CaCl_2$ to exchange cell wall-bound Na^+ and shoots were rinsed in deionized water to remove surface salt. Shoots and roots were separated and blotted; fresh weight (FW) was immediately determined and samples were dried in an oven at 80°C for 2 d to obtain dry weight (DW) (Wu *et al*., 2013). Water contents (WC) of shoots and roots were calculated with the following formula as described by Wu *et al*. (2013):

WC(g/gDW)=(FW-DW)/DW.

Determination of malondialdehyde (MDA) and shoot relative membrane permeability (RMP): MDA concentrations were analyzed using the thiobarbituric acid (TBA) protocol as described by Peever and Higgins (1989) with slight modifications. Shoot RMP was tested using a conductivity meter (DDS-307, Instrument Co., Shanghai, China) according to the method as described by Bao *et al*. (2009). Shoot RMP was calculated using the following equation: RMP (%)=S1/S2×100, where S1 and S2 are conductivity off reshsainfoin shoots and boiled shoots, respectively.

Determination of Na^+ and K^+ concentrations: Na^+ and K^+ concentrations were assayed according to the method as described by Wang *et al*. (2007). Briefly, Na^+ and K^+ were extracted from dried plant tissue (shoots and roots) in 100 mmol/Lacetic acid at 90°C for 2h. Ion analysis was performed using a flame spectrophotometer (2655-00, Cole-Parmer Instrument Co., Vernon Hills, USA). Na^+/K^+ ratios were calculated using the following formula as described by Wu *et al*. (2013, 2015):

Na^+/K^+ ratios=Na^+concentrations/K^+concentrations.

Determination of proline concentration : Proline was assayed according to the method as described by Bates *et al*. (1973) using ninhydrin reagent with slight modifications. Proline concentrations were estimated using an UV-visible spectrophotometer (UV-300OPC, Mapada Co., Shanghai, China) at 520 nm and determined from a calibration curve using L-Proline (Sangon, Shanghai, China) as the standard.

Statistical analysis: Statistical analyses, one-way analysis of variance (ANOVA) and Duncan's multiple range tests, were performed usings tatistical software (SPSS19.0, Chicago, USA).

RESULTS AND DISCUSSION

Phenotypically, proline-treated plants grew better than untreated plants under salinity conditions (Figure1a). Application of proline (P) alone did not influence FW, DW and WC insainfoin plants compared with the control (P>0.05) (Figure1b-d). However, both low salt (L S, 25 mmol/L) and high salt (HS,100mmol/L) significantly reduced FW and DW of shoot and root in plants compared with the control ($P \leq 0.05$) (Figure1b, c). It was also observed that L S remarkably decreased WC in shoot by 20% ($P \leq 0.05$) (Figure1d). HS significantly reduced WC in both shoot and root by 20% and 16% ($P \leq 0.05$), respectively, compared to the control (Figure1d). The reduction in the FW, DW and WC of plants at salt stress showed an inhibition of growth. This growth inhibition might presumably be due to osmotic effects of salinity in the earlier stage or ionic stress, which affects plant growth much later (Munns and Tester, 2008). However, exogenously applied 2.5 mmol/L proline significantly improved growth parameters under either LS or HS ($P \leq 0.05$) (Figure1b-d). Similar results were observed in olive (*Oleaeuropaea*) (Ahmed *et al*., 2010), bean (*Phaseolus vulgaris*) (Abdelhamid *et al*., 2013), barley (*Hordeum vulgare*) (Agami, 2014), and rice (*Oryza sativa*) (Wutipraditkul *et al*.,2015). It was possible that proline could be absorbed by the developing seedling, where it maintained the status of water by enhancing water influx and decreasing water efflux under salinity conditions (Chen and Murata, 2008).

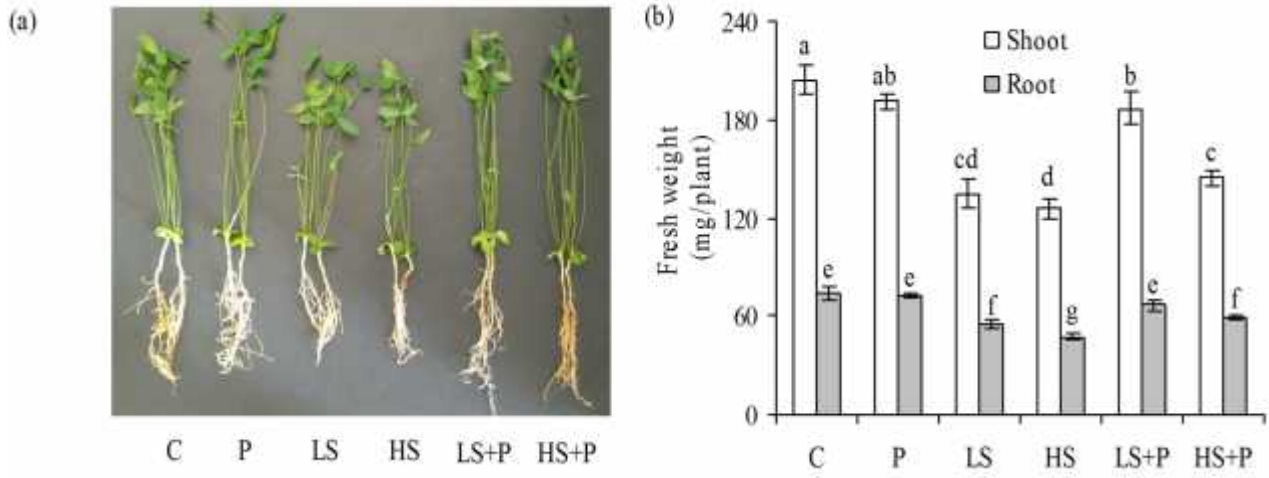
As MDA is the end product of membrane lipid peroxidation, MDA concentrations represent the degrees of cell membrane damage in plants suffered from salinity stress (Bao *et al*., 2009). In this study, compared with the control, proline or LS had no significant effect on MDA concentrations in both shoot and root of plants (P>0.05) (Figure2a). It was clear that high salt remarkably increased the concentrations of MDA in shoot compared with the control ($P \leq 0.05$) (Figure 2a). Similar results were found in lucerne (*Medicago sativa*) (Bao *et al*., 2009) and sugar beet (*Beta vulgaris*) (Wu *et al*., 2013). However, the HS+P treatment significantly reduced MDA concentrations compared with the HS treatment alone ($P \leq 0.05$) (Figure2a). A significant reduction of shoot RMP, which indicates the stability of cell membrane (Bao *et al*.,2009), was also found in plants treated with HS+P, compared with that in plants treated with HS alone ($P \leq 0.05$) (Figure2b).These results indicated that exogenously applied proline could mitigate

oxidative damage and maintain cell membrane stability under high salinity stress.

Among the most common effect of salinity is growth inhibition by increasing the accumulation of Na^+ and reducing the uptake of K^+, and Na^+/K^+ ratios showed the inverse relationship with plant growth (Nounjan *et al*., 2012). Controlling intracellular Na^+ and K^+ homeostasis is of fundamental importance for plants adaptation to salinity conditions (Guo *et al*., 2013, 2015). It was well known that a low Na^+/K^+ ratio is related with plant resistance to salinity (Munns and Tester, 2008). In the present study, no significant differences in Na^+ concentrations were observed between control and proline treatmental one (P>0.05). Both LS and HS significantly increased Na^+ concentration in both shoot and root compared to control, whereas to a lesser degree in LS-treated plants than in HS-treated plants ($P\leq$ 0.05) (Figure 3a). It was also observed that HS+P remarkably reduced Na^+ accumulation in shoot and root of plants compared with the HS treatment ($P\leq$ 0.05) (Figure 3a). The HS treatment significantly decreased shoot K^+ concentrations by 11% compared to control ($P\leq$0.05) (Figure 3b). It was observed that LS and HS remarkably increased shoot and root Na^+/K^+ ratio in plants compared with control ($P\leq$ 0.05). In addition, Na^+/K^+ ratio was significantly lower in HS+P-treated plants than in HS-treated plants ($P\leq$ 0.05) (Figure 3c). There were evidences that exogenous application of proline decreased the accumulation of Na^+ under salinity condition and enhanced K^+/Na^+ ratios (Heuer, 2003; Ahmed *et al*., 2011; Nounjan *et al*., 2012). Thus, exogenous application of proline might be lead to a reduction in Na^+ uptake and salt-induced toxicity. This could explain why proline alleviates adverse effects of salinity on growth of sainfoin seedlings. These results suggested that exogenously applied proline played a crucial role in regulating Na^+/K^+ ratio under salinity condition, which was reflected in higher water content and reduced membrane damage under salt condition.

Proline accumulation is thought as an important physiological parameter for plant adaptation to salt conditions (Wu *et al*., 2013). Salinity remarkably enhanced the levels of proline in different salinity sensitive and/or tolerant plant species, with higher levels in salinity tolerant plants (Bavei *et al*., 2011). In this study, low-and high-salt, or exogenous proline significantly enhanced concentrations of proline in both shoot and root of sainfoin plants compared to control ($P \leq$ 0.05) (Figure4). Increased concentration of proline under salinity condition was also observed in sunflower (*Helianthus annuus*) plants (Wu *et al*., 2015). It was well documented that accumulation of proline under salinity stress may be caused by decreased protein synthesis or by increased proteolysis (Abdelhamid *et al*., 2013). High level of proline under stress condition was of benefit to plants, as proline reduced the osmotic potential of tissues, and thus involved in the osmotic adjustment (Farkhondeh *et al*., 2012). Besides, it has been shown that proline also protects enzymes and increases membrane stability under salinity stress (Wutipraditkul *et al*., 2015). It was clear that exogenously applied proline to low-or high-salt solution increased concentration of proline of shoot in plants by 21% or 44%, respectively ($P\leq$ 0.05) (Figure4). These results implied that exogenous proline enhanced salt tolerance of plants by increasing accumulation of proline.

In conclusion, low-and high-salt markedly inhibited the growth of sainfoin plants, whereas exogenous application of proline can obviously mitigate salt induced toxicity in plants. The mitigation of toxicity may be caused by regulating Na^+/K^+ ratio and increasing accumulation of proline.



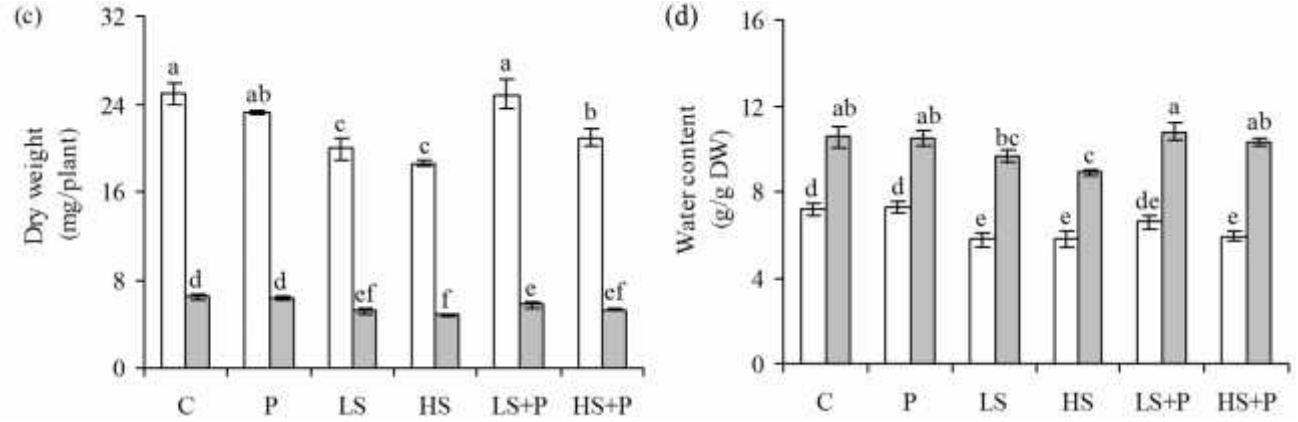


Figure1.(a) Morphology, (b) fresh and (c) dry weight, and (d) water content in 15-day-old sainfoin (*O. viciaefolia* Scop.) seedlings exposed to control (C, neither salt nor proline), proline (P, 2.5mmol/L), low salt (LS,25mmol/LNaCl), high salt (HS, 100mmol/LNaCl), low salt plus proline (LS+P,25mmol/LNaCl plus2.5mmol/Lproline) and high salt plus proline (HS+P,100mmol /LNaCl plus2.5mmol/L proline) for7d. Three plants were pooled in each replicate (*n*=8). Data are mean±SE (standarderror) and bars indicate SE. Columns with different letters indicate significant differences at *P* ≤ 0.05 (Duncan's test). DW – dry weight.

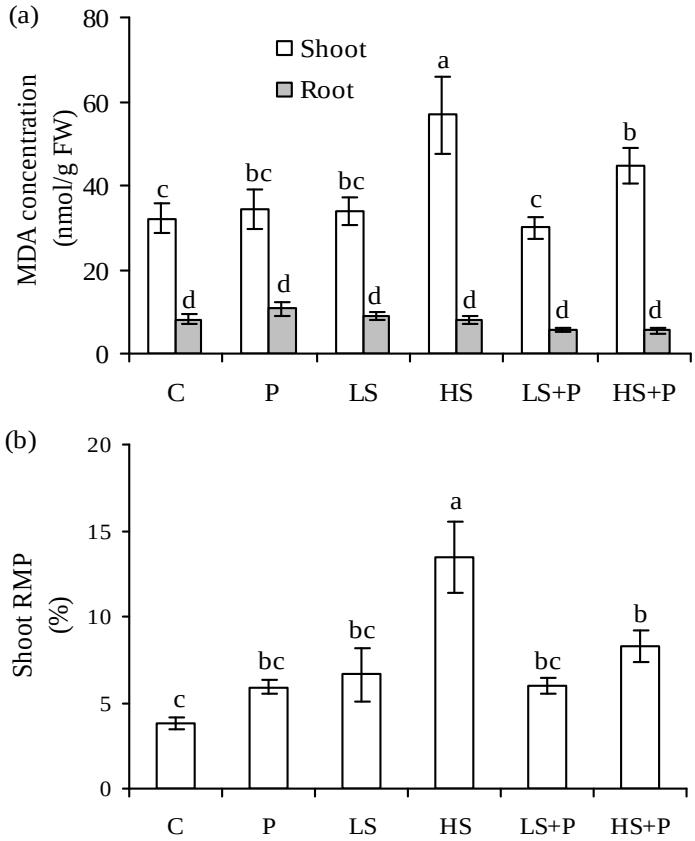


Figure2.(a) MDA concentration and (b) shoot RMP in 15-day-old sainfoin (*O. viciaefolia* Scop.) seedlings exposed to control (C, neither salt nor proline), proline (P, 2.5 mmol/L), low salt (LS, 25 mmol/L NaCl), high salt (HS, 100 mmol/L NaCl), low salt plus proline (LS+P, 25 mmol/L NaCl plus 2.5 mmol/L proline) and high salt plus proline (HS+P, 100 mmol/L NaCl plus 2.5 mmol/L proline) for 7 d. Three plants were pooled in each replicate (*n* = 8). Data are mean ± SE (standard error) and bars indicate SE. Columns with different letters indicate significant differences at *P* ≤ 0.05 (Duncan's test). MDA-malondialdehyde, FW – fresh weight, RMP – relative membrane permeability.

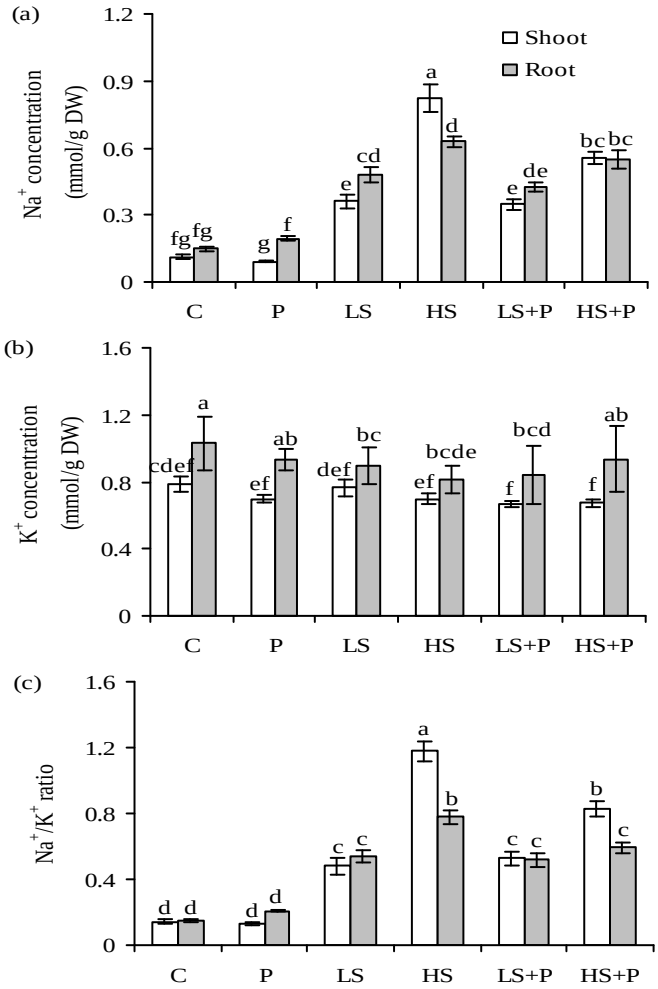


Figure 3.(a) Na^+ and (b) K^+ concentration, and (c)Na^+/K^+ ratio in 15-day-old sainfoin (*O. viciaefolia* Scop.) seedlings exposed to control (C, neither salt nor proline), proline (P, 2.5 mmol/L), low salt (LS, 25 mmol/L NaCl), high salt (HS, 100 mmol/L NaCl), low salt plus proline (LS+P, 25 mmol/L NaCl plus 2.5 mmol/L proline) and high salt plus proline (HS+P, 100 mmol/L NaCl plus 2.5 mmol/L proline) for 7 d. Three plants were pooled in each replicate (*n* = 8). Data are mean ± SE (standard error) and bars indicate SE. Columns with different letters indicate significant differences at *P* ≤ 0.05 (Duncan's test). DW – dry weight.

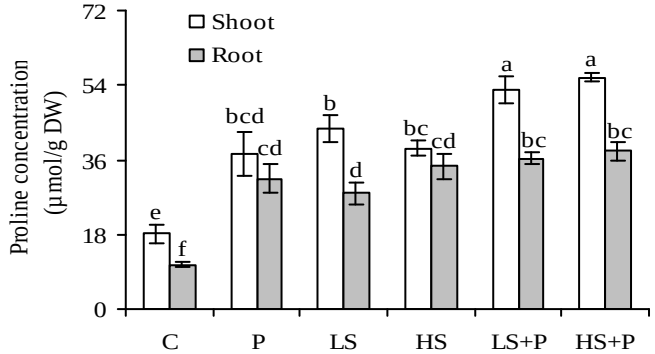


Figure4. Proline concentration in 15-day-old sainfoin (*O. viciaefolia* Scop.) seedlings exposed to control (C, neither salt nor proline), proline (P, 2.5 mmol/L), low salt (LS, 25 mmol/L NaCl), high salt (HS, 100 mmol/L NaCl), low salt plus proline (LS+P, 25 mmol/L NaCl plus 2.5 mmol/L proline) and high salt plus proline (HS+P, 100 mmol/L NaCl plus 2.5 mmol/L proline) for 7 d. Three plants were pooled in each replicate (*n* = 8). Data are mean ± SE (standard error) and bars indicate SE. Columns with different letters indicate significant differences at *P* ≤ 0.05 (Duncan's test). DW-dry weight.

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