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Effects of proline and glycinebetaine on *Vicia faba* responses to salt stress

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Abstract

Plants of bean (*Vicia faba* L. cv. Calvor 103) were salt-stressed with NaCl and $CaCl_2$ in concentrations inducing soil osmotic potentials (ψ_{soil}) from 0 to -1.2 MPa and were sprayed with proline (8.7 μM) and glycinebetaine (8.5 μM) solutions. Bean plants respond to increasing soil salinity by decreased leaf relative water content and osmotic potential. Salinity decreased the contents of dry mass, chlorophyll, soluble and hydrolysable sugars, soluble proteins and enhanced content of total free amino acids, Na^+, Ca^{2+} and Cl^-. The ratio of K^+/Na^+ was decreased on salinization. The membranes of leaf discs from salt-stressed plants appeared to be less stable under heat stress (51 °C) than that of unstressed plants. The reverse was true for discs placed under dehydration stress (40 % polyethylene glycol 6000). Proline and glycinebetaine application reduced membrane injury, improved K^+ uptake and growth. Also both solutes increased chlorophyll contents.

Additional key words: amino acids, bean, chlorophyll, membrane stability, osmotic adjustment, proteins, relative water content, sugars.

Introduction

Accumulation of ions in the leaves under salt stress causes a rapid reduction in net photosynthesis and hence in growth. Excess of Na^+ and Cl^-, creates ionic imbalances that may impair the selectivity of root membranes and induce potassium deficiency. Membranes are vulnerable targets of stress-induced cellular damage and the extent of membrane damage is commonly used as a measure of tolerance to various stresses in plants, such as freezing, heat, drought and waterlogging. Water and salt stress cause accumulation of compatible solutes such as proline or glycinebetaine. Proline has

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Abbreviations: AA - amino acids; Chl - chlorophyll; d.m. - dry mass; f.m. - fresh mass; G - glycinebetaine; HS - hydrolysable sugars; ψ_s - leaf osmotic potential; ψ_{soil} - soil osmotic potential; P - proline; PEG - polyethylene glycol; RWC - relative water content; SP - soluble proteins; SS - soluble sugars.

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been reported to accumulate in tissues/organs of plants subjected to drought, salt, waterlogging, temperature or heavy metal stress. Glycinebetaine accumulates under salt stress in wheat (Mac Donnell and Wyn Jones 1988) and in various species of *Caryophyllaceae*, *Convolvulaceae*, *Solanaceae* and *Compositae* (Weretilnyk *et al.* 1989). The temporary enhancement of these compounds is important in the response to stress. Proline and glycinebetaine may act as non-toxic osmotic solutes, and as enzyme protectants, stabilizing the structures of macromolecules and organelles.

The ameliorating effects of proline and glycinebetaine on growth, and plant metabolism under salt-stress still remains incompletely understood. Therefore, in the present study we have investigated the effects of exogenous application of proline and glycinebetaine on the growth, stability of leaf membranes, leaf relative water content, chlorophyll content and leaf osmotic potential of *Vicia faba* plants grown under salinity. In addition, the contents of soluble and hydrolysable sugars, soluble proteins, total free amino acids, and some mineral ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-) in shoots were also determined.

Materials and methods

Plants: Bean (*Vicia faba* L. cv. Calvor 103) plants were grown in plastic pots containing 1 400 g air-dry soil (sand/clay 2:1 v/v) at the experimental farm of the Faculty of Science, Assiut University. The plants (five per pot) were irrigated twice with 100 cm^3 full nutrient solution (Downs and Hellmers 1975).

Osmotic potential (ψ_{soil} = 0 to -1.2 MPa) of the soil solution was adjusted by irrigating the plants (5-week-old) with solution of NaCl and $CaCl_2$ in concentrations inducing determined osmotic potentials (prepared according to Lagerwerff and Holland 1960, Lagerwerff and Eagle 1961). Each pot received an amount of solution equivalent to the volume of water necessary to raise its soil moisture content to field capacity to eleminated the effect of the matric potential component. Treatment with proline and glycinebetaine were started after two weeks of adjustment to assigned stress level. Three pots were assigned at random at every stress level. Proline and glycinebetaine solutions (8.7 and 8.5 μM, respectively) were applied three times at 5-d intervals by spraying the shoots. The control plants were sprayed with distilled water. Plants were harvested a week after last application.

Membrane stability test: The stability of leaf membrane, was assessed by determining leakage of electrolytes from leaf discs exposed to heat (51 °C) and dehydration (40 % PEG) stress for 20 h (for details see Gadallah 1995b).

The degree of injury was calculated according to the following formula:

$$\text{Injury [\%]} = 1-[1-(T_1/T_2)/1-(C_1/C_2)] \times 100$$

where T_1 and T_2 represent the first and second measurements of the electrical conductance on the treatment samples, and C_1 and C_2 the first and second measurements on the control.

Relative water content and osmotic potential: For measuring leaf relative water content (RWC), the method of Weatherley (1950) was adopted. Leaf osmotic potential was measured cryoscopically and was calculated as described by El-Sharkawi and Abdel-Rahman (1974).

Contents of dry matter, chlorophylls, sugars and proteins: For shoot dry matter determination the fresh plants were dried in an aerated oven at 70 °C to constant mass. Chlorophyll *a* and *b* contents were determined according to Todd and Basler (1965). Soluble sugars (SS), and hydrolysable sugars (HS) were determined according to Buysse and Merckx (1993). Total free amino acids (AA) and soluble proteins (SP) were determined according to Lee and Takahashi (1966) and Lowry *et al.* (1951), respectively.

Mineral ions: Shoot Ca^{2+} and Mg^{2+} contents were determined by an atomic absorption spectrophotometer (Model *AA-630-O$_2$, Shimodzu Corporation*, Kyoto, Japan). Na^+ and K^+ were analyzed by a flame photometer *M7D* (*Carl-Zeiss*, Berlin, Germany). Contents of chlorides were determined by $AgNO_3$ titration method as described by Johnson and Ulrich (1959).

Statistical analysis: To evaluate the effects and relative role of single factors and their interactions on the parameters tested statistical analysis included: analysis of variance (*F* values), coefficient of determination (η^2), and linear correlation coefficient (*r*) (Ostle 1963).

$$\eta^2 = \frac{\text{sum of squares due to the factor}}{\text{total sum of squares due to the treatment combinations}}$$

Results

Stability of leaf membranes: PEG solution applied to leaf discs isolated from unstressed plants causes 16.53 % injury. Discs from salt-stressed plants were less injured than those of unstressed plants. Application of proline and glycinebetaine either independently or in combination (P+G) significantly reduced percent injury caused by PEG in both the unstressed and stressed plants (Table 1).

Heat stress induced 26 % injury in leaf discs from unstressed plants. This injury was enhanced by salinity. Membranes of plants sprayed with P and G were more stable than untreated control (Table 1).

Growth, chlorophyll content and leaf relative water content: In the absence of P and G, lowering of ψ_{soil} resulted in significant inhibition of shoot growth (dry mass production), loss of chlorophyll and lowered leaf RWC. Application of P and G completely relieved the effects of salinity on these parameters. The effect was pronounced at all stress levels (Table 2).

In plants not receiving P or G shoot growth was negatively correlated with Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^- (r = -0.99, -0.88, -0.99, -0.95 and -0.99, $P < 0.05$). The most

pronounced effect of P and G was the lack of the significant correlation with K^+ (in the presence of P and G independently) and with Ca^{2+} (in the presence of P).

Table 1. Effect of foliar application of proline (P) and glycinebetaine (G) on membrane stability (percent injury) in leaf discs of *Vicia faba* plants grown at soil osmotic water potentials (ψ_{soil}) 0 to -1.2 MPa, and dehydrated in 40 % PEG or heated to 51 °C. Means of 3 replicates ± SE. The same letters in columns indicated no significant differences (at $P < 0.05$).

Stress	ψ_{soil} [-MPa]	Control	P	G	P+G
Dehydration	0.0	16.53±0.97 a	0.70±0.29 d	4.33±0.58 c	1.49±0.33 c
	0.3	7.77±0.99 c	2.28±0.30 c	5.91±0.17 ab	0.50±0.20 d
	0.6	11.52±0.76 b	9.03±0.34 a	5.97±0.52 a	3.96±0.27 bc
	0.9	10.14±0.48 b	3.73±0.20 bc	4.73±0.21 bc	4.80±0.29 b
	1.2	7.38±0.57 c	4.29±0.62 b	4.75±0.99 b	7.86±0.20 a
Heat	0.0	26.07±1.87 e	21.00±2.70 b	11.39±1.57 cd	18.04±1.71 b
	0.3	31.92±2.24 d	27.36±2.10 ab	31.47±2.42 a	14.05±1.68 b
	0.6	42.18±2.44 c	18.85±1.76 b	26.21±1.73 b	30.61±1.98 ab
	0.9	54.19±1.89 b	26.72±1.70 ab	14.97±1.42 c	14.34±1.74 b
	1.2	67.96±1.24 a	28.27±1.85 a	10.43±1.64 d	32.09±1.91 a

Osmotic potential: In plants not receiving P or G, reduced ψ_{soil} (Table 2) caused progressive decrease in leaf osmotic potential (ψ_s). P and G treatment significantly increased ψ_s in both unstressed and stressed plants except at ψ_{soil} -0.3 MPa where plants receiving P + G showed lower ψ_s than untreated control.

Soluble carbon and nitrogen: The contents of soluble sugars (SS), hydrolysable sugars (HS) and soluble proteins (SP) of salt stressed plants were substantially lower than those of unstressed plants (Table 3). On the contrary, total free amino acids (AA) increased progressively with salinization. While SP and AA contents were reduced by P and G application (ψ_{soil} -1.2 MPa with P and G was an exception), HS was enhanced. Plants at ψ_{soil} 0 to -0.9 MPa receiving P had lower SS than those of the control, while at higher stress the opposite was found. G enhanced SS accumulation at ψ_{soil} -0.6 and -1.2 MPa but reduced the content at other stress levels. Addition of both P + G increased SS content at lower and moderate stress levels and decreased it in unstressed and highly stressed plants.

Mineral ions: The contents of Na^+, Ca^{2+}, and Cl^- (Table 4) significantly increased with increased salinity. Salinity had negligible effect on the contents of K^+ and Mg^{2+} at ψ_{soil} -0.03 and -0.6 MPa, but lower ψ_{soil} (-0.9 to -1.2 MPa) resulted in increased contents. The ratio of K^+/Na^+ was decreased about 2-fold between ψ_{soil} 0 and -1.2 MPa. Spraying with P and G greatly reduced Na^+, Ca^{2+} and Cl^- accumulation over the entire range of stress and slightly reduced Mg^{2+} content. Stressed plants receiving P and G independently or P + G had higher K^+ content, but the opposite was found in unstressed plants. P and G application increased the K^+/Na^+ ratio over the entire ψ_{soil} range.

Table 2. Changes in chlorophyll (Chl) *a* and *b* contents, dry mass per plant, leaf relative water content (RWC) and leaf osmotic potential (ψ_s) in *Vicia faba* plants in response to decreased soil water osmotic potential (ψ_{soil}), and treatments with proline (P) or/and glycinebetaine (G). Means of 3 replicates ± SE. The same letters in columns indicated no significant differences (at $P < 0.05$).

	ψ_{soil} [-MPa]	Chl *a* [mg g^{-1}(d.m.)]	Chl *b* [mg g^{-1}(d.m.)]	Dry mass [g $plant^{-1}$]	RWC [%]	ψ_s [-MPa]
Cont.	0.0	9.13±0.49 a	5.60±0.33 a	3.36±0.08 a	84.96±1.49 a	1.28±0.02 d
	0.3	5.18±0.26 b	3.72±0.28 b	2.82±0.06 b	79.38±1.57 b	1.76±0.03 cd
	0.6	3.95±0.08 c	3.42±0.08 bc	2.32±0.06 c	74.33±0.97 c	1.89±0.03 c
	0.9	2.96±0.30 cd	2.74±0.10 c	1.93±0.04 cd	69.64±1.89 d	2.54±0.05 b
	1.2	2.40±0.15 d	2.01±0.08 c	1.53±0.07 d	61.95±1.88 e	4.08±0.06 a
P	0.0	10.22±0.26 c	7.60±0.20 c	4.24±0.07a	82.15±2.16 b	0.86±0.02 c
	0.3	10.09±0.63 c	8.21±0.32 c	3.62±0.06 b	91.79±2.23 ab	1.10±0.04 c
	0.6	19.71±0.84 a	18.93±0.52 a	3.58±0.04 bc	92.12±2.17 a	1.10±0.03 c
	0.9	9.54±0.25 c	8.32±0.41 c	3.28±0.05 bc	81.50±1.50 bc	2.11±0.06 b
	1.2	17.40±0.74 b	13.50±0.74 b	2.73±0.05 c	76.66±1.87 c	2.94±0.06 a
G	0.0	21.23±0.72 a	15.60±0.90 a	4.41±0.06 a	81.09±2.16 b	0.98±0.04 c
	0.3	11.09±0.38 d	9.03±0.40 c	4.07±0.04 ab	86.47±1.48 a	1.10±0.01 bc
	0.6	16.36±0.65b	13.84±0.82 b	3.88±0.05 b	86.42±2.05 ab	1.15±0.03 bc
	0.9	12.86±0.33 c	9.00±0.23 c	3.49±0.06 bc	81.59±1.69 ab	1.29±0.04 b
	1.2	12.04±0.31 c	9.87±0.54 c	2.97±0.04 c	76.54±1.79 b	2.66±0.05 a
P+G	0.0	16.61±0.56 a	12.16±0.63 a	5.38±0.07 a	77.41±1.52 b	0.66±0.03 c
	0.3	13.41±0.29 bc	10.57±0.37 b	4.38±0.05 c	83.31±1.78 ab	2.82±0.04 a
	0.6	13.95±0.47 bc	8.88±0.39 c	4.76±0.06 b	79.23±1.47 ab	0.97±0.05 c
	0.9	14.33±0.58 b	9.65±0.35 bc	3.70±0.05 d	84.90±1.63 a	0.82±0.07 c
	1.2	11.44±0.41 c	8.19±0.23 c	3.23±0.04 e	84.04±1.53 ab	2.18±0.08 b

Discussion

Salt stress induced high rates of solute leakage from leaf discs exposed to heat (51 °C) stress. Such an effect is assumed to be closely related to membrane damage. Evidence of toxic effects of salt on plant membranes have been well documented (*e.g.* Leopold and Willing 1984). On the other hand membranes of salt-stressed plants were more tolerant to dehydration stress (PEG treatment) than those of unstressed plants (Table 1). The lower injury can be explained by the protecting effect of minerals accumulated inside the cells (*e.g.* Tal and Shannon 1983). Membrane destabilization caused by high temperature was greatly reduced in the presence of P and G. These solutes provide protection against destabilization of proteins and membranes (Jolivet *et al.* 1982, Zhao *et al.* 1992, Gadallah 1995b).

Salinity induced lower biomass accumulation and chlorophyll content is in accordance with our previous findings (Gadallah 1996, Gadallah and Ramadan 1997). P-treated plants had more chlorophyll and produced more biomass which is in agreement with the results of Shaddad (1990) and Gadallah (1995a). Stressed plants

Table 3. Changes in soluble sugars (SS), hydrolysable sugars (HS), and total free amino acids (AA) contents [mmol g^{-1}(d.m.)] and soluble protein (SP) content [mg g^{-1}(d.m.)] in *Vicia faba* plants in response to decreased soil water osmotic potential (Ψ_{soil}), proline (P) and glycinebetaine (G). Means of 3 replicates ± SE. The same letters in columns indicated no significant differences (at $P < 0.05$).

	ψ_{soil} [-MPa]	SS	HS	AA	SP
Cont.	0.0	0.38±0.005 a	0.09±0.003 a	1.25±0.05 c	21.06±0.80 a
	0.3	0.36±0.002 ab	0.03±0.001 d	1.47±0.05 c	16.06±0.80 b
	0.6	0.32±0.003 b	0.01±0.001 e	1.75±0.03 b	14.04±0.68 c
	0.9	0.31±0.001 bc	0.05±0.002 c	2.00±0.08 ab	13.23±0.54 c
	1.2	0.24±0.004 c	0.07±0.002 b	2.14±0.09 a	16.83±0.48 b
P	0.0	0.33±0.006 a	0.15±0.003 b	0.78±0.02 c	14.48±0.76 ab
	0.3	0.20±0.004 b	0.12±0.003 c	1.15±0.05 b	12.03±0.72 b
	0.6	0.26±0.003 b	0.24±0.006 a	1.10±0.04 bc	16.19±0.93 a
	0.9	0.30±0.004 ab	0.10±0.003 d	1.34±0.06 ab	13.14±0.63 b
	1.2	0.29±0.005 ab	0.14±0.002 bc	1.48±0.04 a	11.68±0.85 b
G	0.0	0.32±0.003 c	0.15±0.003 b	0.58±0.01 d	14.82±0.61 a
	0.3	0.19±0.004 e	0.05±0.001 e	0.93±0.03 c	12.24±0.65 b
	0.6	0.47±0.006 a	0.14±0.002 c	1.34±0.02 bc	12.17±0.73 b
	0.9	0.28±0.002 d	0.13±0.002 d	1.40±0.05 b	11.53±0.54 b
	1.2	0.37±0.001 b	0.16±0.004 a	1.80±0.07 a	11.50±0.42 b
P+G	0.0	0.34±0.005 bc	0.18±0.005 ab	1.14±0.06 bc	16.92±0.48 a
	0.3	0.43±0.007 a	0.19±0.006 a	1.26±0.08 bc	14.55±0.83 bc
	0.6	0.36±0.004 b	0.05±0.001 c	0.96±0.03 c	14.87±0.70 b
	0.9	0.30±0.005 c	0.12±0.002 bc	1.33±0.05 b	11.52±0.49 c
	1.2	0.19±0.002 d	0.13±0.002 b	2.18±0.05 a	12.66±0.86 c

receiving G also accumulated more chlorophyll than their untreated analogues. This could be due to increasing the stability of chloroplast membranes (Mamedov *et al.* 1990) and protection of photosystem 2 by glycinebetaine (Papageorgiou *et al.* 1991). However, P+G improved growth and chlorophyll content even in absence of salinity, probably due to the ability of these solutes to affect activity of some enzymes (Bandurska 1993, Solomon *et al.* 1994) and protein synthesis (Kadpal and Rao 1985). These solutes as reservoirs of C and N (Fukutaku and Yamada 1984) could be another explanation for growth enhancement.

Application of P and G improved water status of stressed plants. Such effects could be due to inhibition of water efflux via effects of solutes on membrane stability (Table 1) and reduced transpiration via effects on stomatal regulation (Raghavendra and Reddy 1987). Furthermore, these solutes may be involved in the osmoregulation (Jolivet *et al.* 1983, Laliberte and Hellebust 1989, Delauney and Verma 1993).

Soluble sugars content markedly declined with reduced ψ_{soil} perhaps through inhibition of carbon metabolism in salt-stressed plants. Stressed plants generally had low contents of soluble proteins and higher concentration of free amino acids. This could be due to inhibition of amino acids incorporation into proteins under salinity. Accumulation of free amino acids may be an important adaptive response to salt-

stress. Plants receiving P and G had lower soluble sugars and higher HS. This could be due to SS interconversion into reserve sugars (HS). However, both P and G enhanced SS accumulation in highly stressed plants (-1.2 MPa).

Table 4. Changes in mineral content [mmol g^{-1}(d.m.)] in *Vicia faba* plants in response to decreased soil water osmotic potential (Ψ_{soil}), proline (P) and glycinebetaine (G). Means of 3 replicates ± SE. The same letters in columns indicated no significant differences (at $P < 0.05$).

	ψ_{soil} [-MPa]	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-
Cont.	0.0	1.24±0.07 d	0.30±0.005 b	0.10±0.002 e	0.09±0.003 d	0.45±0.04 d
	0.3	1.88±0.05 c	0.29±0.004 b	0.13±0.002 d	0.11±0.004 cd	0.81±0.03 cd
	0.6	2.12±0.06 b	0.31±0.003 ab	0.17±0.003 c	0.12±0.003 c	0.90±0.02 c
	0.9	2.64±0.04 ab	0.33±0.002 ab	0.21±0.003 b	0.16±0.005 b	1.16±0.05 b
	1.2	2.82±0.08 a	0.35±0.003 a	0.22±0.004 a	0.18±0.006 a	1.29±0.03 a
P	0.0	0.75±0.01 c	0.20±0.001 b	0.08±0.001 c	0.06±0.001 d	0.36±0.01 c
	0.3	1.41±0.02 bc	0.48±0.003 b	0.11±0.003 b	0.08±0.001 c	0.72±0.01 b
	0.6	1.55±0.02 b	0.51±0.005 b	0.10±0.004 bc	0.09±0.002 bc	0.66±0.03 bc
	0.9	2.11±0.03 ab	0.63±0.006 a	0.17±0.004 ab	0.10±0.002 b	1.17±0.04 ab
	1.2	2.38±0.04 a	0.58±0.007 ab	0.18±0.003 a	0.14±0.001 a	1.21±0.05 a
G	0.0	0.92±0.02 c	0.23±0.001 d	0.08±0.001 e	0.05±0.001 e	0.45±0.02 c
	0.3	1.58±0.04 b	0.52±0.002 cd	0.10±0.002 d	0.07±0.001 c	0.75±0.03 b
	0.6	1.48±0.03 bc	0.53±0.005 c	0.12±0.003 c	0.06±0.003 d	0.67±0.05 bc
	0.9	1.76±0.05 ab	0.62±0.003 a	0.14±0.005 b	0.09±0.005 b	0.91±0.06 ab
	1.2	2.07±0.05 a	0.65±0.005 b	0.17±0.006 a	0.14±0.005 a	1.06±0.02 a
P+G	0.0	0.52±0.01 c	0.22±0.002 c	0.06±0.001 d	0.04±0.001 d	0.36±0.01 c
	0.3	1.69±0.02 b	0.61±0.005 ab	0.11±0.001 c	0.07±0.001 c	0.65±0.01 b
	0.6	1.53±0.03 bc	0.51±0.004 b	0.10±0.003 cd	0.08±0.002 b	0.57±0.03 bc
	0.9	1.81±0.03 ab	0.66±0.006 a	0.15±0.004 b	0.08±0.003 b	0.87±0.03 ab
	1.2	2.23±0.05 a	0.63±0.007 ab	0.19±0.005 a	0.10±0.003 a	1.12±0.05 a

Contents of Na^+, Ca^{2-}, and Cl^- were higher in salt stressed plants than in unstressed plants, due to increased uptake, reduced translocation or a decreased growth. It is accepted that competition exists between Na^+ and K^+ leading to a reduced level of internal K^+ at a high external NaCl concentration (*e.g.* Gorham *et al.* 1990, Botella *et al.* 1997). In our study the imposed stress was achieved by the combination of NaCl and $CaCl_2$ in the external medium. Therefore the increase K^+ content at higher stress levels means that Ca^{-2} is important for maintenance of K^+ transport in the presence of Na^+. K^+/Na^+ ratio decreased steadily with increasing salt addition. This is in agreement with our previous findings (Gadallah 1996).

The contents of Na^+, Ca^{2+} and Cl^- were substantialy lower in plants treated with P and G. The effect of proline and glycinebetaine application, probably occurs through reduced stomatal opening (Raghavendra and Reddy 1987) and decreased transpiration rate, causing less ions to be carried through the transpiration stream, leading to the decrease in their accumulation in shoots. At -1.2 MPa P+G only affected mildly Na^+ and Cl^- accumulation in leaves, suggesting that improved growth

was not a consequence of decreased ion accumulation.

The results indicate, that proline and glycinebetaine application counteracted the deleterious effect of salt stress on most parameters measured, helped the plants to avoid Na^+ toxicity, improved cell membranes stability and K^+ uptake under salinity, and effectively increased K^+/Na^+ ratio. Improvement of growth and water status of stressed bean plants makes it possible to recommend the treatment of plants, grown under saline conditions with these solutes.

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