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Understanding osmotic stress tolerance in leaves and nodules of two *Phaseolus vulgaris* cultivars with contrasting drought tolerance

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Abstract Drought and salinity are environmental constraints that affect crop yields worldwide. In nature, both stresses are multifaceted problems that are usually associated with other adverse circumstances which limit plant performance such as water shortage and nutrient deficits. In order to assess common features of both stresses, the effects of mannitol-induced osmotic stress were monitored using two *Phaseolus vulgaris* cultivars, Cv. 'Flamingo' (tolerant) and Cv. 'Coco Blanc' (sensitive) which differed in their drought and salinity tolerance. Growth, water relations, organic and inorganic compound accumulation and soluble protein contents were measured in leaves and nodules of these N_2-fixing plants. The aim of the present study was to check whether osmotic stress tolerance is associated with accumulation of some of these compounds either in leaves, nodules or both organs. At the whole-plant level, Cv. 'Flamingo' showed a better maintenance of plant biomass and shoot water status. At the cell level, this was related to a better osmotic adjustment ability both in leaves and nodules and also to a better adjustment of the cell wall elasticity. At the metabolic level, the contrasting accumulation of the different amino acids in nodules of each cultivar suggested that amino acids pathways can be regulated to different degrees under stress conditions. At the metabolic level, it seems that symbiosis in the sink organ (the nodule) plays a crucial role in conferring drought and salinity tolerance in the common bean.

Keywords Common bean · Osmotic adjustment · Mannitol · Carbon compounds · Nitrogen compounds · Symbiotic nitrogen fixation

Abbreviations

ε	Bulk modulus of elasticity
Ψ_o	Osmotic potential
Ψ_p	Turgor potential
Ψ_w	Water potential
CE	Capillary electrophoresis
DAS	Days after seedling emergence
DW	Dry weight
FW	Fresh weight
RWC	Relative water content
SOA	Sum of organic acids
TAA	Total amino acids content
TSS	Total soluble sugars

1 Introduction

Although beans and other legumes are basic staples in the human diet particularly in tropical and subtropical areas, their production and availability are not sufficient. A main reason is the increasing size of arid zones as well as adverse climatic and agronomic conditions such as drought and salinity in those regions (Barron and De-Mejia 1998). As much as 60% of bean production in the developing world occurs under conditions of significant drought stress (Graham and Ranalli 1997). Such environmental stresses limit crop growth and productivity by imposing osmotic stress on plants. Different plants use different strategies to counteract osmotic stresses

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(salinity and drought). The ability of plants to cope with osmotic stress is an important determinant of crop distribution and productivity so it is important to understand the mechanisms that confer tolerance.

Osmotic adjustment is one of the important mechanisms that alleviate the detrimental effects of water stress. Plants can readjust their osmotic potential to prevent water losses and protect their metabolic function. This is achieved either by the uptake of inorganic ions from the external solution or by de novo synthesis of compatible solutes (Shabala et al. 2000; Manchanda and Garg 2008). The reduction in osmotic and water potential can be correlated with the accumulation of solutes such as cations (Almansouri et al. 2000; Javed 2002), anions (Cl^- and SO_4^{2-}) (Almansouri et al. 2000; Javed 2002), free proline content (Al-Khayri 2002; Al-Khayri and Al-Bahrany 2002), soluble carbohydrates (Liu and Staden 2001; Javed 2002), phenols (Gonzalez and Garcia 1988) and amino acids (Gilbert et al. 1998). Amino acids can play an important role in plant stress tolerance serving as readily available energy sources, as nitrogen source during limited growth and photosynthesis, as detoxification mechanisms to deal with excess ammonia under periods of stress or as stabilizers of enzymes and/or membranes (Gilbert et al. 1998). Finally they may help to remove active oxygen species and regulate intracellular pH (Ronde et al. 1999; Alia et al. 2001). The accumulation of solutes leads to a reduction in osmotic potential at cellular level and hence helps plants to maintain growth under stressful environment. However, organic solute accumulation and inorganic uptake differ significantly in their time scale: Immediate changes in ion uptake are believed to provide quick osmotic adjustment while biochemical synthesis of solutes occurs more slowly as the stress progresses (Hu and Schmidhalter 2005; Lobato et al. 2008; Munns and Tester 2008).

Differential tolerance of the cultivars, Cv. 'Flamingo' (tolerant) and Cv. 'Coco Blanc' (sensitive) was demonstrated (Sassi et al. 2008a) These authors showed that nodule carbon metabolism and antioxidant defence was involved in the better stress performance of Cv. 'Flamingo' (Sassi et al. 2008b). However, no detailed study was done at the whole plant level to understand the molecular basis of stress tolerance better in *Phaseolus vulgaris*. The present study, using these two cultivars, aimed to investigate whether osmotic stress tolerance is associated with an osmotic adjustment strategy.

2 Material and methods

2.1 Plant material and culture conditions

Seeds of two cultivars of common bean (*Phaseolus vulgaris* L.), Cv. 'Coco Blanc' and Cv. 'Flamingo', were selected. Cv. 'Coco Blanc' originated from local bean populations in Tunisia and Cv. 'Flamingo', originated in Colombia and was provided by CIAT. Seeds were surface sterilized in 80% ethanol for 30 s, 5% sodium hypochlorite for 2 min and washed ten times in sterilized distilled water. Sterilised seeds were pre-germinated in agar (0.9%). They were transferred to 1 L glass bottles wrapped with aluminium foil to maintain darkness in the rooting environment. The roots of selected uniform seedlings were gently passed through the hole of a rubber stopper on the bottle neck, and a cotton wool was fitted at the hypocotyl level to maintain the root system suspended in the nutrient solution. The latter contained 0.25 mM KH_2PO_4, 0.7 mM K_2SO_4, 1 mM $MgSO_47H_2O$, 1.65 mM $CaCl_2$, 22.5 μM Fe for macronutrients, and 6.6 μM Mn, 4 μM Bo, 1.5 μM Cu, 1.5 μM Zn, 0.1 μM Mo for micronutrients. For the first week, the nutrient solution was supplemented with 2 mM urea, and thereafter was renewed every 2 weeks without urea. Seeds were inoculated with 1 mL of *Rhizobium tropici* CIAT 899 containing approximately 10^8 cells mL^{-1} when transferred to bottles. The pH of the nutrient solution was maintained near neutrality by adding 0.2 g L^{-1} $CaCO_3$. It was aerated with a flow of 400 mL min^{-1} of filtered air via a compressor and "spaghetti tube" distribution system. Plants were grown in a temperature-controlled glasshouse with night/day temperatures of circa 20/28°C and a 16 h photoperiod with additional lights of 400 μmol m^{-2} s^{-1} (PPF).

2.2 Osmotic treatment

Osmotic stress was applied by means of 50 mM mannitol, an osmotic component used generally to generate water deficit stress when added to nutrient solution. This mannitol concentration was equivalent to a −0.15 MPa soil water potential (Ψ_w). Mannitol was progressively added as 25 and 50 mM at 15 and 18 days after seedlings emergence (DAS) in order to avoid any osmotic shock. This corresponds to the initial period of nodule formation. At the beginning of flowering, 30 DAS, plants were harvested for growth and water status parameters were determined. Leaves and nodules were frozen and stored at −80°C for further analysis.

2.3 Dry weight determination

After harvest, leaves, stems and roots were separated and weighed. Nodules from each individual root were collected, weighed and their number was determined. Dry weights (DW) of the different organs were determined after drying for 3 days at 70°C.

2.4 Water relations

Leaf and nodule water content was calculated as (FW−DW)/DW, where FW and DW represent the fresh and dry weight,

respectively. The relative water content of leaves and nodules (RWC) was determined as RWC = (FW−DW) (TW−DW)$^{-1}$, where TW is turgid weight obtained after incubation in deionized water, at 4°C for 16 h and 4 h for nodules and leaves respectively.

Leaf water potential was measured in fully expanded mature leaves at the end of the osmotic stress period using a pressure chamber (Soil Moisture Equipments Corp., Santa Barbara, CA, USA) according to Scholander et al. (1965).

The bulk modulus of elasticity (ε) was obtained from the initial part of the P-V curve:

$$\varepsilon = \frac{d\Psi_p}{d\,RWC} RWC_{sym}$$

describing the decrease of turgor potential (Ψ_p) with the relative symplastic water content (RWCsym) (Tyree and Jarvis 1982).

Osmotic potential (Ψ_o) was determined with a vapor pressure osmometer (Wescor Inc. 5500, Logan, UT).

2.5 Carbohydrate extraction and determinations

Fresh material was exhaustively extracted in boiling 80% (*v*/*v*) ethanol. Ethanol-soluble extracts were dried in a Turbovap LV evaporator (Zymark Corp, Hopkinton, MA, USA) and soluble compounds were redissolved with 4 ml of distilled water, mixed and centrifuged at 20,000 g for 10 min. Sucrose and mannitol were analyzed in the aqueous phase by high-performance capillary electrophoresis (CE) in a Beckman Coulter PACE system 5500 (Beckman Instruments, Fullerton, CA, USA) as described by Marino et al. (2006).

2.6 Total soluble sugars determination

Total soluble sugars were quantified using the anthrone method (see Aydi et al. 2010). The 20 mg DW homogenate in deionized water was incubated in a water bath at 70°C then centrifuged at 3,000 g for 10 min. 100 μL of the supernatant was added to 4 ml of anthrone solution and incubated in a boiling water bath. The absorbance of the samples was determined spectrophotometrically according to Aydi et al. (2010).

2.7 Determination of organic acids and inorganic ions

Organic acid content (malate, α-ketoglutarate, oxalate and citrate) and inorganic ion content (Na^+, K^+, Cl^-, Mg^{2+}, SO_4^{2-}, Ca^{2+}, NH_4^+) was determined by ion chromatography in a DX-500 system (Dionex, Salt Lake City, UT, USA) by gradient separation with a Dionex IonPac AS11 column as described in Gálvez et al. (2005).

2.8 Amino acid analysis

Frozen nodules and leaves were crushed with liquid nitrogen to a fine powder and subsequently homogenized with HCl 1 M. The homogenate was incubated 10 min at 4°C and centrifuged (20,000 g, 4°C, 10 min). The supernatant was neutralized with NaOH to pH 7–9 and internal standards (norvaline and homoglutamic acid) were added. Amino acids were derivatised with FITC (fluorescein isotiocianate) 1 mM dissolved in acetone/borate 20 mM pH 10. Single amino acid content was determined by CE in a Beckman-Coulter PA-800 system. The applied potential was −20 kV, and the capillary tubing was 50 μM i.d. and 31.4/38.4 cm long. The background buffer was 80 mM borax, 45 mM ∝-ciclodextrine, pH 9.2. Total amino acid content is presented as the sum of single amino acids for each sample, and expressed in a DW basis.

2.9 Statistical analysis

Replicate numbers for each analysis are given in the legends to figures and tables. Results were examined by two-way (cultivar and mannitol treatment) analysis of variance. Least significant difference (LSD) test between means were used to determine the significance ($P<0.05$) of data.

3 Results

3.1 Growth parameters

Root and shoot growth was assessed by measuring DW at the end of the treatment period (30 days) (Fig. 1). In controls no significant differences were detected in shoots, roots and nodules biomass between the cultivars. Mannitol-induced osmotic stress reduced plant growth in both cultivars. However, this depressive effect was more harmful on the sensitive cultivar (Cv. 'Coco Blanc'). Shoot growth reduction exceeded 50% in stressed Cv. 'Coco Blanc' plants while it did not exceed 40% in Cv. 'Flamingo' stressed plants (Fig. 1a). There was no significant change in root DW in Cv. 'Flamingo' as a consequence of osmotic stress (Fig. 1b), while reduction exceeded 40% in Cv. 'Coco Blanc'. Nodules biomass was also more dramatically affected in the susceptible cultivar where reduction reached 80% in nodular DW, whereas this was only 30% in Cv. 'Flamingo' (Fig. 1c).

3.2 Water status

In control leaves of both cultivars, RWC remained close to 80% (Fig. 2a). After 15 days of osmotic treatment, RWC was 65%

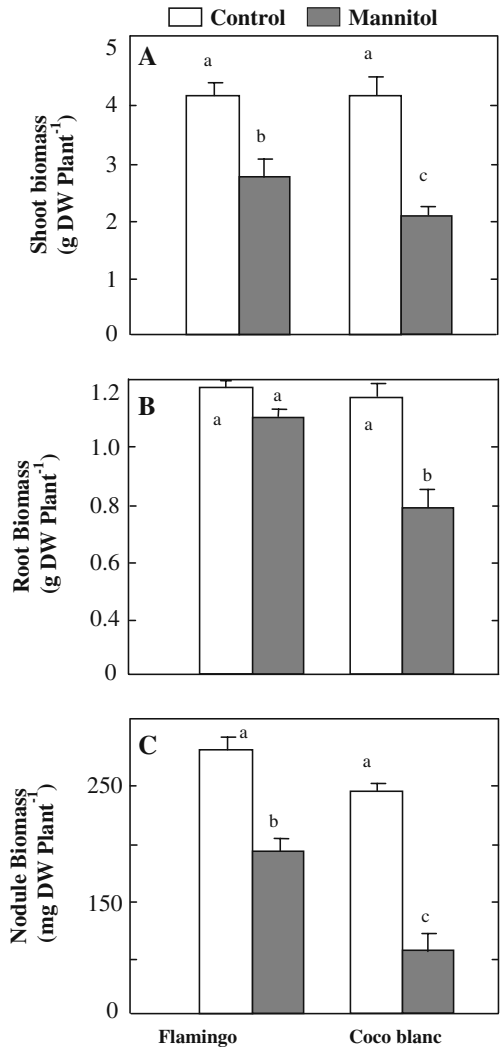

Fig. 1 Effect of mannitol-induced osmotic stress on Flamingo and Coco blanc bean cultivar growth parameters. Controls are represented by white bars, and osmotic stressed treatments as grey bars. **A** dry shoot biomass, **B** dry root biomass and **C** dry nodule biomass. Values represents mean ± SE (n=6). Numbers followed by a different letter within a panel are significantly different at $P \leq 0.05$ according to LSD analysis

in mannitol-treated plants of Cv. 'Flamingo', and only 45% in Cv. 'Coco Blanc'. These results indicate that osmotic stress caused an important reduction in shoot water supply. The same trend was observed in nodules (Fig. 2b). Indeed, data showed decreased nodule RWC in both stressed cultivars. This decrease was higher in Cv. 'Coco Blanc' treated nodules.

Leaf osmotic potential (Ψo) decreased in stressed plants in both cultivars. A minimum value of −2.3 MPa was reached in Cv. 'Flamingo' plants under mannitol-induced osmotic stress (Fig. 3a). Ψ_o decreased in stressed nodules, reaching −1.3 MPa in Cv. 'Coco Blanc' and −1.7 MPa in Cv. 'Flamingo' (Fig. 3b). Therefore, Cv. 'Flamingo' showed a better osmotic adjustment response to osmotic stress both in leaves and nodules.

3.3 Bulk modulus of elasticity (ε)

The bulk modulus of elasticity was significantly higher in stressed plants than in mannitol-free plants (Fig. 4). This reached about a 9-fold increase in Cv. 'Coco Blanc' and about a 17-fold increase in Cv. 'Flamingo' leaves.

3.4 Cations and anions distribution

Osmotic stress significantly decreased the sum of inorganic ions in nodules of stressed plants (Table 1). However, there was only a significant decrease in Cv. 'Coco Blanc' leaves. Reductions did not exceed 25% in Cv. 'Flamingo' leaves and nodules while it reached about 40% in comparable Cv. 'Coco Blanc' organs. In leaves, mannitol-induced reductions were more pronounced in Cv. 'Coco Blanc' in terms of cation content. By contrast, stressed leaves of Cv. 'Flamingo' maintained potassium, calcium and magnesium contents similar to controls. In Cv. 'Flamingo' nodules under mannitol-induced osmotic stress, calcium and sulphate contents decreased after treatment but levels of the other ions showed an increase (magnesium) or remained similar to controls (sodium, potassium, ammonium and chloride) (Table 1). In stressed nodules of Cv. 'Coco Blanc', however, there was a decrease in all the measured inorganic ions.

3.5 Carbon compounds

Under control conditions, Cv. 'Coco Blanc' nodules and leaves exhibited a lower total soluble sugar content (TSS) contents compared with Cv. 'Flamingo' (Table 2). Osmotic stress raised TSS content in leaves and nodules of Cv. 'Coco Blanc', representing 4.5-fold increase in stressed leaves and 1.5-fold increase in nodules. Stressed Cv. 'Coco Blanc' leaves also had a greater mannitol content, while the sucrose content remained the same as controls. No significant changes were observed in Cv. 'Flamingo' organs despite higher accumulation of mannitol in stressed leaves. These concentrations were well below of that of the stressed Cv. 'Coco Blanc' leaves (Table 2).

The sum of organic acids (SOA) was significantly reduced in Cv. 'Coco Blanc' leaves under osmotic stress. This represented a reduction of 42% compared with controls. However, in Cv. 'Flamingo' SOA increased to 115% of the control figure. The situation was mirrored by malate an important organic acid. Cv. 'Coco Blanc' showed a significant decrease in SOA in nodules compared with controls while no significant changes were observed in Cv. 'Flamingo' nodules. Again, malate was always the main organic acid (Table 2).

3.6 Nitrogen compounds

The total amino acids content (TAA) of leaves was similar in Cv. 'Flamingo' and Cv. 'Coco Blanc' under optimal

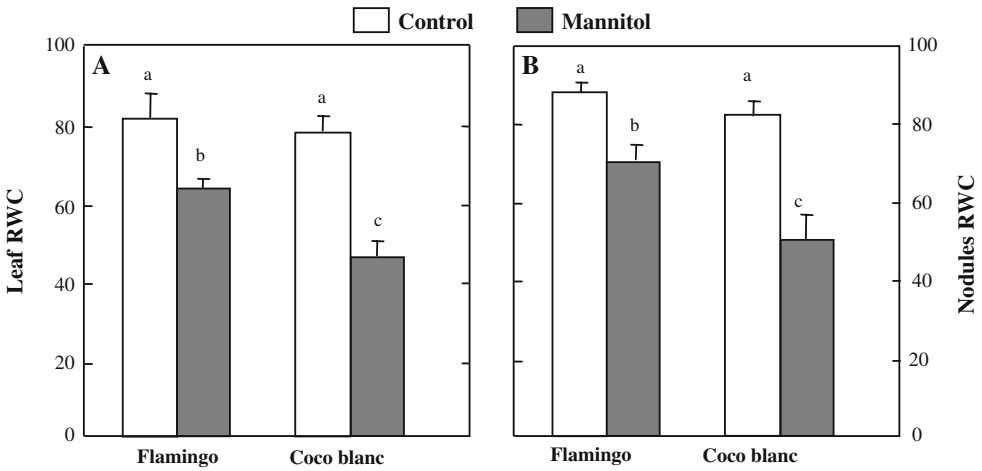

Fig. 2 Effect of mannitol-induced osmotic stress on relative water content (*RWC*) in Flamingo and Coco blanc bean cultivar leaves (**A**) and nodules (**B**). Values represents mean ± SE (n=6)

conditions. Mannitol treatment significantly reduced TAA levels by 33% in Cv. 'Flamingo' leaves and by 65% in Cv. 'Coco Blanc' leaves. Nodule TAA was raised 1.6-fold in Cv. 'Flamingo' following exposurer to mannitol but in Cv. 'Coco Blanc' no significant change occurred (Table 3).

A detailed analysis of the amino acid composition in both leaves and nodules revealed that the reductions of individual AA mirrored the decrease in TAA in stressed leaves (Table 3) with a few exceptions for minor AA and Trp, in particular. The increased TAA content in Cv. 'Flamingo' nodules was attributed to significantly higher contents of Gln, Leu, Trp, Ile, His, Arg, GABA, and Asp-Asn. However the non significant change in TAA in Cv. 'Coco Blanc' seems to hide many content variations. The data showed a dramatic accumulation of GABA and Ile, reaching 7- and 9-fold, respectively, and a slightly increased content of Val, Tyr, Phe and Trp, Pro, Trp. All these changes were associated with remarkable reductions (about 90%) in Gln, Glu and Asp-Asn contents. Total soluble protein exhibited about 25% reduction in leaves of both cultivars. Cv. 'Coco Blanc' was more affected in nodule tissues (−36%) than Cv. 'Flamingo' (−26%) (data not shown)

4 Discussion

4.1 Plant growth

Mannitol induced osmotic stress strongly restricted root and shoot growth despite the fact that the roots were relatively more tolerant (Fig. 1). Consequently, plants grown in mannitol exhibited a higher root/shoot DW ratio than plants growing in a mannitol-free medium. It should be noted that plant water uptake is maximised by adjusting the allocation pattern, namely the plants increase investment in roots (Jackson et al. 2000; Wu and Cosgrove 2000). This may facilitate adaptation to drought (Yin et al. 2005). The severe reduction of growth in the presence of mannitol could be related to a reduction in leaf gas exchange, which is known to be strongly dependent on the water status (Slama et al. 2007).

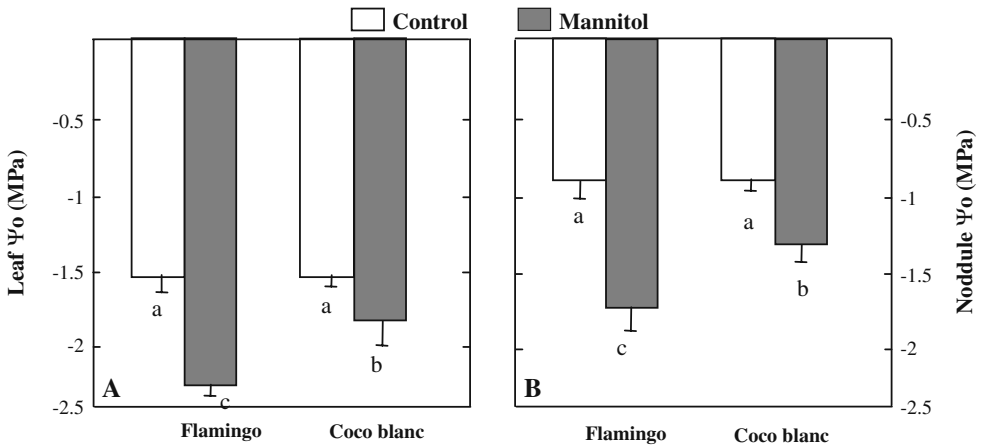

Fig. 3 Variation of osmotic potential (Ψ_o) in response to osmotic stress in leaves (**A**) and nodules (**B**) mediated by 50 mM mannitol. Values represents mean ± SE (n=6)

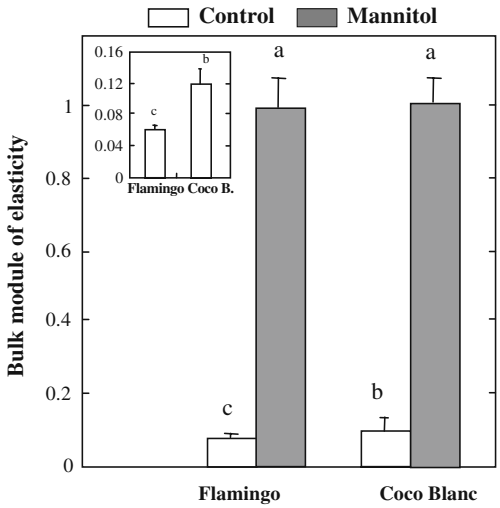

Fig. 4 Effect of mannitol induced osmotic stress (50 mM) on bulk module of elasticity. Controls are represented by white bars and grouped in the small histogram. Values represent the mean ± SE (n= 6). Numbers followed by a different letter within a panel are significantly different at $P \leq 0.05$ according to LSD analysis

4.2 Water relations

Mannitol-induced water deficit produced substantial dehydration that led to decreasing Ψ_o (Fig. 3). The decrease in Ψ_o is considered a potential mechanism of cellular drought resistance as it enables turgor maintenance and growth continuation (Bajji et al. 2000). Cv. 'Flamingo' exhibited lower Ψ_o under osmotic treatment. It was able to uptake more water and then grow more when exposed to decreased Ψ_o, thus it turned out to be a better drought tolerant cultivar than Cv. 'Coco Blanc' (Sassi et al. 2008a). This may be attributed to maintenance of the leaf and nodule water status under stressed conditions (Fig. 2). Several mechanisms could be involved in contributing to water retention. Increased osmotic adjustment is one important mechanism for alleviating the effects of water stress. Plants may readjust their Ψ_o to prevent water loss and protect their metabolic function. In addition, our results showed that the Cv. 'Flamingo' tolerance was associated with higher changes in cell wall elasticity (Fig. 4). An increase in ε (stiffness) is expected when the cell walls become more rigid or thicker. It has been shown that tolerance to low water potential may involve the production of a more rigid cell wall (Chaves et al. 2003). This together with osmotic adjustment is an effective means of counteracting the negative effects of osmotic stress (Navarro et al. 2007).

4.3 Components of osmotic adjustment

4.3.1 Contribution of inorganic acids

Osmotic adjustment can be achieved by the uptake of inorganic ions from the external solution as either cations (Almansouri et al. 2000; Javed 2002) or anions (Cl^- and SO_4^{2-}) (Almansouri et al. 2000; Javed 2002). The addition of mannitol to the nutrient solution resulted in decreased inorganic ions in both organs for both cultivars under osmotic conditions (Table 1). This behaviour was mirrored by K^+ representing the major cation under stressed and control conditions. The decrease of potassium availability to plants with decreasing soil water content is due to decreasing K^+ mobility (Hu and Schmidhalter 2005). Increased concentrations of inorganic ions as a consequence of water deficits have not been commonly found in the tissue of higher plants (Iannucci et al. 2002). This appears to be a response to short-term water stress treatment during the adjustment phase. Osmotic adjustment through K^+ ion uptake is more energy efficient than synthesis of new organic molecules (Hu and Schmidhalter 2005; Munns and Tester 2008).

Table 1 Effect of mannitol induced osmotic stress on inorganic ions content (μmol g^{-1} DW) in leaves and nodules of Flamingo and Coco blanc bean cultivars. Values represent mean ± SE (n=3)

Mannitol (mM)	Leaves				Nodules			
	Flamingo		Coco Blanc		Flamingo		Coco Blanc	
	0	50	0	50	0	50	0	50
Sodium	16.2±3.5 a	0.2±0.02 c	12.4±0.7 a	5.3±0.9 b	20±3.4 a	18.85±1.53 a	20.76±0.48 a	12.83±1.44 b
Potassium	395±8 a	313±4 a	395±8 a	136±9 b	343±15 a	334.4±17.4 a	388.7 ±58.6 a	181.7±0.0 b
Calcium	9±2.0 b	12±0.6 b	22±3.4 a	8±0.9 c	8±0.5 c	6±0.5 d	19±1.3 a	10±0.0 b
Magnesium	26±5 a	36±8 a	23±4 ab	9±1 b	27±2 b	38±10 a	13±1 c	3±1 d
Ammonium	5±1.5 a	3±0.3 ab	2±0.5 ab	2±0.1 b	6±1.2 a	5±1.3 a	7±0.9 a	3±0.5 b
Chloride	321±24 a	194±47 b	247±67 ab	242±7 ab	273±23 a	164±22 ab	210±20 ab	109±4 b
Sulphate	18±0.5 b	39±2.6 a	40±6.8 a	32±8.7 a	304±15.3 a	206±3.3 b	176±18.7 c	143±10.3 d
Ion Sum	795±82 a	606±93 ab	744±46 a	434±7 b	997±12 a	781±5 b	842±65 b	464±16 c

Table 2 Effect of mannitol induced osmotic stress on the accumulation of TSS (Total soluble sugars), sucrose, mannitol, SOA (Sum of organic acids) and malate in leaves and nodules of Flamingo and Coco blanc bean cultivars. All represented values are in μmol g^{-1} DW. Values represent mean ± SE (n=3)

Mannitol (mM)	Leaves				Nodules			
	Flamingo		Coco blanc		Flamingo		Coco blanc	
	0	50	0	50	0	50	0	50
TSS	383±93.7 b	325±124.8 b	121±68.7 c	542±62.9 a	462±313 a	570±66.7 a	289±37.6 b	450±61.3 a
Sucrose	59±7.4 a	57±3.7 a	37±3.5 b	33±12.1 b	124±47.4 a	140±23 a	104±9 b	60±0.3 c
Mannitol	ND	138±33.4 b	ND	316±52.2 a	52±30.8 b	51±12.6 b	ND	75±23.8 a
SOA	176±48.3 a	204±24.2 a	148±48 ab	86±29.4 b	145±24.2 a	169±3.1 a	134±20.2ab	64±7.7 b
Malate	68±3.6 b	118±10.5 a	65±29.3 b	28±12.9 c	87±20.8 a	113±7.3 a	82±22.7 b	36.80±7.99 c

4.3.2 Contribution of carbon compounds

Several organic compounds may also contribute to osmotic adjustment such as de novo synthesis of compatible solutes (Shabala et al. 2000; Manchanda and Garg 2008) like free proline (Al-Khayri 2002; Al-Khayri and Al-Bahrany 2002), soluble carbohydrates (Liu and Staden 2001; Javed 2002), phenols (Gonzalez and Garcia 1988) and amino acids (Gilbert et al. 1998; Lobato et al. 2008). Our results showed that when mannitol was added to the nutrient solution, the concentration of TSS increased significantly in leaves and nodules of Cv. 'Coco Blanc' (Table 2). It was reported earlier that the lowering of Ψ_o by osmolyte accumulation in response to stress improves the capacity of the cells to maintain their Ψ_p at low Ψ_w. This appears to be essential for physiological processes such as photosynthesis, enzyme activity and cell expansion (Tyree and Jarvis 1982; Wu and Cosgrove. 2000; Granier et al. 2000; Kiani et al. 2007). However, it also presents a metabolic cost due to synthesis and compartmentation of osmolytes (Bajji et al. 2000). It seems that plants favour the expression of genes connected with biosynthesis and storage of reserve sugars (including starch) and repress those genes associated with photosynthesis and reserve mobilisation (Ho et al. 2001). Thus it is plausible that the sensitivity of Cv. 'Coco Blanc' may reflect a lower level of photosynthesis under stressed conditions. This suggests that the accumulation of high carbohydrate levels impedes the plants from using more carbon for growth under stressed conditions. Furthermore, the increase in the TSS in Cv. 'Coco Blanc' leaves may represent the inability of this cultivar to export C to nodules due to a lower sink (nodules) strength. This is consistent with a decline in nodule sucrose synthase (Sassi et al. 2008b). Indeed, TSS also accumulated in Cv. 'Coco Blanc' nodules, possibly reflecting a lower nitrogen fixation demand. In these nodules malate concentration also decreased. In general, C-compounds (Table 2) contribute more than N-compounds to the osmotic adjustment: This is a characteristic of nitrogen-fixing plants as opposed to nitrogen-fed legumes (see Frechilla et al. 2000).

4.3.3 Contribution of nitrogen compounds

TAA has been shown to increase under drought conditions in sorghum (Yadav et al. 2005). The metabolism of these compounds may play an important role in plant stress tolerance, by osmotic adjustment, detoxification of reactive oxygen species and by intracellular pH regulation (Ronde et al. 1999; Alia et al. 2001). Our results showed no significant accumulation of TAA in leaves of both cultivars under stressed conditions (Table 3), but rather a significant decrease in these compounds. This fact, together with a decrease in protein content (about 25% reduction in both cultivars, data not shown) seems to reflect the decreased N-availability caused by osmotic stress. A significant accumulation of TAA was observed in stressed Cv. 'Flamingo' nodules, whereas TAA content in Cv. 'Coco Blanc' nodules were almost unaffected. The latter might be attributed to a lower nitrogen fixation activity under osmotic stress of this cultivar (Sassi et al. 2008b). In fact, the actual TAA content of nodules should reflect a balance among amino acids produced by nitrogen fixation and the phloem imports and xylem exports, under osmotic stress. This stress can impair long-distance transport and nodule-plant metabolite exchange. However, if a reduction of water-based export from the nodules were the only reason for the observed metabolic changes, it would imply a general accumulation of metabolites during drought, which does not explain the accumulation of specific compounds found in this study (Larrainzar et al. 2009).

The increased TAA content in Cv. 'Flamingo' nodules was mirrored by a significant accumulation of amino acids associated with primary N assimilation (Gln, Asp-Asn) (6.2 and 2.4 fold, respectively). It is noticeable that Pro content, a compound related to stress development, did not increase as much as the average of TAA in Cv. 'Flamingo' (1.3-fold

Table 3 Effect of mannitol induced osmotic stress on free amino acids distribution (μmol g^{-1} DW) in leaves and nodules of Flamingo and Coco blanc bean cultivars. *TAA* denotes total amino acids. *R* denotes the ratio between the value under osmotic stress and the value under control condition of the same organ and the same cultivar. Values represent mean ± SE (n=4)

Mannitol (mM)	Leaves						Nodules					
	Flamingo			Coco Blanc			Flamingo			Coco Blanc		
	0	50	R	0	50	R	0	50	R	0	50	R
Arg	0.1±0.01 a	0.1±0.01 a	1.0	0.1±0.01 a	0.03±0.002 b	0.5	0.3±0.02 b	0.8±0.1a	2.7	0.4±0.1 b	0.7±0.3 a	1.9
Lys	0.1±0.02 a	0.1±0.01 a	1.0	0.1±0.01 a	0.1±0.001 a	0.6	3.0±0.04 b	4.7±0.5a	1.6	2±0.2 c	2.1±1.1 c	1.0
Cys	3.5±0.5 a	2.9±1.4 ab	0.8	3.7±0.01 a	1.3±0.2 b	0.4	2.3±0.6 b	2.8±0.2 b	1.2	3.7±0.3 a	2.2±0.6 b	0.6
Leu	0.4±0.1 a	0.1±0.01 b	0.3	0.1±0.02 b	0.1±0.01 b	1.0	0.2±0.02 c	1.4±0.04 b	6.1	0.2±0.1 c	4.3±2.5 a	1.6
Ile	0.2±0.02 a	0.1±0.02 b	0.4	0.05±0.02 c	0.1±0.01 b	1.1	0.3±0.1 c	1.0±0.1b	3.3	0.5±0.05 bc	4.1±1.5 a	7.9
Met	0.1±0.01 a	0.02±0.01 c	0.4	0.04±0.000 b	0.02±0.001 c	0.6	1.0±0.05 a	0.7±0.1b	0.7	0.8±0.1 ab	0.2±0.1 c	0.2
Phe	0.4±0.01 a	0.2±0.03 c	0.5	0.3±0.1 b	0.1±0.01 d	0.5	0.9±0.04 ab	1.2±0.1a	1.3	0.5±0.02 b	1.8±1 a	3.4
Trp	0.9±0.02 a	0.5±0.1 b	0.5	0.02±0.02 c	0.2±0.01 c	7.8	0.2±0.1 c	1.0±0.01 b	4.2	1.4±0.2 b	4.1±1.6 a	3.0
His	0.3±0.1 a	0.1±0.02 b	0.5	0.1±0.04 b	0.1±0.01 b	0.7	0.2±0.05 b	0.5±0.1 a	3.0	0.2±0.03 b	0.5±0.1 a	2.3
Tyr	0.8±0.1 a	0.2±0.03 b	0.2	0.2±0.02 b	0.1±0.01 c	0.7	0.6±0.1 b	1.5±0.02 ab	2.3	0.6±0.1 b	2.6±1.3 a	4.0
Val	0.3±0.02 a	0.2±0.03 b	0.6	0.2±0.04 b	0.1±0.01 c	0.5	0.6±0.1 c	1.3±0.1 b	2.3	0.6±0.03 c	3±1.5 a	4.9
Gln	0.3±0.01 a	0.1±0.05 b	0.5	0.3±0.1 a	0.1±0.04 b	0.4	3±1.3 b	18.6±7.0 a	6.2	2.4±1.1 b	0.3±0.1 c	0.1
Pro	0.6±0.1 a	0.3±0.05 c	0.6	0.5±0.002 b	0.2±0.002 d	0.5	3.7±4 b	4.8±0.3 a	1.3	1.9±0.3 c	4.4±2.3 a	2.4
GABA	4.4±0.3 b	2.9±0.4 c	0.7	5.4±0.4 a	2±0.3 d	0.4	5.7±0.4 c	14.4±0.6 b	2.5	4.9±1.1 c	32.6±6.4 a	6.7
Thr	0.5±0.02 a	0.3±0.04 b	0.5	0.5±0.04 a	0.3±0.01 b	0.7	7.1±1.1 b	14.3±1.2 a	2.0	4.1±1.7 c	3.3±1.4 c	0.8
Gly-Ser	3.2±0.1 a	2.0±0.4 b	0.6	2.3±0.1 b	1.5±0.2b	0.6	9.5±3.4 b	14±2.6 a	1.5	4.5±0.2 b	5.9±1.8 b	1.3
Ala	1.0±0.2 b	0.7±0.1 b	0.7	2.7±0.4 a	0.4±0.004c	0.2	6.2±0.2 a	8.5±0.5 a	1.4	7.2±1.4 a	7.4±4.3 a	1.0
Glu	4.3±0.3 b	3.2±0.7 b	0.7	6.8±0.01 a	1.4±0.1 c	0.2	35.7±0.5 a	31.9±0.5a	0.9	34.3±5.5 a	2.5±0.7 b	0.1
Asp-Asn	0.5±0.03 a	0.5±0.1 a	1.0	1.2±0.1 b	0.3±0.02 c	0.2	4.2±0.5 b	10±1.3 a	2.4	7.5±2.2 a	0.7±0.1 c	0.1
TAA	21.7±0.5 a	14.4±3.4 b	0.7	24.5±0.1 a	8.4±0.2 c	0.3	84.7±7.5 b	133.2±5.9 a	1.6	77.9±6.7 b	82.5±27 b	1.1

versus 1.6). This was also lower than the increase observed in Cv. 'Coco Blanc' nodules (2.4-fold). GABA is another compound that has been related to stress tolerance which also showed a greater increase in Cv. 'Coco Blanc' than in Cv. 'Flamingo' nodules. Furthermore, Leu, Ile, Val (the branched chain amino acids) increased in both cultivar nodules upon osmotic stress. ALS activity (the first enzyme in the branched chain amino acids pathway) in *Rhizobium* is high (Gonzalez et al. 1996), when inhibitors of the enzyme (herbicides) are used and this contributes to a better survival of nitrogen fixing compared to nitrate-fed plants (Royuela et al. 1998). Also, the *aap* double mutants of *Rhizobium* are unable to transport assimilated-N from bacteroids to the plant cells of the nodule. As a result the plants suffer from N-deprivation (Lodwig et al. 2003). This may be important in terms of N-cycling in nodules. Other amino acids that also accumulated in Cv. 'Flamingo' nodules under osmotic stress have been recently suggested as new markers for monitoring drought in *Medicago truncatula* nodules (Larrainzar et al. 2009).

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