

# Mechanisms of stress tolerance in xerophyte *Zygophyllum xanthoxylum* and their application in genetic improvement of legume forages

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**Abstract.** Xerophytes, naturally growing in desert areas, have evolved multiple protective mechanisms to survive and grow well in harsh environments. *Zygophyllum xanthoxylum*, a succulent xerophyte with excellent adaptability to adverse arid environments and a fodder shrub with high palatability and nutrient value, colonizes arid areas in China and Mongolia. In this study, we found that *Z. xanthoxylum* grew better responding to salt condition with a typical feature for halophytes and became more tolerant to drought in the presence of moderate salinity (50 mM NaCl); 50 mM NaCl alleviated deleterious impacts of drought on the growth of *Z. xanthoxylum* by improving the relative water content, inducing a significant drop in leaf water potential and, concomitantly, increasing leaf turgor pressure and chlorophyll concentrations resulting in an enhancement of overall plant photosynthetic activity. Subsequently, co-expression of genes encoding the tonoplast  $\text{Na}^+/\text{H}^+$  antiporter (*ZxNHX*) and  $\text{H}^+$ -PPase (*ZxVP1-1*) which involve in leaf  $\text{Na}^+$  accumulation under stress condition by compartmentalizing  $\text{Na}^+$  into vacuoles in *Z. xanthoxylum* significantly improved both drought and salt tolerance in legume forages, *Lotus corniculatus* L. and *Medicago sativa* L.

**Keywords:** Sodium, succulent xerophyte, *Zygophyllum xanthoxylum*, stress tolerance, co-overexpression, legume forages.

## Introduction

Drought is one of the major abiotic stresses constituting a serious threat to agricultural productivity throughout the world (Ben Hassine *et al.* 2010). Some plants, such as xerophytes, however, have evolved multiple protective eco-physiological mechanisms allowing them to survive and grow well in those harsh environments. To meet the challenge of water deficiency for agriculture, there is an increasing interest in studying the eco-physiological behavior of xerophyte species to understand their drought-resistance mechanisms (Chaves and Oliveira 2004; Ashraf 2010). *Zygophyllum xanthoxylum*, a perennial shrub species native to the desert areas of northwest China and Mongolia, is a succulent xerophyte belonging to Zygophyllaceae with excellent adaptability to adverse arid environments (Pei *et al.* 2004). This species has a strong ability for sand-fixing and for soil and water conservation in desert regions (Wang *et al.* 2004). Meanwhile, the high palatability and nutrient value make *Z. xanthoxylum* attractive as a forage crop in local regions, and its leaves, stems, roots and fruits possess high medicinal value (Wu *et al.* 2004). Wang *et al.* (2004) hypothesized that  $\text{Na}^+$  may have positive effects on the growth of *Z. xanthoxylum* in arid environments, since it was found that the plant absorbed a great quantity of  $\text{Na}^+$  from low salinity soil that was transported to leaves under arid field conditions. However, the possible physiological mechanisms involved in these observations had not been explored.

The present work was initiated to reveal the possible eco-physiological mechanisms of  $\text{Na}^+$  accumulation involved in drought resistance of *Z. xanthoxylum* and apply the functional genes in genetic improvement of legume forages.

## Methods

For the sand culture experiment of external 5-300 mM NaCl treatments, 3-week-old seedlings plants were treated with modified ½ strength Hoagland nutrient solution supplemented with 5, 25, 50, 100, 200 or 300 mM NaCl, with the 100, 200 and 300 mM NaCl treatments being increased incrementally by 50 mM each day until the final concentrations were achieved. For treatments of water stress in the presence or absence of NaCl (50 mM) in pot experiments, after seeds germinated, seedlings were transplanted into plastic pots filled with 1 kg oven-dried vermiculite and irrigated with modified ½ strength Hoagland nutrient solution.

The water content of the substrate was maintained at 70% of field water capacity (FWC) by weighing, and the rest of growth conditions were the same as that in the sand culture experiments. After 30 days, seedlings were divided into 3 groups: control (C), drought treatment (D) and drought with additional 50 mM NaCl treatment (D+S). The water content of the substrate in the control (C) group was maintained at 70% of FWC by irrigating with modified ½ strength Hoagland nutrient solution during the experimental period. In the D and D+S

groups, the water content of the substrate was maintained at 70% of FWC for 3 days by irrigating with modified ½ strength Hoagland nutrient solution without or with additional 50 mM NaCl, and then water was withheld for 5 days to induce drought stress gradually. When the water content of the substrate was reduced to 30% of FWC, this value was maintained by irrigating with the corresponding solution. After 7 days, plants were harvested for physiological analysis. The co-transformation of *ZxNHX* (Wu *et al.* 2011) and *ZxVP1-1* (which encodes tonoplast H<sup>+</sup>-PPase of *Z. xanthoxylum* and is an orthologous gene of *AVP1* from *Arabidopsis*) into *L. corniculatus* L. and lucerne was accomplished by the means described by Cheng *et al.* (2010) and Weeks *et al.* (2008), respectively. The salt-tolerance and drought-resistance of transgenic plants was assayed according to the method described by Bao *et al.* (2009).

## Results

### Moderate concentrations of NaCl could stimulate growth of *Z. xanthoxylum* and alleviate the deleterious impact of water deficit on photosynthesis and water status of *Z. xanthoxylum*

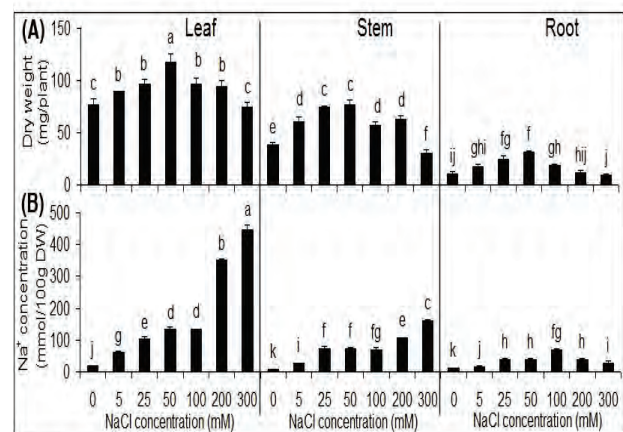
Firstly, 3-week-old seedlings were treated with a series concentration of external NaCl (0 - 300 mM) by sand culture experiments. The results showed that the addition of 25-100 mM NaCl significantly stimulated plant growth (Fig. 1A), which was closely related to high Na<sup>+</sup> accumulation especially in leaf (Fig. 1B), and optimal growth was observed in the presence of 50 mM NaCl (Fig. 1A), indicating that *Z. xanthoxylum* required moderate Na<sup>+</sup> to achieve its maximum growth potential.

Then seedlings were subjected to water stress (30% of FWC) in the presence or absence of NaCl (50 mM) in pot experiments. The results indicated that 50 mM NaCl could alleviate deleterious impacts of water deficit on the growth of *Z. xanthoxylum*, by improving the relative water content, inducing a significant drop in leaf water potential and, concomitantly, increasing leaf turgor pressure and chlorophyll concentrations resulting in an enhancement of overall plant photosynthetic activity (i.e. photosynthetic rate and water use efficiency) (Table 1; Table 2). The contribution of Na<sup>+</sup> to the total osmotic potential varied from 8% in the control to 13% in plants subjected to water deficit and, surprisingly, to 28% in plants grown in the presence of 50 mM NaCl under water deficit; whereas the contribution of K<sup>+</sup> significantly decreased from 13% to 8% (Table 2). Furthermore, NaCl (50 mM) could alleviate the inhibition of water deficit on the activity of photosystem II in *Z. xanthoxylum* (data not

shown). These findings suggest that, under arid environments, *Z. xanthoxylum* is able to accumulate high concentration of Na<sup>+</sup> in leaves under arid environments and use it directly as an osmoregulatory substance, which is coupled with an improvement in leaf hydration and photosynthetic activity. In addition, the ability of NaCl to improve plant performance may be due to the induction of significant increases in chlorophyll concentrations and the resistance of photosystem II to water deficit.

### Co-overexpression of *Z. xanthoxylum ZxNHX* and *ZxVP1-1* enhanced salt and drought tolerance in transgenic *L. corniculatus* and lucerne by increasing cations accumulation

Finally, we cloned the *ZxNHX* and *ZxVP1-1* genes from *Z. xanthoxylum*, which encode the tonoplast Na<sup>+</sup>/H<sup>+</sup> antiporter and H<sup>+</sup>-PPase, respectively, and involve in leaf Na<sup>+</sup> accumulation under stress condition by compartmentalizing Na<sup>+</sup> into vacuoles in *Z. xanthoxylum*, then co-transferred them into important legume forages, *L. corniculatus* and lucerne. Transgenic plants grow well in the presence of NaCl and also under a water-deprivation condition, while wild-type plants exhibit chlorosis and growth inhibition, even death (Fig. 2). Compared with wild-type plants, transgenic *L. corniculatus* plants accumulate more Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup> in leaves and roots (Fig. 3), transgenic lucerne plants exhibit similar cations accumulation trends (data not shown), indicating that co-overexpression of *ZxNHX* and *ZxVP1-1* could enhance salt and drought tolerance in transgenic legume forages by increasing cations accumulation.



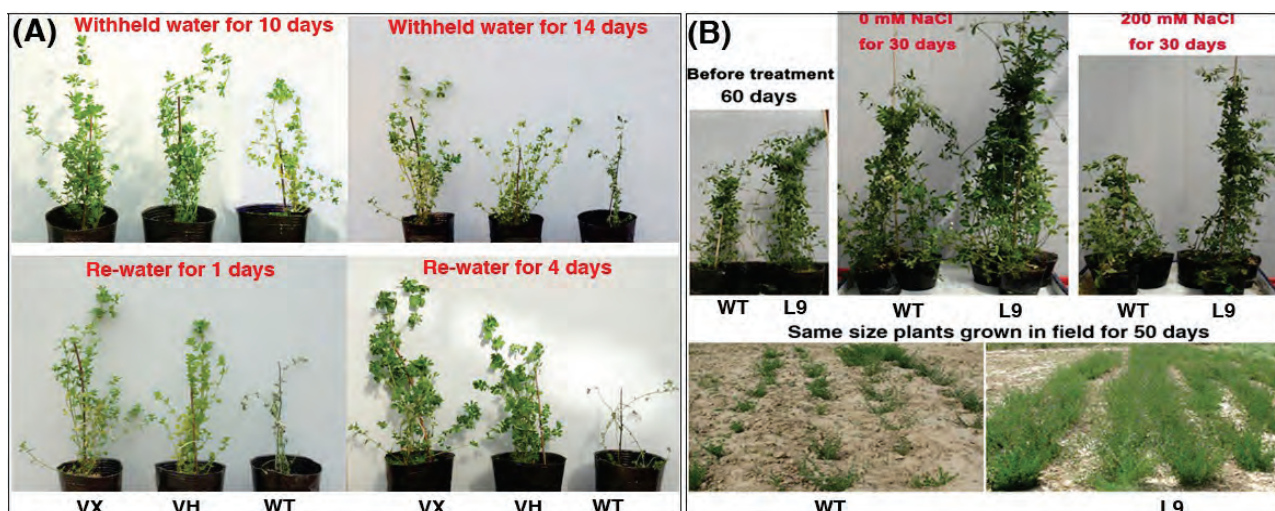
**Figure 1.** Dry weight of leaf, stem and root (A) and Na<sup>+</sup> concentrations in leaf, stem and root (B) of *Z. xanthoxylum* exposed to 5, 25, 50, 100, 200 and 300 mM NaCl.

**Table 1.** Relative growth rate (RGR), net photosynthesis rate (Pn), water use efficiency (WUE), leaf area, chlorophyll concentrations in leaves of *Z. xanthoxylum* seedlings. Treatment: control (C) (70% FWC), drought stress (D) (30% of FWC) and drought stress together with salt (D+S) (30% of FWC with 50mM NaCl) for 7 d.

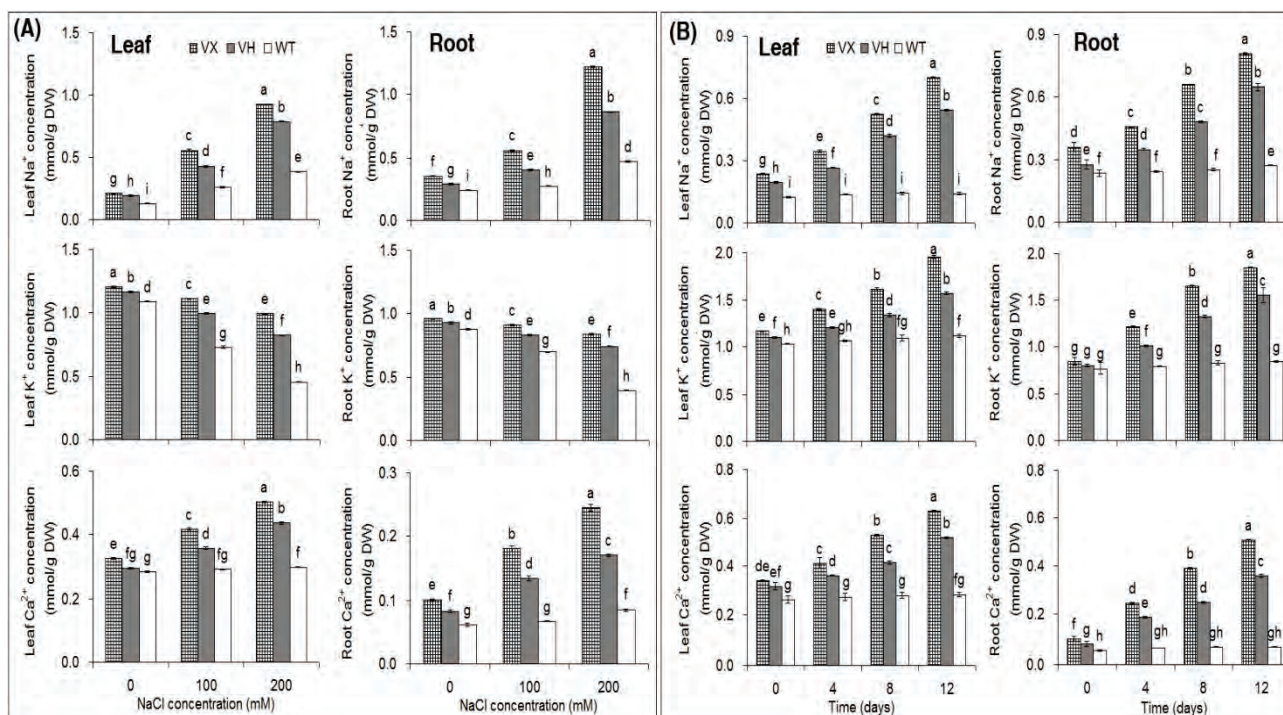
Treatments	RGR (g/kg/d)	Pn (μmolCO <sub>2</sub> /m <sup>2</sup> /s)	WUE (μmolCO <sub>2</sub> /molH <sub>2</sub> O)	Leaf area (cm <sup>2</sup> /plant)	Chlorophyll a (mg/g FW)	Chlorophyll b (mg/g FW)
C	206±6 a	16.1±0.7 a	45.9±2.5 a	17.4±1.0 a	0.96±0.02 a	0.46±0.04 a
D	139±7 c	8.1±0.4 c	35.4±3.0 b	11.0±0.9 c	0.72±0.03 c	0.31±0.02 c
D+S	184±12 b	14.0±0.3 b	44.0±3.1 a	15.7±0.5 b	0.91±0.01 b	0.38±0.04 b

**Table 2.** Leaf water potential ( $\Psi_w$ ), turgor pressure ( $\Psi_t$ ),  $\text{Na}^+$ ,  $\text{K}^+$  concentrations and the contributions of  $\text{Na}^+$  and  $\text{K}^+$  to osmotic potential ( $\Psi_s$ ) of *Z. xanthoxylum* seedlings. Treatments: control (C) (70% FWC), drought stress (D) (30% of FWC) and drought stress together with salt (D+S) (30% of FWC with 50 mM NaCl) for 7 d.

Treatments	$\Psi_w$ (MPa)	$\Psi_t$ (MPa)	$\text{Na}^+$ concentration (mmol/g DW)	$\text{K}^+$ concentration (mmol/g DW)	Contribution of $\text{Na}^+$ to $\Psi_s$ (%)	Contribution of $\text{K}^+$ to $\Psi_s$ (%)
C	-0.45±0.02 a	0.50±0.02 a	0.39±0.06 c	0.59±0.05 a	8	13
D	-1.09±0.07 b	0.24±0.07c	0.64±0.04 b	0.59±0.07 a	13	13
D+S	-1.31±0.03 c	0.41±0.02 b	2.13±0.09 a	0.58±0.04 a	28	8



**Figure 2.** Co-expression of *ZxNHX* and *ZxVPI* genes enhanced salt and drought tolerance of the transgenic *L. corniculatus* (A) and improved the salt tolerance and improved the growth of transgenic lucerne (B). WT: Wild type plants; VX: transgenic *L. corniculatus* co-expressing *ZxNHX* and *ZxVPI-1*; VH: transgenic *L. corniculatus* expressing *ZxVPI-1*; L9: Transgenic lucerne co-expressing *ZxNHX* and *ZxVPI-1*.



**Figure 3.** Cation concentrations in leaf (left) and root (right) of transgenic and wild-type *L. corniculatus* under different NaCl concentrations for 10 days (A) and during withholding water for 0, 4, 8 and 12 days (B), respectively. WT, wild-type plants; VX, transgenic line co-expressing *ZxNHX* and *ZxVPI-1*; VH, transgenic line expressing *ZxVPI-1*.

## Conclusion

Under arid environments, xerophyte *Z. xanthoxylum* is able to accumulate high concentration of Na<sup>+</sup> in its leaves and use it directly for osmotic adjustment, which was coupled with an improvement in leaf hydration and photosynthetic activity.

Co-overexpression of tonoplast Na<sup>+</sup>/H<sup>+</sup> antiporter and H<sup>+</sup>-PPase genes from xerophyte *Z. xanthoxylum* could enhance salt and drought tolerance of important legume forages, which might have resulted from increased Na<sup>+</sup> sequestration in vacuole as well as other cations accumulation in the cell, thus providing a feasible way for improving the stress tolerance in forages and crops using the excellent genes from desert plants.

## Acknowledgements

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