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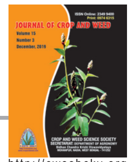

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Response of black gram to seed biopriming with facultative halophilic bacteria under salinity

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ABSTRACT

Under high salinity conditions, plant growth-promoting halophilic bacteria (PGPHB) can thrive and greatly encourage plant growth. The use of PGPHB is minimal and less discussed in sustainable agriculture and abiotic stress control. The current investigation focuses on improving the growth of black gram by inoculating with halophilic bacteria under salinity stress.Four PGPHB bacteria, viz., Bacillus safensis strain Lewis_Bac_3 (HB-5), Pseudomonas stutzeri strain MN1 (HB-13), Staphylococcus xylosus strain C5 (HB-18) and Pseudomonas sp. (GP-21: reference strain) were inoculated to black gram seeds to evaluate their plant growth-promoting ability at 4 dS m^{-1} *and pH >8.5. An increase in root length, plant height and the number of branches reported in consortium treatment (*T_8*) indicate that salinity did not affect black gram photosynthesis and nutrient absorption in consortium treatment. Corroborating evidence revealed higher nodulation and total Nitrogen and phosphorous content in the same treatment than control. Due to salinity stress, decreased blooming was reported in the control; conversely, consortium treatment showed 29.3 flowers/plant. A positive correlation with yield was demonstrated by the number of pods and seeds per pod of black gram. In addition, there also a strong association between pods per plant and the number of flowers per plant, nutrient content, and length of the root. The decrease in control plot yield was due to shoot and root development resulting from insufficient nutrients availability. In this study, we also found a positive correlation between % P in plants and yield. Hence, we conclude that PGPH bacteria help to reduce salt stress and significantly increase black gram growth and yield under mild salinity stress.*

Keywords: *Bacillu* ssp., PGPHB, *Pseudomonas* sp., root length, salinity

Black gram (*Vigna mungo* L.) is the third most predominant pulse crop in India; however, the area under cultivation is continuously decreasing year by year due to several reasons. Generally, sown as a fallow crop after rice in south India, forced flooding of rice crop leads to the increased salinity ultimately reduced yields in black gram. Black gram is highly susceptible to salinity in addition to physical stresses like high temperature, low temperature, freezing, drought, heavy metals, and hypoxia (Priyadharshini *et al.,* 2019). Among all, water stress caused by either drought or salinity declines the yield (Kijne, 2006). Unanticipated water stress occurs in saline and sodic soils due to more dissolved salts and too much sodium. Soil, a non-renewable resource, plays a key role in crop growth and yield; however, is degraded by agricultural, physical, and commercial pollution (Maximillian *et al.,* 2019). It was currently estimated that 20 % of the world's arable land is under threat of Salinity (Butcher *et al.,* 2016).

Soil salinity is a dynamic and global problem escalated by the low rainfall and increased temperatures in the semi-arid and arid regions of world (Shrivastava and Kumar, 2015). Salinity stress leading to loss of entire crop in susceptible crops or loss of economic parts up to 70 % was reported in wheat, maize, barley, and rice (Acquaah, 2007). Moreover, recent predictions pinpointed that climate change has severe unpleasant effects on soil salinity and highlighted the dramatic increase of salinity over a few decades, which necessitates the renewal of existing technologies or developing novel technologies that are sustainable and ecofriendly. Soil salinity reduces the 2000 ha of cultivable arable land every year on a global basis (Shahid *et al*., 2018) and in plants, this stress disrupts photosynthesis and alters the homeostasis of cells by producing reactive oxygen species (Miller *et al.,* 2010). Reclamation of saline soils is an arduous task mainly involving mechanical and chemical inputs, requiring lot of investment and a lot of human effort. Scientists endeavor to breed suitable varieties/hybrids for saline soils, and less adaptability makes it impossible to handle the area under salinity.

Inoculation of crops with bio-inoculants is essential since they facilitate the cultivation of crops in saline soils by improving salinity tolerance and hence, restoring yields (Lugtenberg *et al.,* 2013). Halophilic bacteria from extreme environments such as saline soils, oceans and deserts have been shown to induce salinity tolerance

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in plants. For example, *Pseudomonas fluorescens* strain isolated from the Saharan region promoted root growth in maize (*Zea mays*) under salt stress (Zerrouk *et al.,* 2016). Similarly, wheat (*Triticum aestivum*) inoculated with a halophilic bacterium *Serratia* sp. Sl-12, improved salinity tolerance and increased biomass (Singh and Jha, 2016). Halophilic microorganisms occupy many habitats such as salted foods, hypersaline soils, saline lakes, saltern ponds, and deserts (Ramos, 1993). Saline soils mostly have halotolerant microorganisms rather than halophilic microorganisms (Priyadharshini *et al.,* 2019); nonetheless, latest studies highlighted the abundance of halophilic bacteria in saline soils. Halophilic bacteria are rich sources of genes that transfer resistance or tolerance in plants or give protection to roots against biotic and abiotic stress through several known mechanisms, in addition to plant growth promotion. We aimed to alleviate the salinity stress in black gram using halophilic bacterial isolates at E" 4 dS m^{-1} salinity stress in the present investigation.

A composite of 50 soil samples was collected from saline soils in and around Raichur and Gangavathi, Karnataka, India.Then the soil samples were shade dried, powdered, and passed through a two mm sieve and preserved for Nitrogen (N), Phosphorous (P), Potassium (K), Organic carbon (OC), pH, and Electrical Conductivity (EC) estimation by using standard procedures. This experiment was carried out in Gangavathi, Karnataka, at the Agricultural Research station research plots. Three replications of nine treatments were used in this experiment.Treatments were imposed by treating the black gram seeds with the individual cultures and a consortium of *B. safensis* and *P. stutzeri, P. stutzeri* and *S. xylosus,* and *B. safensis, P stutzeri* and *S. xylosus.* Reference strain (*Pseudomonas* sp.) obtained from the Department of Agricultural Microbiology, University of Agricultural Sciences, Raichur was used in a single treatment (T_9).The recommended dose of fertilizer and 75 % of RDF treated plots were considered as controls. The data obtained from the field experiment (*in vivo*) was analyzed using randomized block design (RBD). Based on the tukey's pairwise and 95 % confidence method, all the values were clustered.

Fifty bacterial isolates were isolated and purified from rhizosphere soils and screened for their plant growth-promoting (PGP) attributes such as Zinc, phosphate solubilization and potassium release, and IAA production. Among the fifty isolates, four halophilic bacteria were selected based on their *in vitro* PGPR characteristics, such as *Bacillus safensis* strain Lewis_Bac_3 (HB-5)*, Pseudomonas stutzeri* strain MN1 (HB-13)*, Staphylococcus xylosus* strain C5 (HB-18), and *Pseudomonas* sp.(GP-21: reference strain) were used from the earlier studies of Nagaraju *et al.* (2020). In brief, these isolates were capable of producing 1 aminocyclopropane-1-carboxylic acid (ACC) deaminase and solubilized zinc (Zn) and phosphorous (P) solubilization, Potassium (K), as well as showed antagonistic activity against *S. oryzae* and *R.solani*. They can also produce IAA at 10 % NaCl concentration and have optimal growth at 3 % NaCl concentration.

Individual bacterial inoculants were multiplied in the flasks by inoculating them into the nutrient broth (supplemented with 3 % NaCl) and incubated at 37 ^{o}C for four days at 100 rpm in a shaking incubator. Cultures (10^9CFUml^{-1}) were added to pre-sterilized lignite powder @ 1/3 of its water holding capacity, followed by incubation for 24 hrs at 37 ^{o}C. Seeds of black gram variety,TAU-1 were obtained from the Seed Unit, College of Agriculture, Raichur, India. The seeds were sterilized as per the procedure given by Han *et al.* (2006); in brief, seeds were soaked in 1% sodium hypochlorite (NaOCl) for 30 sec followed by five times distilled water rinsing. A thin layer of bacterial inoculum (lignite mixed with culture) was coated on seeds using 10 % sugar solution as an adhesive, while the control was not treated with any microorganisms. This experiment was taken up in saline fields (15°27'24.0"N, 76°31'53.5"E) at Agricultural Research Station, Gangavathi, Koppal, India, where seeds were placed in the soil in a spacing of 0.3 m × 0.1 m.

Plant samples were analyzed for total Nitrogen (N) and Phosphorous (P) in black gram. Soil organic carbon analysis was carried out by using a standard protocol given by Piper (1966) and Jackson (1973). For organic carbon analysis, the soil samples were passed through 0.2 mm sieve. Biometric observations were made at regular intervals (30, 45 and 60 days after sowing) such as plant height, root length, branches, fresh and dry weight of shoot, Fresh and dry weight of root, flowers, pods, and root nodules. nutrient uptake of N and P were calculated based on the following formulae.

Nutrient uptake (kg ha^{-1}) = Nutrient concentration (%)/100 x Total Biomass yield (kg ha^{-1})

The saline soils were analyzed for significant nutrients, pH, EC, and organic carbon. The soils had nitrogen, phosphorous, and potassium in the ranges of 111.88-291.5, 21.8-99.8, and 211.8-625.3. Salinity stress decreased the black gram shoot length in control at30, 45 and 60 days after sowing. Among the different combinations followed, co-inoculation with *B. safensis*, *P. stutzeri* and *S. xylosus* enhanced the plant growth significantly over the control. Plant height was increased up to 60 days after sowing. Maximum plant height was reported in *B. safensis*, *P. stutzeri,* and *S. xylosus* treated plants with 18.6, 36.3, and 48.87 cm, respectively. Initial plant growth in control was comparable with other inoculated treatments; however, after 15 days, plant growth was trifling. Similarly, root length was also recorded highest in *B. safensis*, *P. stutzeri* and *S. xylosus* treated plants at 30, 45, and 60 DAS (Fig. 1), lowest root length was recorded in 75 % Recommended Dose of Fertilizer (RDF) treated plants.

The maximum number of branches were reported in co-inoculated treatment *B. safensis*, *P. stutzeri* and *S. xylosus,*which was at par with *Bacillus safensis* and *Pseudomonas stutzeri* treatment (13.3 branches/plant) inoculation. The lower number of branches were observed in 75 % RDF (5.6 branches/plant) treatment. At 60 DAS increase in the number of branches in all the treatments was observed, *B. safensis*, *P. stutzeri* and *S. xylosus* treated plants with 29.3 branches/plant, *B. safensis* and *P. stutzeri* with 26.3 branches/plant, *P. stutzeri* and *S. xylosus* with 24 branches/plant, *B. safensis* with 23.6 branches/plant, *S. xylosus* with 23.3 branches/plant and *Pseudomonas* sp. with 22.67 branches/plant.

Nodulation is very sensitive to salinity; however, significant nodules were observed at 45 DAS in T_8 (26.3 nodules/plant) and the lowest was reported in 75 % RDF (13.6 nodules/plant). Nodulation was significantly less in un-inoculated controls (Fig. 2). Nitrogen (N) and phosphorous uptake in black gram were influenced by co-inoculation of *B. safensis, P. stutzeri* and *S. xylosus*, plant uptake of N and P was recorded as 83.63 and 15.08 kg ha^{-1} respectively (Fig. 4.). The lower amount of nitrogen uptake was observed in the treatment control (41.59 kg ha^{-1}), and the lowest amount was noticed in 75 % RDF (34.34 kg ha^{-1}).

The reduction in biomass of black gram was clearly observed in un-inoculated control at ~4 dS m^{-1}. Fresh weight reduction was observed up to 33 % in un-inoculated control, and reduction in fertilizer by 25 % reduced the yield by 10.6 % under saline conditions. Inoculation with *B. safensis, P. stutzeri* and *S. xylosus* to black gram showed the highest fresh weight of 16.1 g/plant at the same salinity level. Root biomass was observed to be smaller in all treatments than in the shoot biomass. Total plant dry weight was also significantly benefited by the inoculation of plants with a growth-promoting halophilic bacterial consortium, which was not observed in un-inoculated controls. A consortium of *B. safensis, P. stutzeri,* and *S. xylosus* showed a profundal effect than the rest of the combinations on dry weight,and it was observed to increase up to 49.6 % compared to un-inoculated control (Fig. 5.).

Root shoot ratio was significantly higher with the inoculation of reference strain, 75 % RDF application showed increased root shoot ratio when compared to 100 % RDF application. Relative shoot weight percentage was highest with 100 % RDF and 75 % RDF treatments; conversely, relative root weight percentage was observed highest in the reference strain treated plants. Co-inoculation with halophilic bacteria increased relative root weight percentage than mono-inoculated and uninoculated controls.

The number of flowers was highest at 30 days after sowing (DAS) in all the treatments in black gram. Inoculation of facultative halophilic bacteria showed a positive effect on number of flowers. Reduction of fertilizer application by 25 % RDF, reduced the flowering up to 45.32 % in uninoculated control. Mono-inoculation of plants with *P. stutzeri* did not show any positive effect on blooming; instead, fewer flowers were reported than un-inoculated control with 100 % RDF. Conversely, mono-inoculation with *B.safensis* resulted in an increase in the number of flowers than un-inoculated control. Co-inoculation of plants with facultative anaerobic halophilic bacteria (*B. safensis, P. stutzeri* and *S. xylosus*) enhanced the flowering by 14.7 %. The number of flowers at 45 DAS was lower than the 30 DAS, and it was 86.68 and 67 % in control and T_8 treatments, respectively.

The total number of pods was significantly different in consortium-treated plants and non-consortium-treated plants.The highest number of pods was observed with the inoculation of *B. safensis, P. stutzeri* and *S. xylosus* (38.3 pods/plant); a lower number of pods was observed in the 75 % RDF treatment (17.6 pods/plant). Salinity-induced stress on pod yield was clearly observed in un-inoculated treatments (Fig. 3). The difference in the number of pods from control to T_8 (*B. safensis, P. stutzeri* and *S. xylosus*) was 41.74 %, reduction in fertilizer dose by 25 % of RDF reduced the number of pods by 20.8 % in uninoculated control (Fig. 6.).

The maximum number of seeds per pod was recorded at the harvest stage in the co-inoculated treatment *i.e.,*9 seeds/pod was observed in the *B. safensis, P. stutzeri* and *S. xylosus* treated plants. The lower number of seeds per pod was noticed in the 75 % RDF treatment (5 seeds/pod). An appraisal of data showed yield increase under salinity stress within co-inoculated treatments compared to uninoculated treatments and monoculture inoculations. It was found that T_8 yielded 13 q/ha, and the lowest yield was observed in the treatment 75 % RDF (9.44 q/ha).

Four halophilic bacteria capable of producing ACC deaminase, IAA, HCN in the presence of 3 % NaCl (w/v) under in *vitro* conditions were used in the present investigation. In addition, they were also capable of solubilizing zinc phosphate and zinc carbonate and released potassium from potassium aluminosilicate mineral at 10 % NaCl (w/v). Black gram seeds inoculated with *B. safensis, P. stutzeri*, and *S. xylosus* (individually and consortium) and sown in saline soil (pH> 8.5 and EC= <4) at ARS, Ganagavathi. Interestingly, a consortium of *B. safensis, P. stutzeri*, and *S. xylosus* increased the black gram biomass, nodulation, and yield parameters, whereas 75 % RDF alone did not show significant salt stress damage. In addition, the plant's uptake of nitrogen and potassium did not affect by the inoculation of consortium (*B. safensis, P. stutzeri*, and *S. xylosus*). Several researchers confirmed the beneficial activities of halophilic and halotolerant bacteria in several crops (Kadyan *et al.,* 2013; Furkan, 2016; Kearl *et al.,* 2019). In the present study, consortium treatment increased the plant height by13.58 % over the control (100 % RDF) at 60 DAS. It might be due to phosphorous and zinc solubilization, potassium release and production of growth hormones by halophilic bacteria (Buddhi *et al.,* 2014). A significant reduction in plant height was reported to control (75 % RDF), lack of ACC deaminase activity hindered the plant height. Similarly, reduced shoot length was observed in uninoculated control by Paranychianakis and Chartzoulakis (2005), Rodríguez *et al.* (2005), Hasan *et al.* (2017).

Root length had a positive linear correlation with the plant height, showed 30.68 % increase in root length over the control (100 % RDF).We found 75 % RDF treatment had 6 % reduced root length than 100 % RDF treatment. This summarizes that consortium treatment increased five times more root length than fertilizer dose alone. Fabaceae family is more sensitive to salinity stress, predominantly black gram (Munns and Tester, 2008). Impact of salinity stress on black gram was gradually decreased with the inoculation of a consortium of halophilic bacteria corresponding, increase in shoot length and root length was reported in the same treatments. In the present study, consortium treatment (*B. safensis*, *P. stutzeri* and *S. xylosus*) showed maximum shoot length, root length, and the number of branches. Reduction of plant height, root length in 75% RDF treated plants was due to the accumulation of salts in plants, reduced water uptake and reduced photosynthesis (Juan *et al.,* 2005). Salinity increases the Na and Cl concentration in leaves of black gram (Butcher *et al.,* 2016). The difference between consortium-treated plants and non-treated plants arose due to the protection of plant roots from salinity stress by halophilic bacteria (Parida and Das, 2005). The reduction in the number of branches in non-consortium (75% RDF treatment) treated plants was up to 42.03% and 35.21% in 100% RDF treatment. A decrease in the number of branches in control was noticed in black gram (Raptan *et al.,* 2001).

The fresh and dry weight of the inoculated plants was significantly higher than the uninoculated control. 40.45% higher fresh weight was found in consortium treated plants over the control (100 % RDF) and 49.63% higher fresh weight than 75% RDF treated plants. Similarly, 58.19% higher dry weight was recorded in consortium treatment (T_8) than control (100 % RDF). Clear stunting and reduced dry weight of black gram were reported under salinity stress by Parida and Das (2005).The number of flowers was maximum at 30 DAS; fewer flowers were recorded after 45 days of sowing. The number of pods at harvest was highest in consortium treatment (T_8), which was 41.77% higher than control (100% RDF). The number of pods was significantly reduced at 90 mM NaCl concentration (Hasan *et al.,* 2017). The number of pods declined as salt concentration increased (Hossain *et al*., 2008), which might be due to the reduced nutrient availability and decreased photosynthetic activity, as reported by Furkan, (2016).

The number of effective nodules was significantly higher (40.48%) in T_8 at 45 DAS. Enhanced nitrogen and phosphorous uptake were reported in co-inoculated treatment over the mono-inoculated and uninoculated control treatments. The application of insoluble phosphorous along with the phosphate solubilizing bacteria enhanced the phosphorous uptake (Jiang *et al.,* 2018). In this study, we used zinc, phosphorous solubilizing, and potassium releasing halophilic bacteria hence increased nutrient absorption by plants was reported. An increase in nitrogen and phosphorus uptake in black gram was reported after inoculation with PSB and *Rhizobium* (Tanwar *et al.,* 2003). The land configuration has a tremendous effect on the nitrogen and phosphorous uptake; the ridge and furrow method in saline soils enhanced the uptake (Vikas *et al.,* 2017). Micronutrient availability also plays a major role in the uptake of nutrients, primarily N and P (Shanti *et al.,* 2008). The corresponding yield increase in consortium-treated plants was also reported in this study. The reduction in yield in control was due to salt stress, Raptan (2000) reported a slight reduction in the grain yield at optimal salt concentrations. Under higher salinity, reduced food material transport to the grains/pods reduces seeds per pod (Raptan, 2000). Halophilic bacteria helped black gram plants to adapt to nearly 4 dS m^{-1} salinity by producing ACC deaminase and nutrient compensation. Our observations are typical examples of yield-enhancing halophilic bacteria.

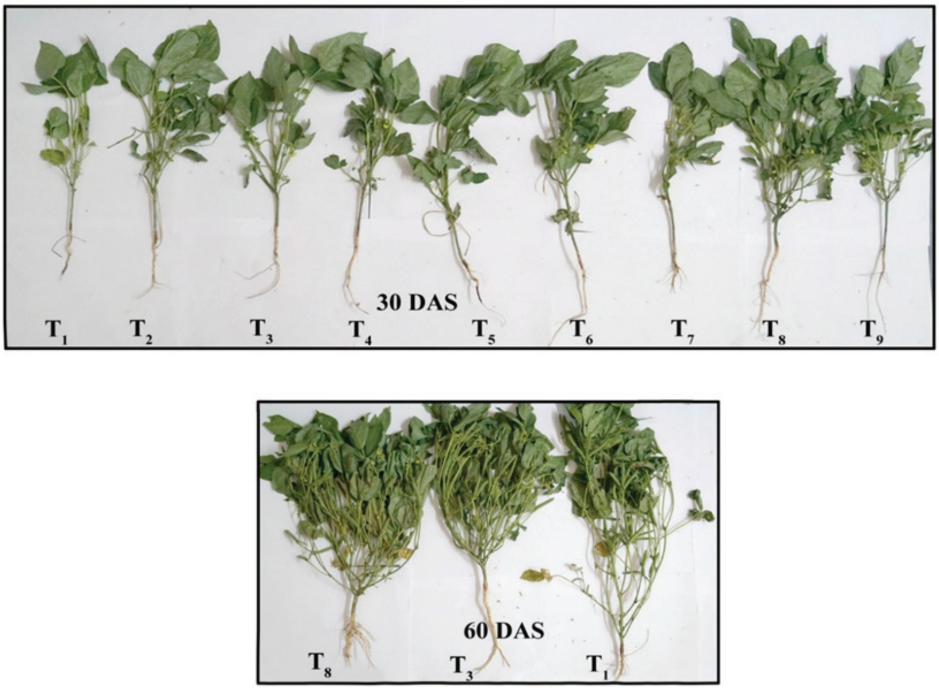

Fig. 1: Influence of halophilic bacterial inoculation on shoot length

Fig. 2:**Influence of halophilic bacterial inoculation on nodulation**

Investigations on salinity stress mitigation and crop improvement under salt stress conditions are meager for black gram; nonetheless, several studies were carried out exclusively on chemical-based methods which sequentially arouse the environmental concern. Halophilic bacteria greatly improved the flowering and seed setting in the present study, which subsequently increased the black gram yield over the controls. This effect was statistically significant. More salinity stress was experienced by control plants and resulted in lower yields. Halophilic bacteria aided the black gram plants under salinity stress by supplying nutrients and relieving salinity stress through ACC deaminase production. Increased nutrient uptake was reported in the

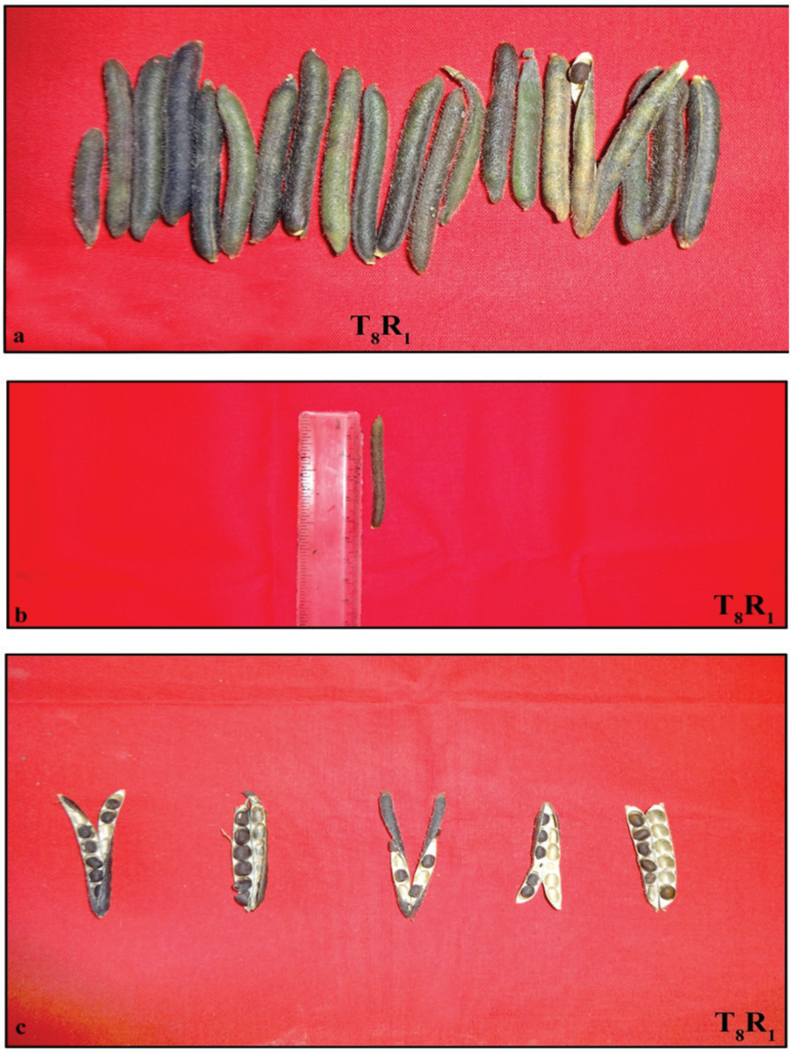

Fig.3: Influence of halophilic bacterial inoculation on number of pods, pod length, and seeds per pod

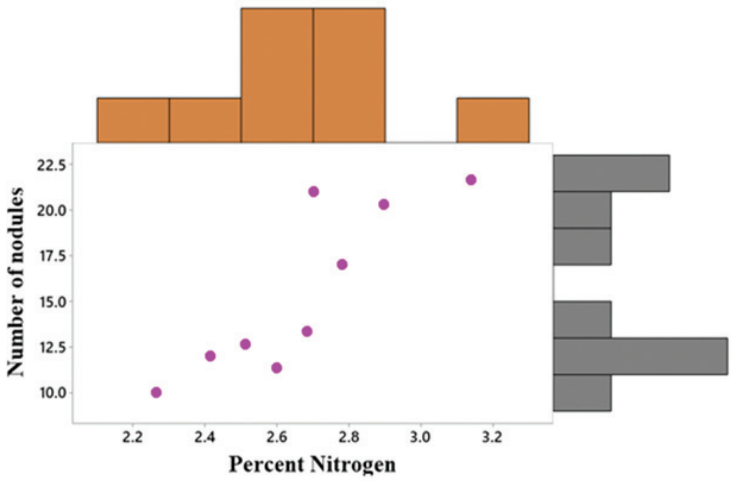

Fig. 4: Marginal plot: nitrogen uptake and number of nodules have a positive correlation

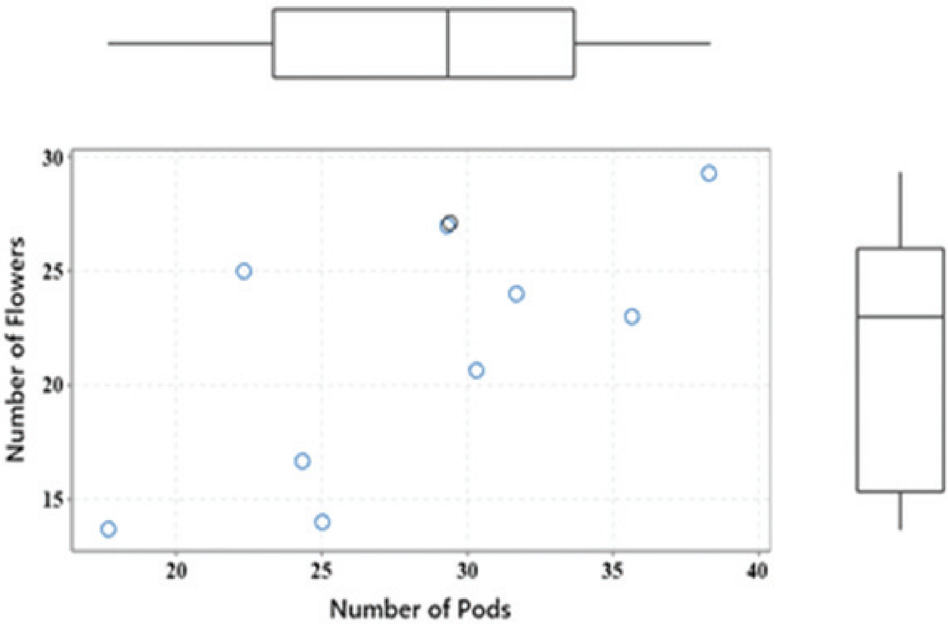

Figure 5: Marginal plot: Number of flowers showed a positive correlation with the number of pods

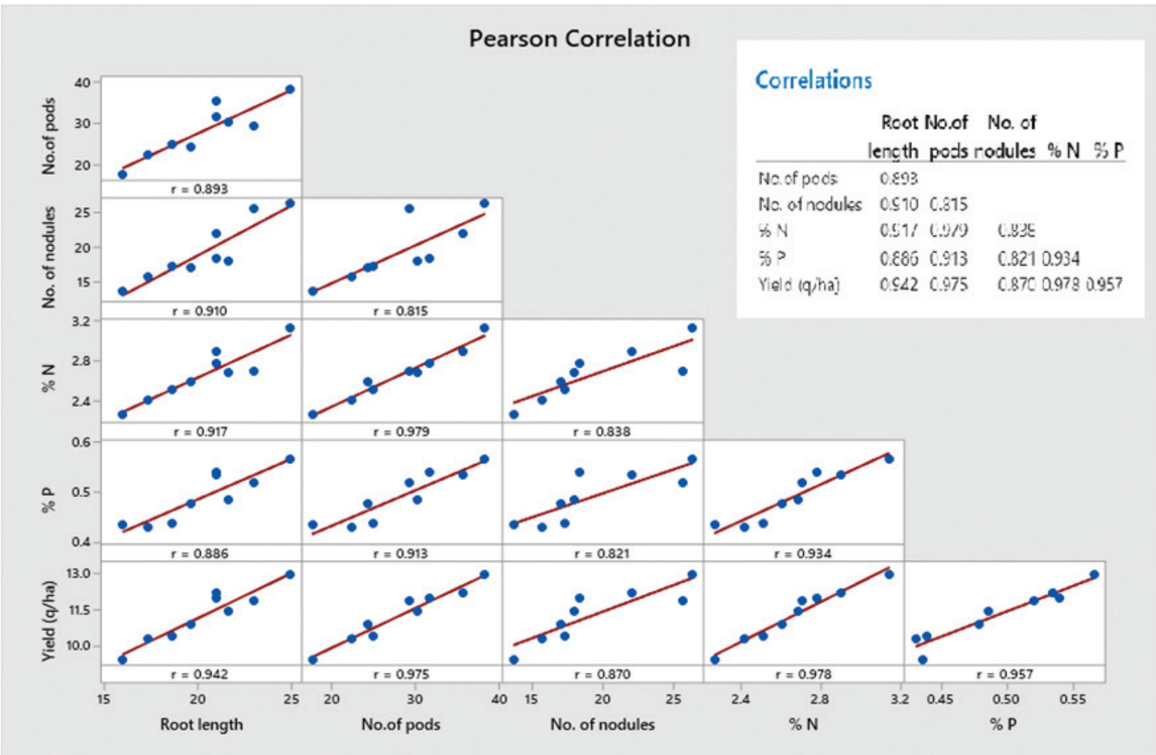

Figure 6: Pearson correlation (matrix): All the parameters are positively correlated; the number of pods positively correlates with root length. Percent phosphorous and number of pods have less correlation. Red lines represent the regression

consortium-treated plants. Based on our findings, we conclude that the best alternative for chemical treatment is the use of halophilic bacteria in saline soil management. This technique is environmentally benign and cost-effective. Environment friendly microbial-based scientific principles are not promulgated and are not encouraged owing to their unobtrusive and repercussive nature. Our study promotes the sustainable management of saline soils and crop production. However, this study is limited by not covering the molecular mechanisms underlying these changes in plants, which potentially influences the biology of plants and in turn, their survival.

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