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Salicylic acid improves seed germination through modulating antioxidant enzymes under salt stress in chickpea (*Cicer arietinum*)

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ABSTRACT

An experiment was conducted to investigate the role of Antioxidant Enzymes (AOE) in Salicylic Acid (SA) induced salt-stress tolerance during chickpea (*Cicer arietinum* L.) seed germination at ICAR- Indian Institute of Seed Science, Mau during 2018. Results showed the differential germination performance of salt-sensitive (PG 186) and salt-tolerant (CSG 8962) chickpea cultivars under three levels (0, 75, 150 mM NaCl) of salt stresses. Salt stress adversely affected the seed germination and traits of early seedling establishment in PG 186 cultivar than in CSG 8962. Sand matrix priming (SMP) of seeds using SA @ 150 ppm improved the germination (up to 2 times) and other seed quality parameters in sensitive cultivar under higher (150 mM NaCl) salt-stress level. Further investigating the role of SA on modulating AOE, a negative influence of SA on major AOE (POX, CAT and GR) was observed in cotyledons of PG 186 genotype under high salt stress. However, the level of APX was observed to be constitutively higher in tolerant CSG 8962 chickpea genotype. The summary of results suggested that, SA alleviates oxidative stress through reducing major AOE in cotyledon to improve chickpea seed germination.

Keywords: Anti-oxidative enzymes, Chickpea, Genotypes, Salt stress, Salicylic acid

Survivability of seed is highly uncertain under salt stress due to high osmotic and specific ionic stress (Arefian *et al.* 2017). Researchers considered vegetative and flowering stage of chickpea as most sensitive stages to salt stress as this stage determines yield (Choudhary *et al.* 2018). Hence, most of the prior art information related to effect of salt stress and mechanism of tolerance, i.e. osmotic (ability to extract water), oxidative (homeostasis of ROS) and specific ion toxicity (Na^+/Cl^- ion accumulation) were limited to vegetative (leaves) and reproductive (flowers) stages of chickpea (Farjam *et al.* 2014, Hirich *et al.* 2014, Khan *et al.* 2017). However, several earlier researchers reported on effects of salt stress (reduction in germination, vigour, speed of germination) during seed germination in chickpea (*Cicer arietinum* L.) genotypes (Eyidogan and Oz 2007, Turner *et al.* 2013, Choudhary *et al.* 2018). However, it is not clear from the available data, how cotyledons of chickpea genotypes protect seeds from different salt induced stresses (osmotic, oxidative and specific ion) during germination.

Seed priming is one of the most useful physiological approaches that could adapt glycolphyte species to grow under saline conditions (Vicente and Plasencia 2011). While adopting different signalling molecules (H_2O_2, ASC, SA, NO, BABA, H_2S) and phyto-hormones as priming agents, researchers have improved the germination performances of seeds of various crop plants under salt stress (Rehman *et al.* 2015, Ibrahim 2016). However, no study reported how SA primed seeds and/or tolerant chickpea genotypes balanced ROS for protecting seeds from oxidative stress as well as promoting seed germination under salt stress. Since, Super Oxide Dismutase (SOD) produces H_2O_2 and other AOE involved in removal of H_2O_2, the balance between SOD and system I [Peroxidase (POX)-Catalase (CAT)] or system II [Ascorbate Peroxidase (APX)–Glutathione Reductase (GR)] AOE in seeds is considered as crucial for determining the steady-state level of H_2O_2. Keeping all the above facts, our study aimed to investigate the wholesome status of AOE in cotyledons of chickpea seeds during final count of germination (8 DAS) in SA assisted improvement in chickpea seed germination under severe salt stress.

MATERIALS AND METHODS

The present study was conducted at ICAR-Indian Institute of Seed Science, Mau during 2018. Seeds of diverse chickpea genotypes, viz. salt susceptible var. PG

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186 and salt-tolerant var. CSG 8962 were obtained from ICAR-Indian Institute of Pulses Research, Kanpur. The seeds were surface sterilised with 3% (v/v) sodium hypochlorite containing 0.1% (v/v) Tween-20 and washed thoroughly with sterile distilled water three times. Shade dried seeds were primed using moist sand hydration method (Vijayakumar *et al*. 2013) with three different concentrations (50, 100 and 150 ppm) of salicylic acid. Chickpea seeds and moist sand (2 mm mesh sieved and autoclaved sand at 121ºC in 15 *psi* pressure for 30 min) were mixed in the ratio of 1: 20 on w/w basis. Priming was done at 60 % WHC and 20ºC for 8 hr in a sealed plastic container (24 cm × 10 cm × 15 cm). After priming, the primed seeds were separated from sand, dried back to original moisture content under shade for 48 h and used for the experiment. The unprimed and sand matrix primed (without SA) seeds were used as a control and internal control, respectively. The experiment was laid out in three factorial completely randomized design (CRD) with following treatment, viz. unprimed seeds, sand matrix primed seeds, sand matrix priming using SA @ 50 ppm, 100 ppm and 150 ppm.

Seed-germination test: Seed-germination test was done by placing primed and unprimed seeds of two varieties in plastic germination box (24 cm × 10 cm × 15 cm) containing moist sand maintained with 0, 75 and 150 mM NaCl salt stress. Then seeds were incubated at 20ºC for 8 days. Three replicates each consisting of 50 seeds were used for each treatment combination. The germinated seeds were recorded every day for 8 days. An index of the speed of germination is then calculated by adding the quotients of the daily counts divided by the number of days of germination. The germination final count was recorded on eighth day and per cent germination was expressed on normal seedling basis. The seedling vigour index was computed by adopting the formula as suggested by Abdul-Baki and Anderson (1973) and expressed in whole number. Seedling Vigour Index-I = Germination (%) × Mean seedling length (cm); Seedling Vigour Index-II = Germination (%) × Mean seedling dry weight (mg).

Antioxident enzyme assays: The cotyledons (200 mg) of 8 days old seedling were incised separately and homogenized in 2 ml of 100 mmol/l potassium phosphate buffer (pH 7.5) containing 0.5 mmol/l EDTA and 0.1% Triton X-100. Additional 1 mM ascorbic acid was used into the homogenisation buffer for APX assay. The extract was filtered through muslin cloth and centrifuged at 15000 g for 15 min at 4ºC. The supernatant was used for the assay. Superoxide dismutase was estimated according to the method of Beauchamp and Fridovich (1971) with suitable modification. Three ml of reaction mixture containing 1.5 M sodium carbonate, 200 mM methionine, 2.25 mM nitro-blue tetrozolium, 3 mM EDTA, 100 mM potassium phosphate buffer, riboflavin (60 μM) and 0.1 ml of enzyme extract were used to estimate the enzyme activity in unit of enzyme per mg of protein. The reaction solution maintained in a glass test tube and irradiated under light (15 W fluorescent lamp) for 15 min and absorbance was recorded at 560 nm.

Catalase was assayed by measuring the disappearance of H_2O_2 at 240 nm for 1 min according to Aebi (1984). Enzyme activity was computed by calculating as per extinction coefficient of H_2O_2 (€ = 43.6/mM/cm). Peroxidase was assayed by measuring the increase in optical density at 470 nm for 1 min due to the oxidation of guaiacol to tetra guaiacol (Fielding and Hall 1978). Enzyme activity was computed by calculating as per extinction coefficient of its oxidation product, tetra-guaiacol (€ = 26.6/mM/cm). Enzyme activity was expressed as μmol tetra guaiacol formed/min/mg protein. Glutathione reductase was assayed as per the method of Smith *et al.* (1988). The activity was expressed as total absorbance (Δ A_{412})/mg protein/min. Ascorbate peroxidase was assayed by recording the decrease in optical density due to ascorbic acid at 290 nm (Nakano and Asada 1981).

Experimental design and statistical analysis: The experiment was laid out in factorial Completely Randomized Design (CRD) with three factors, viz. salt stress (@ 3 levels, i.e. 0, 75 and 150 mM NaCl); genotypes (@ 2 levels, i.e., salt susceptible PG 186 and salt-tolerant CSG 8962); seed

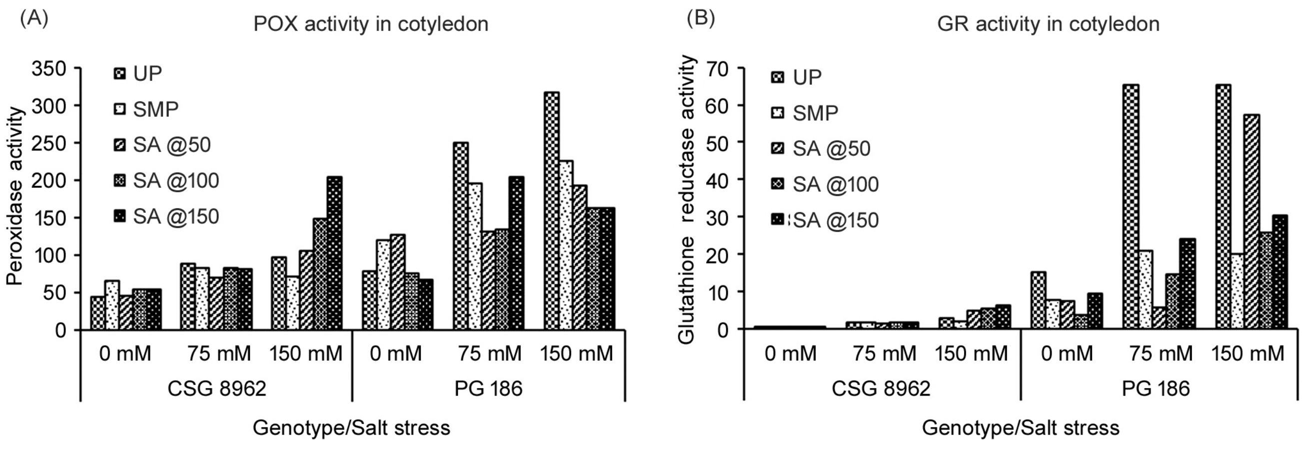

Fig 1 Differential variation of antioxidant enzyme activity in tolerant and susceptible chickpea cultivars. (UP)-Un-primed seeds, (SMP)-Sand Matrix Primed Seeds; (SA @ 50) – Sand Matrix Primed seeds using Salicylic acid @ 50 ppm; (SA @ 100) – Sand Matrix Primed seeds using Salicylic acid @ 100 ppm, (SA @ 150) – Sand Matrix Primed seeds using Salicylic acid @ 150 ppm.

priming (@ 5 levels, i.e. un-primed, sand matrix priming (SMP), SMP using SA 50 ppm, SMP using SA 100 ppm and SMP using SA150 ppm). Three factorial analysis of variance was done by using SAS version 9.4.

RESULTS AND DISCUSSION

Effect of salt stress on seed quality parameters: Based on literature, two diverse chickpea (PG 186 [S] and CSG 8962 [T]) genotypes were selected for conducting this experiment (Flowers *et al.* 2010, Turner *et al*. 2013, Kumar *et al.* 2017). Our results showed that, salt stress had an adverse effect on all basic seed quality parameters and reduced the seedling performance of chickpea seeds (Ibrahim 2016). The comparative analysis of susceptible and tolerant cultivars clearly revealed that the adverse effect of salt stress was severe in susceptible PG 186 cultivar than in tolerant CSG 8962 cultivar. While comparing the performance of PG 186 seeds in control and 150 mM NaCl salt stress, a great reduction in all major seed quality parameters like germination (up to 78.8%), speed of germination (up to 94%), seedling length (up to 77.8%), seedling dry weight (up to 82.3%), seedling vigour index-I (up to 97.6%) and seedling vigour index-II (up to 98%) were recorded. In contrast, CSG 8962 exhibited higher germination percentage (59%) in 150 mM salt stress with lesser significant reduction in speed of germination, seedling length, vigour etc. The effect of salt stress on different seed quality parameters was found to be statistically significant between tolerant and susceptible genotypes.

Effect of salt stress on AOE: Our results on chickpea seed's ability to tolerate oxidative stress (via AOE) observed a drastic increase in AOE (mainly SOD, CAT POX and GR) in cotyledons of un-primed seeds of PG 186 subjected to salt stress. It was also noted that number of units of those AOE (SOD, CAT, POX and GR) were also higher in cotyledons of susceptible PG 186 genotype than in CSG 8962. These higher and increased activities of ROS scavenging system in PG 186 seeds in response to salt stress might have counter balanced H_2O_2 signal, where germinations were also reduced up to 78.8%. The non-significant changes observed in AOE activity in cotyledons of CSG 8962, reconfirmed this fact that AOE mediated ROS homeostasis playing a significant role in seed germination. Similar observations were also recorded in seeds of pea (Barba-Espin *et al*. 2012) and soybean (Shu *et al.* 2017) during germination. In support of this hypothesis, few reports suggested the use of H_2O_2 as an external priming/spraying chemical for ameliorating adverse effects of salt stress (Gondim *et al.* 2010, Ellouzi *et al.* 2017, Li *et al.* 2017).

Among the ROS scavenging system, quantity of APX was always higher in cotyledons of CSG 8962 than in PG 186. This suggests that, salt tolerance at germination stage involves more complex mechanism of regulation that promotes selective ROS scavenging system to balance ROS for signalling germination cues as well as for providing oxidative stress tolerance. In support of this, earlier reports confirm that, APX activity is comparatively insensitive to salt stress than CAT in seedling of diverse crops (Caverzan *et al.* 2012). Since, APX activity mainly depends on ASC pools (as electron donor) under GR inactivated conditions, this also proved that tolerant genotypes might have utilised efficient scavenging mechanism, i.e. both enzymatic (APX)

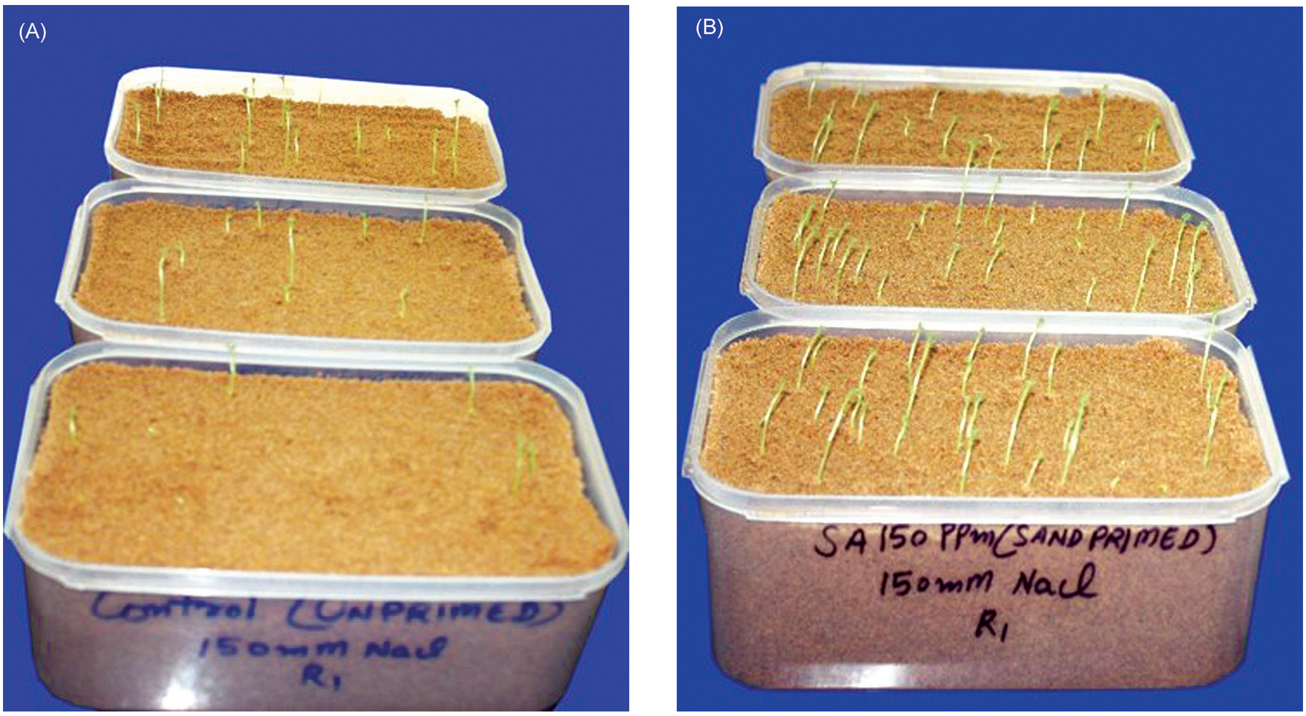

Fig 2 A. Effect of salt stress on germination of un-primed PG 186 seed under150 mM NaCl. B. Effect of sand matrix seed priming using SA @150 ppm on improved seed germination of PG 186 under 150 mM NaCl stress.

and non-enzymatic antioxidant (ASC) (Arefian *et al.* 2017). Few reports also supported use of ASC as priming/spraying agent to alleviate drought or salt induced osmotic stresses (Farjam *et al.* 2014).

Effect of SA priming on seed quality parameters under salt stress: A significant positive effect of sand matrics priming using SA (@150 ppm) was observed in susceptible PG 186 cultivar, where germination was improved 2.4 times (from 19% in un-primed and 46% in SA primed seeds @150 ppm) under 150 mM salt stress (Fig 2). In contrast, any significant influence of sand matrix priming using SA was not observed in CSG 8962 genotype, where 59% germination was recorded under the increased salt stress (150 mM). Apart from seed germination, SA was also found to positively influence the speed of germination and mean seedling length in PG 186 genotype. However, the increased mean seedling length was not concomitantly recorded with increased mean seedling dry weight. Among the treatments, Sand matrix priming using SA @ 150 ppm was found to be the best treatment for improving the seed germination of susceptible cultivar. As few literatures reported that SA negatively arrest seed germination of crop plants beyond 1mM (~150 ppm) concentration (Hernandez *et al.* 2017), we did not further increased SA concentration for improvement of chickpea seed germination. The positive effects of seed priming and its role in improving germination, speed of germination and crop performance under salt stress were reviewed in diverse crop plants (Vicente and Plasencia 2011, War *et al.* 2011, Rehman *et al.* 2015, Ibrahim 2016).

Effect of SA on AOE in cotyledons under salt stress: The amelioration effect of SA on AOE activity was observed in cotyledons of contrast genotypes under 0 and 150 mM NaCl salt stress. In cotyledons, SA significantly increased SOD and decreased POX (Fig 1A) and GR enzyme (Fig 1B) activities in PG 186, where seed germination was also improved up to 46% under salt stress (Fig 2). Statistically, non-significant changes were observed in APX activity in cotyledons of PG 186. However, significantly higher units of APX enzyme activity were observed in tolerant CSG 8962 genotype at 150 mM salt stress only. Earlier, several authors reported the use of SA as priming or spraying agents to alleviate the adverse effect of salt stress (War *et al.* 2011, Shu *et al.* 2017). Scientific reports suggested that, the improved seed germination performance of SA primed seeds was primarily attributed to SA's dynamic role in modulating ROS (H_2O_2) through AOE for reduced oxidative stress and balancing the ROS for cross talk signalling with ABA and GA biosynthetic pathway (Rajjou *et al.* 2006, Lee *et al.* 2010, Vicente and Plasencia 2011, Wojtyla *et al.* 2016, Hernandez *et al.* 2017).

Similarly, in our experiment, SA positively increased SOD and negatively decreased GR and POX enzyme activity in seeds of PG 186 genotype. This significant increase in SOD enzyme activity might have contributed to dismutase superoxide (ROS) to H_2O_2 and complementary reduction in POX and GR might have homeostasis the ROS for germination signalling cross talk. Earlier, Lee *et al.* (2010) related SA's positive influence on germination as SA's ability to reduce oxidative stress by promoting antioxidant enzyme (SOD, POX and Catechol) activity in salicylate hydroxylase (NahG) over expressed transgenic lines of *Arabidopsis thaliana*.

Our results showed that, SA promotes seed germination under salt stress possibly by providing oxidative stress tolerance to embryo so as to synthesis GA for stored reserves mobilisation and utilisation. Still further confirmation studies on status of embryo oxidative stress (ROS and AOE activity) from germinated and non-germinated seeds and dynamics of GA concentration are required to understand the role of oxidative stress tolerance in chickpea seed germination. Besides all, it is hypothesised that germination is relative to vegetative and reproductive growth, resistant to salt stress at germination stage will provide more scope of getting wider genotypes for screening reproductive stage salt tolerance. As, till date, the genotypes having tolerance to germination stage salt stress were mostly used for screening salt-stress tolerance in chickpea.

AOE mediated oxidative stress tolerance was studied in cotyledons of germinating chickpea seeds, as till date mechanism of homeostasis of H_2O_2 in cotyledons is not clear that decides salt stress alleviation during germination. Summary of results such as, constitutive higher activity of APX with lesser significant reduction (insensitivity) of activity due to salt stress in cotyledons tissues of CSG 8962 and the inverse relationship found between APX and other AOE suggest that, oxidative stress tolerance in germinating cotyledons involves contrast and more complex mechanism of regulation that promotes selective (APX-GR or non-enzymatic) systems of ROS scavenging that may be different in diverse genotype and developmental stages etc. Conclusively, SA is mediating the modulation of AOE to improve the chickpea seed germination under salt-stress condition.

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