



# Journal of Biological Sciences

ISSN 1727-3048

**science**  
alert

**ANSI***net*  
an open access publisher  
<http://ansinet.com>

## Effect of Salt Stress on $\alpha$ -amylase Activity, Sugars Mobilization and Osmotic Potential of *Phaseolus vulgaris* L. Seeds Var. 'Cocorose' and 'Djadida' During Germination

<sup>1</sup>Ahmed Adda, <sup>1</sup>Zineb Regagba, <sup>1</sup>Ahmed Latigui and <sup>2,3</sup>Othmane Merah

<sup>1</sup>University of Tiaret, Laboratory of Plant Physiology Applied to Soilless Crop, Algeria

<sup>2</sup>Universite of de Toulouse, INP-ENSIACET, LCA (Laboratoire de Chimie Agro-industrielle),  
F-31030, Toulouse, France

<sup>3</sup>INRA, UMR 1010 CAI, F-31030 Toulouse, France

**Abstract:** The influence of different treatments of NaCl (0, 50, 100, 150 and 200 mM) on water uptake,  $\alpha$ -amylase activity, total soluble sugars, germination and root length of two bean (*Phaseolus vulgaris* L.) cultivars (Cocorose, Djadida) were studied during germination. Increase in NaCl stress reduced moderately seed's water uptake. The effect of salinity on  $\alpha$ -amylase activity depends on the concentration of NaCl. This activity increased with time (48 and 72 h) at lower stress salinity (50 and 100 mM NaCl) and decreased when salt concentration reached 150 mM. However, soluble sugars content augmented significantly inducing therefore, a significant decrease in the osmotic potential of bean seeds during germination. Nevertheless, lower concentrations of NaCl (50 and 100 mM) did not affect the rate of seed germination. In contrast, this trait was significantly reduced at 150 and 200 mM NaCl. Radicle length growth was the most affected parameter by salinity treatment. These results suggest that for both treatment of 50 and 100 mM NaCl, seed germination was not affected by salinity and the  $\alpha$ -amylase activity was increased. At 150 and 200 mM NaCl these traits were markedly affected. Moreover, increasing concentrations of NaCl in the medium was accompanied by an increase of the soluble sugar contents in bean seeds which lead to decrease their osmotic potential.

**Key words:** Salt stress, germination,  $\alpha$ -amylases activity, simple sugars, bean (*Phaseolus vulgaris* L.)

### INTRODUCTION

Salinity and water deficit are the major abiotic factors limiting plant growth and productivity (Adda *et al.*, 2005). The effects of salinity on plant growth and productivity depend on their intensity and timing during crop cycle (Munns, 2002; Khan and Gulzar, 2003; Peel *et al.*, 2004).

Adaptation of plants to salinity during germination and early seedling stages is crucial for the plant establishment. In fact, these stages are the most vulnerable to the salinity effects for beans (Kaymakanova, 2009). Salt stress reduces seed germination by decreasing the water absorption (Dodd and Donovan, 1999; Jamil *et al.*, 2006), limits the mobilization of reserves (Lin and Kao, 1995) and disturbs the synthesis of the protein in the embryo (Ramagopal, 1990).

The hydrolysis of starch reserves of the cotyledons is one of the major activities initiated during the early stages of germination. Mobilized soluble sugars, play an important role in cell osmotic adjustment during germination (Gorham *et al.*, 1981). They may regulate the expression of some genes involved in this phase (Yu *et al.*, 1996; Aubry *et al.*, 2003; Teulat-Merah *et al.*, 2011). The  $\alpha$ -amylase plays a key role in starch

degradation and liberation of soluble sugars. However, the enzymatic activity and the hydrolysis of the starch are strongly influenced by salt stress during germination (Lin and Kao, 1995; Kaur *et al.*, 1998).

The main objective of this study was to study the effects of different concentrations of NaCl on seeds germination of two varieties of beans (Cocorose and Djadida) contrasted for their salt tolerance. The study allows explaining the impact of salinity on the activity of  $\alpha$ -amylase in starch hydrolysis. It also attempts to distinguish the contribution of sugars issued from the hydrolysis in changes of osmotic potential and the realization of various stages of seed germination subjected to different NaCl concentrations in the medium.

### MATERIALS AND METHODS

**Seeds germination condition and treatments:** The experiment was conducted at the University of Tiaret (35° 23' 17" N, 1° 19' 22" E, altitude of 1080 m northwest of Algeria).

Seeds of common bean (*Phaseolus vulgaris* L.) cultivars Cocorose (salt sensitive) and Djadida (salt tolerant), were surface sterilized by immersion in

5% (v/v) NaClO<sub>4</sub> solution for 4 min, rinsed four times with sterile distilled water. Twenty seeds per cultivar were placed on filter paper (Whatman N°2) in Petri dishes (9 cm) containing 5 mL of distilled water (control) and 50, 100, 150 and 200 mM of NaCl corresponding to 0.592, 0.767, 0.919 and 1.073 MPa, respectively and allowed to germination at 25°C. Each treatment includes six replicates.

### Traits measurements

#### Kinetic of seed imbibition, germination rate and root

**length:** Water uptake was recorded during 96 h every 12 h. It was calculated according to the following equation:

$$\text{Water uptake (\%)} = \left[ \frac{W_2 - W_1}{W_1} \right] \times 100$$

where,

W<sub>1</sub> was the initial seed weight before imbibition and W<sub>2</sub> was the weight of seeds after water absorption. Seed germination was evaluated after 108 h. Seeds were considered as germinated when the radicle reached 2 mm. The germinated seeds were counted and germination percentage was determined. Also, the radicle length (mm) was measured.

**α-Amylases extraction and assay:** Extraction of α-amylase was carried out on germinating seeds placed after 48-72 h and their activity was measured. Ten grams of cotyledons were ground in 50 mL of acetate buffer solution (pH 4.8) containing 50 mM of sodium acetate and 20 mM of CaCl<sub>2</sub> (Kaur *et al.*, 1998). The extract was centrifuged at 10000 g for 10 min at 4°C. The supernatant was then heated at 75°C for 15 min to inactivate β-amylase according to Guglielminetti *et al.* (1995). The extract was used for estimating the activity of α-amylases according to the method described by Almansouri *et al.* (2001). This technique involved using 1 mL of enzyme extract, supplemented with 0.5 mL of starch solution 1%. It was diluted in acetate buffer containing 0.05 mM of sodium acetate and 0.02 mM of CaCl<sub>2</sub> (pH 4.8). The whole was mixed by vortex and incubated for 15 min at 35°C. The reaction was stopped by adding 0.5 mL of 3,5 Dinitrosalicylic Acid Reagent (DNS). The assembly was placed in a boiling water bath for 5 min and cooled. The absorbance of the reaction mixture was determined at 540 nm in spectrophotometer (Jenway 73 series model spectrophotometer) against maltose as standard. The α-amylase activity was determined in μ moles of reducing sugars formed min<sup>-1</sup>g<sup>-1</sup> FW.

**Determination of soluble sugars contents:** Soluble sugar content was evaluated on 100 mg of cotyledons of germinating seeds (72 h) from all treatments (0, 50, 100, 150

and 200 mM of NaCl). The cotyledons were homogenized on 5 mL of 80% ethanol for 24 h (Sidari *et al.*, 2008). The extract obtained was diluted 10 times with 80% ethanol. From the resulting solution, 2 mL were mixed with 4 mL anthrone reagent (200 mg anthrone, 200 mL of sulfuric acid W/W). After stirring, samples were placed in boiling water bath for 10 min and cooled for 30 min in the dark. The light absorption of the samples was estimated at 585 nm using a Jenway 73 series model spectrophotometer.

Soluble sugars contents were determined using glucose standard and expressed in mg g<sup>-1</sup> FW of cotyledons.

**Determination of osmotic potential:** Cotyledons are placed in Eppendorf tubes, dipped in liquid nitrogen and ground with Retsch MM 400 mixer mill (USA) and centrifuged. Osmotic potential was determined on 10 mL of supernatant using Wescor Vapro 5520 model osmometer (USA).

**Statistical analysis:** They was performed using two way ANOVA (for p<0.01) from the Statistica 8.0 package. Mean comparison was performed by Duncan test at 5% level of significance.

## RESULTS

**Effect of salinity and cultivar on water uptake, seed germination and radicle length:** Salt treatments and cultivar affected significantly water uptake, seed germination and radical length (Table 1). Moreover, interaction cultivar × salt treatment was significant. Seed imbibition was not changed by the salt stress intensity (Fig. 1). However, the water uptake by Cocorose was higher than Djadida during different times. For example, 96 h after sowing, the rate of imbibition of the control reached 111.79 and 94.42% for Cocorose and Djadida, respectively. At higher salt treatment (200 mM NaCl), these rates were reduced by 1.82 and 1.35% for Cocorose and Djadida, respectively.

No significant difference was observed for the rate of seed germination between control and treatment at 50 and 100 mM of salt (Fig. 2). In contrast, higher salt treatment reduced markedly this rate (Fig. 2). The radicle length was reduced significantly by the salt stress intensity (Table 2). However, this reduction depended on the concentration applied and genotypes tested (Table 1). Compared to control, salt treatment at 50 mM decreased the length of radicle by 16.8 and 29.42% for Djadida and Cocorose, respectively. This reduction was more pronounced for the treatments 100, 150 and 200 mM for

Table 1: Mean square values and significance of NaCl treatment, genotype and their interaction effects on germination (%) radicle length, osmotic potential,  $\alpha$ -amylase activity and soluble sugars content measured on two cultivars bean

Trait	Source of variation		
	Cultivar	NaCl	Interaction cultivar $\times$ NaCl
Seed germination (%)	260.4***	2969.1***	70.8***
Root length	104.41***	585.76***	23.06***
Osmotic potential	0.0904***	1.013***	0.0025ns
$\alpha$ -Amylase activity (48 h)	140.39***	23.98***	4.34***
$\alpha$ -Amylase activity (72 h)	330.13***	165.7***	28.44***
Sugar contents	1.80**	13.07***	0.515*

\* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , ns: not significant

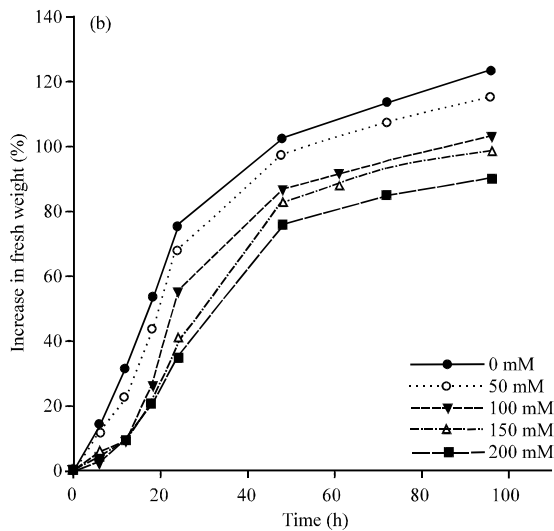
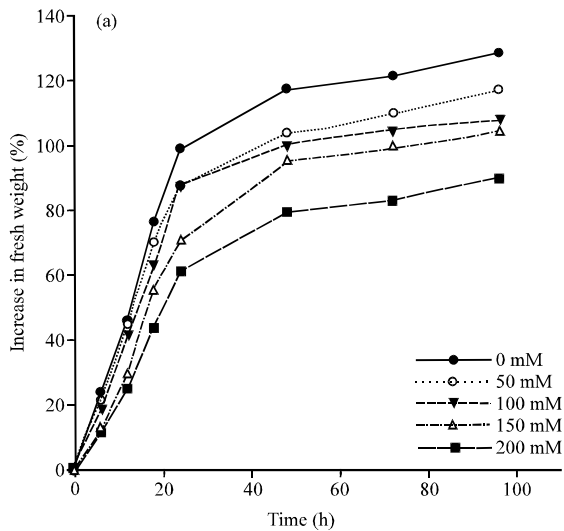


Fig. 1(a-b): Effect of salinity on seed water uptake during germination of two cultivars of *Phaseolus vulgaris* L., (a) Cocorose and (b) Djadida

both genotypes (Table 2). Djedida was less affected than Cocorose for 50, 100 and 150 mM, while Cocorose showed higher radical length at 200 mM.

Table 2: Mean values of radicle length and sugar content seeds under different NaCl treatments

Cultivar	NaCl (mM)	Radicle length (cm)	Sugar content (mg g <sup>-1</sup> FW)
<b>Djadida</b>	0	17.14 <sup>a</sup>	6.72 <sup>a</sup>
	50	14.25 <sup>b</sup>	7.38 <sup>b</sup>
	100	10.25 <sup>c</sup>	7.92 <sup>c</sup>
	150	5.42 <sup>d</sup>	8.62 <sup>d</sup>
	200	2.83 <sup>e</sup>	9.69 <sup>e</sup>
<b>Cocorose</b>	0	24.34 <sup>a</sup>	6.93 <sup>b</sup>
	50	17.18 <sup>b</sup>	8.01 <sup>c</sup>
	100	10.56 <sup>c</sup>	8.72 <sup>c</sup>
	150	6.00 <sup>d</sup>	8.98 <sup>c</sup>
	200	5.00 <sup>d</sup>	9.42 <sup>d</sup>

Means indicated by different letters (within each NaCl treatment) are significantly different (at 0.05 probability level) by the Duncan comparison test

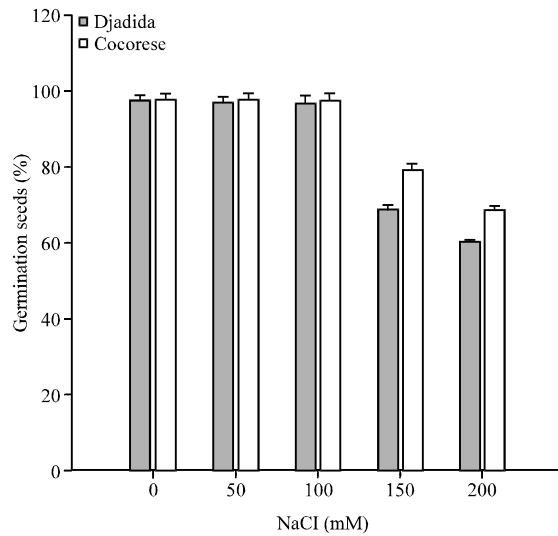


Fig. 2: Effect of salinity (NaCl) concentrations (0, 50, 100, 150 and 200 mM) on seed germination (%) of two cultivars of *Phaseolus vulgaris* L., Cocorose and Djadida

**Effect of salinity and cultivar on biochemical traits:** All biochemical traits were significantly affected by salt treatment and cultivar. Interaction cultivar  $\times$  salt treatment was also significant for all traits except for (Table 1). The

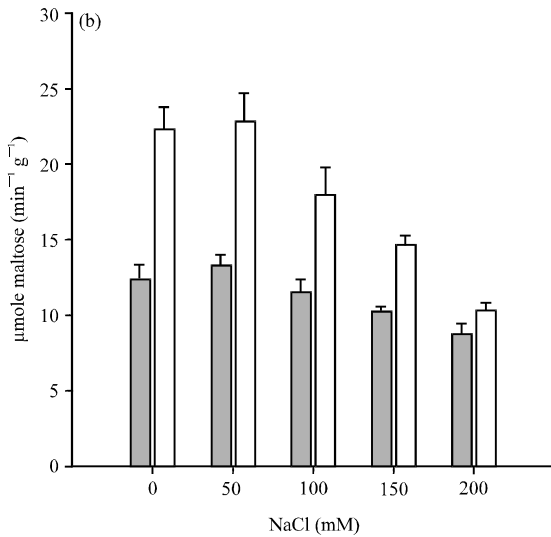
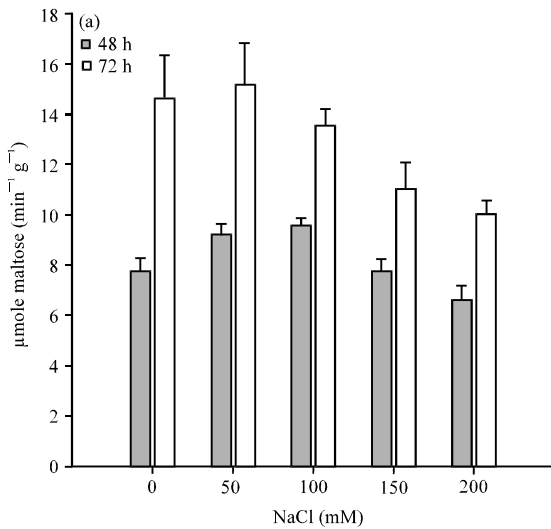


Fig. 3 (a-b): Effect of salinity (NaCl) conc (0, 50, 100, 150 and 200 mM) on activity of  $\alpha$ -Amylase in seeds after 48 and 72 h of germination of two cultivars of *Phaseolus vulgaris* L., (a) Djadida and (b) Cocorose

activity of  $\alpha$ -amylase was affected by salt stress intensity and period of germination (Fig. 3). The impact of salinity was intensified with time of germination in both genotypes and all salt treatments (Fig. 3). In the control treatment,  $\alpha$ -Amylase activity from 48-72 h increased by 87 and 81% for Djadida and Cocorose, respectively. Djadida presented higher  $\alpha$ -Amylase activity whatever the intensity of salt treatment. Moreover, the difference in  $\alpha$ -Amylase activity between 48 and 72 h was more important for Djedida than for Cocorose at higher salt stress intensity (Fig. 3).

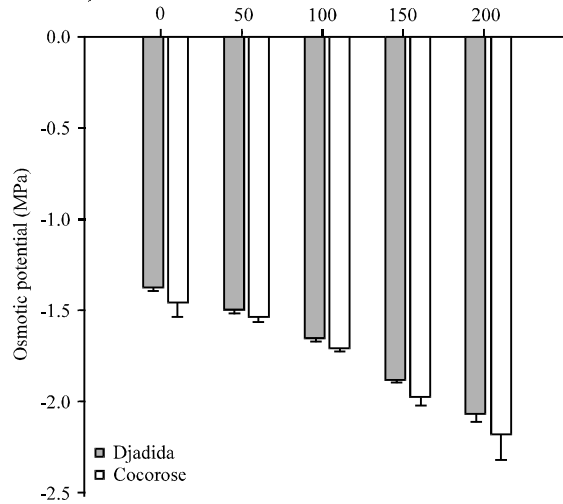


Fig. 4: Effect of salinity (NaCl) conc. (0, 50, 100, 150 and 200 mM) on osmotic potential of seeds of two cultivars of *Phaseolus vulgaris* L., Djadida and Cocorose

The results indicated that the concentration 50 mM and intensities linked to the time increased  $\alpha$ -Amylase activity compared to that of the control. Thus,  $\alpha$ -Amylase activity was greater after 48 h of germination than after 72 h. During the first period, it was 17.40 and 9.18%, respectively for Djadida and Cocorose, against 1.72 and 1.87% for the second. Beyond this level, the activity gradually decreased regarding to the concentration of NaCl and the duration of germination. The inhibition of the activity was more pronounced in the treatment of 200 mM and after 72 h of germination time. It was valued at 45.74 and 52.65%, respectively in Djadida and Cocorose.

The results (Table 2) showed that the sugar content of germinating seeds depended on genotype, salt treatments and their interaction. The increase of NaCl concentration induced a relative rise in the sugar content of seeds of both genotypes. Indeed, by comparison with control sugar content increased from 9.43% (50 mM) to 44.23% (200 mM). The range of sugar content augmentation was quite similar for both genotypes.

Higher NaCl content induced highly significant decrease in osmotic potential of germinating seeds and the sugar accumulation (Fig. 2 and Table 2). The two genotypes responded differently to salt treatment. In Djadida, values of osmotic potential markedly decreased with salt stress intensity compared Cocorose (Fig. 4).

## DISCUSSION

Seed germination starts with uptake of water by quiescent seed and terminates with elongation of the

embryonic axis. The germination is exposed to different environmental stresses, where drought and salinity are the most severe ones. Our results show that salinity levels of 100 and 200 mM caused a marked decrease in the rate of seed germination (Fig. 2).

This result confirmed previous reports (El-Tayeb, 2005; Othman *et al.*, 2006; Jamil *et al.*, 2006; Al-Saady *et al.*, 2013) which showed a reduction in seed rate germination due to higher levels of NaCl. The effect of salinity on radicle length was more marked. Indeed, the increase in salinity reduced the length of the radicle (Table 2).

The  $\alpha$ -Amylase activity estimated after 48 and 72 h at low NaCl concentration 50 mM increased. These results coincided with those of Dodd and Donovan (1999), Sidari *et al.* (2008) and Mei and Song (2008). Beyond this concentration, salinity presented a reducing effect on the activity. Siddiqui and Khan (2011) showed that from 100 mM of NaCl,  $\alpha$ -Amylase activity is greatly reduced. These traits provide important criteria to characterize the response of plants to abiotic stress as salinity (Adda *et al.*, 2005; Othman *et al.*, 2006; Jamil *et al.*, 2006). However, cultivars did not responded similarly to salinity which mirrored the existence of broad genetic variability within this species. Moreover, difference in water uptake and seed germination (Fig. 1) were found between Cocorose and Djadida as observed in several species (Othman *et al.*, 2006; Jamil *et al.*, 2006; Al-Saady *et al.*, 2013; Ogiwara and Terashima, 2010). The large genotypic variability observed for studied traits may offer valuable tools to investigate mechanism of salt tolerance during germination.

Seed germination begins with the water uptake by seed and ends with the radicle protrusion and elongation. Barroco *et al.* (2005) suggested that cell elongation is necessary and can be generally accepted as a sufficient phenomenon for the completion of this process.

During germination, the osmotic potential of the radicle cells becomes more negative because of the accumulation of solutes mainly sugars (Bewley, 1997; Munns, 2002; Othman *et al.*, 2006; Ogiwara and Terashima, 2010). The decrease in osmotic potential would lead to increased water uptake, where resulting turgor and cell extension (Mei and Song, 2008; Jamil *et al.*, 2006).

Higher concentration of salt reduced osmotic potential (and probably water potential) in the medium which limited water absorption by germinating seeds and thus reduced germination as already reported by Maas *et al.* (1983) and Al-Saady *et al.* (2013). Therefore, the rise of salinity was accompanied by a significant decrease in the osmotic potential of cotyledons (Fig. 4).

Which leads to maintain water uptake by seeds and rate germination. We suggest that accumulation of sugars in germinating seeds exposed to high NaCl concentrations may explain allow utilization of these sugars in germination process. In fact, they may be used in osmotic adjustment and synthesis of energy necessary for growth (Bewley, 1997; Gill *et al.*, 2003). This osmotic adjustment maintains cell turgor which results in a reduction in the growth rate. The maintain of cell turgor was probably reached by the rise of sugar content which resulted in lowering of the osmotic potential of seeds subjected to salt stress (Table 2). Nevertheless, increase in sugar content was maintained despite the reduction in the  $\alpha$ -Amylase activity mainly beyond 100 mM NaCl, These results are in accordance with those reported in wheat same results were shown in several plant species, (Jamil *et al.*, 2006; Othman *et al.*, 2006; Zheng *et al.*, 2008).

## CONCLUSION

Increase in NaCl stress affected negatively seed water uptake and  $\alpha$ -Amylase activity depending on the concentration of salt. This fact allowed to a decrease of germination level mostly at higher salt treatments. However, soluble sugars content raised significantly which leads to a significant decrease in the osmotic potential of bean seeds during germination. In contrast, this trait was significantly reduced at 150 and 200 mM NaCl. Radicle length growth was the most affected parameter by salinity treatment. These results suggest that for both 50 and 100 mM NaCl treatments, seed germination was not affected by salinity and the  $\alpha$ -Amylase activity was increased. Beside, at 150 and 200 mM NaCl an opposite situation for seed germination and  $\alpha$ -Amylase activity was observed.

Finally increasing concentrations of NaCl in the medium was accompanied by an increase of the soluble sugar contents in bean seeds which leads to decrease their osmotic potential and therefore maintained water uptake.

## REFERENCES

- Adda, A., M. Sahnoune, M. Kaid-Harch and O. Merah, 2005. Impact of water deficit intensity on durum wheat seminal roots. *Comptes Rendus Biol.*, 328: 918-927.
- Al-Saady, N.A., A.J. Khan and R. Lakshmi, 2013. A study on germination rate, dry matter weight and amylase activity of *Medicago sativa* L. (alfalfa) under induced NaCl stress. *Adv. Crop. Sci. Technol.*, Vol. 1. 10.4172/2329-8863.1000108

- Almansouri, M., J.M. Kinet and S. Lutts, 2001. Effect of salt and osmotic stresses on germination in durum wheat (*Triticum durum* Desf.). *Plant Soil*, 231: 243-254.
- Aubry, C., M.C. Morere-Le Paven, A.L. Chateigner-Boutin, B. Teulat-Merah and C. Ricoult *et al.*, 2003. A gene encoding a germin-like protein, identified by a cDNA-AFLP approach, is specifically expressed during germination of *Phaseolus vulgaris*. *Planta*, 217: 466-475.
- Barroco, R.M., K. Van Poucke, J.H. Bergervoet, L. De Veylder, S.P. Groot, D. Inze and G. Engler, 2005. The role of the cell cycle machinery in resumption of postembryonic development. *Plant Physiol.*, 137: 127-140.
- Bewley, J.D., 1997. Seed germination and dormancy. *Plant Cell*, 9: 1055-1066.
- Dodd, G.L. and L.A. Donovan, 1999. Water potential and ionic effects on germination and seedling growth of two cold desert shrubs. *Am. J. Bot.*, 86: 1146-1153.
- El-Tayeb, M.A., 2005. Response of barley grains to the interactive effect of salinity and salicylic acid. *Plant Growth Regul.*, 45: 215-224.
- Gill, P.K., A.D. Sharma, P. Singh and S.S. Bhullar, 2003. Changes in germination, growth and soluble sugar contents of *Sorghum bicolor* (L.) Moench seeds under various abiotic stresses. *Plant Growth Regul.*, 40: 157-162.
- Gorham, J., L.L. Hughes and R.G. Wyn Jones, 1981. Low-molecular-weight carbohydrates in some salt-stressed plants. *Physiol. Plant.*, 53: 27-33.
- Guglielminetti, L., J. Yamaguchi, P. Perata and A. Alpi, 1995. Amyolytic activities in cereal seeds under aerobic and anaerobic conditions. *Plant Physiol.*, 109: 1669-1676.
- Jamil, M., D.B. Lee, K.Y. Jung, M. Ashraf, S.C. Lee and E.S. Rha, 2006. Effect of salt (NaCl) stress on germination and early seedling growth of four vegetables species. *J. Central Eur. Agric.*, 7: 273-283.
- Kaur, S., A.K. Gupta and N. Kaur, 1998. Gibberellin A<sub>3</sub> reverses the effect of salt stress in chickpea (*Cicer arietinum* L.) seedlings by enhancing amylase activity and mobilization of starch in cotyledons. *Plant Growth Regul.*, 26: 85-90.
- Kaymakanova, M., 2009. Effect of salinity on germination and seed physiology in bean (*Phaseolus vulgaris* L.). *Biotechnol. Biotechnol. Equipment*, 23: 326-329.
- Khan, M.A. and S. Gulzar, 2003. Germination responses of *Sporobolus ioclados*: A saline desert grass. *J. Arid Environ.*, 53: 387-394.
- Lin, C.C. and C.H. Kao, 1995. NaCl stress in rice seedling: Starch mobilization and the influence of gibberellic acid on seedling growth. *Bot. Bull. Academia Sinica*, 36: 169-173.
- Maas, E.V., G.J. Hoffman, G.D. Chaba, J.A. Poss and M.C. Shannon, 1983. Salt sensitivity of corn at various growth stages. *Irrigation Sci.*, 4: 45-47.
- Mei, Y. and S. Song, 2008. Early morphological and physiological events occurring during germination of maize seeds. *Agric. Sci. China*, 7: 950-957.
- Munns, R., 2002. Comparative physiology of salt and water stress. *Plant Cell Environ.*, 25: 239-250.
- Ogiwara, H. and K. Terashima, 2010.  $\alpha$ -amylase activity and soluble sugar supply from endosperm in relation to varietal differences in seedling establishment under low-temperature conditions in rice (*Oryza sativa* L.). *Plant Prod. Sci.*, 13: 321-330.
- Othman, Y., G. Al-Karaki, A.R. I-Tawaha and A. Al-Horani, 2006. Variation in germination and ion uptake in barley genotypes under salinity conditions. *World J. Agric. Sci.*, 2: 11-15.
- Peel, M.D., B.L. Waldron, K.B. Jensen, N.J. Chatterton, H. Horton and L.M. Dudley, 2004. Screening for salinity tolerance in Alfalfa. *Crop Sci.*, 44: 2049-2053.
- Ramagopal, S., 1990. Inhibition of seed germination by salt and its subsequent effect on embryonic protein synthesis in barley. *J. Plant Physiol.*, 136: 621-625.
- Sidari, M., C. Santonoceto, U. Anastasi, G. Preiti and A. Muscolo, 2008. Variations in four genotypes of lentil under NaCl-salinity stress. *Am. J. Agric. Biol. Sci.*, 3: 410-416.
- Siddiqui, Z.S. and M.A. Khan, 2011. The role of enzyme amylase in two germinating seed morphs of *Halopyrum mucronatum* (L.) Stapf. in saline and non-saline environment. *Acta Physiologiae Plantarum*, 33: 1185-1197.
- Teulat-Merah, B., M.C. Morere-Le Paven, C. Ricoult, C. Aubry and D. Peltier, 2011. cDNA-AFLP profiling in the embryo axes during common bean germination. *Biol. Plant.*, 55: 437-447.
- Yu, S.M., Y.C. Lee, S.C. Fang, M.T. Chan, S.F. Hwa and L.F. Liu, 1996. Sugars act as signal molecules and osmotica to regulate the expression of  $\alpha$ -amylase genes and metabolic activities in germinating cereal grains. *Plant Mol. Biol.*, 30: 1277-1289.
- Zheng, Y., A. Jia, T. Ning, J. Xu, Z. Li and G. Jiang, 2008. Potassium nitrate application alleviates sodium chloride stress in winter wheat cultivars differing in salt tolerance. *J. Plant Physiol.*, 165: 1455-1465.