Effect of Salt Stress, Proline, and Polyamines on Seed Germination of *Opuntia streptacantha*♦

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ABSTRACT

In this study we conducted experiments in order to determine whether polyamines (putrescine, spermidine, and spermine) and proline treatments help to break seed dormancy of *Opuntia streptacantha* and also if they facilitate seed germination under salt stress. The results showed that 1mM proline stimulated a better germination percentage as compared to untreated seeds, although the difference was not statistically significant. With respect to polyamines, they did not break seed dormancy. On the other hand, salinity affected seed germination at the higher concentrations (50 and 75 mM NaCl) used, whereas, at 25 mM NaCl, results were similar to the control. The germination percentage in the treatments with proline (1 and 10 mM) and with polyamines (1µM each) diminished significantly when increasing the concentration of NaCl. The exception was spermine treatment, which alleviates seed germination at the higher concentration of NaCl used (75 mM).

Keywords: *Opuntia streptacantha*, Polyamines, Proline, Salt stress, Seed germination.
Abbreviations: Spd−Spermidine; Spm−Spermine ; PAs−Polyamines; Pro−Proline; Put−Putrescine.

1. INTRODUCTION

Seed germination is a process that begins with the uptake of water by the dry seed and it finishes with elongation of the embryo axis (Bewley and Black 1994). This process is affected by adverse factors such as salinity, which is one of the major abiotic stresses that affect plant growth and development, especially in arid and semiarid regions (Neumann 1997, Chinnusamy et al. 2005, Cony et al. 2006). This stress condition could affect the germination of seeds when high osmotic potential is created, which prevents water uptake (osmotic effect) or causes toxic effects of Na^+ and Cl^- ions (Okçu et al. 2005 and references therein). Plants respond and adapt to stress to survive by complex molecular responses, including the

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accumulation of compatible solutes, the production of protective proteins, and the expression of different sets of genes, which are part of the plant signaling and defense system against high salt stress (Zhu 2001, Sairam and Tyagi 2004). Proline (Pro) is one of the most widely distributed osmolytes in stress conditions not only in plants but also in other organisms (Delauney and Verma 1993; Bartels and Sunkar 2005). Pro has diverse functions, such as stabilization of subcellular structures (proteins and membranes), it functions as a hydroxyl radical scavenger and serves as a source of carbon and nitrogen (Kavi-Kishor et al. 2005). In addition**,** Pro could act as a component of signal transduction pathways that regulate stress responsive genes (Khedr et al. 2003). Poljakoff-Mayber et al. (1994), suggested a possible role of Pro during seed germination, since they found that dry seeds of *Kosteletzkya virginica* contain a significant amount of betaine but low Pro, but during germination and in the presence of NaCl the betaine content decreased, while the Pro content increased. Furthermore, Pro application in *Zygophyllum simplex* seeds alleviates either the breaking innate seed dormancy or the effect of low salinity on seed germination (Khan and Ungar 1997).

On the other hand, polyamines (PAs) are polycationic compounds of low molecular weight that have been implicated in various physiological processes, such as rhizogenesis, somatic embryogenesis, pollen formation, flowering and initial fruitlet abscission, dormancy, senescence and the response to abiotic and biotic stresses (Bouchereau et al. 1999, Jiménez-Bremont et al. 2006, 2007, Rodríguez-Kessler et al. 2006, 2008). In addition, it has been suggested that PAs may play a role in the accumulation of seed storage proteins and in the seed maturation (Santanen and Simola 1999) and that they help to promote seed germination (Prakash and Prathapasenan 1988, Sińska and Lewandoska 1991, Zeid and Shedeed 2006). PAs biosynthesis increases greatly in response to stresses and its function is presumed to be protective with a role in scavenging free radicals (Ha et al. 1998, Mansour 2000). NaCl salinity causes both ionic imbalance and water stress, creating membrane damage (Tiburcio et al. 1994). PAs prevent uptake of Na^+, loss of K^+, and leakage of amino acids and electrolytes from plant tissues (Chattopadhayay et al. 2002). It was suggested that PAs contribute to maintaining cellular cation-anion balance and help in the stabilization of the membrane integrity through their low molecular mass and polycationic nature (Smith 1985).

Several reports in Cactaceae have shown that an increase of the salt concentration diminishes the germination percentage (Romero-Schmidt et al. 1992, Nolasco et al. 1996, Vega-Villasante et al. 1996). In the case of *Opuntia* species, there are studies about chemical and mechanical pretreatments used to stimulate seed germination and thus break seed dormancy (Olvera-Carrillo et al. 2003, Mandujano et al. 2005, 2007, Altare et al. 2006). However, it is not known if PAs and Pro exogenous application breaks seed dormancy or if they protect seed germination under salt stress conditions. For this reason, in this present study we examined the effect of PAs such as putrescine (Put), spermidine (Spd) and spermine (Spm), and Pro in breaking seed dormancy and also if they facilitate seed germination of *Opuntia streptacantha* under salt stress.

2. MATERIALS AND METHODS

2.1 Material of seeds and germination conditions

Opuntia streptacantha seeds were collected of "Cardona" variety cactus pears, of the region of Villa de Zaragoza of the state of San Luis Potosí, México (Silva-Ortega et al. 2008). Seeds collection was January 2005, and they were stored at room temperature during twenty-four months. Seeds were sterilized with 30% (v/V) commercial sodium hypochloride solution for 30 min and with 70% (v/V) ethanol for 4 min, and rinsed three times in sterile distilled water between each treatment.

2.2 Treatments of seeds

Seeds were subjected to the following treatments: (a) PAs (1 μM Put, Spd or Spm), (b) Pro (1 or 10 mM), (c) and for salt treatment (25, 50, or 75 mM NaCl). We also used mixtures of the previous treatments: (d) Mix of PAs (Put, Spd, and Spm) with each Pro concentration, (e) PAs (Put, Spd, and Spm) with each salt solutions and (f) each Pro concentration with each salt solutions.

For each treatment five replicates, with twenty seeds per replicate, were used. The seeds were placed in Petri dishes containing a perlite support. For the control we added 20 ml of sterile distilled water and for the treatments we added 20 ml of each of the solutions previously described. These experiments were carried out in a growth chamber at 25°C with a photoperiod of 12 h light/12 h dark cycle. The number of seeds germinated was recorded every two days during 2 months, considering that a seed was germinated when it showed the radicle emerged.

2.3 Statistical analysis

To explore potential differences in seed germination percentage, a one-way ANOVA was used, with treatment as the main factor. Before analysis, the data were transformed using square-root arcsine, as it is recommended for the percentage data (Sokal and Rohlf 1995). Differences among treatments were explored through orthogonal contrasts.

3. RESULTS AND DISCUSSION

3.1 Breaking seed dormancy

It has been found that many Cactaceae have prolonged seed dormancy, with low or null germination (Rojas-Aréchiga and Vázquez-Yanes 2000, Flores et al. 2005, 2006, 2008). Specifically, one genus of this family considered as having seed dormancy is *Opuntia*. The first species found to have dormant seeds was *Opuntia auranthiaca* (Archibald 1939) and, up to the present, seed dormancy has been found in 28 *Opuntia* species (see Table 1).

Here we show that in the untreated seeds of *O. streptacantha* the germination percentage was low (14%), meaning that this cactus species presents seed dormancy (Figure 1A). This result is similar to that reported for seeds of other species of the *Opuntia* genus (Table 1). Several dormancy-breaking treatments, including scarification with sulfuric acid, mechanical scarification, after-ripening, cold stratification, leaching, passage through the digestive tract of animals, soaking in hot water, and some combinations of these treatments have been suggested to enhance germination of *Opuntia* seeds. However, these treatments do not always promote germination and, in some cases, treated seeds have germinated to lower percentages than those in the control (Orozco-Segovia et al. 2007). Gibberellic acid (GA_3) alone seems to be only moderately effective in promoting seed germination of *Opuntia* spp. (Wang et al. 1996). However, it induces a high percentage of germination in combination with other treatments such as rinsing/washing, chemical or mechanical scarification (Orozco-Segovia et al. 2007).

3.2 Effect of the application of proline and of polyamines on the germination.

Due to the low germination percentage obtained for *O. streptacantha*, in the present study we evaluated the effect of exogenous application of Pro and PAs on breaking seed dormancy. Our results showed that at 1 mM Pro treatment the germination percentage was higher than the control; however, this difference was not significant ($F = 1.500$, $P > 0.5$). Also, at 10 mM Pro, we observed that there was not a significant difference in the germination percentage with respect to the control ($F = 0.001$, $P > 0.5$; Figure 1A). It has

been reported that Pro treatments (1 and 0.1 mM) alleviated the innate dormancy of *Zygophyllum simplex* seeds and germination reached 40-60% compared to 12% in the control (Khan and Ungar 1997).

The PA treatments used in this experiment did not stimulate the breaking seed dormancy, because there were not significant differences in germination percentages among PAs and the control (Figure 1B). It was observed that with Put treatment the germination percentage was slightly higher than the control, but this difference was not significant ($F = 0.004$, $P > 0.5$). It has been reported that Put promotes the germination of various species, for example *Oryza sativa* (Prakash and Pratapasenan 1988), *Hordeum vulgare* (Locke et al. 2000), *Citrullus lanatus* (Korkmaz et al. 2004), *Cucumis melo* (Korkmaz et al. 2005) and *Medicago sativa* (Zeid and Shedeed 2006). However, other authors have found that Put inhibits germination. Amoroso-Botelho and Juliano-Gualtieri (2001), reported that the treatment with 0.5 mM Put did not affect the seed germination of *Peltophorum dubium*, while a negative effect was observed at 1.0 mM Put. Moreover, at 0.1 μM and 0.1 mM Put, the germination of the cacti *Turbinicarpus lophophoroides* and *Turbinicarpus pseudopectinatus* was inhibited (Flores et al. 2008). In other plant species, an effect of PAs in the germination has been reported, for example in apple (*Malus domestica*) the exogenous application of Put and Spd (0.1 and 1 mM) stimulated the germination, whereas Spm inhibited it (Sińska and Lewandowska 1991). In *Peltophorum dubium* Spd treatments (0.5 mM and 1.0 mM) did not affect seed germination (Amoroso-Botelho and Juliano-Gualtieri 2001). Thus, we suggest that the effect of PAs in the seed germination depends on the plant species.

We carried out an experiment with the mixture of Pas (1 μM Put, Spd, and Spm) with Pro concentrations (1 and 10 mM). Using the higher concentration of Pro (10 mM) with a mix of PAs (1 μM of each one) a negative effect on seed germination was observed, although it was not statistically significant ($F = 3.025$, $P > 0.05$). The lower concentration of Pro (1 mM) and a mix of PAs (1 μM of each one) showed a similar behavior than the control ($F = 0.446$, $P > 0.05$; Figure 1A).

3.3 Effect of salt stress on the germination.

In a general way, we found that the germination of seeds of *O. streptacantha* was significantly affected by salt stress. The percentage of germination of *O. streptacantha* seeds treated with 25 mM NaCl was similar to the control ($F = 0.045$, $P > 0.05$), while at 50 mM NaCl the germination percentage was lower than the control ($F = 17.633$, $P < 0.0001$) and at 75 mM there was a complete inhibition (Figure 2). The results of this study showed that *O. streptacantha* seed germination is affected by salt stress like in other cacti species. For example, in the "cactus barril" (*Ferocactus peninsulae)*, the "cardón gigante" (*Pachycereus pringlei*) and the "cardón barbón" *(Pachycereus pecten-aboriginum)* the seeds were able to germinate at 25 and 50 mM NaCl, while at 100 mM the percentage of germination diminished and at 200 and 300 mM there was a complete inhibition (Romero-Schmidt et al. 1992, Nolasco et al. 1996, Vega-Villasante et al. 1996).

3.4 Effect of the application of proline during the germination of seeds under salt stress.

In general, the germination percentage in the treatments with Pro (1 and 10 mM) significantly diminished when increasing the NaCl concentration, leading to an inhibition of germination at the higher concentration (75 mM) (Figure 3). Our results are similar to those reported by Poljakoff-Mayber *et al*. (1994) in *Kosteletzkya virginica*, in which the exogenous application of Pro (10 mM) did not improve germination under salt stress conditions (100 mM NaCl). Khan and Ungar (1997) reported that Pro application in *Zygophyllum simplex* seeds alleviates the effect of low salinity (25 mM NaCl), and induced seed germination, being more effective at the lower Pro concentration (0.1mM) than at the higher concentration (1mM). However, at higher salinity treatments (75 and 125 mM NaCl) Pro was ineffective in alleviating the dormancy of seeds. On the other hand, although in our study the exogenous application of Pro did not improve seed germination under stress, it is important to mention that we have shown a

significant Pro accumulation in cladodes of the same species (*O. streptacantha*) under salt stress (Silva-Ortega et al. 2008), suggesting that the effect of Pro depends on the developmental stage of the plant.

3.5 Effect of the application of polyamines during the germination of seeds under salt stress.

The application of exogenous PAs (Put, Spd, or Spm) did not produce a significant difference in the percentage of germination respecting control seeds as described above (Figure 4). In the case of simultaneous application of each PAs and salt stress treatments, we observed that the germination percentage significantly diminished as the salt concentration increased together with the addition of Put and Spd. However, Spm treatment alleviated seed germination (4%) at the higher concentration of NaCl used (75mM); it showed a recovery of 28.57%, as compared to the percentage of germination of control plants (14%). At this concentration of NaCl, seed germination was completely inhibited by salt stress, whereas at these same conditions the application of Put and Spd, or Pro did not stimulate germination.

Our results are in agreement, specifically Spm, with the idea that polyamines may be involved in salt stress protection. In a previous study, we have observed an accumulation of Spm in a bean tolerant cultivar at higher concentrations of NaCl used (150 and 400 mM), suggesting that Spm might be part of the mechanism conferring salt tolerance (unpublished data). Zapata et al. (2004) reported a significant increase in Spm and Spd levels during germination of different plant species such as lettuce (*Lactuca sativa* L.), pepper (*Capsicum annuum*) and broccoli (*Brassica oleraceae* L. var. Italica Plenk.) under conditions of salt stress (150 mM NaCl). A protective role for Spm in abiotic stress such as high salt or drought stress has been shown in the *Arabidopsis* spermidine synthase (acl5/spms) double mutant that does not produce Spm. This Arabidopsis Spm-deficient mutant is hypersensitive to high concentrations of NaCl and also to drought stress; and this phenotype was rescued by exogenously applied Spm (Yamaguchi et al. 2006, 2007). This mutant caused a defect of Ca^{+} homeostasis, evidencing a protective role for Spm in the context of responses to ionic stress (Yamaguchi et al. 2006). On the other hand, Zhu et al. (2006) reported that the exogenous application of PAs, specifically Spd inhibits Na^{+} transport from roots to shoots in barley seedlings under conditions of high salinity, suggesting that PAs could be a mechanism for alleviating salt stress. In a recent report, Zhao et al. (2008) showed that exogenous application of PAs, specifically Spd blocked the inward Na+ and K+ currents (especially Na+ currents) in barley root epidermal and cortical cells. In root xylem parenchyma, the inward K+ current was also blocked by extracellular Spd, while the outward K+ current was enhanced. Thus, by repressing Na+ influx into roots and by preventing K+ loss from shoots, PAs improved K+/Na+ homeostasis in barley seedlings to cope with high saline conditions.

4. CONCLUSIONS

1. *Opuntia streptacantha* presents seed dormancy, which could not be broken down by the different treatments used (Pro, Pas, and Pro plus PAs).

2. The treatments with higher salinity (50 and 75 mM NaCl) affected dramatically the seed germination, whereas the treatment of 25 mM NaCl allowed a similar germination percentage as the control.

3. Germination under salt stress conditions could not be alleviated by the application of different treatments (Pro and PAs), excepting by Spm which promoted 4% of seed germination at 75 mM NaCl.

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Table 1. *Opuntia* species with seed dormancy.

Species	Authors
O. atrispina	Pendley (2001)
O. auranthiaca	Archibald (1939)
O. camanchica	Pendley (2001)
O. chisoensis	Pendley (2001)
O. compressa	Baskin and Baskin (1977)
O. decumbens	Godínez-Álvarez and Valiente-Banuet (1998)
O. discata	Potter (1984)
O. echios var. *gigantea*	Wiggins and Focht (1968)
O. edwardsii	Potter et al. (1984)
O. engel	Pendley (2001)
O. engelmannii	Potter et al. (1984); Pendley (2001)
O. ficus-indica	Wang et al. (1996); Altare et al. (2006); D'Aubeterre et al. (2006)
O. humifusa	Cook (1942)
O. imbricata	Pilcher (1970)
O. joconostle	Sánchez-Venegas (1997)
O. kleiniae	Pilcher (1970)
O. leucotricha	Olivares et al. (1999)
O. lindheimeri	Pilcher (1970); Potter et al. (1984)
O. littoralis	Silverstein (2005)
O. macrocentra	Mandujano et al. (2007)
O. macrorhiza	Timmons (1942)
O. microdasys	Mandujano et al. (2007)
O. poly	Pendley (2001)
O. polyacantha	Smreciu et al. (1988)
O. rastrera	Mandujano et al. (1997, 2005)
O. spinossibacca	Pendley (2001)
O. streptacantha	This study
O. stricta	Lotter et al. (1999); Reinhardt et al. (1999)
O. tomentosa	Olvera-Carrillo et al. (2003), Orozco-Segovia et al. (2007)

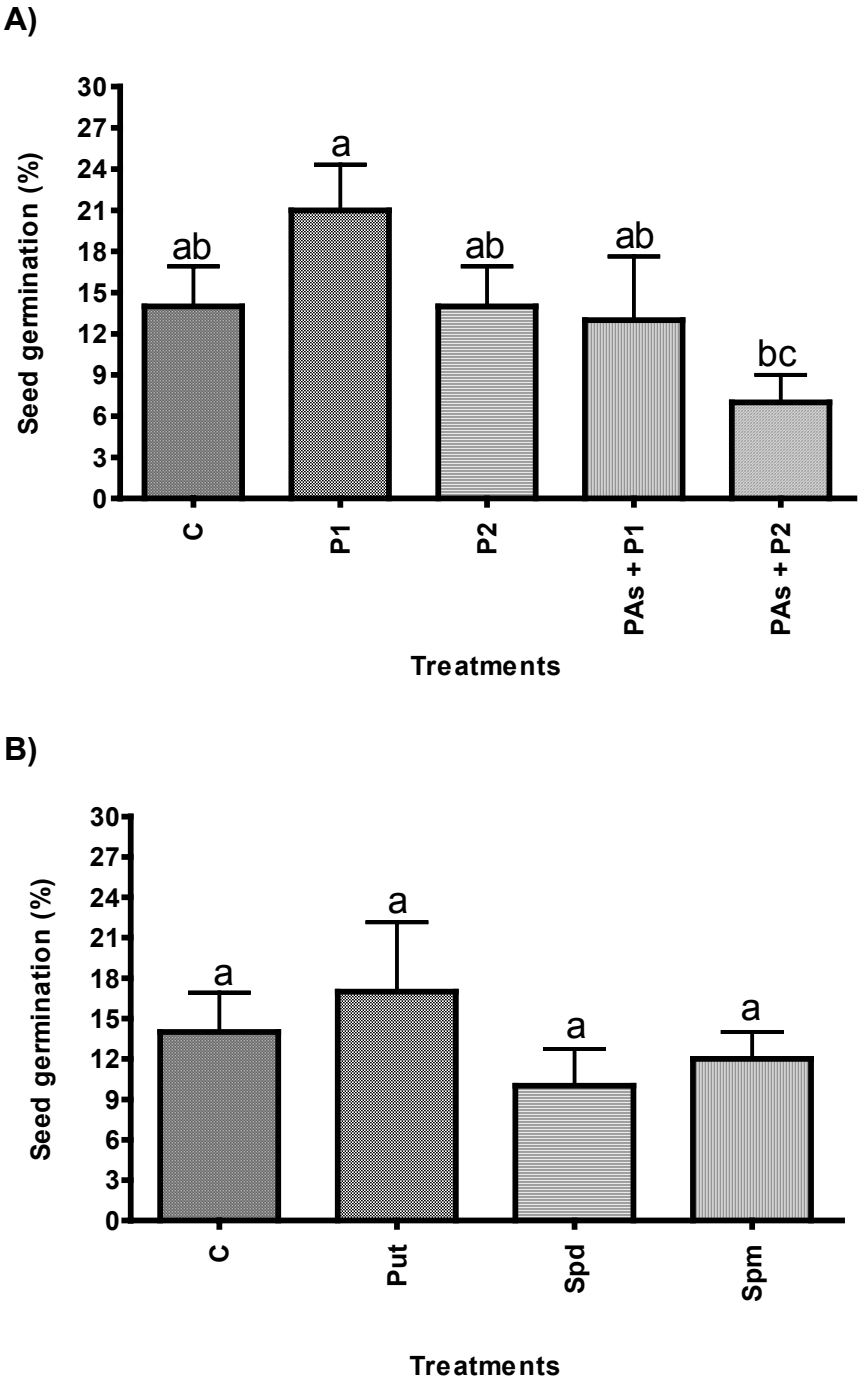

Figure 1. 1A) Germination percentage (Means ± SE) of *O. streptacantha* seeds with 1 and 10 mM Pro and with a mixture of PAs (1 µM: Put, Spd and Spm).
C = Untreated seeds (Control), PAs (Polyamines) = Put (Putrescine), Spd (Spermidine) and Spm (Spermine) mix, P1 = 1mM Pro (Proline), and P2 = 10 mM Pro (Proline).
1B) Germination percentage (Means ±SE) with each PAs.
Different letters represent significant differences between treatments.

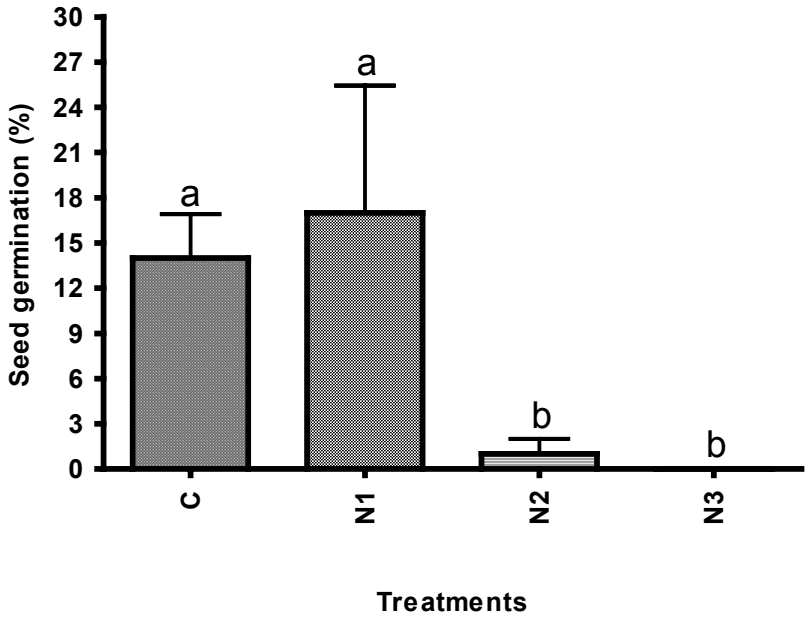

Figure 2. Germination percentage (Means ± SE) of *O. streptacantha* seeds under salt stress. C = Untreated seeds (Control), N1 = 25 mM NaCl, N2 = 50 mM NaCl, and N3 = 75 mM NaCl. Different letters represent significant differences between treatments.

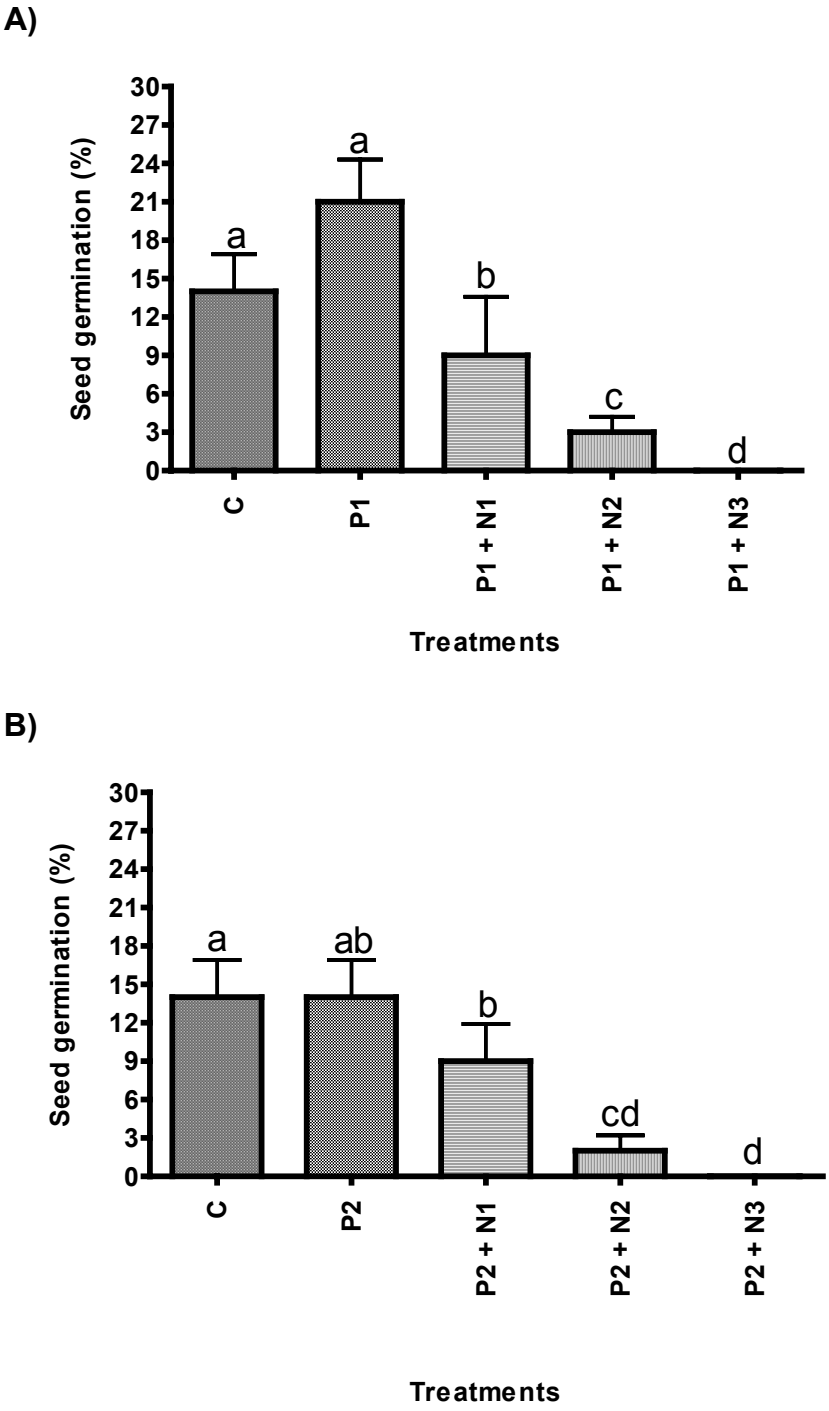

Figure 3. Germination percentage (Means ± SE) of *O. streptacantha* seeds under salt stress with exogenous added 1 mM (Fig. 3A) and 10mM (Fig. 3B) of Pro (Proline).
C = Untreated seeds (Control), P1 = 1 mM Pro, P2 = 10 mM Pro, N1 = 25 mM NaCl, N2 = 50 mM NaCl, and N3 = 75 mM NaCl.
Different letters represent significant differences between treatments.

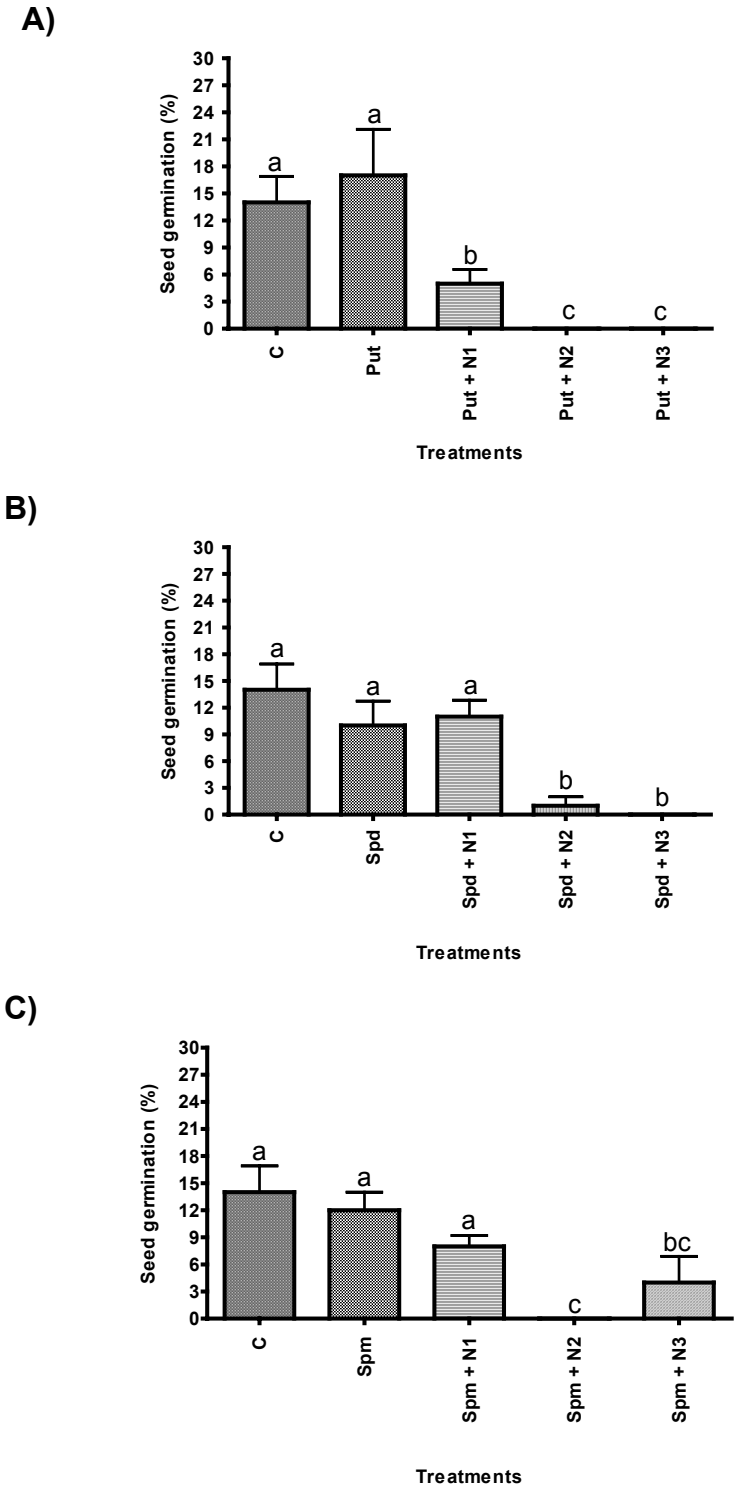

Figure 4. Germination percentage (Means ± SE) of *O. streptacantha* seeds under salt stress with exogenous added PAs: Put (Fig. 4A), Spd (Fig. 4B), or Spm (Fig. 4C). C = Untreated seeds (Control), Put = 1 µM Putrescine, Spd = 1 µM Spermidine, Spm = 1 µM Spermine, N1 = 25 mM NaCl, N2 = 50 mM NaCl, and N3 = 75 mM NaCl. Different letters represent significant differences between treatments.