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Halopriming Imparts Salt Tolerance by Reducing Oxidative, Osmotic Stress and DNA Damage in Five Different Legume Varieties

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ABSTRACT

Background: Salinity challenges legume production worldwide. To maintain the overall legume production, seed halopriming has been adopted as a cost-effective, farmer friendly technique, minimizing noxious effects of NaCl on plant growth.

Methods: Nonprimed and haloprimed seeds were grown under different NaCl concentrations and harvested after 21 days. NaCl-induced alterations on physio-biochemical attributes and DNA damage were studied.

Result: NaCl exposure in nonprimed seedlings exhibited growth inhibition, depletion in water contents, increased accumulation of H_2O_2, MDA and proline causing DNA damage. Conversely, in primed seedlings, these toxic effects were altered and extent of DNA damage reduced. Decreased catalase activity in nonprimed seedlings failed to detoxify the ROS generated under salinity inducing DNA damage whereas in NaCl-treated haloprimed seedlings, improved catalase activity helped to overcome such adversities favouring improved growth of all tested legume varieties.

Key words: DNA damage, Halopriming, Legumes, Reactive oxygen species, Salinity.

INTRODUCTION

Salinity, a brutal abiotic stress, limits crop productivity worldwide (Gupta and Huang 2014). India, the largest producer and consumer of pulses, contributes to 54% of total production. Legumes are rich in proteins and can fix atmospheric nitrogen to improve soil fertility. Plant growth is hampered under NaCl stress due to osmotic imbalance which eventually hinders water uptake from soil. Increased Na^+ uptake inhibit protein synthesis and disrupt various enzymatic functions (Abd Elgawad *et al*., 2016). These cumulatively attribute to generation of reactive oxygen species (ROS) within plants causing oxidative damages to macromolecules thereby interrupting plant cellular functions (El-baky *et al*., 2003). To withstand such consequences, plants recruit ROS scavenging enzymes for detoxification and elevate levels of compatible solutes to cope with salinity stress.

Seed invigoration is a controlled hydration process where seeds are exposed to low water potential that restricts germination, but permits pre-germinative biochemical and physiological changes that favours the germination process and post-germination events (Jisha and Puthur, 2014; Chakrabarty *et al*., 2022). Upon rehydration primed seeds may exhibit faster rates of germination and greater tolerance to environmental stresses Seed halopriming imparts stress tolerance to plants by enhancing biochemical events under salt-stressed conditions (Biswas *et al*., 2022). Apart from its cytotoxic effects, compartmentalization of sodium ions serves as energetically cheap osmoticum, during conditions when osmolyte production is energetically impossible for plants (Chakrabarty *et al*., 2020).

Present research work was focussed on assessing influence of NaCl on growth, physio-biochemical parameters and oxidative stress-induced DNA damage in nonprimed and haloprimed seedlings of five legumes having differential sensitivity towards NaCl. The results of the study may help to improve our knowledge on the mechanism(s) taking place within haloprimed seedlings imparting improved salt tolerance.

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MATERIALS AND METHODS

Seeds of *Cajanus cajan* L. var. *Rabi*, *Cicer arietinum* L. var. Anuradha, *Lathyrus sativus* L. var. Nirmal, *Lens culinaris* Medik. var. Ranjan and *Vigna mungo* L. var. Sulata were collected from Pulse and Oilseed Research Institute, Behrampore, West Bengal, India. Seeds were surface

sterilized with 5% NaOCl solution for 10 minutes and rinsed with distilled water. For priming, seeds were soaked in 50mM NaCl for 2 hours and dried back to its original moisture content. The time duration for seed priming was standardized under laboratory conditions based on trial and error method. Primed and nonprimed seeds were allowed to grow in hydroponic solution (Biswas *et al*., 2022) with corresponding concentration of NaCl (50 mM, 100 mM and 150 mM). Seedlings grown only in hydroponic solution (0 mM NaCl) served as experimental control. Plants were grown under controlled experimental conditions (200 µmol $m^{-2}s^{-1}$ light intensity, 16/8 hours light/dark photoperiod, relative humidity (RH) of about 80-85%) to understand the influence of NaCl on different legume varieties.

Plant growth parameters *viz*., root and shoot lengths, biomass and water contents were recorded from shoot and root of tested legume varieties (Majumder *et al*., 2018). H_2O_2 content was estimated according to Velikova *et al*. (2000), MDA content was estimated according to Hodges *et al*. (1999) and proline estimation was done according to Bates *et al*. (1973). Comet Assay was performed according to Arya and Mukherjee (2014) to assess DNA damage.

Statistically significant values were determined using one-way analysis of variance (ANOVA) and Dunnett's analysis. *p*-values were considered significant at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ and $p < 0.0001$. Pearson's bivariate correlation analysis was done using PASW Statistical software SPSS 18.0 (Version 18.0.0.282).

RESULTS AND DISCUSSION

Influence on growth, biomass contents and water contents

All legume seedlings exposed to NaCl stress encountered significant reduction in growth, biomass and moisture contents. On an average, root lengths diminished by about 10%, 13%, 32%, 49% and 36% and shoot length decreased by about 9%, 9%, 24%, 39% and 34%, respectively in *Vigna*, *Lathyrus*, *Cicer, Cajanus* and *Lens* over corresponding nonprimed controls. Pretreatment of seeds before germination, prevented growth inhibition by about 3%, 9%, 12%, 34% and 12% in root and 5%, 6%, 15%, 27% and 14% in shoot of *Vigna, Lathyrus, Cicer, Cajanus* and *Lens*, respectively over corresponding nonprimed controls. Dunnett's multiple comparison revealed significant and maximum growth inhibition of nonprimed ($p < 0.0001$) root and shoot of *Lens* under 50 mM NaCl unlike primed ones ($p > 0.05$). Primed root and shoot of *Cicer, Lathyrus* and *Vigna* exposed to 150 mM NaCl, showed non-significant growth reduction ($p > 0.05$) contrasting to nonprimed seedlings ($p < 0.001$) (Fig 1).

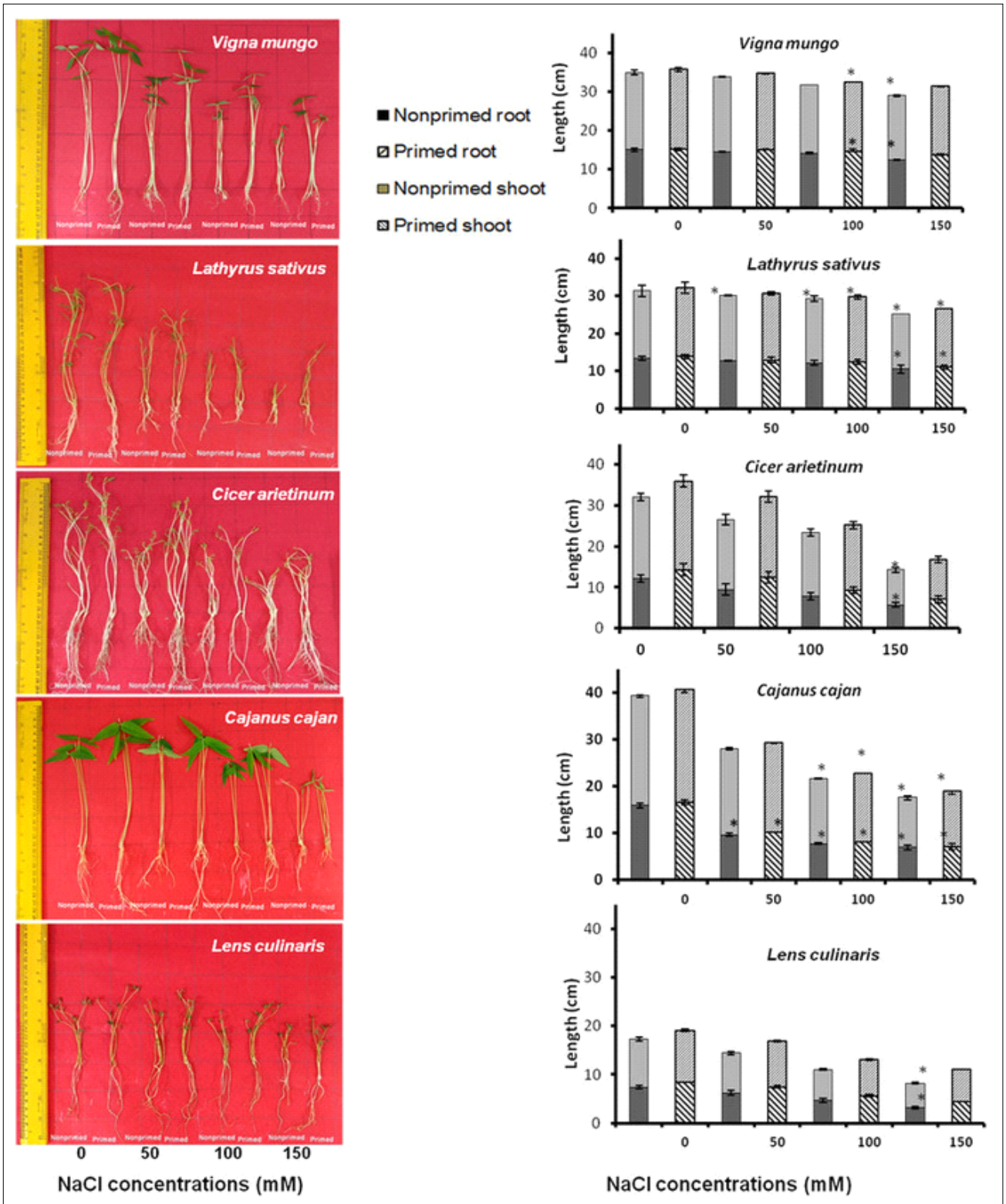
Biomass degradation was highest in nonprimed seedlings of *Lens* (54%), while it was lowered in primed seedlings (44%). Similar trend was observed for nonprimed *Cajanus* (50%) and *Cicer* (44%), compared to primed seedlings (40%, 35% respectively) over control. Primed seedlings of *Vigna* and *Lathyrus* showed minimum biomass degradation compared to their nonprimed seedlings. Degradation was least in *Vigna* (19%) (Fig 2). Similar observation has been reported in ryegrass species having differences in innate tolerance level under salinity (Nizam 2011).

Plant water status is an important attribute identifying plant tolerance level. Among the test varieties, *Vigna* showed least water loss under salinity followed by *Lathyrus, Cicer, Cajanus* and *Lens*. Seedlings raised from nonprimed seeds of *Vigna*, *Lathyrus*, *Cicer, Cajanus* and *Lens* exhibited, on an average, 4%, 7%, 7%, 9% and 9% depletion in water contents, respectively, in comparison with their respective nonpretreated control. Seedlings raised from haloprimed seeds were less vulnerable and water contents was replenished, on an average, to about 2% in *Vigna* and *Cajanus;* about 5% in *Cicer*, *Lens* and *Lathyrus* .Significant reduction in water contents occurred in nonprimed seedlings of *Vigna* ($p = 0.0061$), *Lathyrus* ($p < 0.0001$), *Cajanus* ($p < 0.0001$) and *Cicer* ($p = 0.0319$) under 50 mM NaCl. This was reversed in primed seedlings ($p > 0.05$). Significant changes occurred under 100 mM ($p < 0.001$) and 150 mM ($p < 0.0001$) salt exposure in all nonprimed varieties. Primed seedlings of *Cajanus* ($p = 0.0001$) and *Lens* ($p = 0.0029$) significantly restored a part of water loss under 100 mM NaCl (Fig 2).

Amongst the five varieties, *Vigna* showed the maximum tolerance when grown under salinity followed by *Lathyrus*, *Cicer*, *Cajanus* and *Lens*. Previous report related plant water status with inherent salt tolerant capacity of plants, which could be correlated to above findings (Vaktabhai and Kumar, 2017). Biswas *et al*. (2022) reported halopriming relieved salt stress, similar to our results where seedlings raised from primed seeds exhibited improved plant growth and biomass. Additionally, in our study, halopriming proved more beneficial for the more sensitive varieties, *Lens* and *Cajanus* compared to the less sensitive ones, *Vigna, Lathyrus* and *Cicer*. A positive correlation among growth, biomass and plant water status of primed seedlings ($r = 0.98$ in *Lens*, $r = 0.962$ in *Vigna*, $r = 0.94$ in *Cicer*, *Lathyrus*, $r = 0.823$ in *Cajanus*) was obtained.

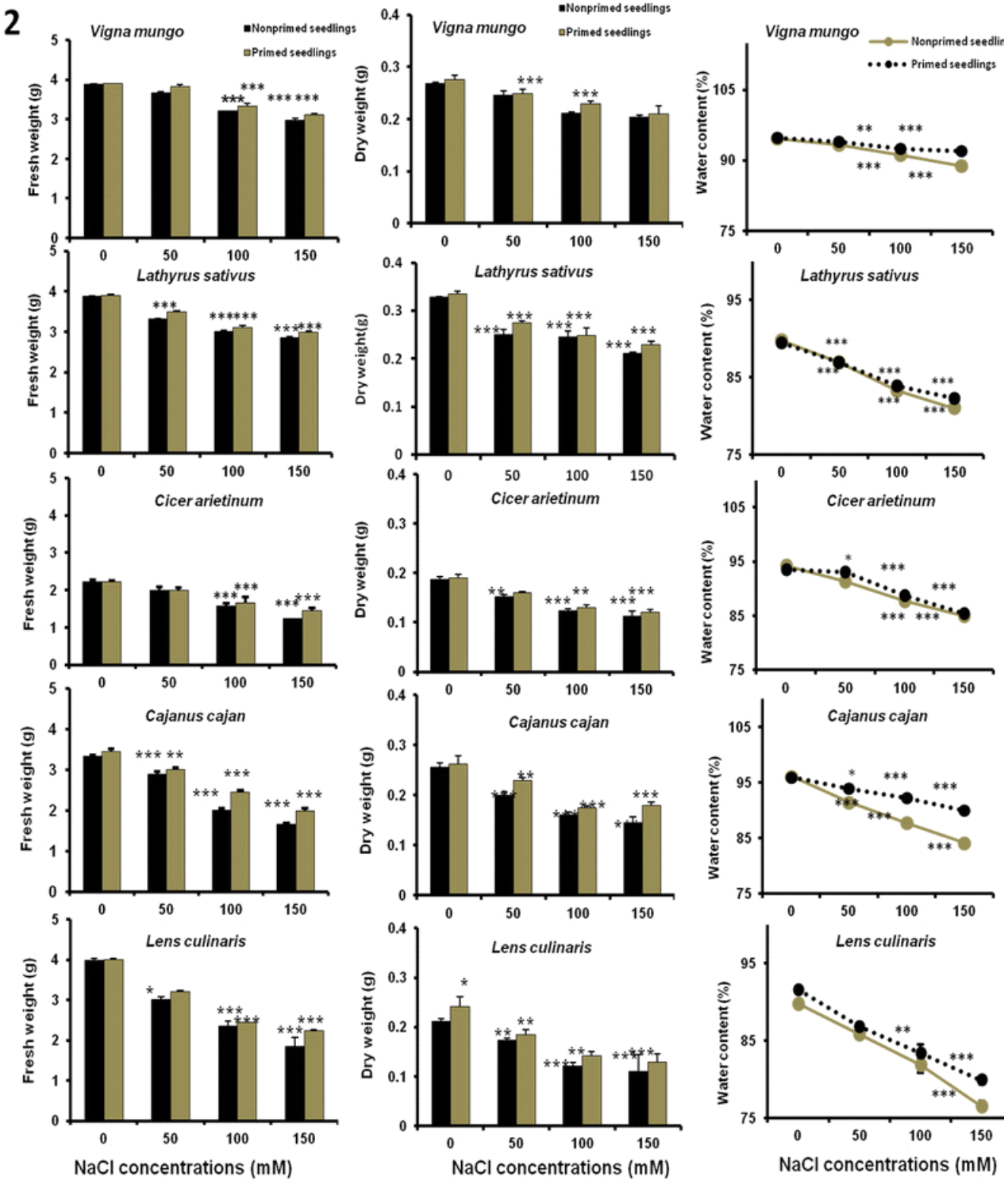
Influence on oxidative stress markers and antioxidant enzyme activity

H_2O_2 contents increased in nonprimed seedlings to 18%, 25%, 30%, 66% and 64% in root and to 16%, 24%, 27%, 42% and 55% in shoot of *Vigna*, *Lathyrus*, *Cicer*, *Cajanus* and *Lens* respectively over nonprimed control. Haloprimed root and shoot, exhibited lower H_2O_2 accumulation accounting to about 8%, 14%, 18%, 35%, 33% in root, while in shoot, it was reduced to 7%, 12%, 14%, 21%, 21% in *Vigna*, *Lathyrus*, *Cicer*, *Cajanus* and *Lens* respectively, on an average, over nonprimed control (Fig 3a). Statistical analysis revealed significant rise in H_2O_2 level at 50 mM NaCl in nonprimed root of *Cicer, Lens* and *Cajanus* ($p < 0.001$) but the significance level reduced in primed ($p < 0.01$) root. In 150 mM NaCl-treated nonprimed root of *Lathyrus* ($p < 0.0001$) and *Vigna* ($p < 0.001$), increase in H_2O_2 level was significantly altered. But, under 150 mM NaCl, elevation was much lesser ($p < 0.01$) in primed root of *Lathyrus* and *Vigna*.



The values are means of three experiments performed with two replicates in each treatment ± standard error. *, **,*** Statistically significant from the Dunnett's multiple comparison analysis at a significance level of $p \leq 0.05$, ≤ 0.01 and ≤ 0.001 as compared to nonprimed control.

Fig 1: Effect of NaCl on morphology, root and shoot length (cm) of 21 days old primed and nonprimed seedlings of five legume cultivars.

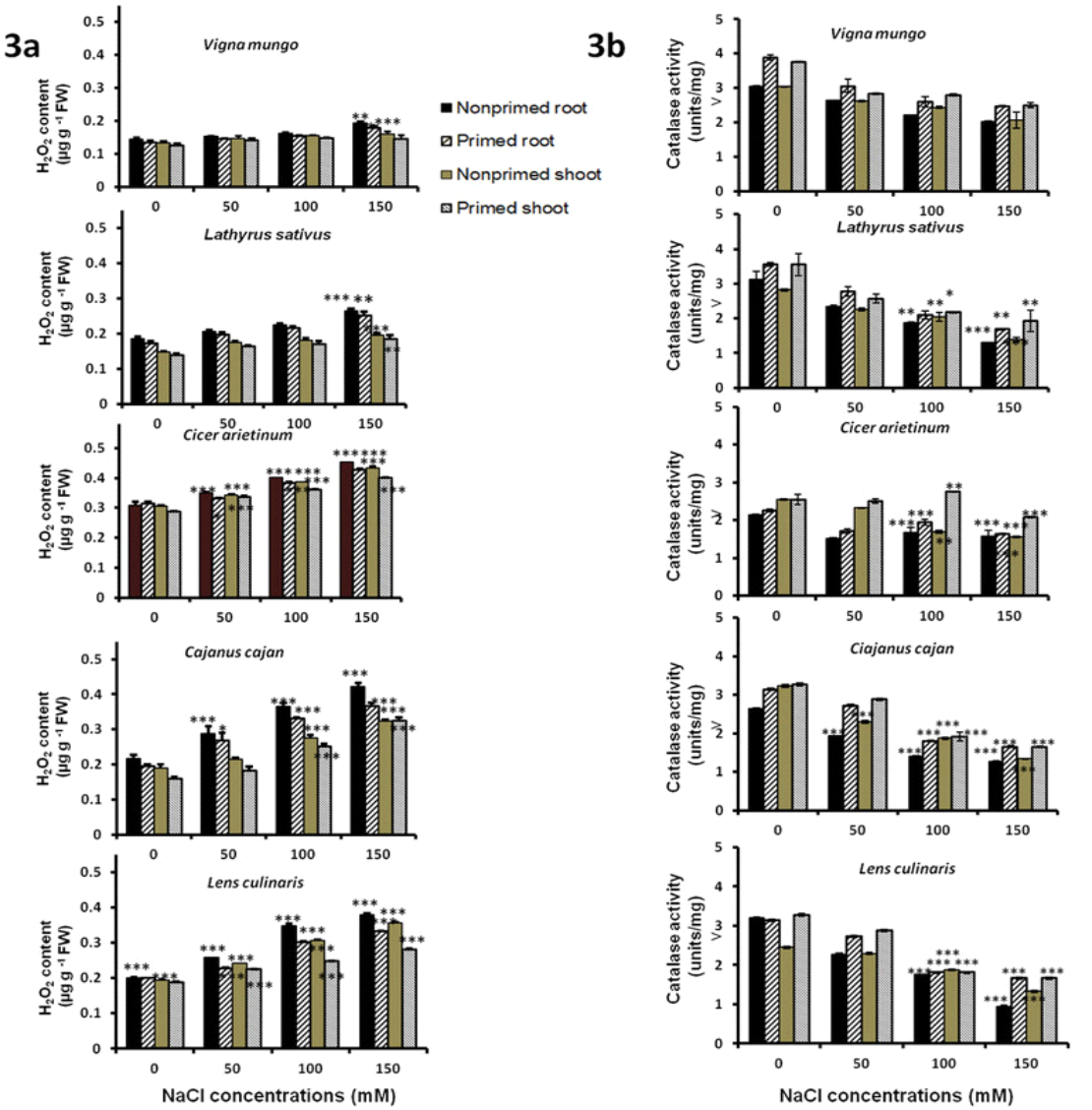


The values are means of three experiments performed with two replicates in each treatment ± standard error. *, **,*** Statistically significant from the Dunnett's multiple comparison analysis at a significance level of $p \leq 0.05$, ≤ 0.01 and ≤ 0.001 as compared to nonprimed control.

Fig 2: Effect of NaCl on fresh weight (mg), dry weight (mg) and water content (%) of 21 days old primed and nonprimed seedlings of five legume cultivars.

Negative correlation amongst H_2O_2 contents and growth (r=-0.7 in *Vigna* and *Cicer*, r=-0.9 in *Lens, Lathyrus* and *Cajanus*) and water content (r = 0.9 in all varieties) (Fig 4) indicated that H_2O_2 imposed toxic influence under salinity. However, halopriming prevented H_2O_2 accumulation exhibiting improved growth in all cultivars. Less amount of H_2O_2 accumulation in salt-tolerant *Lycopersicon pennellii* (Mittova *et al*., 2002), corroborates with our present observation. *Vigna* accumulated least H_2O_2 and thus was found to get minimally affected by NaCl, followed by *Lathyrus*, *Cicer, Cajanus* while *Lens* showed maximum accumulation (Fig 3a). Surprisingly, by limiting H_2O_2 accumulation to greater extent, halopriming was more effective for the probable sensitive cultivars *viz*. *Lens, Cajanus, Cicer* and *Lathyrus* as compared to *Vigna*.

Catalase activity declined significantly (p<0.0001) with increasing concentrations of NaCl in nonprimed seedlings. Catalase activity declined by about 24%, 25%, 41%, 41% and 48% in nonprimed root and by about 24%, 25%, 41%, 41% and 48% in nonprimed shoot of *Vigna*, *Lathyrus*, *Cicer*, *Cajanus* and *Lens,* respectively. In primed root this inhibition could be restricted to 1%, 11%, 11%, 19% and 27% and in primed shoot to about 2%, 3%, 9%, 24% and 27% over their respective nonprimed control seedlings (Fig 3b).



The values are means of three experiments performed with two replicates in each treatment ± standard error. *, **,*** Statistically significant from the Dunnett's multiple comparison analysis at a significance level of p≤0.05, ≤0.01 and ≤0.001 as compared to nonprimed control

Fig 3: Effect of NaCl on **a.** H_2O_2 content (µg g^{-1} FW) and **b.** catalase activity (units mg^{-1} FW) of 21 days old primed and nonprimed seedlings of five test legume cultivars.

Halopriming could revive the hampered catalase activity and thus, scavenged H_2O_2 better, clearly evident in the bivariate plot (Fig 6).

Malondialdehyde (MDA) levels were elevated in nonprimed root, by 13%, 18%, 43%, 48% and 61% while in nonprimed shoot the rise was about 9%, 33%, 33%, 39% and 54% in *Vigna*, *Lathyrus*, *Cicer*, *Cajanus* and *Lens* over their respective controls. However, in haloprimed root, the elevation decreased to 1%, 13%, 26%, 28% and 35% while in haloprimed shoot, it reduced to about 2%, 13%, 24%, 25% and 30% over their respective controls. In nonprimed seedlings, significant increase was noted at 50 mM (p<0.01), 100 mM (p<0.0001) and 150 mM (p<0.0001) but in *Vigna*, significant change was only noted in shoot under 150 mM NaCl exposure (p<0.0097). Levels of significance were lowered in haloprimed root and shoot of all varieties (p<0.01) and in *Vigna* changes were non-significant at 100mM and 150 mM NaCl treatments (Fig 5a).

MDA contents were positively correlated (Pearson's) to H_2O_2 contents (r=0.538 in *Vigna, r*~0.8 *Lathyrus*, *Cicer, Cajanus* and *Lens*) and negative correlated to water contents(r=0.9) and growth(r=0.9) in all the varieties. Halopriming efficiently checked H_2O_2 production, limiting lipid peroxidation(r<0.5). A slope value (m) of 42.86 for nonprimed seedlings in the bivariate plot of H_2O_2 content versus MDA content compared to a lower slope value (m=35.71) for primed seedlings indicated stress releasing effect of halopriming (Fig 6).

Proline accumulation increased under NaCl stress, by 14%, 23%, 37%, 58% and 52% in root and to 13%, 22%, 25%, 40% and 45% in shoot of *Vigna*, *Lathyrus*, *Cicer*, *Cajanus* and *Lens* respectively, on an average, over controls. Haloprimed root and shoot exhibited lower proline accumulation amounting to 2%, 5%, 10%, 17% and 16% in haloprimed root, while in haloprimed shoot, it was reduced to 1%, 15%, 11%, 16% and 19% in *Vigna*, *Lathyrus*, *Cicer*, *Cajanus* and *Lens* respectively, over nonprimed controls. Amongst the test legumes seedlings, root and shoot of *Vigna* responded least to salinity. Significant rise in proline contents occurred in nonprimed root(p<0.0001) and shoot (p<0.01) of *Cajanus, Lens, Cicer* and *Lathyrus* under 50 mM, 100 mM and 150 mM NaCl treatments. Under 100 mM NaCl treatment, rise in proline content was found to reduce in haloprimed root and shoot of *Lens* and *Cicer* (p<0.01). In primed root and shoot of *Vigna* (p<0.01) and *Lathyrus* (p<0.0001) the increment was much less compared to their respective nonprimed root and shoot (Fig 5b).

In our study, a positive correlation was obtained amongst proline, MDA and H_2O_2 contents (r≈0.9 in all varieties) indicating rise in proline level occurs with increasing oxidative stress in nonprimed seedlings. Proline accumulation was least in nonprimed NaCl-stressed *Vigna* indicating its salt tolerance ability, followed by *Lathyrus, Cicer, Cajanus* and *Lens* (Fig 5b). Similar trend was observed in cotton cultivars exposed to 240 mM NaCl wherein, tolerant variety accumulated less proline (Zhang *et al*., 2014; Mondal and Chakrabarty, 2020). Mechanistically, under salinity, halopriming possibly triggered proline

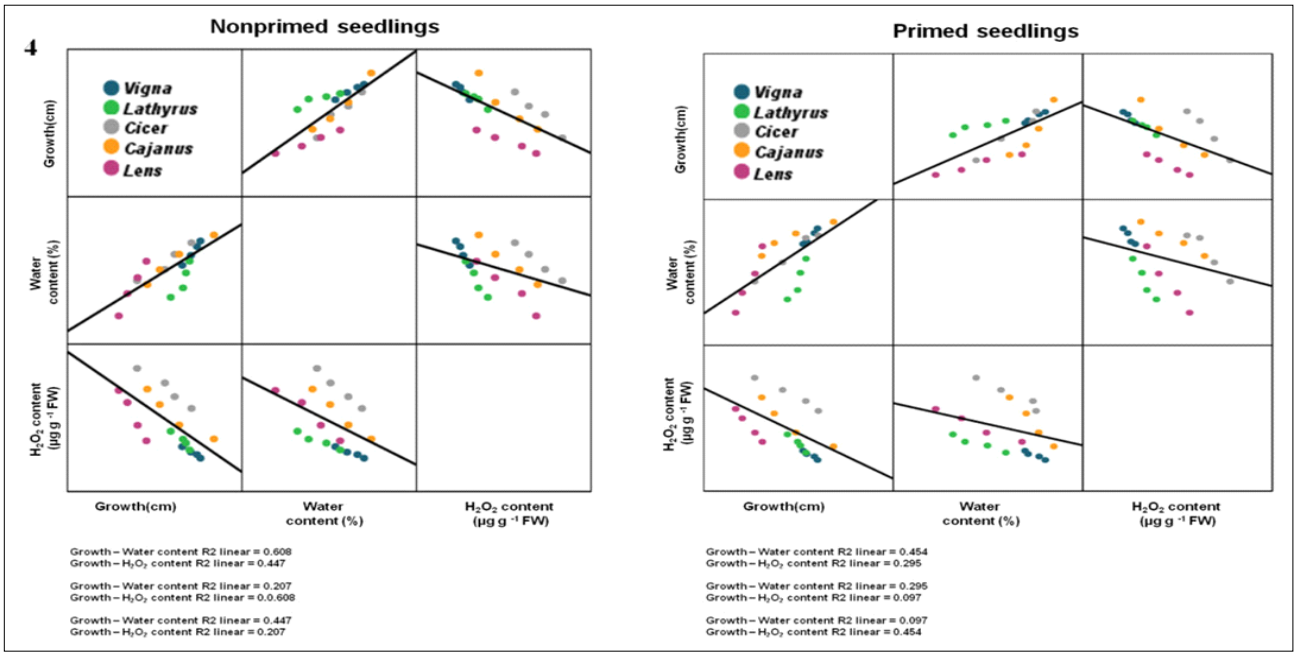
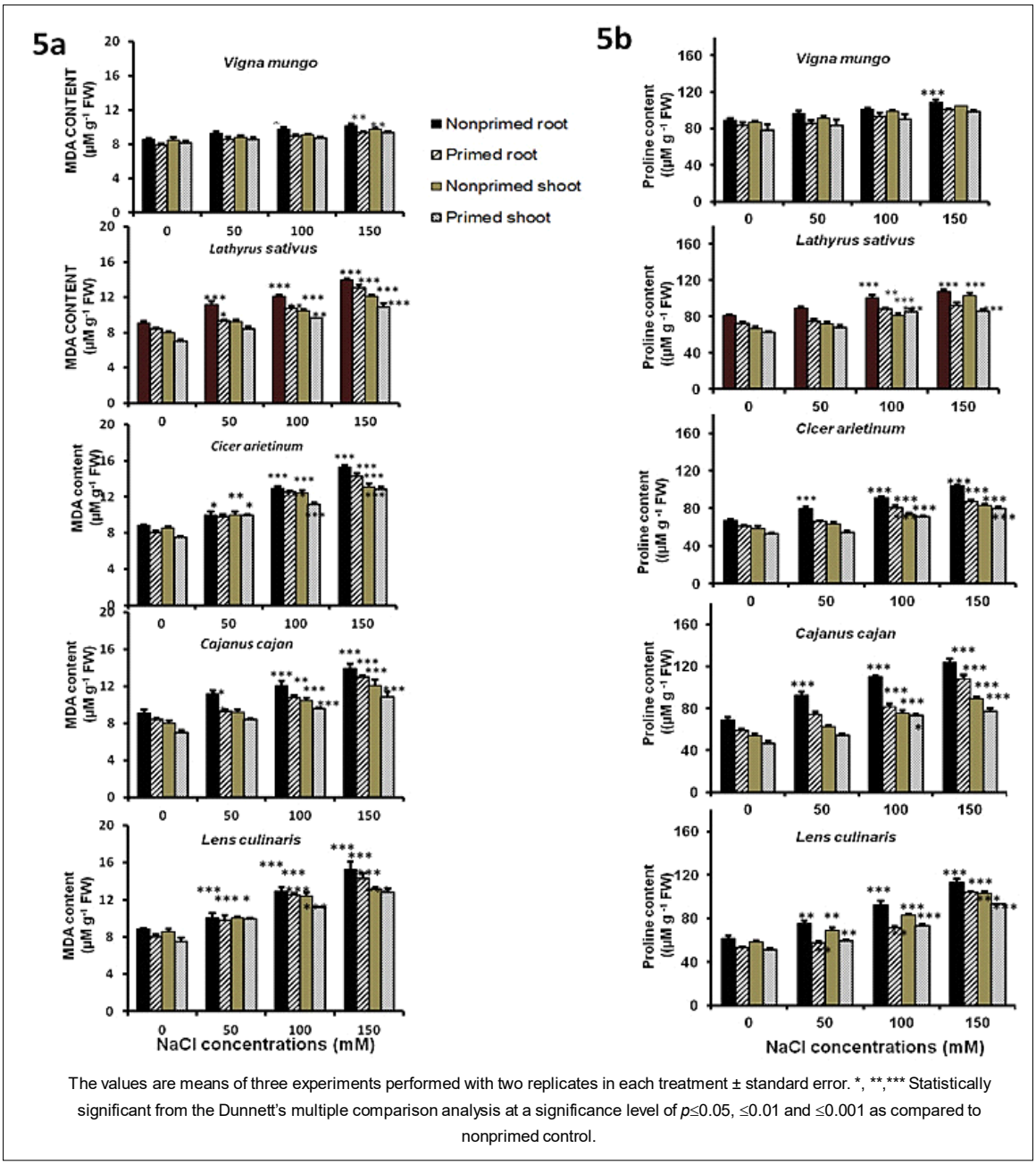


Fig 4: Scatterplot matrix showing interrelationship amongst H_2O_2 content, growth and water content of primed and nonprimed legume varieties.

breakdown rate in the mitochondria to minimize magnitude of osmotic stress conferring tolerance; as observed in KNO_3 primed rice exposed to salinity (Theerakulpisut *et al*., 2017). Elevation in proline and H_2O_2 contents has been graphically indicated by a higher gradient value (*m*=171.43) in nonprimed seedlings while enhanced rate of proline breakdown in primed seedlings was marked by lower gradient value (*m*=142.86) (Fig 6).

Influence on DNA damage

In our study, all varieties responded significantly at 150 mM NaCl; therefore, comet assay was performed with root and shoot grown under 150 mM NaCl. Our results depicted that DNA damage was less than 10% in nonprimed *Vigna* but, priming limited it to less than 5%. Nonprimed *Lathyrus*, *Cajanus* and *Cicer* showed nearly 25% DNA damage. Primed seedlings of same varieties showed significant



The values are means of three experiments performed with two replicates in each treatment ± standard error. *, **,*** Statistically significant from the Dunnett's multiple comparison analysis at a significance level of $p \leq 0.05$, ≤0.01 and ≤0.001 as compared to nonprimed control.

Fig 5: Effect of NaCl on a. MDA (µg g^{-1} FW) and b. Proline (µM g^{-1} FW) of primed and nonprimed seedlings of five legume cultivars.

decline in DNA damage to about less than 10% for *Lathyrus* and *Cicer* and 15% for *Cajanus*. DNA degradation was highest in nonprimed root and shoot of *Lens*, however, primed seedlings recorded lower tail % DNA under stress (10%) jointly. DNA damage was statistically significant in *Lens, Cajanus, Cicer* and *Lathyrus* at $p<0.0001$, while in *Vigna* it was much lower ($p=0.0049$). Lower level of DNA damage ($p<0.01$) after pretreatment in *Lens, Cajanus, Cicer* and *Lathyrus* indicated halopriming induced stress release (Fig 7a).

Previous reports showed that, DNA recombination/DNA repair mechanism gets induced on applying different salt concentrations which could be a possible way to recover or survive under stress (Collins 2004). This was probably achieved by halopriming technique. In our study, primed seedlings showed reduced DNA damage as their nuclei formed comets that visually resembled class 0 comets, similar to nuclei of untreated seedlings (Kumaravel *et al*., 2007). This was probably achieved either by blocking excess H_2O_2 accumulation or by effectively inducing catalase activity. Positive impact of halopriming on salt-induced DNA damage could be correlated with the amount of H_2O_2 and MDA along with CAT activity and proline contents. Statistically, a lower slope value of H_2O_2 and MDA contents versus DNA damage plot ($m=1.0$) for pretreated samples, indicated reduction in DNA damage after halopriming over nonprimed seedlings ($m=1.2$) (Fig 7b). On the other hand (Fig 8) reveals the positive effect of proline and catalase in form of a matrix scatterplot.

Amongst the test varieties, *Vigna* and *Lathyrus* exhibited lower percentages of NaCl-induced inhibition on growth, biomass and moisture contents that resulted in lower accumulation of H_2O_2 in roots and shoots as compared to other three legumes. Also, lower levels of H_2O_2 accumulation in *Vigna* and *Lathyrus* limited the degree of cell membrane injury that corresponded with the lower extent of DNA damage as compared to *Lens*, *Cajanus* and *Cicer*. Thus, present findings suggest that based on the test parameters, *Vigna* and *Lathyrus* responded least to NaCl stress as compared to other varieties, probably indicating their partial

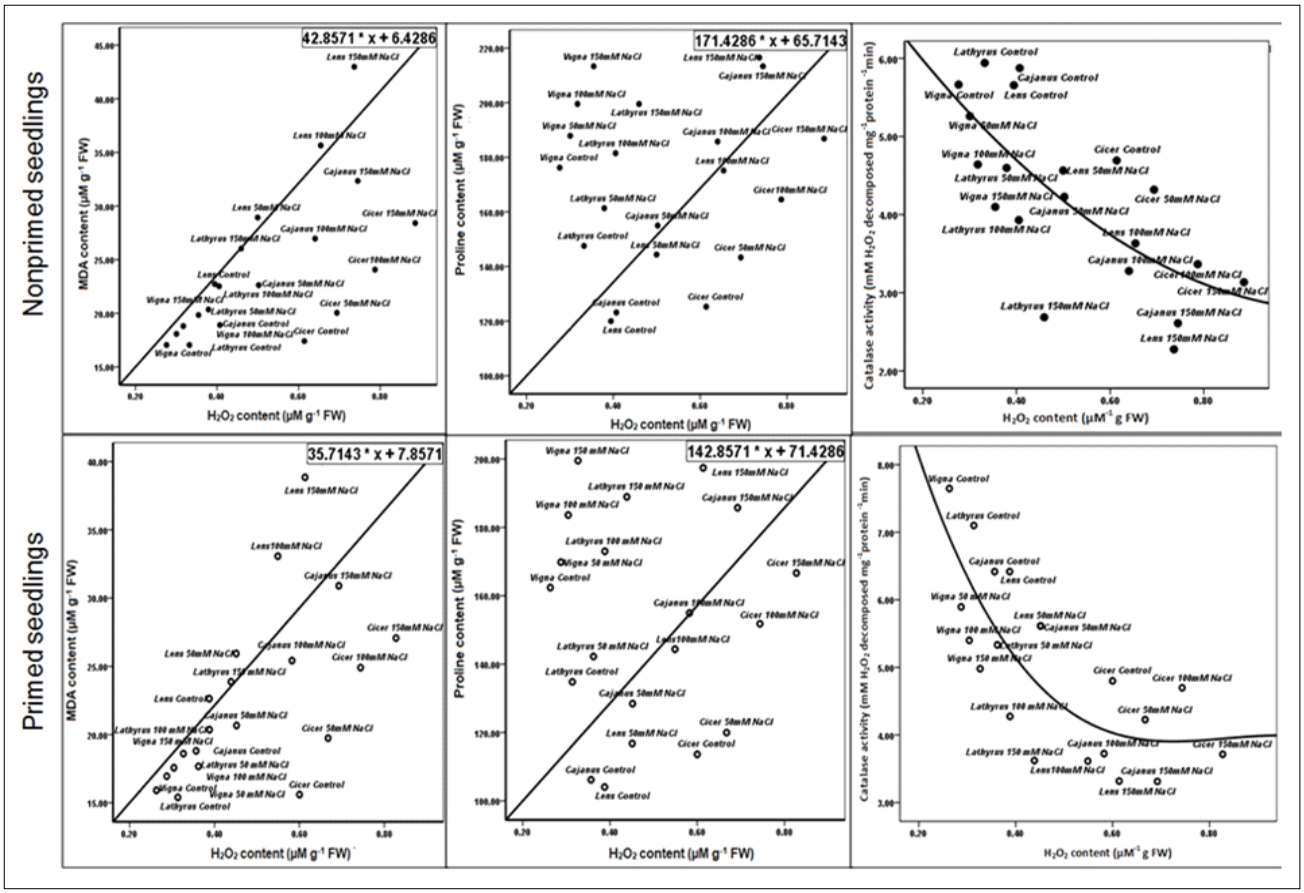
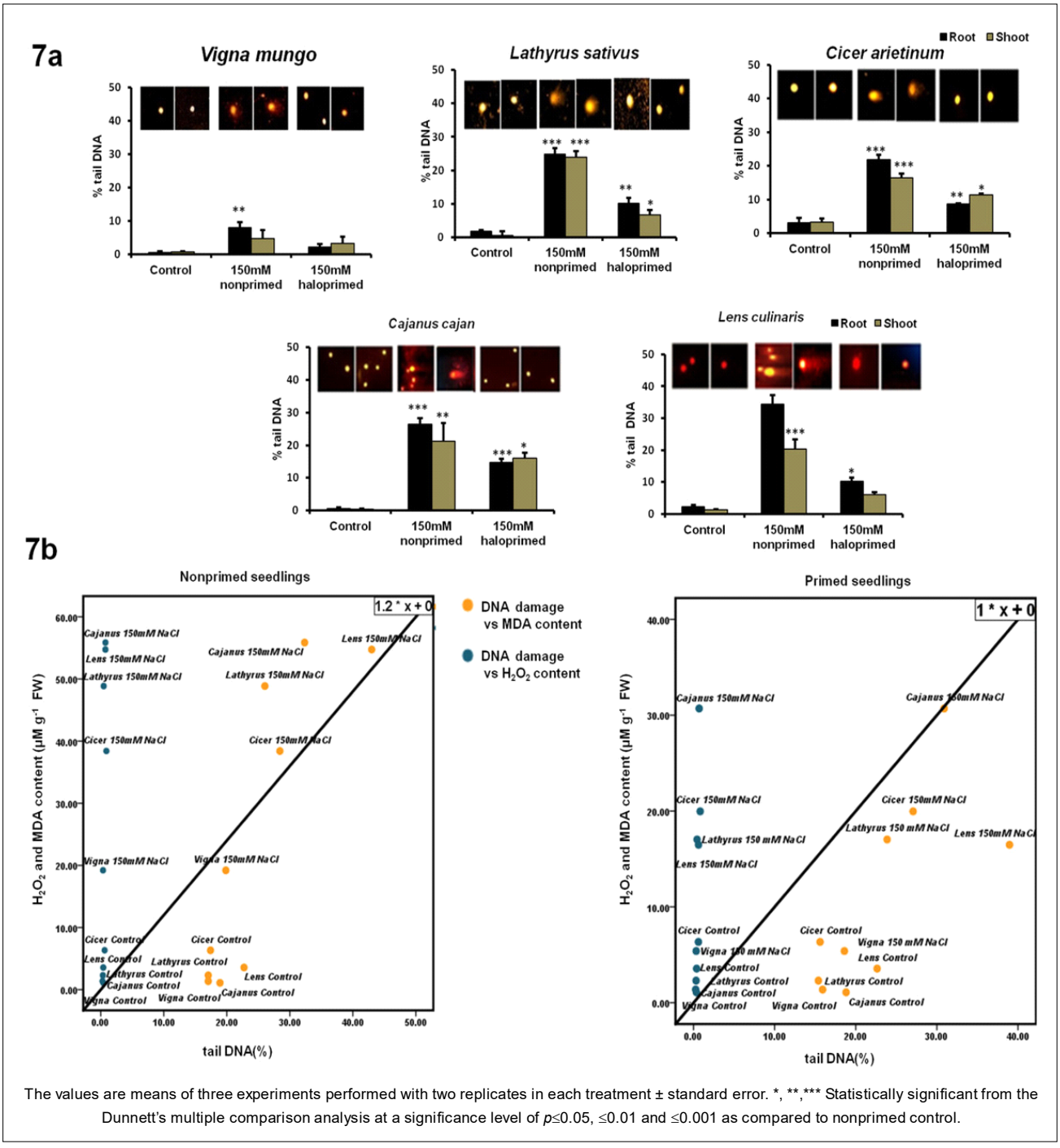


Fig 6: Bivariate scatterplot showing relation between proline content and H_2O_2 content; MDA content and H_2O_2 content; catalase activity and H_2O_2 content in primed and nonprimed legume varieties.

salt-tolerant nature. Similar physiological responses has been observed in salt-tolerant varieties of common bean genotypes *viz.*, KEB-049, KEB-CB053 and Mac-33 over salt-sensitive genotypes *viz.*, KEB-055 and KEB-050 under salinity (Kouam *et al.*, 2017). Reports by Chen *et al.* (2007) in 23 asparagus bean cultivars under salinity also corresponded to our observations. However, in the present work, seed halopriming helped to overcome the NaCl-induced adversities by reducing oxidative stress and DNA damage in the test legume varieties to variable extents.



The values are means of three experiments performed with two replicates in each treatment ± standard error. *, **,*** Statistically significant from the Dunnett's multiple comparison analysis at a significance level of $p \leq 0.05$, ≤ 0.01 and ≤ 0.001 as compared to nonprimed control.

Fig 7: a) Comet character in nuclei isolated from nonprimed and primed 21 days old legume seedlings and graphical representation of tail DNA (%) **b)** Relationship amongst H_2O_2 content and MDA content with tail DNA (%).

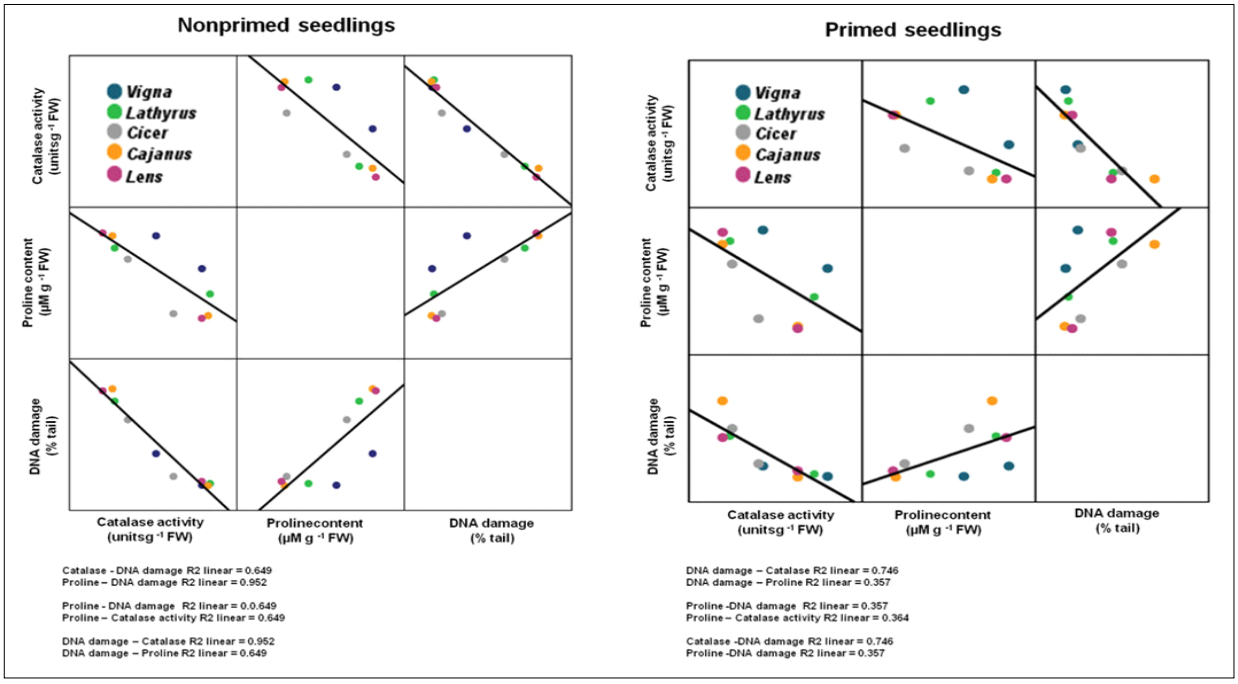


Fig 8: Scatterplot matrix showing interrelationship amongst proline content and catalase activity with DNA damage of primed and nonprimed legume varieties.

CONCLUSION

Salinity retarded growth by triggering oxidative stress in all legumes, impact being more on nonprimed seedlings. Present study revealed that amongst the five tested legume varieties, growth of *Lens*, *Cajanus* and *Cicer* were more adversely affected under NaCl stress as compared to *Lathyrus* and *Vigna*. This not only indicates salt-sensitive nature of *Lens*, *Cajanus* and *Cicer* but also partial salt tolerant nature of *Vigna* and *Lathyrus* seedlings grown under salt stress. Also, seed halopriming was able to alleviate the adversities experienced by all the test seedlings grown under NaCl stress. Salinity tolerance in nonprimed seedlings was achieved by osmolyte accumulation whereas in haloprimed seedlings, it was achieved by increasing catalase activity that promoted cellular detoxification. This lowered membrane degradation causing limited interaction of DNA with Na^+ and Cl^- ions reducing the extent of DNA damage. Based on our study, enhanced rate of ROS detoxification by catalase was the major mechanism adopted by haloprimed seedlings to survive under salinity. Thus, pre-germination treatment acclimatised seeds favouring germination and post-germinative events conferring improved growth under salinity.

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Conflict of interest

All authors declared that there is no conflict of interest.

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