Genetics and Plant Physiology – 2012, Volume 2 (3–4), pp. 177–186

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EFFECTS OF EXTERNAL CALCIUM SUPPLY ON THE PHYSIOLOGICAL RESPONSE OF SALT STRESSED BEAN (*PHASEOLUS VULGARIS* L.)

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Received: 30 January 2013 **Accepted:** 13 June 2013

Summary: Salt stress effects on growth parameters, water status and mineral balance in shoots and roots of common bean (*Phaseolus vulgaris* L *cv.* Djadida) were studied. Plants irrigated with nutrient solution were subjected to increasing concentrations of NaCl (100 mM and 200 mM) as well as 100 mM NaCl + 50 mM $CaCl_2$ and 100 mM NaCl + 100 mM $CaCl_2$. After 10 days of salt treatment, plants were harvested, water status was estimated, shoots and roots were separated, then growth parameters and mineral balance were analyzed. Salinity caused stunted growth; both root and shoot biomass weight was reduced. Similar to biomass production, total water uptake also decreased. Our results showed that plants exposed to NaCl took up high amounts of Na^+ whereas the uptake of K^+ and Ca^{++} was significantly reduced in shoots and roots. Although the K^+/Na^+ ratio decreased consistently in shoots and roots, it was markedly higher in shoots as compared to roots. The addition of Ca^{++} to the soil solution depressed the adverse NaCl effects and produced an extreme K^+/Na^+ ratio in tissues thus making plants less susceptible to osmotic and specific ion injury. Besides, yield biomass was increased and water status was improved.

Citation: Taïbi Kh., F. Taïbi, M. Belkhodja. Effects of external clcium supply on the physiological response of salt stressed bean (*Phaseolus vulgaris* L.). *Genetics and Plant Physiology*, 2012, 2(3–4), 177–186.

Keywords: Salinity; sodium; calcium; water status; growth; mineral nutrition; *Phaseolus vulgaris* L.

INTRODUCTION

The adverse effects of salt stress are usually less severe on salt-tolerant plants such as cotton than on the salt-sensitive species such as beans (Ajmal khan et al., 2006). Generally, exposure to salt stress triggers many common reactions in plants that lead to cellular dehydration with concomitant osmotic changes (Sairam and Tyagi, 2004). Salinity reduces the ability of plants to utilize water and causes a reduction in growth, as well as changes in plant metabolic

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processes (Munns, 2002). Plant growth is affected through osmotic inhibition of water uptake by roots or by specific ion effects (Marschner, 1995). Plant mineral nutrition is crucial to plant growth and development and, as a consequence, to agriculture and human health (Munns et al., 2006). Specific ion effects may lead to direct toxicity or, alternatively, the insolubility or competitive absorption of ions and may affect the plant's nutritional balance through increasing Na^+ uptake or decreasing Ca^{++} and K^+ uptake (Neel et al., 2002). This is attributed to the fact that Na^+ competes with Ca^{++} for binding sites essential for cellular function (Tester and Davenport, 2003). As salt stress occurs frequently and can affect most of the habitats, understanding the mechanisms of plant tolerance to high salt stress is a fundamental environmental research topic especially in arid regions (Wang et al., 2009). Significant ameliorative effects of Ca^{++} on Na^+ plants toxicity have been reported to improve water transport (Knight et al., 1997), mineral nutrition (Lazof and Bernstein, 1999) and growth for most plants (White and Broadley, 2003).

Common bean (*Phaseolus vulgaris* L.) is one of the major vegetable crops for human nutrition in the world (Bayuelo-Jiménes et al., 2002b). Beans are grown in a wide range of environments from sea level to high elevations (Pessarkli, 1993). However, common bean and other legumes are regarded as appropriate crops for the enhancement of bioproductivity and the reclamation of marginal lands, because they not only yield nutritious fodder, protein rich seeds and fruits, but also are known to enrich soil with nitrogen in symbiotic association with rhizobium (Neel et al., 2002). They therefore, contribute a lot to the improvement of soil fertility in the semi-dry lands where most of the soils are already salinized (Bayuelo-Jiménes et al., 2002a).

The main objective of this study was to evaluate the physiological response of common bean to salt stress. We evaluated plant tolerance to NaCl and in order to raise Ca^{++} concentration in the medium we used a salt mixture of NaCl + $CaCl_2$. To determine the predictive screening parameters that can be applied to bean plants, we focused mainly on biomass production, water content, mineral balance in shoots and roots, and mineral ratios as practical physiological markers.

MATERIALS AND METHODS

Plant material and culture

Seeds of common bean (*Phaseolus vulgaris cv*. Djadida) released by the Algerian Technical Institute of Crop Production were evaluated for salt stress. Seeds were surface sterilized with 5% (w/v) commercial bleach sodium hypochlorite solution (NaOCl) three times for 30 min with gentle stirring, washed subsequently in deionized water, then germinated in sand. After 7 days, healthy and uniform seedlings with fully developed trifoliate leaves were transplanted in plastic pots of 20 cm and 15 cm top and bottom diameters, respectively, and 18 cm height, with holes in the bottom, filled with a mixture of sea sand, organic matter and soil (2:1:1).

Plants were grown under a glasshouse controlled conditions with day and night temperatures of 27°C and 19°C, respectively and about 14 h photoperiod. Alternatively and at an interval of 2 days, plants were irrigated with distilled water

and nutrient solution of Hoagland and Arnon (1938) adjusted to pH 5.5. All pots were arranged into five treatments with five replicates each. Each pot contained one seedling which was watered at 60% of field capacity to avoid salt precipitation around roots.

Salt solutions were applied once when plant shoots and roots reached sufficient plant material for analysis (about 30 days). Salt treatments were applied by adding NaCl to the nutrient solution at concentrations of 100 mM and 200 mM while a mixture of 100 mM NaCl + 50 mM $CaCl_2$ and 100 mM NaCl + 100 mM $CaCl_2$ was added to the nutrient solution for the two combined treatments in order to raise Ca^{++} concentration to compete Na^{++} in the medium.

Growth and water status determination

At the end of the 10-d period after salt treatment, plants were harvested, root and shoot systems were separated and immediately, the roots were washed gently under tap water to clean salt and organic matter. Fresh weight (FW) of shoots and roots was determined by analytical balance. The samples were oven-dried at 80°C for 48 h, then the dry weight (DW) was recorded. Water content (WC) was estimated using the formula:

WC(%)= (FW-DW)/(FW)x100.

Analysis of ionic content

Oven-dried samples were ground into a fine powder in a porcelain mortar. To determine mineral concentrations, 100 mg plant samples were ashed at 550°C in a muffle furnace for 5 h and then digested with 2 ml of 20% HCl (6 N) for 5 min at 60°C using a heating block. This hot water extract was cooled and filtered using filter paper and finally diluted to a volume of 50 ml with distilled deionized water. The Na^+ and K^+ concentrations of plant tissue were assayed by a flame emission spectrometer while Ca^{++} concentrations were determined by atomic absorption spectrometry (Perkin Elmer model 360).

Statistical analysis

The homogeneity variance of the data was assessed and conformed to the model which would permit analysis of variance (ANOVA) of the data set. Results on growth, water status and ionic content of plants were analyzed using the General Linear Model (GLM) procedure implemented in the statistical software SPSS (SPSS Inc, Chicago, USA) by one-way analysis of variance (ANOVA). The treatment mean values were compared by post hoc least significant difference test. The term significant indicates differences for which $P<0.05$ under the confidence level $\alpha=95\%$.

RESULTS

The analysis of variance for the parameters studied during the experiment showed that salt stress had different effects on the biomass yield, water relations and plant ion uptake.

Growth measurements and water status

Differences in FW were highly significant among the salt treatments ($P<0.01^{**}$). There was an increase in root (RFW by 50%) and shoot (SFW by 10%) fresh weight after treatment with 100 mM NaCl. Fresh weight dropped considerably in shoots (30%) while it remained higher

in roots (10%) compared to control when salinity doubled in the medium (200 mM NaCl) (Table 1). The maximum values for FW were obtained after the addition of Ca^{++} ($P<0.01^{**}$).

Shoot dry weight (SDW) decreased significantly with increasing NaCl concentration ($P<0.01^{**}$). However, root dry weight (RDW) increased slightly (15%, $P<0.05^{*}$) under the lower salt concentration and decreased (15%, $P<0.05^{*}$) thereafter. Plants treated with 100 mM NaCl + 50 mM $CaCl_2$ had lower DW than non-treated plants (10%, $P<0.05^{*}$), whereas after the addition of 100 mM $CaCl_2$ to the medium DW increased reaching the control level ($P>0.05$).

Water content was also altered differently in plant tissues under salt conditions. A significant decrease of shoot water content was observed when NaCl concentrations increased in the medium. For root water content, a smaller rise was recorded under the higher NaCl concentration; nevertheless, it decreased when this concentration increased twice. The addition of Ca^{++} as $CaCl_2$ improved notably water content in both shoots and roots.

Ionic content

The concentration of Na^+ in root tissues (43.2 $mg.g^{-1}$ DM) was higher than in shoots (13.6 $mg.g^{-1}$ DM) (Table 2). Na^+ content was significantly affected by salinity treatments ($P<0.01^{**}$). The accumulation of Na^+ under saline conditions was even higher, approximately 45% and 95% in shoots against 6% and 14% in roots, respectively under the first and second NaCl concentration tested. In roots, the application of 50 mM $CaCl_2$ to the salt medium lowered Na^+ concentration to the control level, whereas in shoots, Na^+ concentration was lowered but remained significantly higher compared to the control (25%). Treatment with 100 mM $CaCl_2$ decreased Na^+ content in both shoots and roots.

Our results showed that K^+ level was two-fold in shoots (40.7 $mg.g^{-1}$ DM) than in roots under control conditions. As salinity increased, K^+ concentration decreased significantly ($P<0.01^{**}$) by 10% in all tissues under 100 mM NaCl and then declined to 15% and 5% in shoots and roots, respectively when the salt concentration doubled in the medium. Treatment with 50 mM $CaCl_2$ increased significantly K^+ content ($P<0.05^{*}$) by 4%

Table 1. Biomass yield and water status of *Phaseolus vulgaris* plants under salinity stress.

		Control	NaCl		100 mM NaCl	
			100 mM	200 mM	50 mM $CaCl_2$	100 mM $CaCl_2$
Fresh weight [mg/plant]	Shoots	5.9±0.9	6.6±2.4	4.3±1.2**	6.5±1.2**	6.8±1.6**
	Roots	4.1±1.2	6.4±2.6**	4.5±2.2**	4.5±0.1**	4.9±1.1**
Dry weight [mg/plant]	Shoots	1.2±0.1	1.1±0.3	0.9±0.3	1.1±0.2	1.3±0.2
	Roots	0.7±0.1	0.8±0.2	0.6±0.2	0.6±0.1	0.7±0.1
Water content [%]	Shoots	85.1±2.5	83.3±3.7	75.2±5.9**	84.0±2.6	87.0±2.2
	Roots	82.2±3.1	86.9±4.7**	79.9±9.6**	82.8±0.9	84.1±1.8

Table 2. Ionic content in *Phaseolus vulgaris* plants under salinity stress.

		Control	NaCl		100 mM NaCl	
			100 mM	200 mM	50 mM $CaCl_2$	100 mM $CaCl_2$
Sodium [mg/g DM]	Shoots	13.7±2.3	20.0±2.2**	26.8±1.3**	17.0±0.5**	9.0±0.3**
	Roots	43.2±1.6	45.7±2.5	49.0±3.5**	41.2±2.0	35.2±2.1**
Potassium [mg/g DM]	Shoots	40.6±4.0	36.6±1.0**	34.8±2.7**	42.5±1.8	44.0±2.3**
	Roots	22.8±1.5	25.0±1.0**	21.8±1.0	34.3±1.3**	54.8±1.5**
Calcium [mg/g DM]	Shoots	41.3±1.8	27.5±1.8**	19.8±1.0**	42.0±0.5	44.0±0.3**
	Roots	20.0±1.8	20.0±1.8	5.3±0.8**	25.6±1.0**	35.5±1.3**
K^+/Na^+	Shoots	2.9	1.8	1.3	2.5	4.8
	Roots	0.5	0.5	0.4	0.8	1.5

and 50% in shoots and roots, respectively compared to the control. The highest concentrations of K^+ were found in the presence of 100 mM $CaCl_2$ under salinity. Shoots acquired 8% of additional K^+ while this content increased to 140% in roots compared to the control.

The K^+/Na^+ ratio was significantly influenced by salinity treatments. The K^+/Na^+ ratios decreased with increasing NaCl level ($P<0.01^{**}$). The addition of 50 mM $CaCl_2$ lowered the K^+/Na^+ ratio slightly below the control level in shoots, whereas in roots the ratio raised by 60%. The increase of supplemented Ca^{++} raised the K^+/Na^+ ratio by 60% in shoots and three-fold in roots compared to the control. The concentrations of Ca^{++} decreased in shoots by 33% and 52% under the two salt concentrations tested, respectively, but these values increased to the control level (41.3 $mg.g^{-1}$ DM) after the addition of Ca^{++}. In roots, Ca^{++} content was not affected by 100 mM NaCl (20.0 $mg.g^{-1}$ DM, $P>0.05$) but it decreased drastically (70%, $P<0.01^{**}$) when this concentration doubled to 200 mM NaCl. The addition of Ca^{++} to the soil solution resulted in an increase of Ca^{++} levels in roots to 25% and 75% and slightly in shoots to 1% and 5%, respectively.

DISCUSSION

Even though NaCl constitutes the major abundant salt in most salt-affected soils, other salts could play an important role in plant salt tolerance (Marschner, 1995). Salinity had adverse effects on the biomass yield of the tested genotype, reductions in plant biomass under saline condition were indicative of severe growth limitations in bean (Gama et al., 2007). The effects of NaCl on both dry and fresh matter production of bean were also examined in several studies, which demonstrated that NaCl stress significantly reduced total dry and fresh matter yield, but the severity of stress differed depending on salinity levels (Pessarkli, 1993; Gama et al., 2007). In several legumes, such as soybean (Grattan and Maas, 1988), faba bean (Belkhodja, 1996) and bean (*Phaseolus vulgaris* L.)

(Wignarajah, 1992), salinity was found to reduce shoot and root weights. In the present study, both root (RDW) and shoot dry weight (SDW) of the evaluated genotype were adversely reduced as salinity concentration increased like in many other bean cultivars (Pessarkli, 1993) and a number of other crops (Kumar et al., 1994; Avnimelech et al., 1994; Chauhan, 1995).

It has long been accepted that the major causes of plant growth inhibition under salinity stress are osmotic inhibition of plant water absorption and specific ion effects including toxicities and imbalances (Serrano et al., 1999). It would appear that the growth response at moderate salinities might be the consequence of an increased uptake of solutes that are required to induce cell expansion since this maintains the pressure potential in plant tissues (Zhu et al., 2002). At high salinities, growth reduction might be caused either by a reduced ability to adjust osmotically as a result of saturation of the solute uptake system, or because of excessive demand for the energy requirements of such systems (Zhu, 2003). Other factors, such as nutrient deficiencies, may also play an important role (Marschner, 1995). It is hypothesized that increased medium salinity could restrict the synthesis of plant growth promoters such as cytokinins and increase the production of inhibitors such as abscisic acid (Xiong and Zhu, 2003).

It is possible that under salt stress the plant spends more photosynthetic energy on root production in search of water and/or reducing water loss and thus maintains relatively high water relations (Kafkafi, 1991). Our results showed that the total water uptake decreased with increasing salinity level (Table 1) and the decrease patterns were similar to those of dry matter reduction as reported by Pessarkli (1993). The reduction in water uptake by other plants and other bean cultivars grown under salinity was mentioned by Salim (1991) who reported that plant root permeability decreased significantly under salinity. This may explain the reduction in the water-uptake rate and may contribute to a reduction in nutrient absorption resulting in retarded plant growth and decreased matter production under salinity.

It has been reported that Na^+ is more toxic to the plant tissues than Cl^- but the growth of most plants is stimulated at low Na^+ concentrations (Tester and Davenport, 2003). As in most of other glycophytes, a consistent increase in Na^+ as well as a decrease in K^+ and Ca^{++} in both shoots and roots of *Phaseolus vulgaris cv.* Djadida were found with increasing NaCl concentration in the growth medium. Salinity charged considerably plants with Na^+ noting that shoots acquired the higher accumulation levels. In addition, the concentration of Cl^- in the shoots was higher than in the roots since the accumulation of Cl^- in the shoots was markedly higher than that of Na^+ (Lazof and Bernstein, 1999). Such a pattern of toxic ions accumulation was earlier reported in a number of plant species referred to as salt includers (accumulators) (Munns et al., 2000). There is abundant evidence that salinity alters ion transport and contents in plants (Cramer, 1997). It has been generally observed that plants exposed to NaCl take up high amounts of Na^+, whereas the uptake of K^+ and Ca^{++} is significantly reduced (Rengel, 1992; Cramer, 1997). However, reasonable amounts of K^+ and Ca^{++} are required

by plants to maintain the integrity and functioning of cell membranes (Davenport et al., 1997). The maintenance of adequate K^+ in plant tissue under saline conditions seems to be dependent upon selective K^+ uptake and selective cellular K^+ and Na^+ compartmentation and distribution in the shoots (Munns et al., 2000). However, high K^+/Na^+ selectivity in plants under saline conditions is considered as one of the important selection criteria for salt tolerance (Ashraf and Harris, 2004). Tester and Davenport (2003) have pointed out that low K^+/Na^+ ratios could disrupt protein synthesis in the cell, given that Na^+ competes with K^+ for binding sites essential for cellular function, but cannot substitute for K^+ to activate functional enzymes.

The ability of Ca^{++} to form intermolecular linkages gives it an important role in maintaining the integrity and structure of membranes and cell walls and it is also used as a second messenger in many signal transduction pathways within the cell (Bush, 1995). Furthermore, the maintenance of Ca^{++} acquisition and transport under salinity constitutes an important determinant of salinity tolerance (Unno et al., 2002). Although the K^+/Na^+ ratio decreased consistently in the shoots and roots of *Phaseolus vulgaris cv.* Djadida after the increase in NaCl salt level in the growth medium, this ratio was markedly higher in shoots compared to roots. Moreover, supplemental concentrations of Ca^{++} in the medium depressed the adverse NaCl effects and produced an extreme K^+/Na^+ ratio in plants tissues, thus making plants to be less susceptible to osmotic and specific ion injury as well as to nutritional reorders.

Important ameliorative effects of Ca^{++} on Na^+ toxicity in plants have been reported to improve ion selectivity and ion transport (Lazof and Bernstein, 1999) and also, to ameliorate the Na^+-induced inhibition of growth in most plants (White and Broadley, 2003). The effects of supplemental Ca^{++} on the growth of salt-stressed plants were immediate (Zhong and Läuchli, 1993) and were related to the ion activities in the external solution of roots (Yermiyahu et al., 1997). Differences in leaf growth of maize (Cramer, 1992) and bean (Ortiz et al., 1994) were discernable within hours after varying the Na^+/Ca^{++} balance. Water transport into roots and shoots was also affected by the Na^+/Ca^{++} ratio (Knight et al., 1997). It was reported that salinity reduced hydraulic conductance to the leaves of maize plants treated with NaCl salinity (Cramer, 1992). One consequence of Na^+/Ca^{++} interactions is the reduction of K^+ content in salinized plants, which can be prevented with supplemental Ca^{++} (Marschner, 1995). It is now clear that Na^+ can enter cells through ion channels (Tyerman and Skerrett, 1999). In some cases, these channels are more selective for Na^+ than K^+ and the increase in the external Ca^{++} concentration reduces Na^+ conductance through these channels (Tyerman et al., 1997). Ca^{++} entry into cells can also occur through ion channels (White, 1998b). These channels are also permeable to Na^+ (White, 1998a), but it is not certain how Na^+ interacts with Ca^{++} in these channels. K^+ also moves through the Ca^{++} channels and can interfere with Ca^{++} transport (Piñeros and Tester, 1997); it seems likely that Na^+ would do the same. These channels may control the transport of cations to the xylem and therefore, may control cation transport to the shoot. These

channels are preferentially selective for K^+, but Na^+ can also move through them to a lesser extent (White and Broadley, 2001). Movement of Na^+ through these channels would likely reduce K^+ movement through them. These channels also appear to be permeable to Ca^{++} although Ca^{++} entry is predicted to be from the apoplast. In addition to the direct effects of Ca^{++} on ion transport, it may act on transport through a Ca^{++} signaling pathway (White and Broadley, 2001). Thus, it is believed that external supplementary Ca^{++} regulates Na^+ and K^+ transport, mineral balance and as a consequence, the K^+/Na^+ selectivity of the plant (Liu and Zhu, 1998). Although reduction in biomass, water content and mineral imbalance have been reported to have a cumulative effect attributed to salinity stress presuming the includer character of the experimental genotype, it is also clear that external supplement of Ca^{++} can improve plant growth under salinity conditions. The addition of calcium chloride could offer a simple solution to plant production problems caused by salinity. Specific ions accumulation in shoots and roots, and biomass production represent a good criterion to select and identify salinity tolerant genotypes. Nevertheless, indices based on molecular basis constitute the ultimate screening tool and could help a lot to understand the mechanisms underlying such stress responses.

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