

ANATOMICAL STRUCTURES OF *REAUMURIA SOONGORICA* IN ALASHAN DESERT

RUIXIANG LIU*, YINGMEI MA, XINGHUA ZHAO, SHICHAO CHEN, YANJIE MI AND TIANGE WANG

College of Desert Control Science and Engineering, Inner Mongolia Agricultural University, 306 Zhaowuda Road, Saihan District, Hohhot, Inner Mongolia, 010018, P. R. China

*Corresponding author's email: liuruix@126.com

Abstract

Reaumuria soongorica endemic to Alashan desert, Inner Mongolia, China is tolerant to extreme arid and saline soil environment that plays a vital role in maintaining environmental sustainability. It possessed unique anatomical structures capable of adaptation to desert environment. In order to understand the relationship between the anatomical structures and its abiotic stress tolerances, this study was designed to examine leaf, stem, and root anatomical structures of *R. soongorica* from 'Luanjingtang', 'Changliushui', and 'Wuliji' within Alashan desert using paraffin section and scanning electron microscopy analysis. Extreme arid and saline soil profiles of three sampling sites revealed by low soil moisture content and high salt content with negative soil water potential. Extreme arid and saline soil conditions enabled *R. soongorica* to have more leaf mesophyll cells and in return with more stem cortex cell shrinkage and cellular osmotic adjustment compounds using paraffin section method. Thick leaf epicuticle and small mesophyll cell size were also found which is involved in preventing leaf water loss. Low leaf stomata density ($213.68 \pm 71.72 \text{ mm}^{-2}$), high amount of salt glands ($24.5 \pm 0.5 \text{ mm}^{-2}$), and abundant salt crystals on leaf and stem surface as well as xylem vessels were observed under scanning electron microscopy. Specialized multicellular salt gland was structurally formed by five to seven specialized epidermal cells as an 'opened flower' shape to secret extra salt out of leaf. In summary, arid and saline soil in Alashan desert enabled *R. soongorica* to form succulent leaf with thick cuticles, subsidized stomata with low density, and salt glands to decrease leaf transpiration and reduce salt toxicity to maintain active growth.

Key words: *Reaumuria soongorica*, Anatomical structures, Drought tolerance, Desert.

Introduction

Desertification is one of the sources to dust/sand storms in early spring each year which affect public health and environment condition in northern part of China (Cyranoski, 2003). Group effort including France, Japan, Korea, Russia, and the United States has been made in Beijing to discuss the integration of different countries' research on dust storms (Cyranoski, 2003). In Alashan desert, the southwestern portion of 'Gobi Desert' in Inner Mongolia, China has typical desert climate with 30 to 102 mm annual precipitation and gradual gray to brown desert soil with high salinity level (Editorial Committee of Flora Reipublicae Popular Sinicae, Chinese Academy of Science, 1990). High radiation and evaporation, overgrazing, poor land use policy, and increased human activities accelerated desertification of this region (Yang *et al.*, 2004; Cyranoski, 2003). More recent research (Yang *et al.*, 2015) showed that the top soil (30 cm) is desalinized due to wind-induced erosion with extremely low soil moisture content. The deep rooting soil (30-70 cm) has higher soil moisture content and higher salinity level. Drought and salinity are major environmental stresses that restrict plant survival and growth. Identifying native plants, such as xerophytes that evolved with distinguished drought and saline tolerant leaf and stem morphological and anatomical structures play important roles in preventing soil erosion and reducing desertification (Potters *et al.*, 2007). Such xerophytes found in Alashan desert include wormwoods (*Artemisia absinthium*), beancaper (*Zygophyllum xanthoxylum*), *Calligonum mongolicum*, and *Reaumuria soongorica* (Chinese Vegetation Map Compilation Committee, 1979).

Reaumuria soongorica ($2n = 2x = 22$; Wang *et al.*, 2011) is an important deciduous xerophytes which is 10-30 centimeter tall with many branches and plays an

important role in soil and water conservation for sustaining ecological stability in Alashan desert. Grazing animals, such as camel and sheep, also feed on them as source of salt and micronutrient (He *et al.*, 2015; Liu *et al.*, 2007). This species is derived from Iran-Mediterranean area and endemic to Alashan, Tianshan, Pamir-Alai Mountains (Zhang *et al.*, 2014). Evolution facilitates *R. soongorica* with unique leaf and stem anatomical characteristics to survive in arid and saline environment. Understanding the adaptation mechanisms using leaf and stem structures in xerophytes will place the foundation of better utilizing them and transform the knowledge of xerophytes to enable non-xerophytes to be drought and salinity tolerant to restore high salinity sandy steppe to decrease the level of soil erosion.

Researchers have focused on mechanisms of plant survival to arid and saline soils since the early 18th century (Levitt, 1972). A lot of effort has been devoted to study antioxidative enzyme activities (Huseynova *et al.*, 2016), osmoprotectants (Zivcak *et al.*, 2016), and drought tolerant genes (Ni *et al.*, 2009). For instance, study of *R. soongorica* under semi-environmentally controlled rain out shelter showed that superoxide dismutase (SOD) and catalase (CAT) were very effective in scavenging H_2O_2 during drought stress while peroxidase (POD) did not contribute protective effect to *R. soongorica* (Bai *et al.*, 2009). Environmentally controlled study on metabolic changes of *R. soongorica* indicates that malate and proline accumulation might be involved in osmoregulation for drought tolerance (Liu *et al.*, 2007). To understand how the unique leaf and stem anatomical characteristics of *R. soongorica* play a role in adaptation of drought- and saline-soil in Alashan desert for environment restoration and sustainability, studying those unique structures of *R. soongorica* is vital.

Plant anatomical characteristics (e.g., mesophyll cell size, cuticle, and stomata density) are important factors in drought tolerance by regulating CO₂ assimilation (Miyazawa & Terashima, 2001) and transpiration rate (Rascio *et al.*, 1990). Tomato (*Solanum lycopersicum*; Al-Abdallat *et al.*, 2014) and Arabidopsis (*Arabidopsis thaliana*; Aharoni *et al.*, 2004) with altered leaf epidermis and cuticles showed increased amount of wax deposition compared with the wild type to be drought tolerant. In *Arabidopsis*, the drought tolerant plant had 30% fewer stomata density (150 mm⁻²) with increased stomata dimension (23.3 x 14.4 µm) compared with the non-drought tolerant type (245 mm⁻²; 19 x 12.8 µm; Yu *et al.*, 2008). Similar results were found in tobacco plant and the reduced stomata density was correlated with drought tolerance (Yu *et al.*, 2008). In rice (*Oryza sativa*), 25% fewer stomata density (175 mm⁻²) was found in drought and salinity tolerant plants compared with the non-stress tolerant type (225 mm⁻²; Huang *et al.*, 2009). Liu *et al.*, (2007) also compared anatomical structure of *R. soongorica* that preserved under environmentally controlled condition. However, how anatomical structures evolved in the process of plant adaptation in Alashan desert has not been investigated. Thus, demonstrating the native structures of leaf, stem, and roots of *R. soongorica* that adapt to Alashan desert will provide basic knowledge to plant physiology.

Plants also developed strategies to cope with salinity stress, such as salt inclusion, extrusion, and exclusions (Flowers *et al.*, 1986; Munns & Teste, 2008). Fine mapping identified QTL gene that could up regulate Na⁺ exclusion. Transformed barley (*Hordeum vulgare*) and Arabidopsis using this Na⁺ exclusion gene showed significantly higher salt tolerance than wild type (Roy *et al.*, 2013). Halophytes, *Salvadora persica*, had thicker epidermis and spongy parenchyma to adapt saline and anaerobic growing environment (Parida *et al.*, 2016). Other plants are able to exclude salt using salt gland. For instance, *Limonium bicolor* could exclude extra salt from salt gland at the appoplast to be salt tolerant (Feng *et al.*, 2015). However, whether *R. soongorica* tolerate high soil salinity in Alashan desert using salt gland or what type of gland that is used to exclude salt have not been clearly understood. Therefore, this study aimed to examine the leaf, stem, and root anatomical structures of *R. soongorica* collected from three sites within Alashan desert to reveal its potential environmental adaption mechanisms in drought and saline soil.

Materials and Methods

Soil profiling of sampling sites: Plant leaf, stem, and root samples of *Reaumuria soongorica* (n = 10 per location) were collected from its naturally occurring habitats where were 'Wuliji' (105°33' E, 39°1' N) and 'Luanjingtan' (105°29' E, 38°06' N), 'Changliushui' (105°38'E, 38°38'N) in Alashan desert, Inner Mongolia, People's Republic of China (Fig. 1a-d). Three sampling sites are on the east side of Alashan which are dominated by typical desert climate. Three soil samples at the middle

and edge of each plant canopy at the sampling sites were collected for soil profiling. Soil profiling analysis was conducted for measuring soil gravimetric water content (θ_g , %), water potential (MPa), and salinity level (g·kg⁻¹ DW) (Fig. 1e and f). Soil θ_g was calculated by using the formula of net weight between soil fresh and dry weight divides dry weight according to Gardner (1986). Soil water potential was calculated according to Livingston and Topp (2006) by measuring osmotic potential of the solution that dissolved the same amount of soil sample. Soil salinity level was measured by measuring the soluble salt content level in one kilogram of dry soil based on Dahnke and Whitney (1988).

Paraffin section and optical microscopy analysis: Each twig, leaflet, and root samples were excised and fixed immediately in FAA solution (5% formaldehyde, 5% acetic acid, and 90% ethanol [Yongsheng Co., Ltd, Tianjin, China]) and kept in a cooler. They were then transferred to a refrigerator and kept until further processing for paraffin section (Weigel & Glazebrook, 2008).

Fixed samples were dehydrated through a series of dehydration process in which leaf, stem, and root samples were placed in 70%, 80%, and 95% ethanol, respectively, for an hour at room temperature followed by another two hours of soaking in 100% ethanol. Samples were infiltrated by soaking them in solution of xylene (Yongsheng Co., Ltd, Tianjin, China) and 100% ethanol (V: V, 1:1) for one hour which was then infiltrated for another two hours by soaking in pure xylene. Finally, samples were embedded in molten paraffin wax in a wax paper box (Yongsheng Co., Ltd, Tianjin, China) at a 38°C incubator (DHG-9240, Noki Instrument Co., Ltd, Changzhou, China) for 72 hours. After incubation, samples were cooled to room temperature for slicing and staining.

Embedded samples were sliced into 8-µm thick slice using a semi-automated rotary microtome (Leica RM2245, All microscopy, Beijing, China). In order to remove wax from samples for staining, samples were soaked in xylene for 45 min followed by another 5 min soaking in xylene and 100 % ethanol (V: V, 1:1). Sliced leaf samples were then stained with a safranin (Yongsheng Co., Ltd, Tianjin, China) solution for 24 hours and soaked in 100%, 95%, and 80% ethanol for 5 min each, respectively. After staining, stem samples were stained by solid green (Yongsheng Co., Ltd, Tianjin, China) for 3 min followed by soaking them in 80% and 95% ethanol for 5 min each, respectively. Finally, all stained slices were soaked in xylene and 100% ethanol (V: V, 1:1) for 5 min followed by another 5 min soaking in xylene. For optical microscope detection and photographing, stained slices were sealed by using natural gum.

Scanning electron microscopy analysis: *R. soongorica* leaf and stem twigs described above that used for paraffin section analysis were collected simultaneously for scanning electron microscopy (SEM) analysis and were fixed in glutaraldehyde (Yongsheng Co., Ltd, Tianjin, China). Fixed samples were exposed to moderate vacuum for 15 minutes to pull the air out of the tissue and kept in a refrigerator until further processing.

Fixed leaf and stem twigs were washed by removing fixation solution and were then dehydrated by using a serial concentration of ethanol (35%, 50%, 60%, 70%, 95%, 100% and 100% once for an hour at each step; Hess *et al.*, 1966) and isoamyl acetate (Yongsheng Co., Ltd, Tianjin, China). After dehydration, samples were dried using carbon dioxide using a critical point drying method (Anderson, 1951; Bray *et al.*, 1993; Hardy *et al.*, 1995; Neinhuis & Edelmann, 1996) with slight modifications. Dried samples were then coated with ion sputtering gold with the thickness of 20 nm. Finally, HITACHI S-570 (Hitachi High-Technologies Corporation, Japan; Nanotoday, 2006) SEM microscope was used for prepared sample photographing.

Traits quantification and data analysis: Number of stomata, stomata size (μm^2), density (number per mm^{-2}), and salt gland density (number per mm^{-2}) were counted in imaging software (Motic Images Plus 2.0 ML, Motic BA210LED Digital, Motic Electric Group Co., Ltd, Xiamen, China). Thickness of leaf cuticle (μm), epidermis (μm), diameter of mesophyll cells (μm), and spongy mesophyll cell (μm) was measured using imaging software. All data of the measured traits were analyzed in SAS software through PROC GLM procedure in SAS (SAS® 9.4 for Windows, Cary, NC).

Results

Soil profiling of sampling sites: Top soil gravimetric water content at both middle and edge of *R. soongorica* canopy in three sampling sites were not significantly different. Significant differences were found among three sampling sites for root zone soil moisture content in which ‘Luanjingtang’ was higher in soil moisture content (2.7%) compared with ‘Wuliji’ (1.7%) and ‘Changliushui’ (0.9%) at the middle of *R. soongorica* canopy. At the edge of *R. soongorica* canopy, ‘Changliushui’ (0.7%) also showed lower soil moisture content compared with the other two sampling sites (Table 1). Accordingly, the soil water potential in ‘Changliushui’ was significantly ($p<0.05$) lower compared with the other two sampling sites in both top soil and root zone soils at middle and edge of *R. soongorica* canopy (Table 1). Similar to soil water potential, the soil salinity level in ‘Changliushui’ (2.5 and 2.05 $\text{g}\cdot\text{kg}^{-1}$) was significantly higher than the other two sampling sites at both middle and edge of *R. soongorica* canopy. Top soil salinity level (2.25 $\text{g}\cdot\text{kg}^{-1}$) was lower than root zone soil (5.45 $\text{g}\cdot\text{kg}^{-1}$) at both middle and edge of *R. soongorica* canopy for all sampling sites (Table 1).

Table 1. Profiling of soil gravimetric water content (θ_g , %), water potential (MPa), and salinity level ($\text{g}\cdot\text{kg}^{-1}$) for three sampling sites.

Traits	Location	Middle of <i>R. soongorica</i> canopy		Edge of <i>R. soongorica</i> canopy	
		Top soil ^r	Root zone soil ^r	Top soil ^r	Root zone soil ^r
Soil gravimetric water content (%)	Wuliji	0.431 ± 0.032 a	1.657 ± 0.159 b	0.346 ± 0.197 a	1.362 ± 0.432 a
	Luanjingtang	0.584 ± 0.114 a	2.696 ± 0.154 a	0.495 ± 0.331 a	1.946 ± 0.526 a
	Changliushui	0.484 ± 0.165 a	0.864 ± 0.161 c	0.451 ± 0.183 a	0.737 ± 0.179 b
Soil water potential (MPa)	Wuliji	-9.171 ± 0.457 a	-7.687 ± 1.296 a	-10.498 ± 2.017 a	-10.497 ± 2.411 a
	Luanjingtang	-9.965 ± 1.085 a	-7.224 ± 1.257 a	-10.158 ± 1.523 a	-9.105 ± 3.014 a
	Changliushui	-11.058 ± 1.002 b	-10.552 ± 0.969 b	-12.997 ± 0.942 b	-11.737 ± 1.079 a
Soil salinity level ($\text{g}\cdot\text{kg}^{-1}$)	Wuliji	1.94 ± 0.083 b	3.96 ± 0.261 b	1.51 ± 0.136 b	3.45 ± 0.093 b
	Luanjingtang	2.05 ± 0.383 b	4.10 ± 0.256 b	1.49 ± 0.234 b	3.43 ± 0.145 b
	Changliushui	2.52 ± 0.242 a	5.91 ± 0.250 a	2.05 ± 0.123 a	5.13 ± 0.227 a

^r indicates mean ± standard error; the same letter indicates non significant differences among locations at $\alpha \leq 0.05$

Anatomical structure of *Reaumuria soongorica* under optical microscopy: *R. soongorica* is a perennial shrub which is 10 to 30-cm tall with multiple branches and four to six succulent leaves formed on the branch alternatively (Fig. 1c and d). At reproductive stage, white or pink flower can be formed at leaf auxiliary of branch (Fig. 1d). Root system can reach below ground as deep as 70 cm (Fig. 1e and f). Leaf cross section from three sampling sites showed cylindrical and flat shape with one layer of thick cuticle (Fig. 2). The thickness of leaf cuticle in ‘Wuliji’ was 2.14 μm while it was 1.94 μm in ‘Luanjingtang’. Epidermal cells varied in size with uneven surface which was composed of five or six layers of mesophyll cells. The thickness of epidermal cells from ‘Luanjingtang’ was 12.46 μm while in ‘Wuliji’ was 13.88 μm (Table 2). Mesophyll cells from ‘Changliushui’ were less organized with more cell shrinkage and free air spaces compared with ‘Wuliji’ and ‘Luanjingtang’ (Fig. 2).

Tightly packed palisade parenchyma mesophyll cell in ‘Luanjingtang’ had diameter of 51.96 μm while it was 42.24 μm in ‘Wuliji’. The diameter of spongy mesophyll cell from ‘Luanjingtang’ was 69.97 μm while it was 46.1 μm in ‘Wuliji’ (Table 2). More osmotic adjustment compounds were observed in vascular tissues of samples from ‘Changliushui’ and ‘Wuliji’ when compared with sampling from ‘Luanjingtang’ (Fig. 2d, e, and f).

Stem cross-section was irregular and cylindrical which was composed by a uniseriate epidermis, cortex, phloem, and xylem. Vascular and ground tissue differentiated into a wide cortex and pith (Fig. 3a, b, and c). Specifically, samples from ‘Changliushui’ and ‘Wuliji’ showed more purplish staining in cortex tissue compared with samples from ‘Luanjingtang’ (Fig. 3). The epidermal cells were loosely packed with irregular cell in samples from ‘Changliushui’ and ‘Wuliji’ as compared to the samples from ‘Luanjingtang’ which showed tighter phloem cells packed with smaller cell size (Fig. 3d, e, and f). Stem from ‘Changliushui’ and ‘Wuliji’ formed a wider phloem layer compared with sample from ‘Luanjingtang’ (Fig. 3a, b, and c).

Reaumuria soongorica root cross section showed that xylem was detached from endodermis with many disorganized and shrank cortex cells and less phloem for samples from ‘Changliushui’ (Fig. 4a and b). Samples from ‘Wuliji’ showed no detachment of xylem from the endodermis with less cell shrinkage (Fig. 4c).

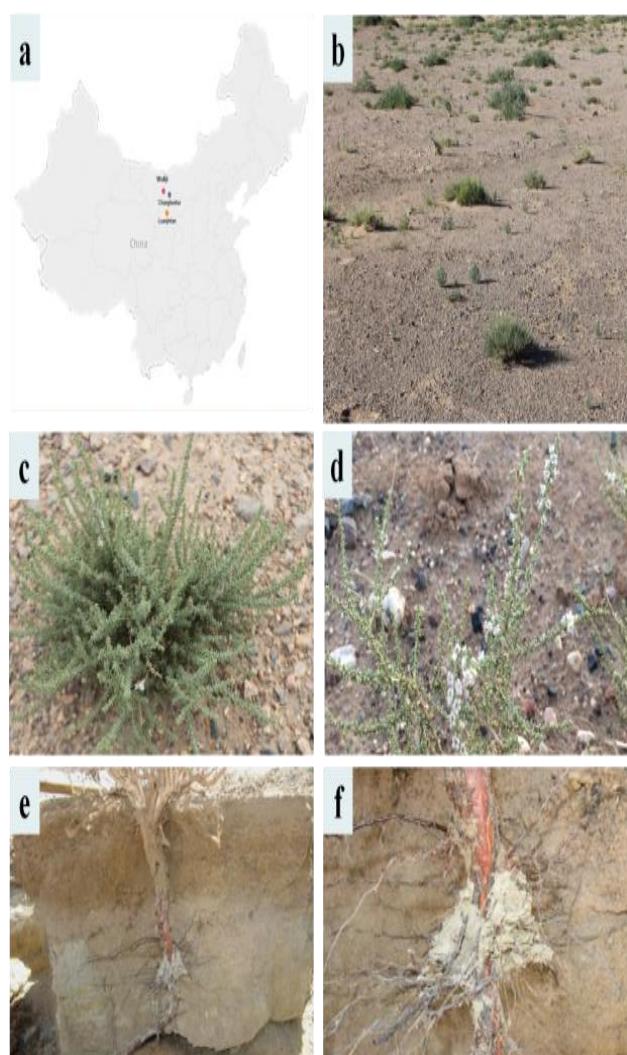


Fig. 1. Three locations that *Reaumuria soongorica* samples were collected at Alashan desert of Inner Mongolia, P. R. China (a). *Reaumuria soongorica* population that formed at Alashan desert of Inner Mongolia, P. R. China (b). A bush of *Reaumuria soongorica* that locates at ‘Luanjintan’ (c). Close view of the morphology of branches with leaf and flower (d). The morphology of tap root in top soil (e). The closer visualization for the morphology of tap root (f).

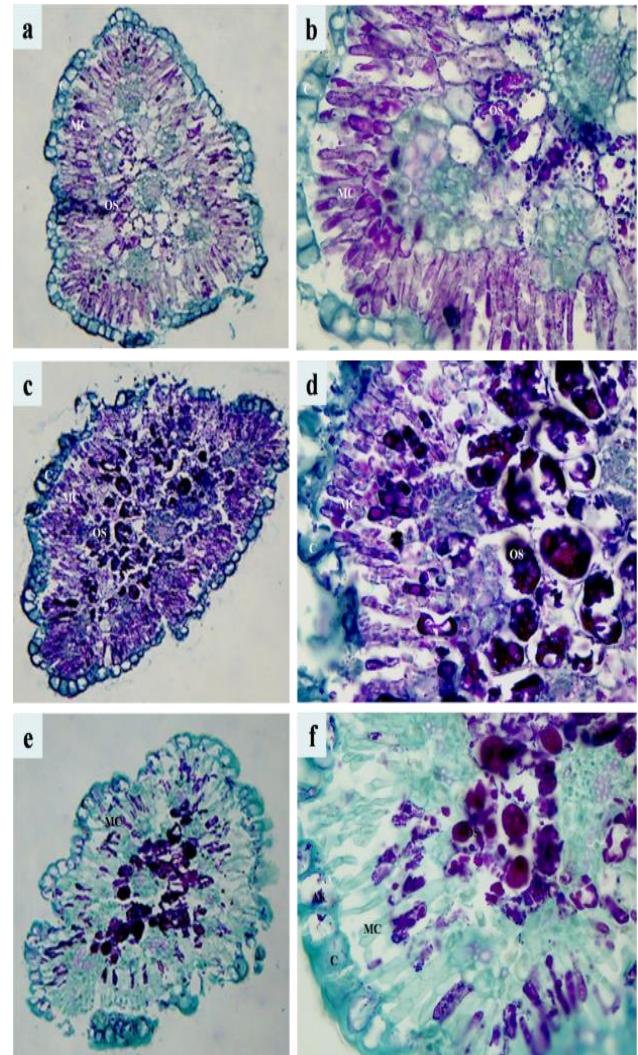


Fig. 2. *Reaumuria soongorica* leaf cross section under 10X magnification that located in ‘Luanjingtan’ (a), ‘Wuliji’ (b), and ‘Changliushui’ (c). Leaf cross section under 40X magnification that located in ‘Luanjingtan’ (d), ‘Wuliji’ (e), and ‘Changliushui’ (f). C = leaf cuticle, MC = mesophyll cells, AS = air space, OS = osmotic solute.

Table 2. Statistical summary of leaf anatomical structures that measured from scanning electronic microscopy. SE: standard error of the mean for each trait.

Traits	Wuliji		Luanjingtan	
	Mean ± SE	Sampling size	Mean ± SE	Sampling size
Cuticle thickness (μm)	2.14 ± 0.57	13	1.94 ± 0.35	19
Epidermis thickness (μm)	13.88 ± 3.19	11	12.46 ± 2.68	20
Mesophyll cells diameter (μm)	42.24 ± 6.05	15	51.96 ± 10.59	20
Spongy mesophyll cells diameter (μm)	46.1 ± 9.25	7	69.97 ± 14.99	16

Table 3. Statistical summary of stomata and salt gland that obtained from scanning electronic microscopy for samples from ‘Changliushui’.

Traits	Stomata size (μm ²)	Stomata density (mm ⁻²)	Stem cell wall thickness (μm)	Salt gland density (mm ⁻²)
Mean	50.14	213.68	42.15	24.5
Standard error	15.49	71.72	5.59	0.5
Sampling size	17	10	16	12

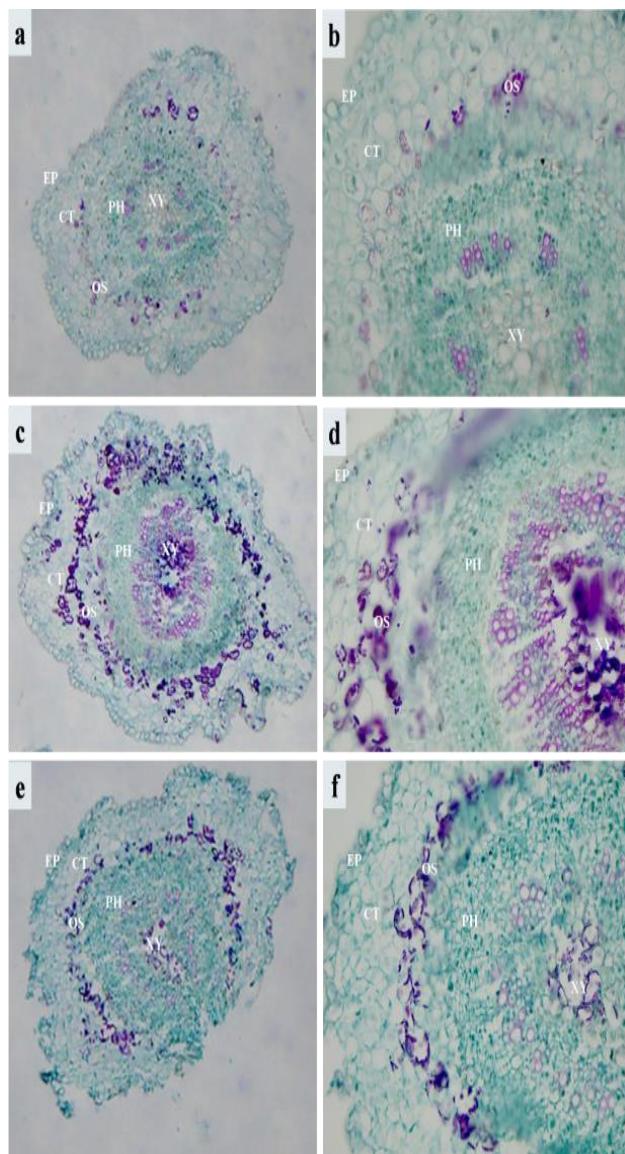


Fig. 3. *Reaumuria soongorica* stem cross section under 10X magnification that located in 'Luanjingtian' (a), 'Wuliji' (b), and 'Changliushui' (c). Leaf cross section under 40X magnification that located in 'Luanjingtian' (d), 'Wuliji' (e), and 'Changliushui' (f). OS = osmotic substance, EP = epidermis, CT = cortex, PH = phloem, XY = xylem.

Anatomical structure of *Reaumuria soongorica* under scanning electron microscopy: Samples from the most severely arid and saline sites 'Changliushui' was used for scanning electron microscopy (SEM) analysis. *R. soongorica* leaf is succulent and linear semi-cylindrical. Densely distributed granular salt crystals were observed on its leaf surface (Fig. 5a and b) while no other trichomes or hairy appendages were detected. Nevertheless, subsided salt glands were found around the salt crystals which was formed by five to seven tightly packed large epidermal cell forming an 'opened flower' shape (Fig. 5c).

Stem cross section from SEM showed similar stem structure as the paraffin section method which indicated epidermis, cortex, and vascular bundle, respectively (Fig. 5d). Stem cell wall thickness was 42.15 μm (Table 3). No trichomes and other hairy appendages were observed on stem epidermis while granular salt crystals were found on the stem epidermis of *R. soongorica* (Fig. 5e). The densities of the salt glands were 25 per square millimeters of stem

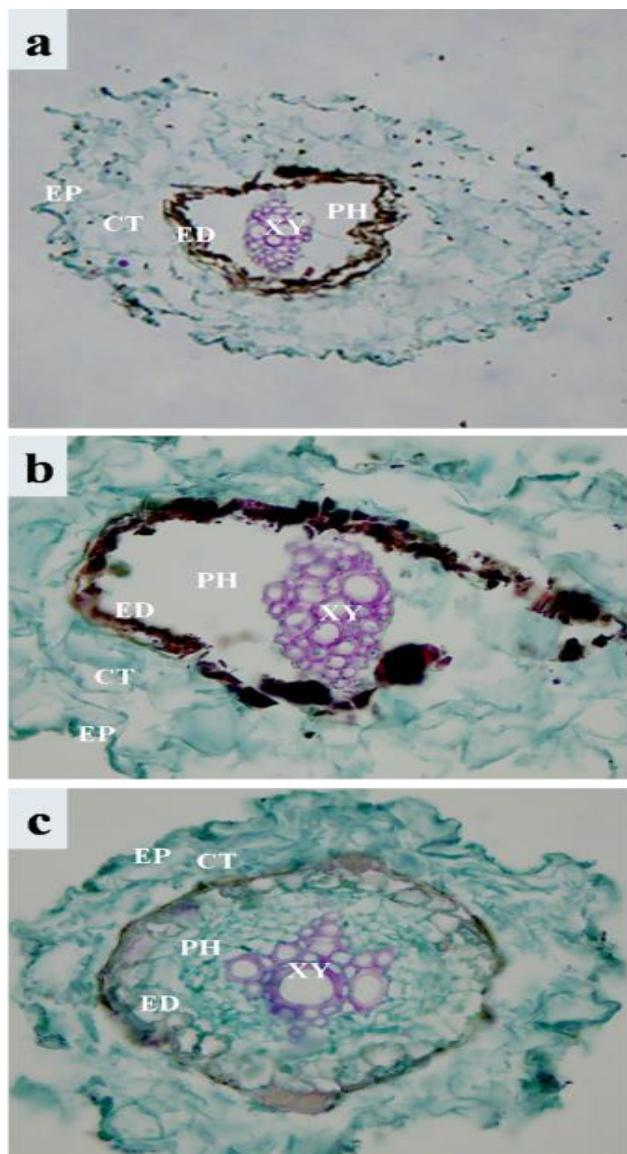


Fig. 4. Cross section of root structure for samples from 'Changliushui' at 10x magnification (a), 40x magnification (b), and 'Wuliji' at 40x magnification (c). EP = epidermis, CT = cortex, ED = endodermis, PH = phloem, XY = xylem.

epidermis area (Table 3). Densely distributed salt vesicles in the xylem of the stem were observed as well (Fig. 5f).

A small amount of subsided stomata were found on the side of the leaf which was formed by two kidney-shaped guard cells (Fig. 6). The size of the stomata was 50.14 μm^2 and density of stomata was about 214 stomata per millimeters of epidermis area (Table 3).

Discussion

***Reaumuria soongorica* plays important role in soil and water conservation in Alashan desert:** Alashan desert in Inner Mongolia, China has typical desert climate with 30 to 120 mm annual precipitation and is dominated by saline and alkaline soil (Li *et al.*, 2013; Yang *et al.*, 2015). Three sampling sites, 'Changliushui', 'Luanjingtian' and 'Wuliji', are the places called "Black Gobi" have extremely low soil gravimetric water content (θ_g) and high levels of soil salinity and pH (Table 1). Due to higher soil salinity level and lower

soil moisture content in 'Changliushui', soil water potential was the lowest compared with the other sampling sites. For instance, the top 30-cm soil layer of 'Changliushui' is desalinized due to wind-induced erosion with only 0.46% soil θ_g . Deep rooting soil in which root branches are located between 30 to 70 cm below ground, the soil water potential (-11.5 MPa) and soil θ_g (0.7%) are lower than the top soil (Table 1). Wind induced soil erosion is one of the contributors to dust storm in China (Wang *et al.*, 2004). However, maintaining current plantation in desert and create man-made forest to prevent soil erosion plays an important role in controlling dust storm (Parungo *et al.*, 1994). Identifying plants that could survive in arid and high radiation environment will be beneficial to choose the right species for man-made forest construction to reduce soil erosion. In consistent with the records of chronological proceedings in Inner Mongolia (Zhu *et al.*, 2004; Zhao *et al.*, 2006), *R. soongorica* is the only plant species that remain green and alive while most of other species went to dormancy under hot and arid summer. It has an extremely strong ability to stabilize sand shifting during high wind-induced sand movement. Nevertheless, how arid and saline environment affected *R. soongorica* to be drought and saline tolerant using unique leaf and stem morph-anatomic structures are the questions that have not been completely answered. Thus, this study will be beneficial to plant cultivation and utilization in desert regions.

Morph-anatomic structures enable *Reaumuria soongorica* to be drought tolerant: Plants develop phenotypic plasticity, for instance, unique leaf or stem morph-anatomic structures to cope with adverse environment to minimize water loss while decreasing salt detoxification as drought and saline tolerant. Expanded and broad leaf which increases transpiration rate to lose more water (Campitelli *et al.*, 2013), however, in *R. soongorica*, succulent and rod-like leaf with small leaf surface area increases individual leaf cell volume to store large amounts of water to withstand drought conditions and detoxifying extra salt. This type of leaf shape also minimizes heating stresses that is caused by intense radiation and heat damage during hot and arid summer (Ehleringer & Forseth, 1980). In addition to leaf shape, leaf epidermis which acts as an essential protective barrier to enhance plant resilience to stress environment via regulating water and CO₂ exchanges. The primary aerial surfaces are covered by cuticle formed by a heterogeneous layer of cutin and wax (Aharoni *et al.*, 2004). The internal structures of *R. soongorica* were preserved very well using paraffin section method and cuticle thickness from more arid 'Wuliji' is higher than less arid 'Luanjingtang' which suggests arid environment in Alashan desert had *R. soongorica* to have increased leaf cuticle to be drought tolerant.

Mesophyll cell organization was also regulated during the process of arid environment selection. In *R. soongorica*, optical microscopy showed tight packing of palisade parenchyma mesophyll cells (Fig. 2). It was similar to what Liu *et al.*, (2007) found that a very tightly packed palisade parenchyma of mesophyll was formed in well-watered leaf while disorganized and irregular shaped cells in drought-stressed plants. Because of more arid and saline condition in 'Changliushui', plant leaf palisade parenchyma mesophyll cells were less turgid with smaller cell diameter and irregular

mesophyll cell organization compared with 'Luanjingtang' and 'Wuliji'. Reduced cell shrinkage and clearer internal structures when compared with what was found by Liu *et al.*, (2007). Leaf, stem, and root samples from more arid areas 'Changliushui' and 'Wuliji' showed more cell shrinkage and irregularity compared with samples from 'Luanjingtang' which might be an indication of damage to the cells of *R. soongorica* under drought and salinity condition. According to Parkhurst (1994) and Nelson *et al.*, (2005), tightly packed spongy mesophyll cells may also lower the intercellular air spaces to reduce internal CO₂ transfer conductance and increase light reaction rate of photosynthesis for plants to be abiotic stress tolerant. It was found that tightly packed spongy mesophyll cells, smaller cell size and thicker cell wall contribute to resistance to drought stress in cotton (*Gossypium hirsutum*; Cutler *et al.*, 1977). In *R. soongorica*, the diameter of leaf spongy mesophyll cell for samples from 'Luanjingtang' is higher than in samples from 'Wuliji' which indicates a larger cell size for *R. soongorica* may lead to larger cell volume with higher turgid to dilute cell concentration for reducing ion toxicity and increase photosynthesis rate to cope with high soil salinity.

Stomata position play important role in regulating plant water status and photosynthesis rate. Highly specialized and ring-shaped stomata are found in rose position with low density in both hydrated and dehydrated leaves of *R. soongorica* under environmentally controlled shelter (Liu *et al.*, 2007). Nevertheless, opened stomata with similar shape but low density and subsided position were found from SEM in this study (Fig. 6). Compared with Liu *et al.*, (2007), such discrepancy might due to differences at sample preservation in which they used phosphate buffer containing 3% glutaraldehyde for 24 h while we used pure glutaraldehyde. According to Pathan *et al.*, (2010), better preparation during sample drying process by using CPD following fixation with glutaraldehyde may also provide excellent preservation of internal leaf structures as close to the natural state as possible. Arid and saline environment might be functioning to create local humidity or reduce exposure to dry air due to subsided stomata in *R. soongorica* to make it drought tolerant.

Stomata size and density also play critical role in regulating plant water loss and CO₂ uptake. For instance, size of stomata in *R. soongorica* leaf is very small (50.14 μm^2) and stomata density is 213 mm⁻² of leaf surface area for samples from 'Changliushui' which is higher than sampling sites of 'Wuliji' and 'Luanjingtang'. A rice plant that is transformed with a zinc finger protein to regulate stomata aperture lead to 25% reduce stomata density to withstand drought and salt conditions (Huang *et al.*, 2009). For instance, rice plant has leaf stomata density of 175 mm⁻² is more drought tolerant than the wild type rice which has 225 mm⁻². In *Arabidopsis*, stomata density is 245 mm⁻² for wild type and 150 mm⁻² for the drought tolerant one (Yu *et al.*, 2008). This comparison implies that *R. soongorica* leaf has the medium amount of stomata density compared with herbaceous plants which might indicate balance between transpiration and CO₂ uptake via stomata size and density for photosynthesis under arid and saline condition of Alashan desert enabling the woody plants to remain alive.

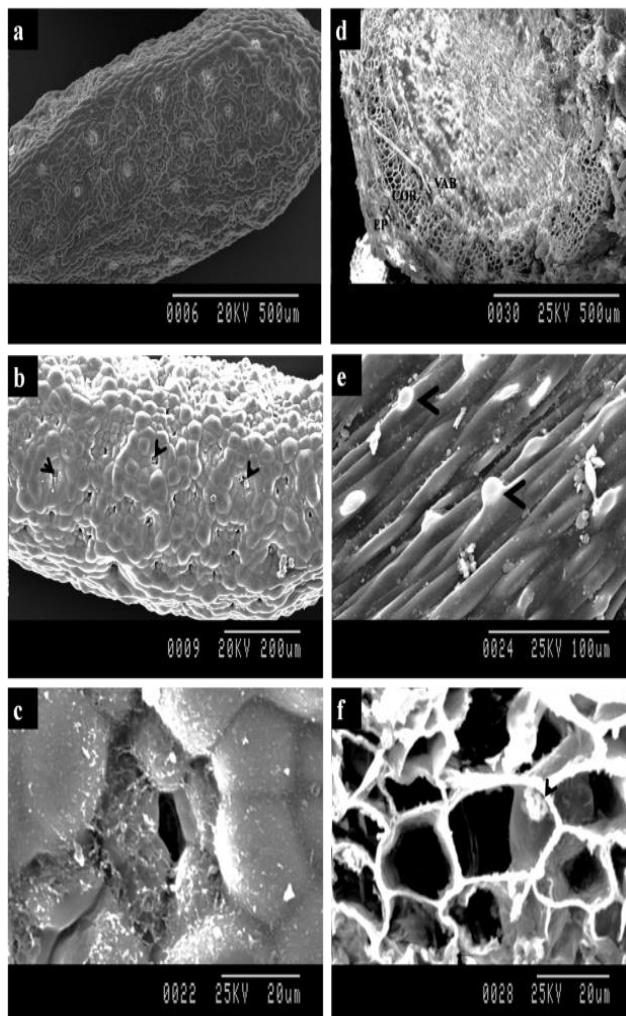


Fig. 5. Succulent leaf with salt crystals that secreted from salt gland (a). Leaf with salt gland and salt crystals indicated by black arrows (b). Salt gland formed by 5-7 large tightly packed epidermal cells forming a 'opened flower' shape under higher magnification (c). Stem cross section showing epidermis (EP), cortex (COR) and vascular bundles (VAB) (d). Salt crystals indicated by arrows on stem epidermis under scanning electron microscopy (e). Salt crystals in cortical parenchyma cells of stem (f).

Salt gland secrets salt from *Reaumuria soongorica* to be saline tolerant: Salt glands are considered as important channels for secreting extra salt to be salt tolerant in plants. For instance, Na^+ or Cl^- exclusion via salt gland is one of the plant salinity adaptation strategies in halophyte (Munns & Tester 2008). The importance of salt gland is revealed by secreting Na^+ out of cells to maintain high ratio of K^+/Na^+ to be salt tolerant in sea lavender (*Limonium bicolor*) (Feng *et al.*, 2015) and grass plants (Taleisnik & Anton, 1988). In *R. soongorica*, Liu *et al.*, (2007) found some glands on leaf that seems to be involved in drought tolerance; however, they conducted experiments in a controlled environment by water withholding which limited salt secretion and they were not able to confirm the function of those glands. Nevertheless, the anatomical structures in this study are well preserved from the prevalent growing environment in 'Changliushui' where it has the lowest soil moisture content and highest salinity level which reveals the

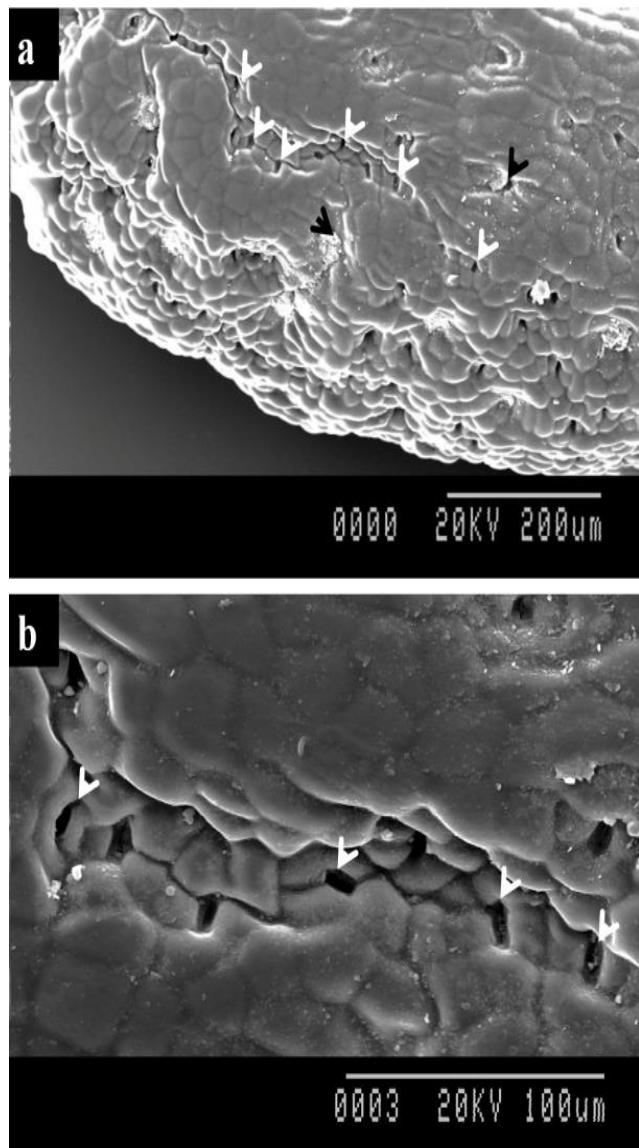


Fig. 6. Salt glands indicated by black arrow and stomata indicated by white arrow are distributed on leaf (a). Subsided stomata on the leaf at high magnification (b).

natural structure for *R. soongorica* under low precipitation and saline soil in Alashan desert. Salt vesicles that secreted from those glands were clearly observed (Fig. 5) and they were suggested to be salt glands for *R. soongorica* due to high level of salts in its habitat (Table 1). Salt vesicle in xylem (Fig. 5d) was also observed while no cross section for SEM was shown in Liu *et al.*, (2007). In consistent with this, Na^+ concentration in top ten cm plant root tip tissues and the highest concentration was also detected at the epidermis and xylem pericycle using quantitative and calibrated X-ray microanalysis (Munns & Tester, 2008). In other xerophytes, such as mangrove (*Kandelia candel*) and resurrection plants (Vicré *et al.*, 2004), secreting salt from salt gland allow them to adapt to salinity environment. Similar to the mechanism of this halophyte, secreted salt crystals were observed on *R. soongorica* stem epidermis and xylem under SEM (Fig. 5). Slat gland structure in grass is bi-cellular while salt gland in *R. soongorica* is multicellular

which is comprised of 5 to 7 tightly packed epidermal cells. Since the identification of salt secreting gland in *R. soongorica* for its survival under dry and saline condition, whether *R. soongorica* could be used for phytoremediation in mining and heavy metal contaminated regions should be further investigated.

Osmotic adjustment is another approach for plants to be abiotic stress tolerant via osmotic protective compounds biosynthesis. For instance, polysaccharide compound pectin could contribute to water storage in drought tolerant plants (Robichaux & Morse, 1990). Due to more drought and saline stress in 'Changliushui' and 'Wuliji' compared with 'Luanjingtan', darker purple dye coloration was observed in cortex tissues when compared with samples in 'Luanjingtan' which might be the staining of osmotic regulating compound in vacuole (Fig. 2). Malate and proline were found in stem of *R. soongorica* to play a role in osmotic adjustment (Liu *et al.*, 2007). However, further research of identifying other possible osmotic adjustment compounds in leaf and stem of *R. soongorica* will be more beneficial to utilize better this species.

The essence of investigating the plant drought tolerant mechanisms that shaped by the environment is to investigate how plants sense and acclimate to abiotic stress condition and to transform this knowledge to other plants for enhanced abiotic stress tolerance. In this study, the anatomical structures of leaf, stem, and root in *R. soongorica* were well preserved and *R. soongorica* evolved with thick leaf cuticles, irregular mesophyll cell organization, subsidized stomata, and salt gland formation to survive in Alashan desert soil with extreme low moisture content and high salinity level. These anatomical structures may be used as indicators for developing and evaluating a more drought and salinity tolerant plant in future. Identifying the underlying molecular regulators to those structural developments could be beneficial to engineering non-xerophytes to be drought and salinity tolerant.

Conclusions

Reaumuria soongorica is adaptive to low precipitation and high saline soil of Alashan desert, China to sustain and restore fragile desert ecosystems. Drought and salinity tolerances in arid and saline soil are two merits of *R. soongorica* in Alashan desert which can be served as candidate for man-made forest to prevent wind-induced soil erosion. This desert environment enable *R. soongorica* have succulent leaf with thick cuticles and sunken stomata to reduce moisture loss and minimize radiation exposure for making good use of limited water resources. Salt secreting gland is formed by five to seven large tightly packed epidermal cells for salinity tolerance. Making use of salt glands as potential target to improve salt tolerance of non-xerophytes will be beneficial to phytoremediation and desert environment protection.

Acknowledgements

The authors thank the technical assistance from HOU Yu, LI Ruanruan, and ZHU Lin. The authors also want to thank the funding support from the National Natural Science Foundation of China (31160108 and 31460124).

References

- Aharoni, A., S. Dixit, R. Jetter, E. Thoenes, V.A. Gert and A. Pereira. 2004. The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when over expressed in Arabidopsis. *Plant Cell*, 16: 2463-2480.
- Al-Abdallat, A.M., H.S. Al-Debei, J.Y. Ayad and H. Shireen. 2014. Over-expression of *SISHN1* gene improves drought tolerance by increasing cuticular wax accumulation in Tomato. *Int. J. Mol. W. Sci.*, 15: 19499-19515.
- Anderson, T.F. 1951. Techniques for the preservation of three dimensional structures in preparing specimens for the electron microscope. *Trans NY Acad. Sci.*, 13: 130-133.
- Bai, J., C.M. Gong, K. Chen, C. Kang, C.M. Gong and J. Bai. 2009. Examination of antioxidative system's responses in the different phases of drought stress and during recovery in desert plant *Reaumuria soongorica* (Pall.) Maxim. *J. Plant Bio.*, 52: 417-425.
- Bray, D.F., J. Bagu and P. Koegler. 1993. Comparison of hexamethyldisilazane (HMDS), peldri 11, and critical-point drying methods for scanning electron microscopy of biological specimens. *Microsc. Res. Tech.*, 26: 489-495.
- Campitelli, B.E., A.J. Gorton, K.L. Ostevik and J.R. Stinchcombe. 2013. The effect of leaf shape on the thermoregulation and frost tolerance of an annual vine, *Ipomoea hederacea* (Convolvulaceae). *Am. J. Bot.*, 100: 2175-2182.
- Chinese Vegetation Map Compilation Committee. 1979. Vegetation map of China. Map (1:10,000,000). Science Press, Beijing, China.
- Cutler, J.M., D.W. Rains and R.S. Loomis. 1977. The importance of cell size in the water relations of plants. *Physiol. Plant.*, 40: 255-260.
- Cyranoski, D. 2003. China plans clean sweep on dust storms. *Nature* 421: 6919.
- Dahnke, W.C. and D.A. Whitney. 1988. Measurement of soil salinity. In: (Ed.): Dahnke, W.C. Recommended chemical soil test procedures for the North Central Region. North Dakota Agricultural Experimental Station Bulletin. North Dakota, USA, pp 32-34.
- Editorial Committee of Flora Reipublicae Popularis Sinicae, Chinese Academy of Science. 1990. Flora of China volume 50. Science Press, Beijing, Beijing, China.
- Ehleringer, J.R. and I. Forseth. 1980. Solar tracking by plants. *Science*, 210: 1094-1098.
- Feng, Z.T., Y.Q. Deng, S.C. Zhang, X. Liang, F. Yuan, J.L. Hao, J.C. Zhang, S.F. Sun and B.S. Wang. 2015. K⁺ accumulation in the cytoplasm and nucleus of the salt gland cells of *Limonium bicolor* accompanies increased rates of salt secretion under NaCl treatment using NanoSIMS. *Plant Sci.*, 238: 286-296.
- Flowers, T.J., M.A. Hajibagheri and N.J.W. Clipson. 1986. Halophytes. *Q. Rev. Biol.* 6: 313-337.
- Gardner, W.H. 1986. Water content. In: (Ed.): Klute, A. Methods of soil analysis. Soil Science Society of America, Inc. Madison, Wisconsin, USA, pp. 167-223.
- Hardy, J.P., V.J. Anderson and J.S. Gardner. 1995. Stomatal characteristics, conductance ratios, and drought-induced leaf modifications of semiarid grassland species. *Amer. J. Bot.*, 82:1-7.
- He, M., K. Zhang, H. Tan, R. Hu, J. Su, J. Wang, L. Huang, Y. Zhang and X. Li. 2015. Nutrient levels within leaves, stems, and roots of the xeric species *Reaumuria soongorica* in relation to geographical, climatic, and soil conditions. *Ecol. Evol.*, 5: 1494-1503.
- Hess, W.M. 1966. Fixation and staining of fungus hyphae and host plant root tissues for electron microscopy. *Stain Technol.*, 41: 27-35.

- Huang, X.Y., D.Y. Chao, J.P. Gao, M.Z. Zhu, M. Shi and H.X. Lin. 2009. A previously unknown zinc finger protein, DST, regulates drought and salt tolerance in rice via stomatal aperture control. *Genes Dev.*, 23: 1805-1817.
- Huseynova, I.M., S.M. Rustamova, D.R. Aliyeva, H.G. Babayev and J.A. Aliyev. 2016. Photosynthesis, antioxidant protection, and drought tolerance in plants. In: Hossain, M.A., S.H. Wani, S. Bhattacharjee, D.J. Burritt & L.S.P. Tran. Drought stress tolerance in plants volume 1: physiology and biochemistry. Springer. pp. 349-378.
- Levitt. 1972. Water stress. In: (Ed.): Levit. Responses of plants to environmental stresses. Academic Press, New York, New York, USA.
- Li, S.J., P.X. Su, H.N. Zhang, Z.J. Zhou and T.T. Xie. 2013. Character and relationships of foliar water and leaf functional traits of desert plants. *Plant Physiol. J.*, 49:153-160.
- Liu, Y.B., G. Wang, J. Liu, X. Zhao, H.J. Tan and X.R. Li. 2007. Anatomical, morphological and metabolic acclimation in the resurrection plant *Reaumuria soongorica* during dehydration and rehydration. *J. Arid Environ.*, 70: 183-194.
- Livingston, N.J. and G.C. Topp 2006. Water potential. Taylor and Francis Group, LLC.
- Miyazawa, S.I. and I. Terashima. 2001. Slow development of leaf photosynthesis in an evergreen broad-leaved tree, *Castanopsis sieboldii*: Relationships between leaf anatomical characteristics and photosynthetic rate. *Plant, Cell and Environ.*, 24: 279-291.
- Munns, R. and M. Teste, 2008. Mechanisms of salinity tolerance. *Ann. Rev. Plant Biol.*, 59: 651-681.
- Neinhuis, C. and H.G. Edelmann. 1996. Methanol as a rapid fixative for the investigation of plant surfaces for SEM. *J. Microsc.* 184: 14-16.
- Nelson, E.A., T.L. Sage and R.F. Sage. 2005. Functional leaf anatomy of plants with crassulacean acid metabolism. *Funct. Plant Biol.*, 32: 409-419.
- Ni, F.T., L.Y. Chu, H.B. Shao and Z.H. Liu. 2009. Gene expression and regulation of higher plants under soil water stress. *Curr. Genomics*, 10: 269-280.
- Parida, A.K., S.K. Veerabathini, A. Kumari and P.K. Agarwal. 2016. Physiological, anatomical and metabolic implications of salt tolerance in the halophyte *Salvadora persica* under hydroponic culture condition. *Front. Plant Sci.*, 7: 351-369.
- Parkhurst, D.F. 1994. Diffusion of CO₂ and other gases inside leaves. *New Phytol.*, 126: 449-479.
- Parungo, F., Z. Li, X. Li, D. Yang and J. Harris 1994. Gobi dust storms and the Great Green Wall. *Geophys. Res. Lett.*, 21: 999-1002.
- Pathan, A.K., J. Bond and R.E. Gaskin 2010. Sample preparation for SEM of plant surfaces. *Mater. Today*, 12: 32-43.
- Potters, G., T.P. Pasternak, Y. Guisez, K.J. Palme and M.A.K. Jansen. 2007. Stress-induced morphogenic responses: growing out of trouble? *Trends in Plant Sci.*, 12: 98-105.
- Rascio, A., M.C. Cedola and M. Toponi, Z. Flagella and G. Wittmer 1990. Leaf morphology and water status in *Triticum durum* under water stress. *Physiol. Plant*, 78: 462-467.
- Robichaux, R.H. and S.R. Morse. 1990. Extracellular polysaccharide and leaf capacitance in a Hawaiian bog species, *Agyroxiphium grayanum* (Compositae-Madiinae). *Amer. J. Bot.*, 77: 134-138.
- Roy, S.J., W. Huang, X.J. Wang, A. Evrard; X.J. Wang, W. Huang and S.J. Roy. 2013. A novel protein kinase involved in Na⁺ exclusion revealed from positional cloning. *Plant, Cell and Environ.*, 36: 553-568.
- Taleisnik, E.L. and A.M. Anton 1988. Salt glands in *Pappophorum* (Poaceae) *Ann. Bot.*, 62: 383-388.
- Vicré, M., J.M. Farrant and M. Driouich. 2004. Insights into the cellular mechanisms of desiccation tolerance among angiosperm resurrection plant species. *Plant, Cell and Environ.*, 27: 1329-1340.
- Wang, X., Z. Dong, J. Zhang and L. Liu. 2004. Modern dust storms in China: an overview. *J. Arid Environ.*, 58: 559-574.
- Wang, X.H., T. Zhang, Z.N. Wen, H. Xiao, Z. Yang, G. Chen and X. Zhao. 2011. The chromosome number, karyotype and genome size of the desert plant diploid *Reaumuria soongorica* (Pall.) Maxim. *Plant Cell Rep.*, 30: 955-964.
- Weigel, D. and J. Glazebrook. 2008. Fixation, embedding, and sectioning of plant tissues. Cold Spring Harbor Protoc. doi: pdb. prot4941.
- Yang, J., A.S. Cushman, X. Song, J. Yang and P. Zhang. 2015. Genetic diversity and drivers of genetic differentiation of *Reaumuria soongorica* of the Inner Mongolia plateau in China. *Plant Ecol.*, 216: 925-937.
- Yang, X.P., K.T. Rost, F. Lehmkuhl, Z. Zhu and J. Dodson. 2004. The evolution of dry lands in northern China and in the Republic of Mongolia since the Last Glacial Maximum. *Quat. Int.*, 118-119: 69-85.
- Yu, H., X. Chen, Y.Y. Hong, Y. Wang, P. Xu, S.D. Ke, H.Y. Liu, J.K. Zhu, D.J. Oliver and C.B. Xiang. 2008. Activated expression of an *Arabidopsis* HD-START protein confers drought tolerance with improved root system and reduced stomatal density. *The Plant Cell*, 20: 1134-1151.
- Zhang, M., X. Hao, S.C. Sanderson, B.V. Vyacheslav, A.P. Sukhorukov and X. Zhang. 2014. Spatiotemporal evolution of *Reaumuria* (Tamaricaceae) in Central Asia: insights from molecular biogeography. *Phytotaxa*, 167: 089-103.
- Zhao, Y.Z. 2006. Vascular plant of plateau ordos. Inner Mongolia University Press, Hohhot. China.
- Zhu, G., Z.P. Li, W.P. Wang and F. Zhong. 2004. The study progress on plant of *Reaumuria* Linn. *J. Gansu Forestry Sci. Tech.*, 29:1-4.
- Zivcak, M., M. Brešić and O. Sytar. 2016. Osmotic adjustment and plant adaptation to drought stress. In: (Eds.): Hossain, M.A., S.H. Wani, S. Bhattacharjee, D.J. Burritt and L.S.P. Tran. Drought stress tolerance in plants volume 1: physiology and biochemistry. Springer, pp. SW 105-144.

(Received for publication November 17 2017)