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EFFECT OF GA3, ABA AND KINETIN ON THE RESPONSE OF THE HALOPHYTE *ATRIPLEX HALIMUS* TO SALINITY DURING GERMINATION

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Abstract

Atriplex halimus is a xerohalophytic perennial shrub native to the Mediterranean and has potential for use in ecological restoration programmes. In this study we investigate in order (i) the effects of increasing concentrations of NaCl (100, 200, 300, 400, 500 and 600mM) on germination of *Atriplex halimus*, (ii) the impact of phytohormones on the biochemical traits of germination, under 300 mM of NaCl. The increase in salinity resulted in the decrease of germination, water uptake and osmotic potential especially at high concentration. Germination in the ABA-treated group, was markedly decreased either in presence or absence of NaCl. However, both GA3 and kinetin were very effective in alleviating salt adverse effects. This was reflected in the increased content of soluble sugars, whereas ABA-treated seeds showed a contradictory effect under salinity. Consequently, a remarkable decrease in alpha amylase activity was observed under ABA and NaCl treatments, while no substantial changes were recorded after addition of kinetin in the absence of NaCl. Furthermore, in stressed seeds this activity was strongly reversed with GA3, Results suggest that these hormones appear regulate the germination under salinity, by aleviating the toxic effect of NaCl and contrubute to osmotic adjustment in this specie.

Key words : *Atriplex halimus,* salt stress, germination, growth regulators, alpha amylase.

Introduction

Salinity is one of the major physical parameter of an environment, which determines the success or failure of plants establishment (Gul *et al*., 2013). It also, negatively affects the quality of soil and limits plant growth and production (Soualem *et al*., 2014). Consequently, the study of salt tolerance during germination early and late growth of plants is important for determining saline limits at each developmental phase (Zapata *et al*., 2004). In addition, it has been reported that salinity decreased as well as delayed germination of most crops (Adda *et al*., 2014).

Many authors have suggested that seeds of most halophytes, especially the genus Atriplex, are very sensitive to elevated salinity during germination and early seedling establishment phases (Abbad *et al.,* 2004). The inability of halophyte seeds to germinate under hypersaline conditions but then initiate germination when salinity decreases, is a criterion of salt tolerance which distinguishes them from most glycophytes (Debez *et al*., 2004), the inhibition of seed germination of halophytes under saline condition and early growth was primarily due to an osmotic effect and not to a specific ion toxicity of either the chloride or sulfate salts (Sosa *et al*., 2005; Yingchao *et al*., 2013).

The inhibitory effect of salt stress on seed germination is alleviated by phytohormones, including cytokinin (Miransaria and smith, 2014), ethylene (Kepczynski and Kepczynska, 1997), GA (Khan and Ungar, 1998; Khan *et al*., 2004). Among the phytohormones, ABA inhibits seed germination, while ethylene and gibberellin acid (GA) antagonize the ABA-induced inhibitory effect on seed germination (Yuan *et al*., 2011). Salt stress inhibits seed germination by reducing water uptake, increasing ABA content and decreasing GA content (Almansouri *et al*., 2001; Kim and Park, 2008; Cao *et al*., 2014). The decreased cytokinin and gibberellic acid and increased

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abscisic acid contents observed in stressed plants, suggested that salt stress induces changes in membrane permeability, as well as water relations (Akter *et al*., 2014). Recently, Kong *et al*. (2017), found that, salt stress increased the ABA content by up regulating ABA biosynthesis genes, and downregulating the ABA catabolism gene, while decreased the GA content by increasing the expression of GA catabolism gene and decreasing that of the GA biosynthesis gene, during germination.

It must be mentioned, however, that germination involves mobilization of reserves, as a whole, rather than being an event associated with one or two enzymes. It has been demonstrated that GA regulates the initiation of germination and the mobilization of storage by increasing amylase activity (Huang *et al*., 2016).

The understanding of the hormonal action on germination process is a major key to improve germination of salt-sensitive species and subsequently their establishment under saline conditions. The present study was carried out in order to (i) examine the effects of increasing salt stress induced by NaCl on germination of the saltbush Atriplex halimus water uptake and osmotic potential, (ii) determine the impact of GA_3, ABA, and kinetin during the germination, under NaCl salinity, focusing on: percentage of germination, solubles sugars contents and activity of alpha amylase.

Materials and Methods

Seeds germination and treatments

The seeds of *Atriplex halimus* were collected from the area of El Mesrane in the province of Djelfa, inland zone; 3°03'E, 34°36'N, and 830 m elevation. After removal of the fruiting bracts, surface seeds were sterilised for 5 minutes in 10% (v/v) sodium hypochlorite solution, and rinsed thoroughly with deionised water. Seeds were then used to investigate the effect of NaCl and phytohormones on seed germination.

Study of germination under salt

Seeds of Atriplex incubated in distilled water (control), or NaCl (100, 200, 300, 400, 500 and 600 mM) corresponding, respectively to an external osmotic potential of 0.41, 0.86, 1.30, 1.5, 1.96 and 2.20 MPa, assessed by the use of a Wescor vapour pressure osmometer. For each of the treatments, four replicates of 50 seeds were incubated in absorbent paper on Petri dishes placed in darkness at 27°C. For each treatment, moisture concentration was recorded at 0 (dry seeds) 6, 12, 18, 24 and 30 hours after the start of imbibition. The water uptake was expressed as the percentage increase in moisture concentration on fresh weight basis. The germination percentage was determined, a seed was considered as germinated when radicle was longer than 2 mm. The osmotic potential of seeds was determined using wescor vapro model osmometer.

Study of germination under salt and Phytohormones treatments

Atriplex seeds were placed randomly in Petri dishes (9.0cm diameter) containing filter paper soaked with solution containing either 0 (control) or 300mM of NaCl. The effects of GA, kinetin and ABA on seed germination in the absence and presence of NaCl were investigated by treating the seeds with the solutions containing GA3, kinetin and ABA at concentrations of 29µM, 46.5µM and 50µM, respectively. Treatments were : (1) control (C): distilled water, (2) GA3 :29µM GA3, (3) Kin : 46.5µM of kinetin, (4) ABA : 50µM ABA, (5) salt stress (S): 300mM NaCl, (6) S + GA3: 300mM NaCl and 29µM GA3, (7) S+Kin : 300mM NaCl and 46.5µM Kinetin (8) S+ABA : 300mM NaCl and 50µM ABA. There were 50 seeds in each Petri dish and the seeds were soaked with 5mL treatment solution and placed at 27°C for 24 hours in a growth chamber in the dark. Seeds were considered to be germinated at the emergence of the radicle and scored. Fresh samples were collected 24 hours after sowing for the assay of alpha amylase, solubles sugars.

Determination of soluble sugars content

Sugars were extracted in 80% ethanol from 1 g of seeds and quantified by the classical anthrone method (Sidari *et al*., 2006) using a spectrophotometer (Jenway 73 series model). Standard curve was established using glucose and results were therefore expressed in $\mu g.g^{-1}$ FW.

Alpha amylase extraction and activity assay

Extraction and activity of amylase were analyzed according to Marambe *et al.* (1992). 1g of germinating seeds was macerated in 20 mL of 0.05 M succinate buffer (pH 5.2) containing 2mM $CaCl_2$. The extract was then centrifuged at 10000g for 20 minutes at 3°C and was filtered through two layers of Miracloth. Filtrate was immediately used as a crude enzyme preparation to determine α-amylase activity using a 0.2% (w/v) boiled starch solution and 0.5mL succinate buffer. Samples were then incubated for 30 minutes at 30°C and 1 mL each of dinitrosalicylic reagent and the final volume was made up to 10 mL with distilled water. The alpha-amylase activity was then determined using a spectrophotometer at 540 nm and results were expressed in U/mg of protein.

Data analysis

All data were subjected to the one-way analysis of variance (ANOVA) to determine the significant differences between treatments, using the software of SPSS (Ver. 16.0 SPSS). The mean pair-wise comparisons were based on the Duncan test.

Results

Effect of salinity concentration on germination

Salt stress induced significant differences on water uptake (fig. 1), seed germination and osmotic potential (table 1). NaCl at 100 Mm was a little effect on seeds germination (16% compared to control). However, the effect was much greater in the high concentrations of salt, when the decreases of the germination were about 74, 85, and 87% in seeds treated by 400, 500 and 600mM respectively. The osmotic potential was reduced significantly by the stress intensity (table 1) compared to control. The maximum osmotic potential was recorded in the seeds under control condition, followed by 100mM. Significant reduction of osmotic potential was verified in plants submitted up to 200mM of NaCl and the effect was more pronounced at highest salinity level (600mM of NaCl).

Effect of salinity and phytohormones on germination

Effects of salt treatment and phytohormones supply on physical and biochemical traits of germination are displayed in table 3. Mean values of each trait in salt and regulators growth supply are presented in table 3. The results showed that the highest ($P < 0.01$) germination percent in distilled water-treated group (control) was reached at 24 hours. However, the imposition of both ABA and NaCl treatments significantly ($P<0.01$) decreased seed germination, with values 65% and 63% respectively, as compared to control. As shown in Table 3, medium without NaCl, addition of GA3 and kinetin, was found to be effective in promoting germination of these seeds which the percentage of germination approached 100% after 24hours. Conversely, the impact of NaCl combined with ABA was more deleterious of seeds germination when the greatest reduction was recorded by more than 70%. Both kinetin and GA3 substantially accelerated the germination and completely reversed the inhibitory effect imposed by salinity and ABA (table 3).

Both NaCl and phytohormones treatments caused a significant ($P< 0.01$) modification in total soluble sugars content of seeds (table 3). The results revealed that addition of 300mM of NaCl and ABA decreased significantly the soluble sugars content, in seed (51% and 57%, respectively) compared to control. In general, in the absence of NaCl, GA3 was more responsive in increasing in soluble sugar content compared to Kinetin. Moreover, it is wealthy noted that the supply of ABA combined with NaCl caused a significant reduction of sugar content at about 80%. Interestingly, exogenous application of GA3 and Kinetin, clearly and significantly improved this biochemical trait under saline condition. These findings indicate the alleviative effect of salt by exogenious supply of GA3 and Kinetin by increasing in sugar content of seeds.

The major amylase activity in seeds is constituted by alpha-amylase (Gupta *et al*., 1993). The activity of alpha amylase in seeds exposed to salt-stressed and ABA were less than in the control seeds. However, addition of GA3 to the culture medium in the absence of NaCl was found to be most effective in promoting the amylase activity than kinetin (table 3). On the other hand, the addition of ABA to NaCl-containing medium significantly decreased ($P< 0.01$) amylase activity about 61%. In contrast, exogenous application of kinetin in the presence of 300mmol of NaCl was found to improve all the depressed effect of salinity but Supply of GA3 had more pronounced effect on this activity, when the greatest increase of this activity was obtained at about 54% compared to the control, suggesting that this later growth regulator alleviate the NaCl-induced suppression of amylase activity during germination.

Discussion

Seed germination is a complex process, commences with water uptake and imbibition, by the dry seed, followed by a series of metabolic changes, and ends with the protrusion of the radicle of the embryo through all the surrounding tissues (Holdsworth *et al*., 2008). The physical process of water uptake leads to the activation of metabolic processes as the dormancy of the seed is broken following hydration. Elevated salinity slows down water uptake by seeds, there by inhibiting their germination and root elongation (Werner and Finkelstein, 1995). The results demonstrated that the increase of NaCl inhibited the processes of imbibition, germination and osmotic potential especially at high concentrations (figure 1). The drop in the water uptake by the seeds is probably caused by the decrease in water potential gradient between the seeds and their surrounding media (Bewley and Black, 1994).

Many environmental factors such as light intensity, low temperature, soil salinity and drought affect seed germination (Huang *et al*., 2016). Although, many previous reports have shown that seed germination declined with increasing salinity levels (Almansouri *et al.*, 2001; Houle *et al.*, 2001) and ABA, GA and cytokinines are key hormones that regulate seed germination (Iqbal *et al.*, 2014).

Salinity affects almost every aspect of seeds physiology and biochemistry. Since the presence of salt in the medium reduces external osmotic potential and thus compromises water absorption, halophyte plant species are exposed in their natural habitats to both ion toxicities and to physiological drought (Flowers and Colmer, 2008).

Our data show that salt stress affects strongly the physical and biochemical traits of germination (table 3). Similar results were obtained by Abbad *et al.* (2004), which observed that the maximum germination of Atriplex halimus was recorded in distilled water whereas salinity inhibits this germination but may not kill seeds. It may be hypothesised that, when the seeds were soaked in NaCl solutions is probably caused a decrease in water potential gradient between the seeds and their surrounding media, consequently inhibited the processes of imbibition of seeds. In this regard, our results are in agreement with those of Ungar (1996) and Prado *et al.* (2000), who found that the seeds and seedlings of several halophytes were less tolerant to salinity than growing plants. As with the majority of halophytes, Atriplex halimus shows a great sensitivity to salinity at the germination stage.

Plant hormones such as abscisic acid, gibberellic acid, and cytokinines are involved in regulating seed germination with or without abiotic stresses (Sarath *et al.*, 2007).

In this study, exogenous gibberellic acid and kinetin increased germination when ABA markedly reduce this rate, especially under saline condition. Possibly these growth regulators either reduce the moisture requirement or enhance water uptake during germination. In this trend, Kaur *et al.* (2003), found that the growth regulators affect water uptake under stress conditions, either by increasing membrane permeability or by increasing the internal concentration of osmotically active solutes. Kong *et al.* (2017) suggested that the inhibited seed germination was possibly due to the increased ABA and decreased GA contents achieved by adjusting the expression of their biosynthesis and catabolism genes.

Sugars also act as regulatory molecules and play a pivotal role in the plant life cycle. Prado *et al.*(2000), reported that seed carbohydrate metabolism under stress condition can be considered a dynamic process involving often concomitantly occurring processes of polysaccharide degradation and synthesis of new compounds. In this context, our results, showing variations

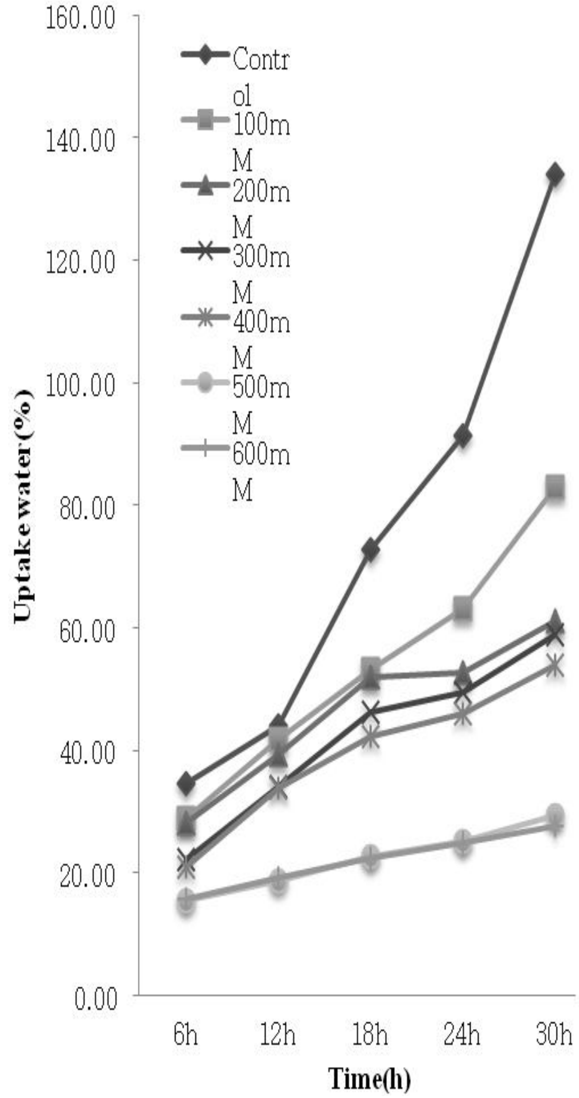

Fig.1 : Effect of salinity (NaCl) concentration on seed water uptake during germination of *A. halimus*.

that occurred in total sugar content during exposure of *Atriplex halimus* seeds to salt stress (table 3). Therefore, Our results showed that imposition ABA and NaCl treatments resulted in a very significant reduction of soluble sugars content. Huang *et al.* (2016), reported similar declines in sugars content on barley under saline conditions.

It is well documented that addition of GA enhance some sugar-degrading enzymes (Briggs, 1998), while ABA has an antagonistic effect to GA (Bewley, 1997). The current study confirms the substantial effect of GA and ABA on sugar solubles content of seeds, and may make further insight into the role of these hormones in seeds during germination. GA3 and kinetin increased sugars content of stressed seeds, under saline condition (table 2). From these results, it became evident that the

Table 1 : Test F value and signification of NaCl treatments on germination and osmotic potentiel.

Trait	Source of variation
	NaCl
Germination percentage	372**
Osmotic potentiel	95,14**

* : p<0,05, ** : p<0,01

Table 2 : Mean values of germination percentage, osmotic potentiel under NaCl concentrations (0, 100, 200, 300, 400, 500 and 600Mm). Each value is the Mean values of four replications of each trait. For a given parameter, values sharing a common superscript letter are not significantly different at P< 0.05

Treatments	Germination percentage (%)	Osmotic Potentiel(Mpa)
Control	98[a]	-0,99[a]
100mM	88[b]	-1,32[b]
200mM	45[b]	-3,36[c]
300mM	41[c]	-3,57[cd]
400mM	32[c]	-4,32[d]
500mM	28[d]	-5,38[e]
600mM	12[e]	-6,58[e]

of polysaccharides present in seeds of various plants (Siddiqui and khan 2011). They playing key role in carbohydrate metabolism of developing and germinating seeds (Muralikrishna and Nirmala, 2005). In this study, it was found that the activity of alpha amylase in germinating seeds was depressed not only by the salinity, but also by the addition of ABA to the medium of germination. The reduction in a-amylase activity may be due to salt toxicity or due to the less imbibition effect, because water as a solvent is necessary to stimulate the activity of this enzyme. According to the finding of Atia *et al*. (2009) and Cao *et al*. (2014), the salt stress inhibition, may be explained by reducing water uptake, increasing ABA content and decreasing GA content.

GA3 and kinetin increased amylase activity in stressed seeds, whereas ABA causes a stong decrease (table 3). A decrease in specific activity of amylase in seeds of halophytes treated with salt has been reported by Siddiqui and Khan (2011). According to Kaur *et al*. 2000, Gibberellic acid and kinetin have been reported to increase percentage germination under saline condition by increasing amylase activity and mobilization of starch in cotyledons. From these results, it became evident that the adverse effect of stress on amylase activity was reversed with the addition of GA3 and kinetin in the medium.

Table 3 : Mean values of germination percentage, soluble sugars contents and alpha amylase activity under NaCl and phytohormones treatment. Each value is the mean values of four replications±ES of each trait. For a given parameter, values sharing a common superscript letter are not significantly different at P< 0.05.

Treatments	Germination percentage (%)	Soluble sugars content (µg.g^{-1} FW)	Alpha amylase activity (U/mg pro.)
Control	97,57±0,27[e]	2,29 ±0,08[g]	7,22 ±0,12[bc]
GA3	93,69±1,48[d]	1,79 ±0,05[f]	6,34±0,02[b]
Kin	90,77±0,20[c]	1,22±0,03[cd]	3,90 ±0,43[a]
ABA	35,81 ±0,97[b]	0,98±0,02[b]	2,55 ± 0,29[a]
S	33,29±1,14[b]	1,12±0,01[bc]	3,64 ±0,18[a]
S+GA3	96,84±0,47[e]	1,56±0,16[e]	11,19±1,0[d]
S+Kin	97,50±0,28[e]	1,42 ±0,04[de]	7,79±0,30[c]
S+ABA	26,51±1,41[a]	0,31±0,02[a]	2,75±0,30[b]
F-test	1261**	68.56**	43.17**

** : significant at p=0.01 level.

adverse effect of stress on sugars content was reversed with the addition of GA3 and kinetin in the medium. However, GA3 was more effective than kinetin. It was thought that enhanced germination by the growth regulators might be mediated through changes in the activities of enzymes of carbohydrate metabolism (Evans *et al*., 2009).

Amylases are a class of hydrolases which are cleave the ortho-glycosidic bonds in amylose, a principal storage

Conclusion

In conclusion, the result of this experiment indicates that NaCl disturbed the mechanism of germination in Atriplex halimus seeds, water uptake, osmotic potential, soluble sugars content and the amylases activity. Exogenously applied GA3 and kinetin, effectively alleviated the adverse effects of salinity, while ABA had the opposite effect. In the presence of NaCl, supply of GA3 and Kinetines increase the activity of alpha amylases witch appears to be involved in the accumulation of soluble sugars and improved the uptake of water, mechanism witch reflected by the increased percentage of germination of seeds. This fact, may be involved in both osmotic adjustment and protection of cellular structures of Atriplex halimus seeds under saline conditions. Our results suggest that exogenous phytohormones could be useful to alleviate salt stress on *Atriplex h.* seeds and seedlings, which in turn may improve early growth during stand establishment and provide basic information for its conservation and reintroduction in salt and arid region

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