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Research Article

Growth and Physiological Responses of *Phaseolus* Species to Salinity Stress

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This paper reports the changes on growth, photosynthesis, water relations, soluble carbohydrate, and ion accumulation, for two salt-tolerant and two salt-sensitive *Phaseolus* species grown under increasing salinity (0, 60 and 90 mM NaCl). After 20 days exposure to salt, biomass was reduced in all species to a similar extent (about 56%), with the effect of salinity on relative growth rate (RGR) confined largely to the first week. RGR of salt-tolerant species was reduced by salinity due to leaf area ratio (LAR) reduction rather than a decline in photosynthetic capacity, whereas unit leaf rate and LAR were the key factors in determining RGR on salt-sensitive species. Photosynthetic rate and stomatal conductance decreased gradually with salinity, showing significant reductions only in salt-sensitive species at the highest salt level. There was little difference between species in the effect of salinity on water relations, as indicated by their positive turgor. Osmotic adjustment occurred in all species and depended on higher K^+, Na^+, and Cl^- accumulation. Despite some changes in soluble carbohydrate accumulation induced by salt stress, no consistent contributions in osmotic adjustment could be found in this study. Therefore, we suggest that tolerance to salt stress is largely unrelated to carbohydrate accumulation in *Phaseolus* species.

1. Introduction

Salinity is considered a significant factor affecting crop production and agricultural sustainability in arid and semiarid regions of the world, reducing the value and productivity of the affected lands [1]. Because soil infertility is often due to the presence of large amount of salt, the identification of plants capable of surviving under these conditions is worth investigating [2]. Currently, there are no economically viable technological means to facilitate crop production under salt stress conditions. Nevertheless, development of genotypes with field tolerance to salinity stress is considered a promising approach, which may help to satisfy growing food demands of developed and developing countries. To improve on salt stress tolerance requires knowledge of the physiological mechanisms and genetic controls of the traits associated with salt tolerance at different plant development stages.

To understand the physiological mechanisms responsible for salinity tolerance, it is necessary to know whether their growth is limited by the osmotic effect of the salt in the soil, or by the toxic effect of the salt within the plants. In the simplest analysis of the response of a plant to salinity stress, the reduction in shoot growth occurs in two phases: a rapid response to the increase in external osmotic pressure, and a slower response due to the accumulation of Na^+ in leaves [1]. In the first *osmotic phase* which starts immediately after the salt concentration around the roots increases to a threshold level (40 mM NaCl for most plants, which is equivalent to ECe of 4 dS/m; [3]), the rate of shoot growth falls significantly. This is largely due to the osmotic effect of the salt outside the roots. The second, *ion-specific*, phase of plant response to salinity starts when salt accumulates to toxic concentrations in the leaves, causing necrosis and reducing the photosynthetic area, resulting in further decline of growth [1, 4].

In the past 2 decades, biotechnology research has provided considerable insights into the mechanism of abiotic stress tolerance in plants at the physiological and molecular levels [4]. Stress tolerance mechanisms may vary from species to species and at different developmental stages [5]. Salt tolerance in crops is based on specific physiological characteristics like shoot or leaf specific ion accumulation or production of specific osmolyte compounds [2].

Ion transport processes are central to the understanding of the complex and multigenic nature of salt tolerance in crop plants [2]. The crucial role of K^+ homeostasis in salt tolerance mechanisms of salinized plants have placed it in center stage [6, 7]. Imposition of salt stress results in a massive efflux of K^+ from cells [8] and significantly reduces the intracellular pools of K^+ [9]. Mitigation of this loss strongly correlates with the level of salt tolerance [8, 10–12].

Plant abiotic stress-tolerance is often associated with increased *de novo* synthesis of so-called compatible solutes [4]. Traditionally, the role of osmolytes in drought and salt tolerance was thought to be as cytosolic osmoticum involved in cellular osmoregulation [4]. However, the measured levels of many compatible solutes often appear to be too low to act as osmolytes [13]. It has been proposed that the role of compatible solutes in cytosolic osmotic adjustment is indirect, through regulatory or osmoprotective functions. The latter may include a possible role for compatible solutes in stabilizing the structure and activities of enzymes and protein complexes, scavenging radical oxygen species and maintaining the integrity of membranes under dehydration stress conditions [2, 14]. Another function of compatible solutes may be in maintaining cytosolic K^+ homeostasis by preventing NaCl-induced K^+ leakage from the cells [10].

In plants, growth is particularly important because survival and reproduction depend on plant size and therefore on growth rate. Relative growth rate (RGR) is therefore a key variable when comparing plant species growing under stressful environments [15]. RGR is determined by two factors, the unit leaf rate (ULR), which is an index of plant photosynthetic-assimilatory capacity per leaf area unit, and leaf area ratio (LAR), which is the amount of leaf area per total plant weight [16]. In some species, salinity mainly affects the leaf elongation and hence the development of photosynthetic surface area (LAR) [17] and photosynthetic capacity in others [18]. Salinity reduction of LAR could be caused by a decrease in SLA (the amount of leaf area per unit leaf weight) and/or a decrease in the proportion of dry matter allocated to the leaf tissue (leaf weight ratio) (LWR) [16]. At the whole plant level, these growth parameters may make it possible to clarify whether genotypic variation in salt tolerance could be attributed to morphological or photosynthetic response [15, 19].

The common bean, *Phaseolus vulgaris* L., is extremely sensitive to salinity, and suffers yield losses at soil salinity of less than $2\,dS\,m^{-1}$ [20]. However, the common bean is regarded as an appropriate crop for bioproductivity enhancement and marginal land reclamation, not only because it yields nutritious fodder, protein rich seeds, but also it is a soil nitrogen enricher in symbiotic association with rhizobium [21]. Common bean is known to exclude Na^+ from the leaves, but takes up Cl^- in proportion to the external concentration [20]. High leaf Cl^- concentrations reduce growth by altering the nutritional balance of the plant, affecting CO_2 assimilation [22, 23], and altering water relations [24]. Although there are several studies demonstrating the effect of salinity on bean growth [25, 26], there is limited genetic variation in cultivated bean germplasm for salinity tolerance [24].

Certain *Phaseolus* species such as wild *P. acutifolius* Gray var. *latifolius* Freem. and *P. vulgaris* L. can be classified as salt tolerant due to their ability to restrict Na^+ ions in roots and leaves [23]. Salt tolerance in both species is also associated with better stomatal control through osmotic adjustment. *Phaseolus* species adjust to high salt concentrations by lowering tissue osmotic potential with an increase of inorganic ions, predominantly Cl^-, Na^+, and K^+ in their leaves [23]. The role of compatible solutes (e.g., soluble sugars) as possible osmolytes have not been well established or discarded on *Phaseolus* species. Many studies have dealt with osmotic potential decrease in common bean as a result of water deficit in the leaf tissue [26], but few differences between the various inorganic ions and compatible organic solutes contributing to osmolyte accumulation [24]. This allows the following hypothesis; assuming that the production of organic solute requires considerable expenditure of energy while accumulation of inorganic ions is inexpensive, it is possible that the ability of *Phaseolus* species to withstand osmotic stress can be attributed to changes in the ratio of organic and inorganic compounds that contribute to osmotic adjustment. Therefore, the object of this study was to evaluate the effects of salt stress on growth, water relations, and gas exchange of different *Phaseolus* species, and at the same time correlate these effects with changes in ionic and soluble carbohydrate accumulation, to better understand the mechanisms of salt tolerance in these species.

2. Materials and Method

2.1. Plant Material and Location. Two wild *Phaseolus* genotypes, *P. vulgaris*, PI325687, *P. acutifolius*, G40169 and two cultivated genotypes, *P. vulgaris*, G04017 and *P. acutifolius*, G40142 were used. These genotypes were classified into three groups: salt-tolerant *P. vulgaris*, PI325687 (PvWT), moderately tolerant *P. acutifolius*, G40142 (PaCT) and salt-sensitive *P. acutifolius*, G40169 (PaWS) and *P. vulgaris*, G04017 (PvCS) based on the ranking in terms of variation on their salinity tolerance defined by total dry weight reduction as a percentage of the unsalinized controls, salt susceptibility index (SSI), and root: shoot ratio (RSR) [27]. Wild species were selected as they are widely distributed throughout the Pacific slopes of Mexico, where saline soils are common. The cultivated *P. vulgaris* is a Brazilian variety "Carioca" (G04017) belonging to the Mesoamerican gene pool with an indeterminate prostrate growth habit (Type III) and small seeds ($\leq 300\,mg\,seed^{-1}$) [21]. The cultivated *P. acutifolius* is grown in semiarid areas of Sonora, Mexico and also has small seeds ($\leq 137\,mg\,seed^{-1}$). Plants were grown on nutrient solution under greenhouse conditions.

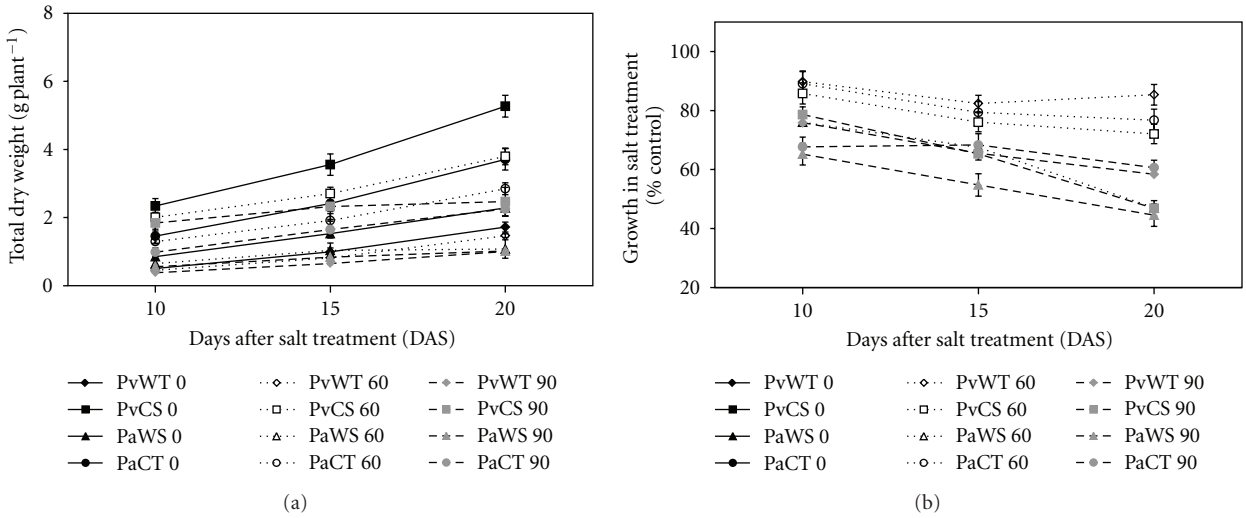

Figure 1: Effects of 0, 60, and 90 mM NaCl on total dry weight (a) and percentage total dry weight (b) in salt relative to control conditions between 10 and 20 days after salt treatment for *P. vulgaris* PI325687 (PvWT), *P. vulgaris* G04017 (PvCS), *P. acutifolius* G40169 (PaWS), and *P. acutifolius* G40142 (PaCT). Each value represents the mean ± SE of six replicates.

Average temperature during the experiment was 26°C, and minimum and maximum temperatures were 22°C, and 34°C, respectively. Relative humidity varied between 50 and 65%.

2.2. Plant Growth. Seeds were surface sterilized with 2.5 g L^{-1} sodium hypochlorite for 5 min and rinsed with sterile distilled water, then they were scarified mechanically and germinated in the dark at 25°C in rolled germination paper (Anchor Paper Co., St. Paul, MN) moistened with 0.5 mM $CaSO_4$. Seven-day-old seedlings of uniform size were transferred to aerated tanks (100 L) containing nutrient solution [28]. Nutrient solution composition, in mM, was: 6 KNO_3, 4 Ca $(NO_3)_2$, 1 $MgSO_4$, 1 $NH_4H_2PO_4$, 0.05 Fe-EDTA, 0.05 KCl, 0.025 H_3BO_3, 0.002 $MnSO_4$, 0.002 $ZnSO_4$, 0.005 $CuSO_4$, 0.005 $(NH_4)_6MoO_2$ $4H_2O$. The solution pH was adjusted daily to 6–6.5. The nutrient solution was aerated continuously and replaced weekly. Plants were grown in this control solution until the emergence of the first trifoliate leaf (7 days after transplanting), at which time salt treatments were added to the solutions. The treatment nutrient solution was identical to that for controls except for the addition of NaCl. Plants were exposed gradually to their final NaCl concentration (0, 60 and 90 mM) through a progression of 30 mM NaCl increments at one-day intervals added shortly before sunset. A randomized complete block design with a split-plot arrangement of treatments and six replications was used with NaCl treatments as the main plot and genotypes as subplots.

2.3. CO_2 Assimilation and Stomatal Conductance. Measurements of net CO_2 assimilation (A_n) and leaf diffusive conductance (g_s) were taken at 9, 14, and 19 days after initiation of salt treatments (DAS) using the second, third, and fifth trifoliate leaf, which were the youngest fully expanded leaves, respectively. Measurements were performed using a LI-COR 6400 infrared gas analysis system (LI-COR, Corp., Lincoln, NE). A portion of the central leaflet was enclosed in a ventilated temperature controlled leaf chamber (6 cm^2). A_n was measured at 34 MPa external CO_2 partial pressure (340 μ mol CO_2 mol^{-1} air) and a vapor pressure deficit (VPD) of 1.8 KPa. The photosynthetic photon flux density (PPFD) was 1200 μ mol m^{-2} s^{-1}, provided by a 6400-02 LED light source. Gas exchange rates were monitored until steady-state rates were attained.

2.4. Leaf Water Relations. Predawn water potential (Ψ_w) of a whole leaf was measured with a pressure chamber (Model 3000, Soilmoisture, Santa Barbara, CA) [29]. Leaf osmotic potential (Ψ_π) measurements were made on the remainder leaf material that was used for leaf water measurements. Osmotic potential (Ψ_π) was determined by pressing frozen thawed tissue with a ground plastic tissue homogenizer. The homogenate was centrifuged for 5 min at 2000 ×g in an Eppendorf micro centrifuge and 10 μL of supernatant was collected for measuring leaf solute potential with a Wescor-5500 vapor pressure osmometer (Wescor, Logan, UT, USA). The osmometer was recalibrated after every pair of readings using commercial standards. Readings were converted to pressure units by using the van't Hoff equation ($\pi = -cRT$), where c is osmolality (mOsmol kg^{-1}), R is the gas constant, and T is temperature (°K). Turgor potential (Ψ_p) was determined as the difference between leaf water potential and osmotic potential. As a measure of osmotic adjustment to salt, a value for osmotic potential at full turgor Ψ_π was calculated as the product of the measured values and relative water content. This accounts for the effects of change in tissue hydration on leaf Ψ_π.

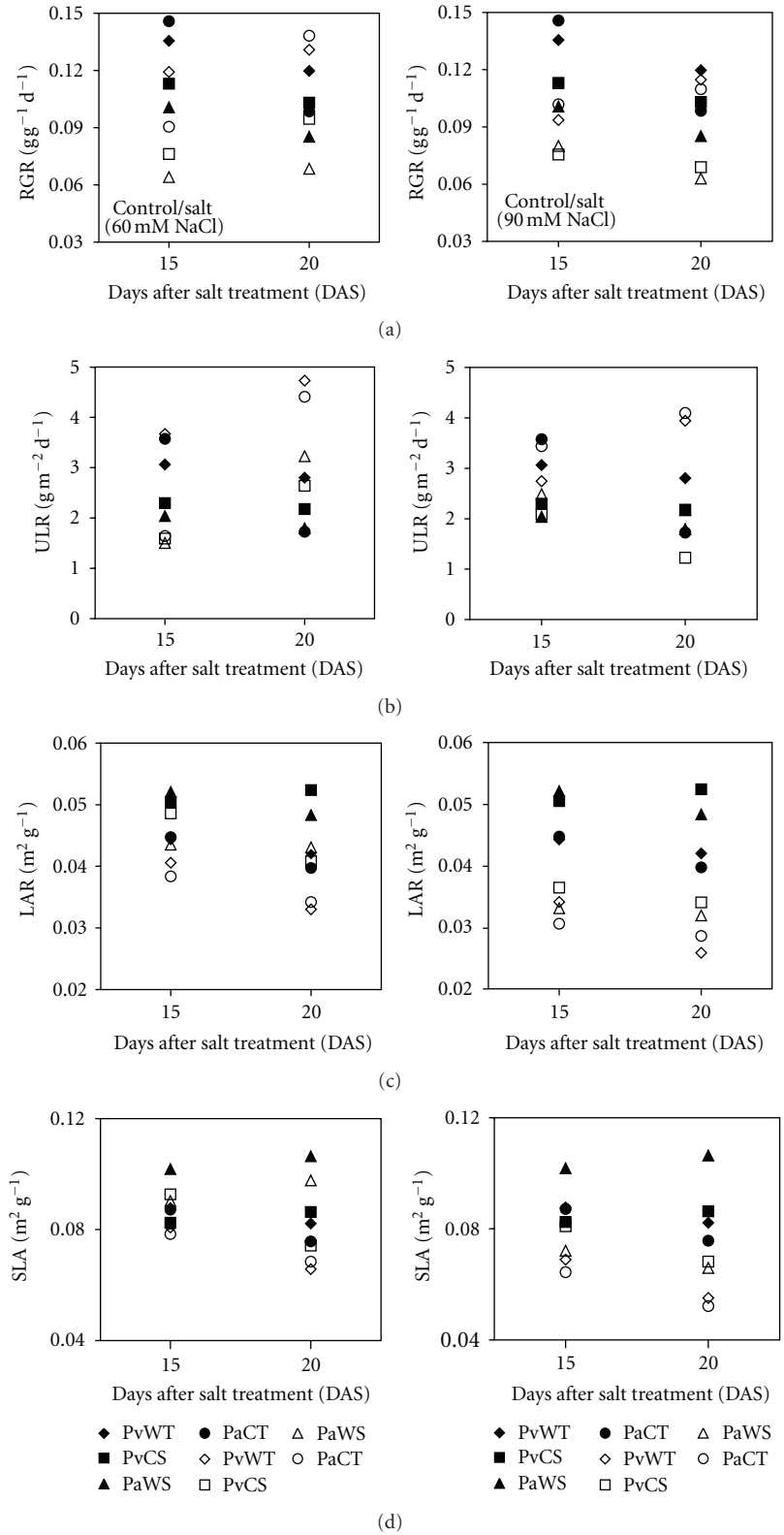

Figure 2: Effects of increasing NaCl levels on relative growth rate (RGR) (a), unit leaf rate (ULR) (b), leaf area ratio (LAR) (c), and specific leaf area (SLA) (d) for *Phaseolus* species, based on the differences between two means (10–15 and 15–20 days). Each value represents the mean ± SE of six replicates. Control (filled symbols) and salt treatments open (symbols).

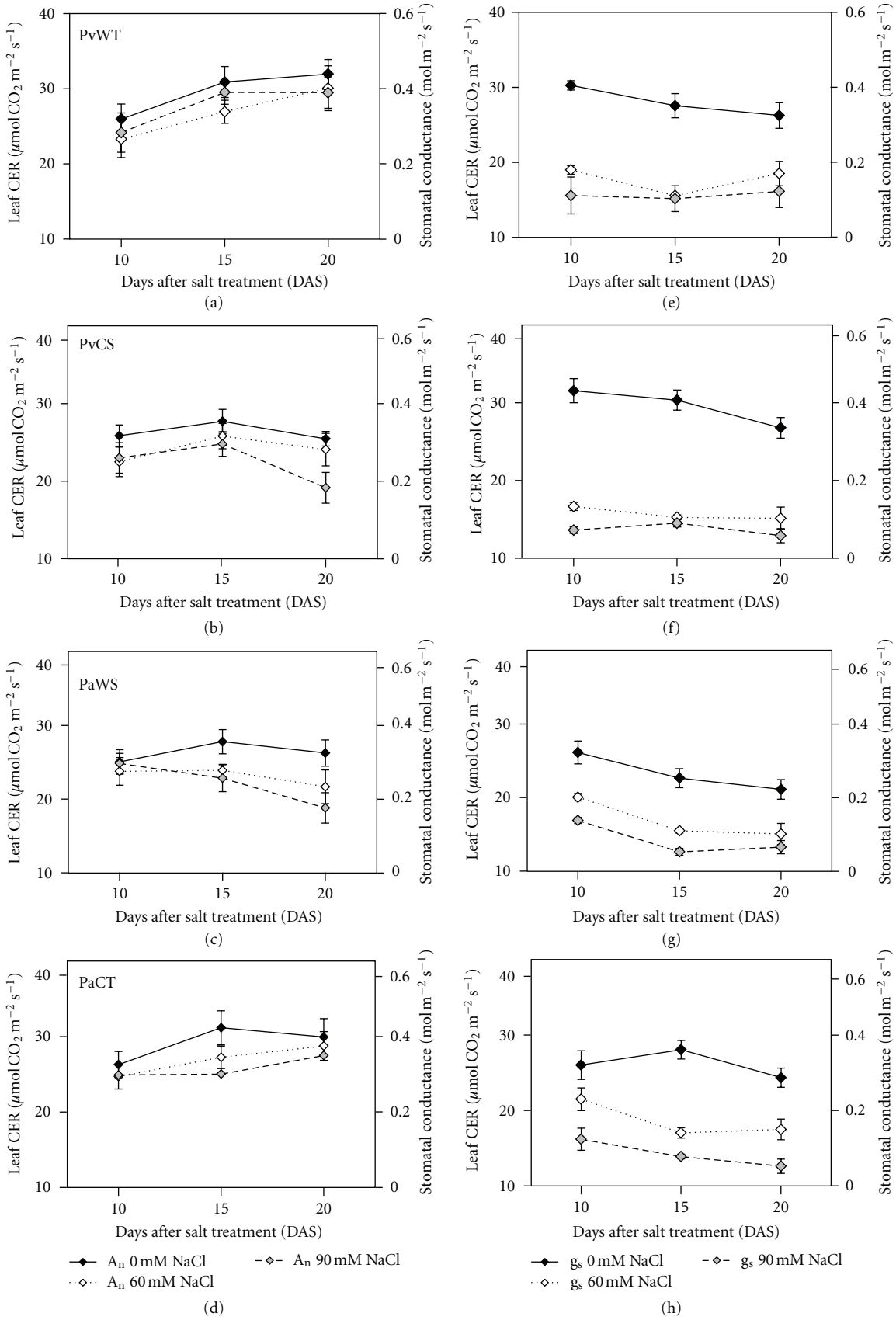

FIGURE 3: Effects of increasing NaCl levels on leaf CO_2 exchange rate (*left axis*; a–d) and stomatal conductance (*right axis*; e–h) between 10 and 20 days of salt treatment for *Phaseolus* species. Each value represents the mean ± SE of six replicates.

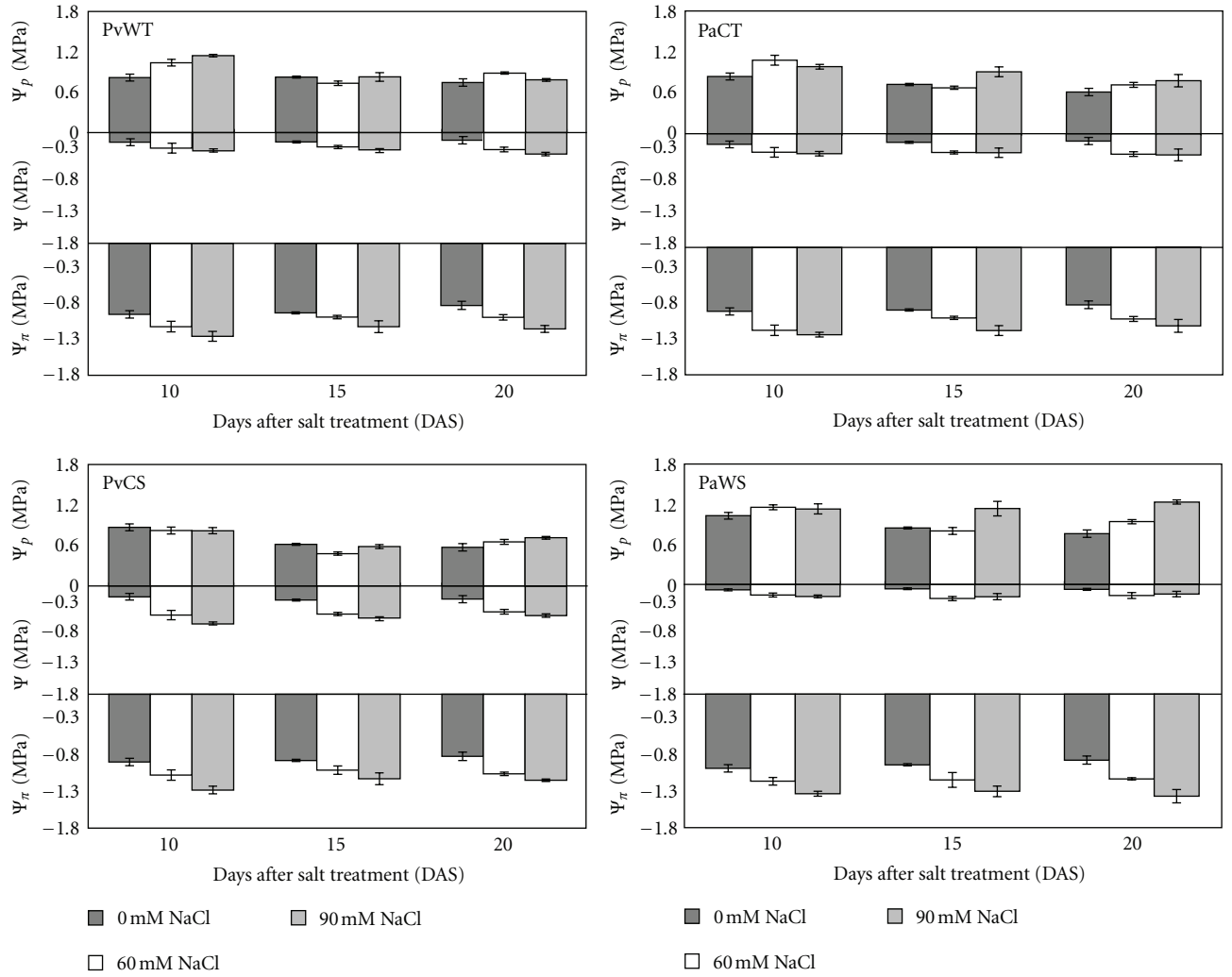

Figure 4: Effects of increasing NaCl levels on leaf turgor pressure (Ψ_p), leaf water potential (Ψ), and osmotic potential (Ψ_π) (in MPa) between 10 and 20 days of salt treatment for *Phaseolus* species. Each value represents the mean ± SE of six replicates.

2.5. Growth Measurements. Plants were harvested at 10, 15, and 20 days after the initiation of salt treatments (DAS) and separated into roots, stem, and leaves. Plant material was dried at 65°C for 96 hours to determine dry weight. Leaf area was measured with a portable leaf area meter (Model LI-3000 A, LI-COR, Lincoln, NE). Growth parameters were calculated according to Hunt [16]. The mean relative growth rate, RGR ($g\,g^{-1}\,d^{-1}$), was calculated as was the rate of increase of total dry weight per unit for each period. Two growth components were determined: the unit leaf rate, ULR (also called net assimilation rate, $g\,m^{-2}\,d^{-1}$), calculated as the rate of increase of total dry weight per unit of total leaf area, and the leaf area ratio, LAR ($m^2\,g^{-1}$), calculated as the ratio between the total leaf area and the total plant dry weight. The leaf weight ratio, LWR ($g\,g^{-1}$), was calculated as the ratio between the total leaf dry weight and the total plant dry weight; and the specific leaf area, SLA ($m^2\,g^{-1}$), was calculated as the mean area of leaf displayed per unit of leaf weight, the RGR being related to these quantities by the equation: RGR = ULR × LAR = ULR × LWR × SLA.

2.6. Elemental Analysis. Tissue was ashed at 500°C for 8 h, followed by dissolution in 1 mM hydrochloric acid [30]. Sodium and potassium concentrations were determined by flame emission using an Atomic Absorption Spectrometer (Varian SpectrAA-220FS; Mulgrave, Australia). Free chloride was extracted from 3 mg of ground material with 50 mL of deionized water and then filtered through 0.22 μm millipore paper [31]. Chloride concentration was determined colorimetrically using an UV/BIS Spectrometer (Lamda 40 Perkin Elmer; Uberlingen, Germany).

2.7. Carbohydrate Analysis. Root, stem, and leaves samples were frozen with liquid nitrogen before storage at −20°C. An enzymatic assay method for nonstructural carbohydrate was used [32]. Soluble sugars were extracted from 15 mg of fine ground plant powder, in 4 mL methanol: water solution, followed by 100 μL chloroform. Two liquid phases were separated from the plant powder after centrifugation (IEC Model GP8R., Needham, MA). After evaporation under

vacuum (CentriVap Labcondo Model 75100, MO, USA), the dried pellet was returned to its soluble form by agitation in water at 4°C. The aqueous extract was then combined with 15 mg polyvinylpyrrolidone (PVP) to eliminate any residual phenols. After repeated shaking, the supernatant was analyzed using the MP plate (The Multtiskan Ascent MP Systems, Labsystems Thermo Fisher Scientific, Helsinki, Finland). Glucose, fructose, and sucrose concentrations were quantified by measuring the production of NADH.

2.8. Statistical Analysis. Prior to analysis of variance, salt treatments data for each variable were analyzed for normality and homocedasticity (homogeneity of covariance matrices) by using Bartlett's tests [33]. Because error variances of some variables were not homogenous, the data was transformed to natural logarithm, root square, or the inverse value. Original or transformed data were further subjected to parametric procedures when both requirements were met. Data was analyzed using the GLM procedure of the Statistical Analysis System [33]. Six replicates per salinity treatment per species per harvesting date and organ tissue were used for analyses of variables. Two-way analysis of variance was used to determine significant differences among species for various traits.

3. Results

3.1. Effect of Salt Stress on Growth. Growth of all four genotypes was reduced by a similar extent by salinity (Figure 1). Biomass production in the absence of salinity differed among genotypes, but the effect of salinity was similar, so that the genotypes that grew most in the control treatment also grew best in the salt treatment (Figure 1(a)).

Salt tolerance is shown in Figure 1(b), as the percent biomass in saline *versus* control conditions. This illustrates that the greatest reduction in plant growth occurred during the first period of salt treatment. There were no significant differences between genotypes, except during the first two harvests when PaWS were significantly more affected than PvCS as a percent of biomass. The final biomass production, after 20 d of salinity, was reduced by 47 to 72% for salt-sensitive genotypes and by 58 to 61% for salt-tolerant ones.

Calculations of total RGR throughout the experimental period showed that plants treated with 60 mM NaCl reduced grow only during the first period (10–15 DAS; Figure 2(a)). For subsequent harvest, there were statistically significant differences ($P \leq 0.0084$) in RGR between genotypes. RGR of salt-tolerant PvWT and PaCT was maintained between 15 and 20 days, whereas in salt-sensitive PvCS and PaWS, RGR declined with increasing salinity (Figure 2(a)). Thus, the effect of salt on final biomass was attributed to less growth in the first weeks of salt treatment.

ULR also declined over time in salt-sensitive genotypes, particularly in salinized plants (Figure 2(b)). For salt-tolerant genotypes (PvWT and PaCT), ULR was maintained across salt treatments and time (Figure 2(b)). LAR was steady during the first period (10–15 days) of salt stress, whereas LAR decreased for all genotypes with increasing salinity at day 20 (Figure 2(c)). Also LWR values were steady over time in all salt treatments and genotypes (data not shown), whereas SLA was significantly decreased ($P \leq 0.0007$) for all salt-stressed genotypes at 90 mM NaCl (Figure 2(d)).

3.2. Photosynthesis and Stomatal Conductance. Salinity and salt stress duration significantly affected photosynthesis (A_n) ($P \leq 0.0025$) and stomatal conductance (g_s) ($P \leq 0.0001$). Salinity and species interaction was not significant ($P \leq 0.1903$), indicating that all species responded similarly to salt stress. A_n was steady with time in control plants throughout the experiment period and decreased in the salt treatment (90 mM NaCl) only in salt-sensitive genotypes (Figures 3(a)–3(d)). No significant differences were detected on stomatal conductance for all genotypes under nonsaline conditions throughout the experiment but declined as salinity and duration intensified (Figures 3(e)–3(h)).

3.3. Ions. Tissue concentration of Cl^- and Na^+ ions increased significantly in response to salt treatments (Table 1). However, the magnitude of the Cl^- increments was always higher than those of Na^+ at all salt treatments. The concentrations of Na^+ increased in plants treated with salt stress until day 15 (0.79 to 1.06 mmol kg^{-1} DW), and then remained constant until day 20 (Table 1), whereas the concentration of Cl^- in plants treated with 60 and 90 mM NaCl increased sharply between day 10 and 20 (1.75 to 2.44 and 1.63 to 5.24 mmol kg^{-1} DW), except in PaCT.

Saline-induced changes in minerals concentration varied with plant organ and ion. In all species, Na^+ concentration increased almost equally in stems and roots, whereas the concentration of Cl^- increased more in stems and leaves than in roots (Table 1). Species differed in leaf Na^+ accumulation. PvWT and PaCT were able to exclude Na^+ from leaves at 60 mM NaCl. In contrast, PaWS accumulated Na^+ in their leaves as salt levels increased (Table 1). Salinity reduced K^+ concentration in the root, stems and leaves of all species (Table 1). However, decrease in K^+ concentration on stems of PvWT and PvCS was greater than leaves and roots (Table 1). Compared to controls, leaf and root K^+ concentration at 20 days with 90 mM NaCl decreased between 24 to 46 and 40 to 72%, respectively. At moderate and high salinity levels, leaf K^+ concentration on PaWS and PaCT was about 28 to 15% higher at day 20 than those observed on PvWT and PvCS genotypes.

3.4. Carbohydrates. Hexoses (glucose plus fructose) of both PvWT and PvCS decreased proportionally to the amount of salt added to the nutrient solution (Table 2). In both PaWS and PaCT, the increase in hexose concentration was linear over salt treatment, attaining values about three times as high as those in control plants, particularly at day 10 (data not shown). Sucrose concentration was also affected by salinity where sucrose of PvWT and PvCS decreased linearly with salt treatments (Table 2). In PaWS and PaCT, however, no significant differences were detected under both salinity levels.

Table 1: Effects of external NaCl concentrations on mineral composition of leaves, stem, and roots of *Phaseolus* species.

Species/Genotype	Leaves			Stem			Root		
NaCl (mM)	mmol kg^{-1} dry weight								
	Na^+	K^+	Cl^-	Na^+	K^+	Cl^-	Na^+	K^+	Cl^-
P. vulgaris PvWT									
0	17 c[z]	1382 a	9 c	27 c	1308 a	16 c	20 b	1302 a	29 c
60	13 b	1021 b	1078 b	222 b	1077 b	658 b	423 a	838 b	663 b
90	159 a	747 c	2153 a	285 a	831 c	1342 a	460 a	778 b	999 a
P. vulgaris PvCS									
0	20 b	1289 a	2 c	22 b	2267 a	2 c	21 c	1403 a	25 c
60	143 a	849 b	767 b	276 a	1527 b	672 b	321 b	806 b	481 b
90	154 a	727 b	1895 a	268 a	1272 c	1262 a	480 a	703 b	879 a
P. acutifolius PaWS									
0	19 c	1430 a	23 a	24 c	1428 a	32 c	18 b	1773 a	45 c
60	166 b	1213 b	933 b	257 b	877 b	443 b	377 a	901 b	452 b
90	223 a	1032 b	1889 c	309 a	704 c	880 a	447 a	495 c	1040 a
P. acutifolius PaCT									
0	14 c	1222 a	22 c	22 b	1188 a	26 c	15 b	2158 a	34 c
60	90 b	952 b	1109 b	289 a	1073 a	418 b	349 a	905 b	630 b
90	267 a	926 b	2201 a	256 a	612 b	843 a	371 a	788 b	965 a
F-values from ANOVA									
NaCl	470***	112***	79***	352***	89*	37***	308***	118***	1111***
Species	12***	17***	13***	0.1[ns]	109***	18***	7**	13.6***	15***
NaCl × Species	29***	3.3**	3.5**	5.1**	7.7***	6.9***	4.7**	11.2***	9.1**

[z]Values are means of six replicates after 20 days of salt exposure. Differences among treatments at $P \leq 0.05$ are given according to Duncan multiple range test. ns: not significant, *, **, *** Significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

Saline-induced changes in soluble carbohydrate concentration were also highly dependent upon the species and plant organ (Table 2). Hexose accumulation on leaves and stems occurred during the first 10 days of salinization in all species (data not shown). However, these accumulations decreased over time in PvWT and PvCS, with the lowest content at day 20 (Table 2). In PaWS and PaCT, similar hexose concentrations were found in leaves and stems in both salt treatments, with the highest concentration at day 20 (Table 2). Hexose concentration in roots also increased with salinity in all species except for PvCS. Sucrose concentration sharply increased in relation to stress intensity and duration in the leaves and stems in PaWS and PaCT at day 15 (data not shown), and remaining constant for the remainder of the study (Table 2). On PvCS, however, sucrose accumulation decreased with time and salinity stress.

3.5. Plant Water Relations. Differences in leaf turgor, water, and osmotic potentials among genotypes were statistically significant at all salt concentration ($P \leq 0.0001$) (Figure 4). All genotypes except PvWS had significantly higher leaf water potentials at increasing NaCl concentrations for most of the experimental period ($P \leq 0.0001$) (Figure 4). Differences on leaf osmotic potential among genotypes were observed only for high salinity at day 20. Generally, the leaf osmotic potential decreased as salt level increased. Osmotic potential of all species ranged from −0.92 to −1.4 MPa for salt treatments. Leaf turgor potential increased between 0.47 and 0.95 MPa at 60 mM NaCl and between 0.70 and 1.2 MPa at 90 mM NaCl (Figure 4).

At the end of the experiment, all genotypes showed significant differences between control and salt-stressed plants in ions and soluble carbohydrates to Ψ_π (Table 3). Among the genotypes, PaWS had the lowest Ψ_π due to ions in both control and high salt stress (Table 3). This genotype had the highest leaf K^+ and Na^+ concentration in the control and salt treatments (Table 1), which contributed to its having the lowest Ψ_π (Table 3). In all genotypes, inorganic ions accounted for approximately 60% of total Ψ_π in control plants. With salt treatment (60 and 90 mM NaCl), the relative contribution from ions remained near 71 and 82% for the salt-tolerant genotypes, whereas it accounted to about 60 and 85% for the salt-sensitive genotypes.

In regard to the degree of osmotic adjustment due to the three ions (Na^+, K^+, and Cl^-), the salt-sensitive genotypes PaWS showed the highest increase in Ψ_π due to ions, 0.32 MPa (Table 3). The other salt-sensitive genotype, PvCS, was quite different, and had the least increase in Ψ_π due to ions, 0.16 MPa. In the salt-sensitive genotype PvCS, the osmotic adjustment due to ions made up about half the total osmotic adjustment (48%), whereas it accounted to about 76% in PaWS (Table 3). In contrast, in salt-tolerant genotypes, the change in Ψ_π due to ions was lower than the change in total Ψ_π (Table 3). Thus, ions made up 80% of the total osmotic adjustment. The salt-induced soluble

Table 2: Effects of external NaCl concentrations on glucose (Glu), fructose (Fru), and sucrose (Suc) of leaves, stem, and roots of *Phaseolus* species.

Species/Genotype	Leaves			Stem			Roots		
NaCl (mM)	mmol kg^{-1} dry weight								
	Glu	Fru	Suc	Glu	Fru	Suc	Glu	Fru	Suc
P. vulgaris PvWT									
0	4 b[z]	5 a	46 a	10 a	15 b	64 a	5 c	8 b	32 b
60	8 a	14 b	38 b	10 a	21 a	51 b	9 b	26 a	38 a
90	1 c	1 c	4 c	3 b	3 c	6 c	13 a	30 a	35 a
P. vulgaris PvCS									
0	6 b	7 b	42 a	19 b	25 b	50 a	5 b	8 b	50 a
60	17 a	22 a	33 b	36 a	44 a	39 b	16 a	34 a	27 b
90	2 c	2 b	3 c	11 b	15 c	6 c	2 b	4 b	4 c
P. acutifolius PaWS									
0	12 c	12 b	41 a	12 b	13 c	47 a	4 c	12 c	48 a
60	22 b	28 a	31 b	15 b	22 b	50 a	10 b	35 b	32 b
90	25 a	29 a	36 b	22 a	31 a	44 a	14 a	50 a	37 b
P. acutifolius PaCT									
0	10 b	12 b	34 a	22 a	24 a	47 a	8 b	15 c	51 a
60	24 a	32 a	30 b	21 a	25 a	38 b	8 b	33 b	30 c
90	24 a	31 a	29 b	21 a	26 a	41 b	15 a	52 a	38 b
F-values from ANOVA									
NaCl	56.9***	57.4***	576***	3.9[ns]	8.8*	261.9***	49.8***	91.2***	77.7***
Species	11.8***	8.5***	2.6*	15.3***	5.4***	9.1***	4.2**	13.3***	4.7**
NaCl × Species	2.3*	1.8[ns]	6.7***	6.7***	3.5**	16.4***	16.2***	9.5***	6.9***

[z]Values are means of six replicates after 20 days of salt exposure. Differences among treatments at $P \leq 0.05$ are given according to Duncan multiple range test. ns: not significant, *, **, *** Significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

carbohydrate accumulation had a small contribution to leaf osmotic adjustment potential. Their contribution to the Ψ_π did not change with the increase in NaCl concentration (9 to 14%), as the increase in leaf soluble carbohydrate content was proportional to the increase in leaf osmolality. Thus, other solutes appear to have decreased.

4. Discussion

Water status is highly sensitive to salinity and is, therefore, a dominant factor in determining plant responses to salt stress [1]. The results clearly showed that water relations of salt-tolerant genotypes were the same as salt-sensitive genotypes and, the genotype with the greatest osmotic adjustment was one of low salt tolerant (PaWS). There was no statistical difference for genotypes in the degree of ion accumulation and osmotic adjustment. Although the greatest change in total osmotic potential occurred in the genotype PaWS which also showed the greatest change in ion accumulation (K^+ and Na^+) (Table 1), the genotype showing the second greatest change in total osmotic adjustment (PvCS) had the least change in ion accumulation. In both PaWS and PvCS, the change in total osmotic potential was lower than that calculated for the ions, so other solutes must have decreased in concentrations in the salt-treated plants. This indicates that NaCl did not limit osmotic adjustment in salt-stressed plants and other organic solutes play an important part, independently, despite the higher energy costs for their synthesis [13].

The salt-induced soluble sugar accumulation had a small contribution to leaf osmotic potential (Table 3). The net increase in soluble carbohydrate fractions contributed about 14% of the measured decrease in leaf osmotic potential. Salinity increased carbohydrate content in leaves of *P. acutifolius* (PaWS and PaCT) (Table 2) but had a limited contribution to osmotic adjustment. Therefore, the hypothesis that assigns soluble carbohydrates a role in maintaining high turgor potential in leaves of *Phaseolus* species under prolonged stress can be dismissed. Despite the contrasting information found in the literature on the role of carbohydrates as osmolytes [34, 35], data presented in this work together with previous research [13, 23, 36] seem to indicate that the osmotic adjustment in *Phaseolus* species under salt stress is mostly dependent upon accumulation of inorganic ions. The important reductions in hexoses and sucrose in leaves of *P. vulgaris* (PvCS) (Table 2) proportional to the degree of salinization could be a consequence of the decrease in CO_2 assimilation (Figure 3) and might account for the impairment in plant growth and metabolism generally found in response to salt stress.

Although there were not genotypic differences in soluble carbohydrate contribution to the Ψ_π, the accumulation of

Table 3: Leaf water relations (MPa) of *Phaseolus* species after 20 days salt exposure[z].

Genotypes	Ψ_π	Ψ_π salt (mM NaCl)		$\Psi_{\pi s60} - \Psi_{\pi C}$	$\Psi_{\pi s90} - \Psi_{\pi C}$
	Control	60	90		
PvWT	−0.94 ± 0.13	−1.11 ± 0.11	−1.21 ± 0.12	0.18 ± 0.04	0.27 ± 0.04
PvCS	−0.88 ± 0.06	−1.13 ± 0.12	−1.22 ± 0.13	0.25 ± 0.05	0.34 ± 0.07
PaWS	−0.97 ± 0.12	−1.21 ± 0.11	−1.39 ± 0.15	0.20 ± 0.04	0.42 ± 0.06
PaCT	−0.87 ± 0.05	−1.10 ± 0.10	−1.18 ± 0.11	0.23 ± 0.03	0.31 ± 0.05
Na + K + Cl					
PvWT	−0.69 ± 0.07	−0.78 ± 0.11	−0.90 ± 0.10	0.09 ± 0.01	0.20 ± 0.05
PvCS	−0.63 ± 0.06	−0.69 ± 0.08	−0.79 ± 0.08	0.07 ± 0.02	0.16 ± 0.03
PaWS	−0.59 ± 0.05	−0.70 ± 0.07	−0.91 ± 0.12	0.11 ± 0.05	0.32 ± 0.09
PaCT	−0.50 ± 0.04	−0.63 ± 0.07	−0.76 ± 0.08	0.13 ± 0.05	0.26 ± 0.08
Glu + Fru + Suc					
PvWT	−0.09 ± 0.03	−0.06 ± 0.02	−0.05 ± 0.02	0.03 ± 0.01	0.04 ± 0.01
PvCS	−0.08 ± 0.02	−0.10 ± 0.04	−0.06 ± 0.02	0.02 ± 0.01	0.02 ± 0.01
PaWS	−0.04 ± 0.01	−0.07 ± 0.03	−0.06 ± 0.02	0.03 ± 0.01	0.02 ± 0.01
PaCT	−0.05 ± 0.01	−0.09 ± 0.04	−0.03 ± 0.01	0.04 ± 0.02	0.02 ± 0.01

[z]Data are for osmotic potential at full turgor (Ψ_π) of control and salt treatments, the degree of osmotic adjustment (($\Psi_{\pi s} - \Psi_{\pi C}$), as the difference of Ψ_π between the salt treatments and control plants), the contribution of Na^+, K^+, and Cl^- and glucose (Glu), Fructose (Fru) and Sucrose (Suc) to Ψ_π at 20 d. Values show means ± s.e. ($n = 6$).

sugars appears to be a common response in *P. acutifolius* (PaCT and PaWS) genotypes when grown under osmotic stress (Table 2). A similar finding in a comparison of rice varieties was reported by Cha-Um et al. [37]. They found that the total soluble sugar content in leaf and root tissues of salt-tolerant rice variety was higher than in the salt-sensitive variety, and that sugars enhance resistance to salt-induced osmotic stress in rice plants. Accumulated soluble sugars including glucose, fructose, and sucrose in the leaf tissues may function as osmoregulant solutes stabilizing photosynthetic pigments and maintaining electron transport functions during light reaction, and O_2 assimilation during dark reaction of photosynthesis. Sugars play a key role in the adaptive processes linked with NaCl-tolerance, such as Na^+ and Cl^- translocation and/or compartmentation, solute synthesis for growth, osmotic adjustment, and protein turn-over [38]. Sucrose has been shown to reduce oxygen activity of *Rubisco* during salt stress [38] and might be of primary importance in antioxidative mechanisms [14]. Further attention to determine if *P. acutifolius* has better osmoprotective functions or more efficient mechanisms to regulate photosynthetic rate parameters should be given.

There were significant differences between genotypes in their growth response to salinity. For salt-tolerant genotypes, RGR was reduced by salinity only in the first period (10–15 d) after salt treatment. After 15 d there was no significant difference in RGR between control and salt treatments. However, for salt-sensitive genotypes, the treatment differences in RGR were steady with time. Similar results were found by Rivelli et al. [39] for wheat grown at 150 mM NaCl for 30 d. The authors found that the greater effect on RGR occurred within the first 10 d of treatment, after which the difference between treatments largely disappeared. However, in an experiment with barley, the treatment differences in RGR were steady with time, over a 9-week period, RGR averaged 0.13, 0.09 and 0.09 for the 0, 100 and 175 mM NaCl treatments, respectively [40].

At the whole-plant level, decreases observed in RGR could be attributed to a photosynthetic response (ULR) and/or morphological changes (LAR), depending on the species [19]. Results confirm that decreases on RGR for salt-sensitive genotypes were related to ULR ($r^2 = 0.95$), indicating that the reduced growth in these species under high salinity was primarily as a result of a decline in leaf photosynthetic rate, as indicated by the lower stomatal conductance ($r^2 = 0.62$). These results support those reported by Romero and Marañón [17] and Bayuelo-Jiménez et al. [23], where the ULR was also found to be highly correlated with RGR for salt-stressed barley and beans, respectively.

In both salt-tolerant and salt-sensitive genotypes any decrease in SLA was consistently associated with a decline in LAR ($r^2 = 0.94$), and consequently in RGR (Figure 2). Growth of salt-tolerant genotypes was affected by a reduced leaf area expansion (smaller and thicker leaves) rather than impairment on CO_2 assimilatory capacity. In salt-sensitive genotypes, however the lower SLA may reflect an overloading of the leaves by inorganic (Cl^-; $r^2 = 0.85$ to $r^2 = 0.98$) solutes, which allow osmotic adjustment but decreased photosynthetic return per unit leaf mass.

Specific leaf area (SLA) is a variable associated with a number of functional aspects of plant physiology, including gas exchange and relative growth rate [19]. Generally, evidence shows that salinity increases the leaf lamina thickness, due to an increase in mesophyll cell size or number of layers [41, 42]. Such salt-induced succulence could lower the resistance to CO_2 uptake and thus increase photosynthetic rates by increasing the amount of internal leaf surface area across which gaseous exchange can occur per unit of

leaf area [41]. We suggest that the lower SLA of the salt-tolerant genotypes may reflect an increase in mesophyll thickness and the internal surface area for CO_2 absorption, which probably compensates for any stomatal assimilation limitation. Although the role of stomatal and mesophyll resistance in controlling the CO_2 diffusion resistance has yet to be proven in salt-tolerant *Phaseolus* species, the proven effects of changes in salt-sensitive *P. vulgaris* is restricted to stomatal conductance [41].

The decrease in conductance in salt-stressed plants could be due to chemical signals coming from the roots or reduced shoot water content [43]. Our data indicate that decreased conductance was not due to leaf water deficit since the calculated turgor was not reduced by salinity, whereas conductance was (Figure 4). This suggests hormonal control originating from the roots [43]. According to Rivelly et al. [39], values of carbon isotope discrimination (Δ) measured on expanding leaf tissue of wheat genotypes was substantially lower for salt-stressed plants than for control plants. This indicates that the effects of salinity on stomatal conductance were greater than effects on photosynthetic capacity. Thus, the reduction of the photosynthetic capacity of salt-tolerant *Phaseolus* species may not reflect an apparent damage to photochemistry and chlorophyll concentrations; however, more information is necessary for conclusion on this point.

It has also been proposed that the reduction of A_n in response to salinity is due to an increase on Na^+ and Cl^- leaf contents [18, 35, 36]. However, other authors reported associated reductions in A_n and g_s with K^+ deficiency [44]. Because salt stress impairs K^+ uptake of plants, it has been suggested that K^+ deficiency might be a contributing factor to salt-induced oxidative stress and related cell damage. Due to impairment in: (1) stomata regulation, (2) conversion of light energy into chemical energy, and (3) phloem transport of photosynthates from leaves into sink organs, photosynthetic CO_2 fixation is limited [44]. It is therefore possible that in salt-sensitive *Phaseolus* species potassium deficiency combined with salt stress induced a reduction in CO_2 photo assimilation and stomata closure (Figure 3).

The results also showed that high foliar concentrations of Cl^- were related with reduced A_n in PaWS ($r^2 = 0.66$) and PvCS ($r^2 = 0.83$) genotypes. It is interesting that leaf Cl^- concentrations of salt-tolerant PvWT and PaCT remained relatively high but did not inhibit photosynthesis (Figure 3). This seems to exclude the possibility that more intense inhibition of leaf growth expansion in salt stressed of these species was caused by Cl^- toxicity on leaves and/or photosynthesis inhibition. Cl^- is an important inorganic ion and might also play key roles in osmotic adjustment. For example, Shabala et al. [45] suggested a role of the hyperosmolarity induced influx of K^+ and Cl^- in plant (e.g., bean) cells that could be sufficient for osmotic adjustment without additional accumulation of organic solutes. Under conditions of saline stress, excess concentration of Cl^- occurs in plants, and the Cl^- channel might be involved in change of cellular Cl^- content for osmotic adjustment.

Osmotic adjustment and turgor maintenance were achieved by inorganic ions uptake, but an imbalance of essential nutrients may also be a factor contributing to the salt-induced decrease in leaf function and consequently in plant growth [1]. Excess NaCl in the external solution induced a reduction in the vegetative growth of salt-sensitive genotypes, which correlated with the accumulation of Cl^- in plant tissues (Table 1). The addition of salt ions (Na^+ and Cl^-) to the nutrient solution was reflected in higher absorption rates of these ions by the plant. However, chloride absorption was higher for salt-sensitive PvCS than for all other species, probably due to the higher proportion of young root zones [25].

Plant growth can be stimulated by low concentrations of sodium, mainly as a result of the effects of Na^+ on cell expansion and cell water balance [7]. Na^+ transport from root to shoot seemed to be more strongly inhibited than absorption, as deduced from the higher concentrations of salt ions measured in roots than in foliar tissues of all *Phaseolus* species (Table 1). On the other hand, the toxic effects of salt ions and/or a deficiency of particular nutrients may inhibit plant growth [4]. Chloride, the other salt ion, has a high mobility within the plant and affects processes related to charge compensation and osmoregulation [1]. For salt-sensitive genotypes, a greater concentration of Cl^- in leaves was associated with reduced plant growth (Figure 2).

A decrease in potassium accumulation in salt-stressed plants appears to be one of the most widespread responses associated with reduced growth [17, 44]. Salinity affected the potassium accumulation in the vegetative phase, probably by significantly reducing the absorption of potassium in roots (Table 1). The K^+ concentration fell continuously in roots and stems of salt-stressed species, while the leaves had similar concentrations to those in control plants at the medium salt stress levels, suggesting a compensation over time, probably by translocation of K^+ from roots and stems to leaves [17], a sustained acquisition despite appreciable overall Na^+ uptake [23], and/or a high K^+ selectivity and/or K^+/Na^+ exchange across the plasmalemma of the root epidermis [6, 9, 10]. The ability to withdraw Na^+ and to retranslocate K^+ seems crucial for salt tolerance [10, 11]. Therefore, the maintenance of higher leaf K^+ concentrations in PvWT could be an important mechanisms underlying superior salt tolerance reported in *P. filiformis* [23] and barley (*H. vulgare* L.) [10].

Legumes are a key component of sustainable agriculture and can offer many economic and environmental benefits if grown more widely in crop rotations because of their ability to fix nitrogen in the root nodules in a symbiotic interaction with soil rhizobia. Due to their capacity to grow on nitrogen-poor soils, they can be efficiently used for improving saline soil fertility and help to reintroduce agriculture to these lands [46, 47]. However, in legumes, salt stress imposes a significant limitation of productivity related to the adverse effects on the growth of the host plants, the root nodule bacteria, symbiotic development, and the nitrogen fixation capacity [46].

Possible approaches to improve productivity under saline stress conditions require a better understanding of the physiological and molecular mechanisms involved in the response to salt stress. These mechanisms include (1) exclusion of Na^+ and Cl^- from plant tissue, (2) inclusion of these ions in inert compartments or tissues, and/or (3) some means

of osmotic adjustment with solutes that are compatible with the metabolic machinery of the cell [46]. Conventional plant breeding based on yield in target environments has increased production; however, physiologically based approaches utilizing molecular tools to identify key genes or provide molecular markers have the potential to take it further [47]. Accurate and selective phenotyping will enable to best use of mechanistic molecular understanding of plant responses to salinity, and mechanisms of adaptation [1].

5. Conclusions

From the present study, we conclude that salt-induced growth reductions in salt-sensitive *Phaseolus* species during vegetative growth are due to a decrease in the specific activity of the leaves (ULR). In contrast, a reduced leaf area expansion per unit of plant biomass (LAR), primarily caused by a decrease in SLA, played an important role in determining RGR of salt-tolerant species. The lower ULR of salt-sensitive species may be a result of decreased photosynthesis due to a decreased leaf water vapor conductance. Leaf water relations, however, seem unlikely to be a growth-limiting factor in *Phaseolus* species. There was little difference between genotypes in the effect of salinity on water relations, as indicated by the estimated turgor. Osmotic adjustment occurred in all *Phaseolus* species, with one of the low salt tolerance genotypes having the greatest osmotic adjustment. A higher level of soluble carbohydrates was found in salt-tolerant species. However, the salt-induced soluble sugar accumulation does not play a significant role in defense against osmotic stress conditions. Salt-sensitive *Phaseolus* species are Na^+ excluders and maintained turgor-driven extension growth by accumulating Cl^- (osmotic adjustment), but subsequent weight gain reductions suggest that this led to ion toxicity.

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