International Journal of Botany

ISSN: 1811-9700

science alert

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International Journal of Botany 7 (1): 53-62, 2011
ISSN 1811-9700 / DOI: 10.3923/ijb.2011.53.62

Comparative Leaf Anatomy and Pressure-Volume Analysis in Plants of *Ipomoea pes-caprae* Experimenting Saline and/or Drought Stress

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Abstract: *Ipomoea pes-caprae* is a vine common in tropical and subtropical coasts. This species appears to be more susceptible to drought as compared with saline stress and when both stresses are combined, the water and carbon balance may be enhanced compared with the two stresses applied independently. In this study, leaf anatomy and parameters obtained by P-V analysis were measured in *I. pes-caprae* experimenting saline and/or water stress. NaCl and PEG were used to induce iso-osmotic pressures in the soil solution (Ψ_{sol}) to -5 and -10 bar and both osmotica were combined to apply simultaneous saline and water stress (S+W) for a total reduction in Ψ_{sol} of -5 bar. Saline and/or water stress increased the lamina thickness and cell size but the effect was higher under high saline stress. Osmotic potential at full (Ψ_{π}^{100}) and at zero (Ψ_{π}^{0}) turgor were significantly lower at high salinity and S+W stress, compared with the other treatments. Osmotic adjustment resulting from net solute accumulation explains 85-98% of the total change in Ψ_{π}. The contribution of dehydration to changes in Ψ_{π} was apparent only under high water stress. Single stress had no significant effect on the modulus of elasticity (ϵ) and apoplastic water fraction. However, plants growing under S+W showed the lowest values of ϵ. Changes in Ψ_{π} and ϵ affected the relative ability of plants subjected to S+W treatment to maintain high turgor pressure at lower relative water content and confirm that the ability of *I. pes-caprae* to grow under lower Ψ_{sol} may be increased if experiments both stresses simultaneously.

Key words: Drought, modulus of elasticity, *Ipomoea pes-caprae*, P-V curve, salinity, water relations

INTRODUCTION

Salinity and drought affect virtually every aspect of plant physiology and metabolism and the tolerance mechanisms overlap (Munns, 2002). Common responses in species experimenting saline or drought stress are the increase in the osmotic adjustment and changes in the cell wall elasticity (Dichio *et al*., 2005; Hasegawa *et al*., 2000; Zheng *et al*., 2010). Both, tissue elastic and osmotic properties, should enhance water uptake from the soil to decrease the wilting point at low water potential (Ψ)_and to promote the maintenance of high turgor pressure (Ψ_p; Pita and Pardos, 2001; Dichio *et al*., 2005; Merchant *et al*., 2007). The leaf osmotic potential (Ψ_{π}) and modulus of elasticity (ϵ), in turn, are influenced by other factors such as solute accumulation, cell size, cell wall thickness and osmotically active water volume (Merchant *et al*., 2007; Navarro *et al*., 2007; Shrestha *et al*., 2007; Sassi *et al*., 2010). The relative contribution of all of these mechanisms in Ψ_p maintenance under low soil water potential may depend on the type, duration and intensity of stress (Munns, 2002; Zheng *et al*., 2010).

Ipomoea pes-caprae is a pantropical perennial vine common in tropical and subtropical coasts, being one of the earliest species to colonise newly deposited dunes (Ripley and Pammeter, 2004). Recent studies suggest that this species is able to tolerate moderate reductions in the soil water osmotic potential (Ψ_{sol}) caused by the presence of NaCl and/or PEG by the accumulation of Na^+ and organic solutes and by a reduction in stomatal conductance, which prevents the development of an excessive tissue water deficit (Suarez, 2011; Sucre and Suarez, 2010). In addition, this species appears to be more susceptible to drought as compared with saline stress and when both sources of stress are applied simultaneously, the water and carbon balance is enhanced compared with the two stresses applied independently (Sucre and Suarez, 2010). Similarly, in other studies it has been found that the presence of salt in the nutrient solution of plants subjected to water-deficit stress allows a fast water balance re-establishment, compared to plants exposed only to drought stress (Richardson and McCree, 1985; Glenn and Brown, 1998; Martinez *et al*., 2005; Liu *et al*., 2008; Slama *et al*., 2008). However, the interaction of saline and water stress strongly reduces the capacity of the plant to recover the water and carbon balance after stress alleviation, as compared with plants subjected to a single source of stress (Omami and Hammes, 2006; Perez-Perez *et al*., 2007; Slama *et al*., 2008).

The leaf water relation, gas exchange and growth parameters have been well established in *I. pes-caprae* by Sucre and Suarez (2010) and Suarez (2011). However, there are few studies that have evaluated these parameters in plants that experiment both stresses simultaneously and the mechanisms involved during a combination of salinity and drought require more study (Martinez *et al*., 2005; Perez-Perez *et al*., 2007; Slama *et al*., 2008; Sucre and Suarez, 2010). In addition, there are not studies that have evaluated the leaf anatomy and/or parameters obtained by P-V analysis in simultaneously salinized and drought stressed plants. P-V analysis allow to determine if the decrease in Ψ_π is a consequence of an accumulation of solutes or a decrease in cell water content and evaluate the contribution of ϵ on the maintenance of high Ψ_p at low Ψ_{sol}, while the evaluation of the number and size of leaf cell allow evaluate the cost associated at the Osmotic adjustment (OA) and explain changes in leaf succulence. Then, the objective of this study was to assess changes in leaf anatomy and parameters obtained by P-V analysis in plants of *I. pes-caprae* experimenting saline and/or water stress. In addition, changes in Ψ and Ψ_π were examined during 12 d after stress relief.

MATERIALS AND METHODS

Plant growing conditions: Plants of *Ipomoea pes-caprae* (L.) R. Br. (Convolvulaceae) collected in the field at Tucacas (10° 48′N, 68° 19′W) Estado Falcon, Venezuela in November 2005. Then, were transferred to a glasshouse facility at Universidad Simón Bolivar, Caracas, Venezuela (10°25'N, 66°50'W) and transplanted to 5 l pots filled with washed sand and watered as needed with Peat-Lite Special Peters® Water Soluble Fertilizers (Scotts Company Marysville, OH, USA) plus an additional supply of $Ca(NO_3)_2$. This nutrient solution, diluted in tap water, contained (mol m^{-3}): NO_3^-, 1.20; NH_4^+, 0.80; P, 0.21; K^+, 0.64; Ca^{2+}, 2.75; Mg^{2+}, 0.01; Fe, 0.003; Mn^{2+}, 0.003; B, 0.0004; Zn^{2+}, 0.0002; Cu^{2+}, 0.0002; Mo^{2+}, 0.0002. The plants were pruned several times were and maintained under these conditions before the experiment was carried out in June 2008.

Thirty plants were randomly separated into six groups of five plants each. Raw marine salt (98% NaCl) and PEG-6000 were selected to induce saline stress and water deficit, respectively, to adjust the iso-osmotic pressures in the culture media to -5 and -10 bar. Water solution osmolality and the quantity of PEG added to the water were measured and adjusted using a Wescor 5500 vapour pressure osmometer (Wescor Inc., Logan, UT, USA). Another group was subjected to saline and water stress simultaneously, NaCl and PEG being added in such quantities that both solutes contributed in the same proportion to a total reduction in Ψ_{sol} of -5 bar. At the end, there were five plants growing in six different treatments: control (C), low (LS) and high (HS) saline, low (LW) and high (HW) water stress and simultaneous saline and PEG stress (S+W). Before starting measurements, plants were kept for 60 d in a glasshouse under natural sunlight with a 12 h photoperiod. The maximum level of photosynthetically active radiation along the experiment was 1511±372 μmol m^{-2} sec^{-1}. Temperatures ranged from 25 to 35°C in daytime and from 15 to 20°C at night. The relative humidity oscillated between 49 and 97%.

At the time of measurement (60 d of stress) the plants had in average 41, 35, 37, 33, 18 and 21 leaves. Differences in the number of leaves per plant were a consequence of a reduced leaf production rate and an increased cumulative number of dead leaves in presence of NaCl and/or PEG compared with control plants, as was found by Sucre and Suarez (2010). All measurements were made on fully expanded leaves that had developed during the imposition of stress.

Leaf succulence and anatomy: Leaf succulence, defined as water content per unit area (W_c/A), was determined at predawn by sampling 6-10 disks from five to ten fully expanded leaves from each of five to six plants from each treatment. The samples were taken at predawn. After measuring fresh mass (M_f), samples were dried for 48 h at 70°C to determine dry mass (M_d). Water content ($W_c = M_f - M_d$) and M_d for each sample is expressed on a leaf area basis. Additionally, samples of laminae were collected from a position approximately half-way between the base and apex from four leaves from each treatment. Transversal sections of leaf segments were observed under a light microscope (Leica, Model Galen III, Leica Inc, Buffalo, NY, USA) and the thicknesses of total leaf, adaxial and abaxial palisade and spongy parenchyma were measured. The number of cell layers of the palisade parenchyma and the cell size of palisade and spongy parenchymae were also measured. At least 20 measurements were taken from each examined leaf.

Tissue water relations parameters derived from P-V curves: Five plants per treatment were kept with their roots surrounded by undisturbed soil and kept covered overnight with a black plastic bag to allow natural rehydration of leaf tissue under pre-established Ψ_{sol}. In non-rehydrated whole plants, the plateau effect and the error in the estimation of average elastic modulus (ϵ) associated with excise dehydrated leaves and/or natural rehydration whole plants is eliminated (Meinzer *et al*., 1986; Kubiske and Abrams, 1990). Next day, pressure-

volume (P-V) analysis was conducted on excised leaves and completed 16-24 h after collection. The leaves were allowed to dehydrate by free transpiration and their mass loss and leaf water potential (Ψ) determined at intervals during dehydration. Leaf mass was measured by using an analytical balance with 0.1 mg precision and Ψ was measured in a pressure chamber (Model 1400, PMS Instruments Co. Corvallis, OR, USA). After the P-V curve generation was completed, the excised leaves were oven dried at 60°C for 48 h to obtain the dry mass.

Results of P-V analysis were calculated by plotting the reciprocal of water potential ($1/\Psi$) against relative water content (RWC) (Tyree and Hammel, 1982). Water content at full turgidity was calculated by linear regression between initial fresh mass and Ψ at and above the turgor loss point and afterwards the RWC corresponding to each balancing pressure was calculated (Kubiske and Abrams, 1990). P-V curves were fitted for each plant using the PCV set of equations of Schulte and Hinckley (1985). The parameters derived were: osmotic potential at full turgor (Ψ_π^{100}; the inverse of the y intercept) in the straight line region of the P-V curve, osmotic potential at zero turgor (Ψ_π^0) and relative water content at zero turgor (RWC^0) as the first point in the straight line region of P-V curves and apoplastic water fraction (V_a) by the *x*-intercept of a regression line on Ψ *vs* RWC. The value of ϵ was calculated following Jones and Turner (1980): $\epsilon = (\Psi_p^{100}-\Psi_p^0)(1-V_a)/(1-RWC^0)$,.

The total number of osmotic solutes in all the living cells of the sample (N_s) was calculated as $N_s = (\Psi_\pi^{100}V_s)/(RT)$ from P-V data, where R and T are the universal gas constant and absolute temperature, respectively (Tyree and Hammel, 1982). Leaf turgor pressure (Ψ_p) was calculated as the difference between Ψ and Ψ_π obtained from the line relating Ψ_π and RWC. Means of each parameter were calculated for each treatment from individual P-V curves for 4-5 replicates.

Leaf osmotic potential changes ($\Delta\psi_\pi$) resulting from net solute accumulation were estimated as the difference between total change in Ψ_π and the change resulting from dehydration (D). Changes in Ψ_π and Ψ_π^{100} ($\Delta\Psi_\pi$ and $\Delta\Psi_\pi^{100}$), induced by saline and water deficit stress, were calculated following Gucci *et al.* (1997) by comparing predawn Ψ_π and Ψ_π^{100} of plants treated with NaCl and/or PEG ($\Psi_{\pi,\,treated}$) with predawn Ψ_π and Ψ_π^{100} of control plants ($\Psi_{\pi,\,control}$), so that $\Delta\Psi_\pi = (\Psi_{\pi,\,treated}) - (\Psi_{\pi,\,control})$ and $\Delta\Psi_\pi^{100} = (\Psi_{\pi,\,treated}^{100}) - (\Psi_{\pi,\,control}^{100})$. In addition, the contribution of D to changes in Ψ_π was calculated as: $D = \_\Delta\Psi_\pi - \_\Delta\Psi_\pi^{100}$. The change in solute concentration resulting from changes in the non-osmotic volume at 100% hydration was not considered.

Recovery experiment: In order to follow the changes in Ψ_π on recovery from stress, the soil was repeatedly flushed with tap water to eliminate the NaCl and PEG in the soil solution. Five-seven leaves from each treatment and expanded previous to the stress relief were sampled 5 and 12 d after re-watering with fresh water. In addition, newly expanding leaves during the relief period were sampled after 12 d. As number and size of leaves expanding during the relief period were too low, only two-three leaves could be sampled. Leaf blades, excluding middle veins, were placed in plastic syringes and frozen in liquid nitrogen and stored for analysis of Ψ_π. To obtain the sap, the frozen samples were thawed (1/2 h) at room temperature before sap extraction to determine leaf osmolality with a vapour pressure osmometer. Thereafter, predawn leaf sap Ψ_π was calculated using the Van't Hoff equation.

Statistical analysis: The statistical significance of the differences between treatments was tested using one-way ANOVA test and an a posteriori test. Least Significant Difference (LSD) and Dunnet's T3 tests were performed as a posteriori tests, when homogeneous and non-homogeneous variance, respectively, was found in the data. All data were tested for normality and homogeneity of variances by the Kolmogorov-Smirnov and the Levene Median test, respectively. All the analyses were carried out using SPPS 12.0 (SPSS Inc., Chicago, IL). Regression between parameters was analysed with Regression Wizard (SigmaPlot 11.0; SPSS Inc., Chicago, Illinois, USA.). A significance value of $p = 0.05$ was used throughout.

RESULTS

Leaf anatomy and succulence: Anatomical observation showed that leaves of *I. pes-caprae* have isolateral characteristics, with presence of stomata on both, abaxial and adaxial surfaces. The mesophyll is composed of chlorenchyma, differentiated into three to six layers of adaxial and abaxial, anticlinally extended palisade cells and isodiametric spongy and colourless cells occupying the middle region of the mesophyll. The palisade and spongy mesophyll represent 55-60 and 40-45% of the total lamina thickness, respectively (Table 1).

In general, saline and water stress induced a significant increase in lamina thickness and cell size (Table 1). However, under a water potential of -5 bar in the nutrient solution (Ψ_{sol}) the increase in tissue thickness and cell size was higher when both stresses were applied simultaneously (S+W) compared with single saline (LS) and water (LW) stress. In addition, the more obvious differences in leaf anatomy were found between control

Table 1: Leaf and tissue characteristics in fully expanded leaves of *I. pes-caprae* subjected to low saline (LS) and low water (LW) stress, high saline (HS) and high water (HW) stress and simultaneous saline and water stress (S+W) for 60 day. Means of 4-5 replicates±SD. Twenty measurements were taken for replicates

Characteristics	C	LS	LW	HS	HW	S+W
WC/A (g m^{-2})	635±45a	720±94ab	657±46a	818±216b	656±84a	682±54a
WC/M_d (g g^{-1})	8.2±0.8ab	9.0±2.4ab	7.4±1.4ab	10.9±2.7a	7.8±1.1ab	6.9±1.1b
M_d/A (g m^{-2})	78.5±10.6a	84.8±25.8ab	91.9±16.5ab	75.2±6.9a	85.6±17.6ab	100.5±13.1b
Lamina thickness (μm)	518±34a	617±36b	600±76bc	855±67d	585±39c	640±56e
Adaxial palisade thickness (μm)	147±20a	165±18bd	164±36bd	236±34c	160±22b	174±25d
Abaxial palisade thickness (μm)	138±20a	196±23b	196±29b	272±61c	169±25d	201±23b
Spongy layer thickness (μm)	232±34a	256±32bc	240±45ac	347±73d	256±34bc	265±38b
Number of adaxial palisade layers	4±1	4±1	4±1	4±1	4±1	4±1
Number of abaxial palisade layers	5±1	5±1	5±1	5±1	5±1	5±1
Colourless cell diameter (μm)	52±10a	57±14bc	65±16cd	73±23d	58±14bc	61±15b
Spongy cell diameter (μm)	35±7a	44±8b	42±9b	65±21c	35±7a	50±11d
Adaxial palisade cell length (μm)	38±7ab	41±6bc	42±9cd	67±21e	37±7a	44±9d
Adaxial palisade cell width (μm)	16±2a	19±3b	20±5b	24±10c	18±3b	20±3b
Abaxial palisade cell length (μm)	37±6a	43±7b	46±13b	68±24c	39±7b	45±8b
Abaxial palisade cell width (μm)	18±3a	22±4b	23±5b	26±7c	20±3d	22±3b

Values followed by different letters differ significantly at $p \leq 0.05$

(C) and high saline (HS) treated plants. The main effect of HS stress on leaf anatomy was the increase of 65, 60, 97 and 49%, respectively, in the total lamina, adaxial and abaxial palisades and spongy parenchyma thickness, compared with C plants. This increase in tissue thickness with salinity was due to an increase of 76-83% in the length of palisade cells and an increase of 40 and 84%, respectively, in the diameter of colourless and spongy cells (Table 1). In correspondence with the anatomical characteristics, leaf succulence (W_c/A) was also affected at HS stress and increased 29% compared with C plants. The dry mass to leaf area (M_d/A) was statistically significant only under S+W and was 28% higher than in the other treatments (83±18 g m^{-2}; Table 1).

Saline and/or water stress effects: Predawn leaf water potential (Ψ) was -3.3, -5.6, -5.6, -9.3, -9.4 and -6.8 bar in C, LS, LW, HS, HW and S+W, respectively. Single saline and water stress led to a decrease in Ψ and it was near equilibrium with soil water potential (Ψ_{sol}); however, under S+W the soil-plant water potential gradient ($\Delta\Psi$) was -1.8 bar. Both osmotic potentials, at full (Ψ_π^{100}) and at zero (Ψ_π^{0}) turgor, were significantly lower in stressed than in non-stressed plants and the magnitude of these decreases was dependent upon the type and degree of stress (Table 2). Because Ψ_π^{100} and Ψ_π^{0} turgor were lowest under HS and S+W, the differences between Ψ_π of control and treated plants ($\Delta\Psi_\pi$) increased either at high saline stress or when saline and water stress were applied simultaneously, in spite of the fact that Ψ_{sol} was -10 and -5 bar, respectively (Table 2).

Compared to the control, the contribution of dehydration (D) to changes in Ψ_π was from 2 to 15% and was highest under HW stress. Osmotic adjustment (OA) resulting from net solute accumulation reached a maximum of 98, 97 and 96% of total $\Delta\Psi_\pi$ under LS, LW and S+W, respectively. Consequently, the degree of OA, measured at full turgor, was always higher at low levels of stress, independent of the type of stress applied. The total number of osmotic solutes in all the living cells of the sample (N_s) was 31, 34, 67, 41 and 68% at LS, LW, HS, HW and S+W, compared with C plants (Table 2).

Stress had no significant effect on the average modulus of elasticity (ϵ) in leaf tissue of plants subjected to saline or water stress, although saline stress tends to increase ϵ in 29 and 24% under LS and HS, compared with C plants, respectively (Table 2). In addition, plants growing under S+W showed the lowest values of ϵ. The differences in tissue osmotic and elastic properties between treatments became apparent when leaf turgor pressure (Ψ_p) was plotted as a function of relative water content (RWC) (Fig. 1). Changes in Ψ_π and ϵ affected significantly the relative ability of plants subjected to S+W treatment to maintain a high and positive Ψ_p at lower RWC (Fig. 1). However, no consistent effect of saline or water stress alone on relative water content at zero turgor (RWC^0) was observed (Fig. 1, Table 2). In all treatments, OA allowed to maintain a higher Ψ_p, over a wider range of Ψ than those observed in control plants (Fig. 2). The partition of water between apoplast and symplast was affected only under HS in such a way that the apoplastic water fraction (V_a) decreased significantly compared with the other treatments (Table 2).

Stress relief and recovery: During recovery from saline and water deficit stress, the predawn Ψ_π returned to control values after 5 days of the stress relief and was in average -2.8±0.6 in all treatments. However, the predawn Ψ_π of leaves expanded during the stress period, remained relatively constant for at least 12 d following stress relief (Fig. 3). Within 5 days after stress relief plants under all treatments started developing new leaves and after 12 days the predawn Ψ_π of these leaves were similar between treatments (Fig. 3).

Table 2: Water relation parameters derived from pressure-volume curves determined on fully expanded leaves of *I. pes-caprae* subjected to low saline (LS) and low water (LW) stress, high saline (HS) and high water (HW) stress and simultaneous saline and water stress (S+W) for 60 d. Osmotic potential at full turgor (Ψ_π^{100}) and turgor loss point (Ψ_π^0), relative water content at turgor loss point (RWC^0), apoplastic water fraction (V_a), average modulus of elasticity (ϵ), number of osmotic solutes (N_s), contribution of dehydration and net solute accumulation to changes in Ψ_π are shown. LS, LW and S+W correspond to a Ψ_{sol} of -5 bar, HS and HW correspond to a Ψ_{sol} of -10 bar. Means of replicates±SD

	Ψ_π^{100} (bar)	Ψ_π^0 (bar)	RWC^0 (%)	V_a (%)	ϵ (MPa)	N_s (mOsm kg^{-1})	Dehydration (bar)	Dehydration (%)	Net solute accumulation (bar)	Net solute accumulation (%)
C	-9.33±0.80a	-11.08±0.85a	90.1±1.0a	36.3±11.0a	5.05±0.72a	240±47a	-	-	-	-
LS	-11.82±0.78b	-13.76±0.92b	90.6±1.8a	33.8±5.6ab	6.54±1.92a	315±20ab	-0.05	1.9	-2.49	98.1
LW	-12.10±0.90bc	-14.79±0.82bc	88.2±2.2ab	34.2±5.7ab	5.25±1.63a	322±46ab	-0.10	3.4	-2.77	96.6
HS	-13.34±1.47c	-15.49±1.60c	89.6±3.0a	25.2±4.7b	6.24±1.55a	402±42b	-0.31	7.1	-4.01	92.9
HW	-12.25±1.41bc	-14.97±1.4bc	88.1±2.6ab	31.5±7.3ab	5.43±1.60a	339±81ab	-0.50	14.7	-2.92	85.3
S+W	-13.18±1.88bc	-16.43±2.01c	85.4±2.9b	25.4±10.2ab	4.46±0.57a	403±116ab	-0.17	4.1	-3.85	95.9

Values followed by different letters differ significantly at $p \leq 0.05$

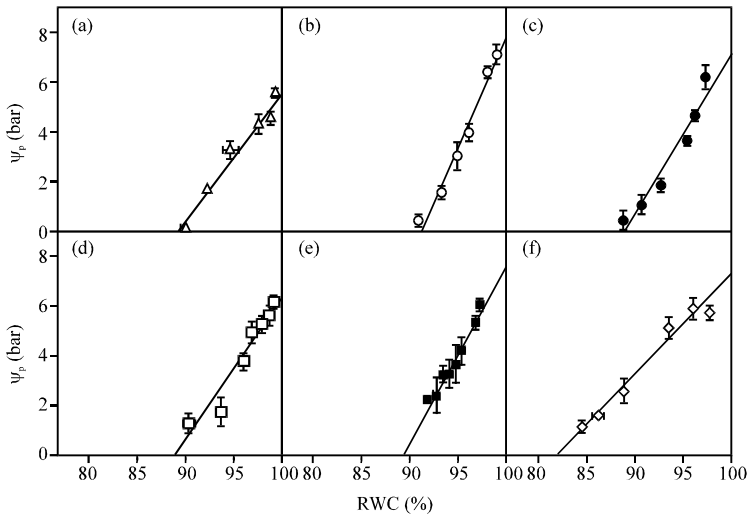

Fig. 1: Relationship between leaf turgor pressure (Ψ_p) and relative water content (RWC), obtained from pressure-volume curves for fully expanded leaves of *I. pes-caprae* subjected to low saline (LS) and low water (LW) stress, high saline (HS) and high water (HW) stress and simultaneous saline and water stress (S+W) for 60 day. (a) Plants grown as control, (b) low and (c) high saline stress, (d) low and (e) high water stress and (f) simultaneous saline and water stress (S+W). Values are means±SE Lines fitted by linear regression were statistically significant at $p \leq 0.05$

DISCUSSION

Leaf anatomy and succulence: Saline and water stress increase the leaf thickness but the effects were significantly higher in the presence of NaCl. Salinity may have promoted the relocation of photosynthates in leaves, which did not occur in drought-stressed plants and as a consequence of this relocation the leaf thickness could increase more than in water stressed plants (Slama *et al*., 2008). In addition, the leaf succulence was only affected in the presence of NaCl in the nutrient solution. In contrast, Slama *et al*. (2008) report that in *Sesuvium portulacastrum* saline and drought stresses had opposite and additive effects on leaf succulence, increasing in saline and decreasing in drought conditions.

A significant increase in succulence under LS, HS and S+W was associated with leaves having similar number of cells with larger size, as compared with control plants. Increased cell size generally occur with salinity and allows that solute saturation may be reached more slowly than in small ones (Suarez and Sobrado, 2000). PEG-induced water stress also increased significantly the cell size although the increase was lower that in presence of salt it in spite of the fact that the depression of cell elongation is the most sensitive plant response to water stress (Hsiao *et al*., 1976). Then, the increase in leaf cell size found in *I. pes-caprae* under water stress may limit to some extent the ability to reach and/or increase the cost associated with the osmotic adjustment because solute accumulation in small cells may be less expensive than net accumulation of solutes in large cells (Hsiao *et al*., 1976; Cutler *et al*., 1977; Jones and Turner, 1980; Sobrado and Turner, 1983; Meinzer *et al*., 1986; Chartzoulakis *et al*., 2002).

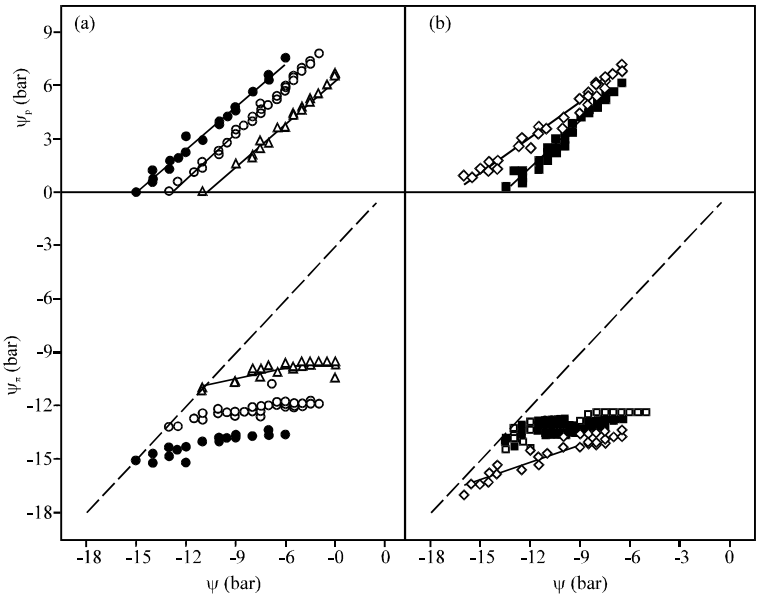

Fig. 2: Leaf turgor pressure (Ψ_p) and osmotic potential (Ψ_π) as a function of leaf water potential (Ψ), obtained from pressure-volume curves in plants of *I. pes-caprae*. Plants were exposed for 60 days to (a) control treatment (white triangles), low (white circles) and high (black circles) saline stress, (b) low (white squares) and high (black squares) water stress and simultaneous saline and water deficit (white diamonds) stress. Lines fitted by linear regression were statistically significant at $p \leq 0.001$

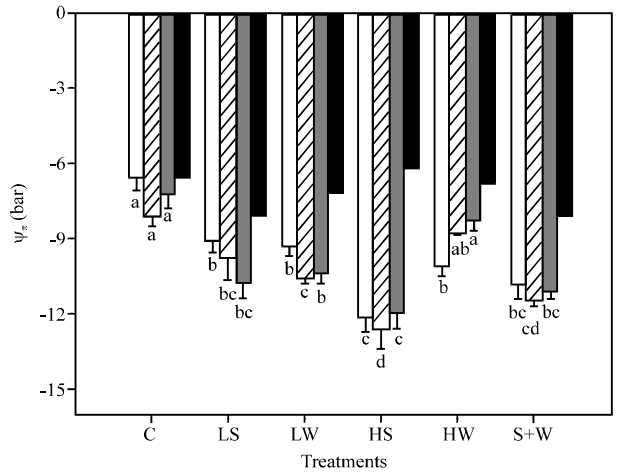

Fig. 3: Predawn osmotic potential (Ψ_π) obtained by osmometry after stress relief. Leaves expanded during stress period and measured just before stress relief (white), 5 days (stripes) and 12 days (grey) after stress relief. Black bars correspond to leaves expanding during the stress relief period and measured after 12 d of stress relief. Plants had been previously exposed for 60 d to control treatment (C), low (LS) or high (HS) saline stress, low (LW) and high (HW) water stress and simultaneous saline and water deficit stress (S+W). Values are means±SE, n = 5-7. Different letters indicate significant differences between treatments, at $p<0.05$. Black bars are the mean of 2-3 leaves

Saline and/or water stress effects: Tissue water relations parameters derived from P-V curves showed that the plants growing in presence of NaCl had adjusted osmotically after 60 days to the two levels of saline stress imposed on them. This became apparent as a decrease in osmotic potential at full turgor (Ψ_π^{100}) and turgor loss (Ψ_π^{0}) with increasing salinity, compared with the control. These changes were enough to maintain a higher turgor

pressure (Ψ_p) than that of control plants under both saline conditions. In the presence of water stress, the plants also showed lower Ψ_π^{100} and Ψ_π^{0} in relation to control plants. However, _when the water potential in the nutrient solution (Ψ_{sol}) increased from -5 to -10 bar, the Ψ_π^{100} decreased only by 1%. In correspondence with these results, Sucre and Suarez (2010) found in the same species and conditions that leaf sap Ψ_π determined by osmometry were at least 0.5 MPa lower than those of the nutrient solution, with the exception of plants experimenting HW stress. Similarly, in many species that have been exposed to both saline and drought stress, the OA is lower in drought than in saline stress (Richardson and McCree, 1985; Shalhevet and Hsiao, 1986; Alian *et al*., 2000; Martinez *et al*., 2005; Rodriguez *et al*., 2005; Omami and Hammes, 2006; Slama *et al*., 2008).

When both stresses were applied simultaneously, the decrease in Ψ_π^{100} and Ψ_π^{0} was higher as compared with plants subjected to a single source of stress, regardless of the level of stress imposed, suggesting that the accumulation of osmotically active solutes was enhanced. Previous studies have also shown that the simultaneous application of saline and water stress further reduces leaf Ψ_π, as compared with a single stress and is associated with higher accumulation of Na^+, proline and sugar and improvement of K^+ use efficiency in leaves (Glenn and Brown, 1998; Martinez *et al*., 2005; Omami and Hammes, 2006; Liu *et al*., 2008, Slama *et al*., 2008; Sucre and Suarez, 2010).

Compared to the control, the contribution of dehydration (D) to changes in Ψ_π was always higher under water stress than under iso-osmotic saline solution. In many species experimenting water stress, the increase in Ψ_π was largely due to a decrease in the water content of the tissue (Meinzer *et al*., 1986; Evans *et al*., 1992; Chartzoulakis *et al*., 2002; Dichio *et al*., 2005; Perez-Perez *et al*., 2007; Slama *et al*., 2008). Maintenance of Ψ_p by passive concentration of solutes lowering Ψ_π^{100} has been found in *Artemisia tridentate* under drought stress and is considered a more advantageous mechanism in arid environments than OA and its associated metabolic cost (Evans *et al*., 1992). In addition, low Ψ_π^{100} can also result from a decrease in the turgid to dry mass ratio (M_t/M_f) and has been found in many species in response to water deficit (Cutler *et al*., 1977; Sobrado and Turner, 1983; Pardossi *et al*., 1998). In this study, the M_t/M_f ratio was not measured, but in a previous study the M_t/M_f ratio in *I. pes-caprae* plants growing under the same conditions for 37 d was 9.4, 11.5, 10.1, 15.7, 8.5 and 11.4 g g^{-1} under C, LS, LW, HS, HW and S+W, respectively (Sucre and Suarez, 2010). Then, lowering Ψ_π^{100} by a reduction in M_t/M_f only may be possible under HW. If the values obtained for the M_t/M_f ratio are used, the change in the non-osmotic volume at 100% hydration explains a reduction of 0.30 bar, equivalent to 8.8% of the reduction in Ψ_π^{100} under HW. As, in general, the contribution of dehydration (D) and M_t/M_f to changes in Ψ_π was relatively small, the increased net solute accumulation was the main component of the changes in Ψ_π in all treatments (85-98%), which is consistent with the fact that the relationship between Ψ_π^{100} and the number of osmotic solutes (N_s) is significant with an $r^2 = 0.83$. In addition, the partition of water between apoplast and symplast may contribute to changes in Ψ_π. However, the apoplastic water fraction (V_a) decreased upon exposure to high saline stress while it was equivalent in the other treatments, confirming that the net solute accumulation is the main mechanism explaining the reduction in Ψ_π.

In general, stress had no apparent effect on the average modulus of elasticity (ϵ) in *I. pes-caprae* under saline and water stress, indicating few changes in the mechanical properties of the cell walls. Despite the fact that the differences in ϵ were not statistically significant, at high saline stress ϵ tends to increase compared with the other treatments. Similarly, in many species experimenting saline stress, tissues became less elastic (larger values of ϵ)_and the tissue water deficit associated with decreases in leaf Ψ could be reduced, mostly through a decrease in Ψ_p, facilitating a continued water uptake from saline soil (Youngman and Heckathorn, 1992; Pardossi *et al*., 1998; Suarez and Sobrado, 2000; Navarro *et al*., 2007; Shrestha *et al*., 2007; Sassi *et al*., 2010). However, in other species saline or water stress has no apparent effect on ϵ (Jones and Turner, 1980; Sobrado and Turner, 1983; Alarcon *et al*., 1993; Erdei and Taleisnik, 1993; Tattini and Gucci, 1999). When both stresses are applied simultaneously, ϵ tends to decrease. A low ϵ has been correlated with drought adaptation and has ecological significance by buffering plants against short term changes in water content (Robichaux *et al*., 1986; Chartzoulakis *et al*., 2002).

At a particular Ψ_or RWC, Ψ_p depends upon Ψ_π and ϵ (Jones and Turner, 1980). Thus, although under S+W the changes in Ψ_π and ϵ were slight compared with the other treatments, the combination of relatively high cell elasticity and OA were apparently responsible for the ability of S+W plants to maintain Ψ_p at a significantly lower value of relative water content at zero turgor (RWC^0) compared with the other treatments (85 vs. 90%, respectively). In plants under single saline or water stress, the reduction in Ψ_π and changes in the elastic properties were not enough to contribute to turgor maintenance during tissue dehydration. However, the OA allowed in all treatments to maintain a higher Ψ_p and over a wider range

of Ψ. Thus, assuming a Ψ_{sol} of -10 bar, the Ψ_p could be 1.6, 2.4, 4.0, 3.1, 3.2 and 4.4 bar under C, LS, HS, LW, HW and S+W, respectively. The maintenance of Ψ_p permits cell elongation, stomatal opening and other processes dependent on turgor pressure (Jones and Turner, 1980; Munns, 2002; Dichio *et al*., 2005). In fact, *I. pes-caprae* subjected to the same treatments for 37 days was able to maintain relatively higher values of photosynthesis and stomata conductance after the OA was reached (Sucre and Suarez, 2010).

Cell size or volume influences tissue elasticity, so lower ϵ values are associated with smaller cells (Steudle *et al*., 1977; Sobrado and Turner, 1983; Meinzer *et al*., 1986; Robichaux *et al*., 1986). In this study, no relationship between ϵ and cell size was observed. However, the effect of cell size on elasticity is not great, since a fourfold increase in cell size was required to halve the elasticity in epidermal bladder cells (Steudle *et al*., 1977) and the tissue elastic properties may be affected by other factors such as the thickness and chemical composition of the cell wall (Cutler *et al*., 1977; Robichaux *et al*., 1986; Sassi *et al*., 2010).

Stress relief-recovery: The present study has shown that upon restoration of Ψ_{sol}, Ψ of plants that were subjected to pre-stress treatments increased rapidly and reached similar values to the controls after 5 days. Similarly, plants of different species that suffered drought or saline stress, showed a rapid (within 2-10 days) recovery of Ψ and regained full turgor after re-watering (Richardson and McCree, 1985; Alarcon *et al*., 1993; Herrera *et al*., 1994; Tattini and Gucci, 1999; Pardossi *et al*., 1998; Chartzoulakis *et al*., 2002; Perez-Perez *et al*., 2007). However, plants subjected to saline and/or water stress maintained a lower predawn Ψ_π after 12 days of stress relief. Similarly, in olive plants experimenting drought stress, the Ψ_π remain lower 6 and 29 days after the beginning of the rewatering period (Dichio *et al*., 2005). Contrarily, in some species the OA reached after saline and water stress is not permanent and the concentration of individual solutes returns to pre-stress levels within a few days after watering (Jones and Turner, 1980; Meinzer *et al*., 1986; Evans *et al*., 1992; Herrera *et al*., 1994; Kerepesi and Galiba, 2000; Omami and Hammes, 2006). Differences related to changes in Ψ_π after stress relief may be associated with the solutes accumulated for OA, due to the relative contribution of these various compounds to the total OA change according to the intensity, type and kinetics of stress exposure (Di Martino *et al*., 2003; Hassine *et al*., 2010). Thus, Na^+ and glycinebetaine have a slow turnover rate and the OA by accumulation of these solutes is permanent (Di Martino *et al*., 2003; Hassine *et al*., 2010). Contrarily, proline and water soluble carbohydrates are solutes with a high turnover rate and may be quickly recycled after removal of temporary stress (Kerepesi and Galiba, 2000; Di Martino *et al*., 2003; Hassine *et al*., 2010).

On the other hand, leaves expanding after stress relief had a similar predawn Ψ_π than the control. Similar results indicate that leaves that emerged during the development of water deficits retained their lower Ψ_π, whereas those that developed during stress relief have a similar Ψ_π to the controls (Sobrado and Turner, 1983). Hence, it could be inferred that saline, water, or both stresses applied simultaneously do not cause permanent alterations in water relation parameters. Similar results have been found in *S. portulacastrum* (Slama *et al*., 2008). The parameters measured in this study during stress relief were insufficient to detect and understand the physiological mechanisms involved in the recovery process and differences between treatments were not detected. However, other studies have shown important differences between single and combined saline and water stress. Omami and Hammes (2006) found in *Amaranthus* that water relation parameters in plants subjected to combined saline and water stress did not fully recover. Perez-Perez *et al*. (2007) have shown in *Citrus* that the interaction of saline and water stress strongly reduces plant capacity to recover photosynthesis after stress alleviation as compared with plants subjected to a single stress and Slama *et al*. (2008) showed that in *S. portulacastrum* the plant ability to recover its leaf development after exposure to a combination of drought and salinity was limited. Thus, simultaneously salinized and drought stressed plants have high levels of leaf Na^+ and Cl^- that limit recovery after re-irrigation with fresh water (Omami and Hammes, 2006; Perez-Perez *et al*., 2007; Slama *et al*., 2008).

CONCLUSIONS

Saline and water stress increase the leaf thickness and was associated with leaves having similar number of cells with larger size, as compared with control plants. Under saline stress an increase in cell size may delay the cell solute saturation while under water stress may increase the cost associated with the osmotic adjustment. The ability to accumulate solutes decreased at high water stress levels, confirming that *I. pes-caprae* is more tolerant to saline stress than to water stress and that it has a low inherent water stress tolerance for long drought periods. In addition, the RWC^0 and ϵ were not affected in comparison with the control under the two stresses evaluated. However, when saline and water stress were

applied simultaneously, there was a tendency to decrease ϵ and the RWC^0. Although these changes are slight, they may suggest that the ability of *I. pes-caprae* to grow under lower Ψ_{sol} may be increased when both stresses are combined. The present study has shown that upon restoration of Ψ_{sol}, Ψ of plants that were subjected to pre-stress treatments increased rapidly and reached similar values to the controls after days, however, results during stress relief were insufficient to detect and understand the physiological mechanisms involved in the recovery process.

ACKNOWLEDGMENTS

Financial support was provided by the Decanato de Investigaciones USB-S1-CB-23-IC.

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