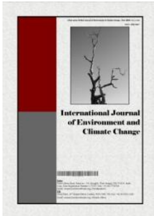

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Response of Black Gram to Seed Biopriming with Facultative Halophilic Bacteria under Salinity

Yalavarthi Nagaraju[1*], Mahadevaswamy[1], R. C. Gundappagol[2] and Nagaraj M. Naik[1]

[1]Department of Agricultural Microbiology, University of Agricultural Sciences, Raichur-584104, India.
[2]AICRP on Pigeonpea, Kalburgi, Karnataka, India.

Authors' contributions

This work was carried out in collaboration among all authors. Author YN carried out the experiment and wrote the first draft of the manuscript, performed the statistical analysis author Mahadevaswamy and RCG designed the study, wrote the protocol and managed the analyses of the study. Author NMN managed the literature searches. All authors read and approved the final manuscript.

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Short Communication

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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 focus of the current investigation is to improve the growth of black gram by inoculating with of 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. Increase in root length, plant height, and number of branches have been reported in consortium treatment (T_8), indicating that salinity does not affected black gram photosynthesis and nutrient absorption in consortium treatment. Corroborating evidence revealed higher nodulation and total nitrogen and phosphorous content in the same treatment, in comparison with control. Due to salinity stress, decreased blooming was reported in control, conversely, consortium treatment showed 29.3 flowers/plant. A positive correlation with yield was demonstrated by number of pods and seeds per pod of black gram. In addition, there is a strong association between pods per plant

**Corresponding author: E-mail: nagarajulvrth62@gmail.com;*

and the amount of flowers per plant, nutrient content, and length of 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 positive correlation between% P in plant and yield. Hence, we conclude that PGPH bacteria helps in the reduction of salt stress and significantly increase black gram growth and yield under mild salinity stress.

Keywords: Bacillus sp.; PGPHB; Pseudomonas sp. Root length; salinity.

1. INTRODUCTION

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 lead 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 [1]. Among all, water stress caused by either drought or salinity declines the yield [2]. Unanticipated water stress occurs in saline and sodic soils due to the presence of more dissolved salts and too much sodium respectively. Soil, a non-renewable resource plays a key role in crop growth and yield however, degraded by agricultural, physical, and commercial pollution [3]. Currently, it was estimated that 20% of Worlds arable land is under threat of salinity [4].

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 [5]. Salinity stress leads to loss of entire crop in susceptible crops or loss of economic parts up to 70% was reported in wheat, maize, barley and rice [6]. Moreover, recent predictions pinpointed that climate change have severe unpleasant effects on soil salinity and also highlighted dramatic increase of salinity over few decades, which necessitates the renewal of existing technologies or to develop a novel technologies which are sustainable and ecofriendly. Soil salinity reduces the 2000 ha of cultivable arable land every year on global basis [7] and in plants this stress disrupts the photosynthesis in turn alters the homeostasis of cells by producing reactive oxygen species [8]. Reclamation of saline soils is an arduous task involving mostly mechanical and chemical inputs, requiring lot of investment as well as involves lot of human effort. Scientists endeavor to breed suitable varieties/hybrids for saline soils will take long time and less adaptability makes it impossible to handle the area under salinity.

Inoculation of crops with bioinoculants is achieving importance, since they facilitate cultivation of crops in saline soils by improving salinity tolerance and hence, restoring yields [9]. Halophilic bacteria from extreme environments such as saline soils, oceans and deserts have been shown to induce salinity tolerance in plants. For example, *Pseudomonas fluorescens* strain isolated from Saharan region promoted root growth in maize (*Zea mays*) under salt stress [10]. Similarly, wheat (*Triticum aestivum*) inoculated with a halophilic bacterium *Serratia* sp. SI-12, improved salinity tolerance and increased biomass [11]. Halophilic microorganisms occupy a wide range of habitats such as salted foods, hypersaline soils, saline lakes, saltern ponds, and deserts [12]. Saline soils mostly have halotolerant microorganisms rather than halophilic microorganisms [1], nonetheless, latest studies highlighted the abundance of halophilic bacteria in saline soils. Halophilic bacteria are rich sources of genes which transfer resistance or tolerance in plants or give protection to roots against biotic and abiotic stress through several known mechanisms, in addition to the plant growth promotion. In the present investigation, we aimed to alleviate the salinity stress in black gram using halophilic bacterial isolates at $\cong$ 4 dS m^{-1} salinity stress.

2. MATERIALS AND METHODS

2.1 Soil Sampling and Analysis

A composite of 50 soil samples were collected from saline soils in and around Raichur and Gangavathi, Karnataka, India (15.456628, 76.532192). Then the soil samples were shade dried, powdered and passed through 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. safenisis* and *P. stutzeri, P. stutzeri* and *S xylosus,* and *B. safensis, P stutzeri* and *S. xylosus.* Reference strain (*Pseudomonas* sp.) obtained from Department of Agricultural Microbiology, University of Agricultural Sciences, Raichur was used in single treatment (T_9). Recommended dose of fertilizer and 75% of RDF treated plots were considered as controls. The data obtained from field experiment (*in vivo*) was analyzed by using randomized block design (RBD). Based on the tukey's pair wise and 95% confidence method, all the values were clustered.

2.2 Bacterial Strains and Inoculum Preparation

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 as well as 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 early studies of Nagaraju et al [13]. 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. solanii*. They also capable of producing IAA at 10% NaCl concentration and have optimal growth at 3% NaCl concentration.

Cultures produced in the flasks by inoculating them in to nutrient broth (supplemented with 3% NaCl) and incubated at 37 ^{0}C for four days at 100 rpm in shaking incubator. Cultures (10^9 cfu ml^{-1}) were added to pre-sterilized lignite powder @ 1/3 of its water holding capacity followed by incubation for 24 hrs at 37 ^{0}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 distil water rinsing. 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 of the microorganism. 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, seeds were placed in the soil at a spacing of 0.3 m × 0.1 m.

2.3 Nutrient Uptake and Biometric Observations

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 [14] and Jackson [15]. 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 conc. in samples (%) / 100] X Total Biomass yield (kg ha^{-1})

3. RESULTS

3.1 Plant Growth Parameters

Salinity stress decreased the black gram shoot length in control at 30, 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 after sowing, at 30, 45 and 60 days of 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 also recorded highest in *B. safensis*, *P. stutzeri* and *S. xylosus* treated plants at 30, 45 and 60 DAS (Plate 1), lowest root length 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. Lower number of branches were observed in 75% RDF (5.6 branches/plant) treatment. At 60 DAS increase in 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.

3.2 Nodulation and Nutrient Uptake

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

3.3 Biomass yield

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 same salinity level. Root biomass was observed to be smaller in all treatments than in the shoot biomass in all treatments. 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. Consortium of *B. safensis, P. stutzeri* and *S. xylosus* showed a profundal effect than the rest of combinations on dry weight and it was observed that increase up to 49.6% when compared to un-inoculated control (Fig. 2).

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 the application of 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 un-inoculated controls.

3.4 Blooming

Number of flowers were 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 un-inoculated control. Mono-inoculation of plants with *P. stutzeri* doesn't showed any positive effect on blooming, instead lower number of flowers were reported than un-inoculated control with 100% RDF. Conversely, mono-inoculation with *B. safensis* resulted in the increase of 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%. Number of flowers at 45 DAS were lower than the 30 DAS, it was 86.68 and 67% in control and T_8 treatments respectively.

3.5 Yield Parameters

The total number of pods was significantly different in consortium treated plants and non-consortium treated plants. Highest number of pods was observed with the inoculation of *B. safensis, P. stutzeri* and *S. xylosus* (38.3 pods/plant), lower number of pods were observed in the 75% RDF treatment (17.6 pods/plant). Salinity induced stress on pod yield was clearly observed in un-inoculated treatments (Plate 3). The difference in 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. 3).

The maximum number of seeds per pod was recorded at 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. 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 when compared to un-inoculated treatments and monoculture inoculations. It was found that T_8 yielded 13 q/ha, and lowest yield was observed in the treatment 75% RDF (9.44 q/ha).

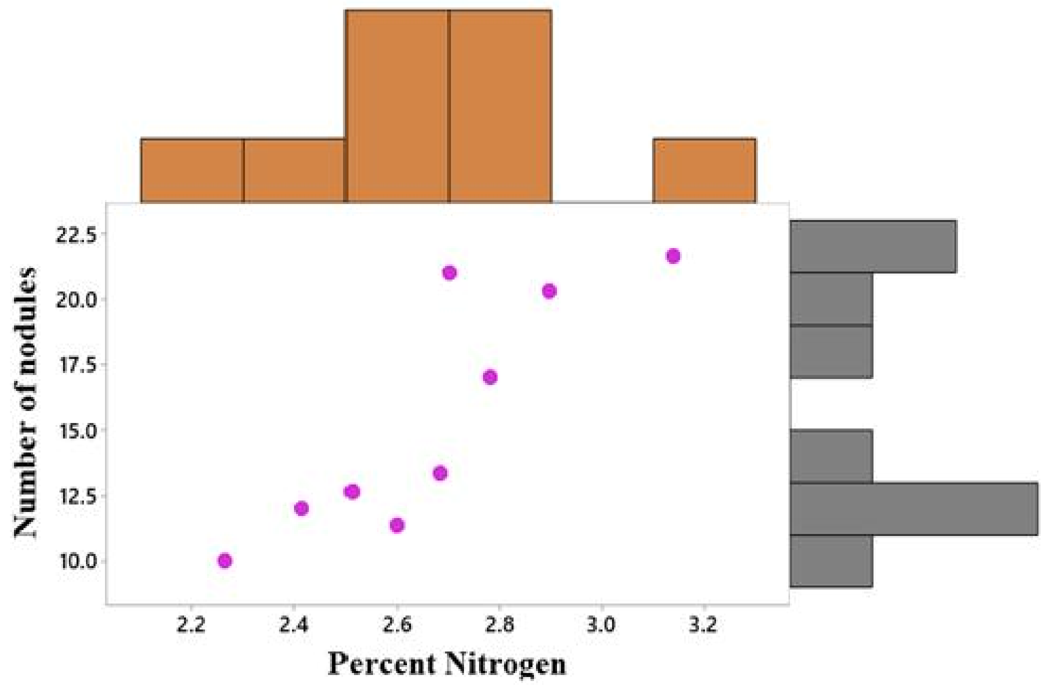

Fig. 1. Marginal plot: Number of nodules showed a positive correlation with the percent nitrogen

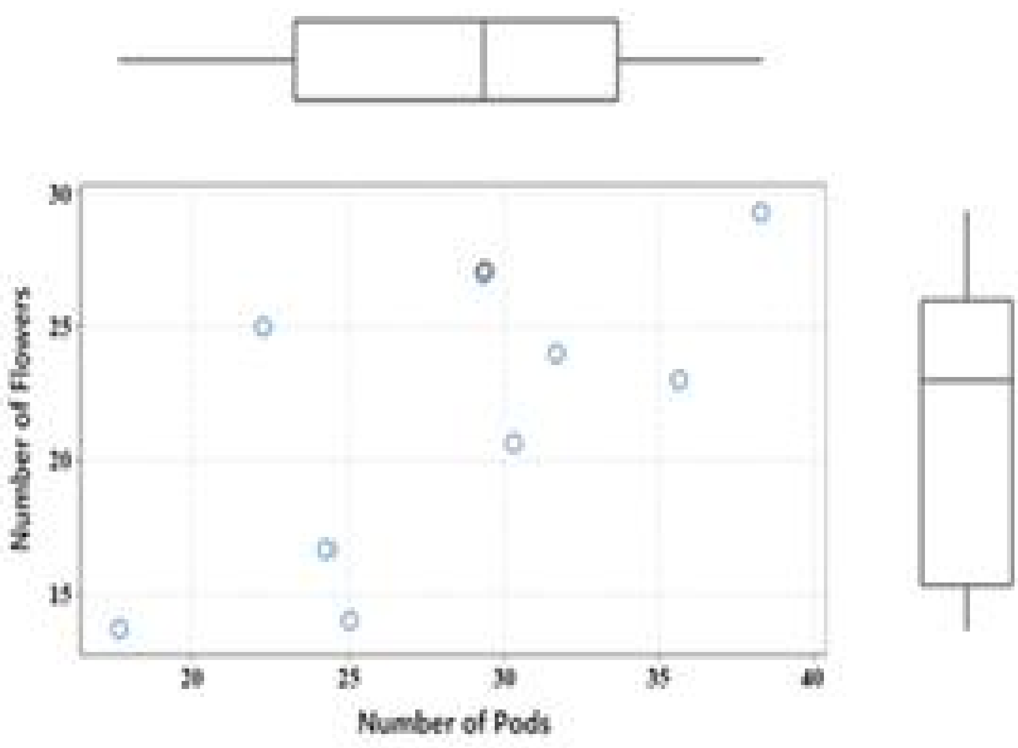

Fig. 2. Marginal Plot: Number of flowers showed a positive correlation with number of pods

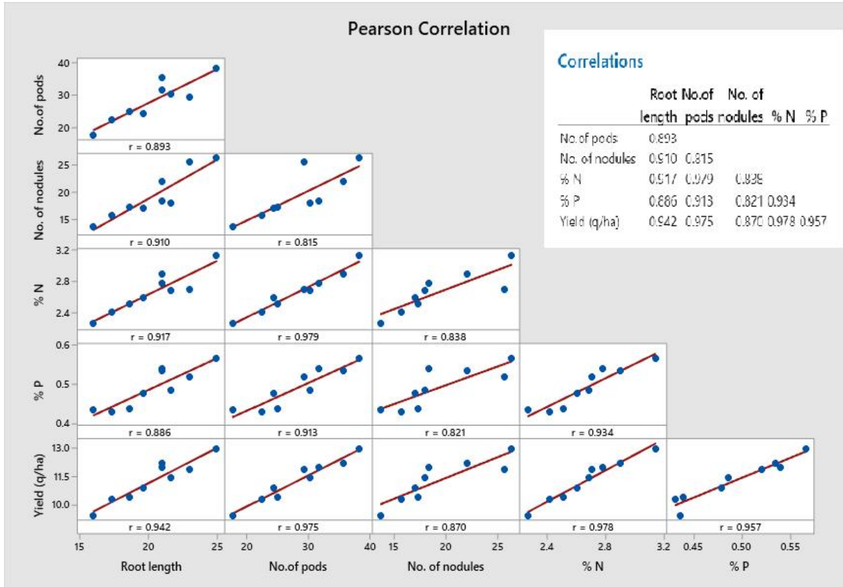

Fig. 3. Pearson correlation (Matrix plot): All the parameters are positively correlated; number of pods has correlation with root length. Per cent phosphorous and number of pods has less correlation. Red lines represent the regression

Plate 1 . Influence of halophilic bacteria on root length of black gram

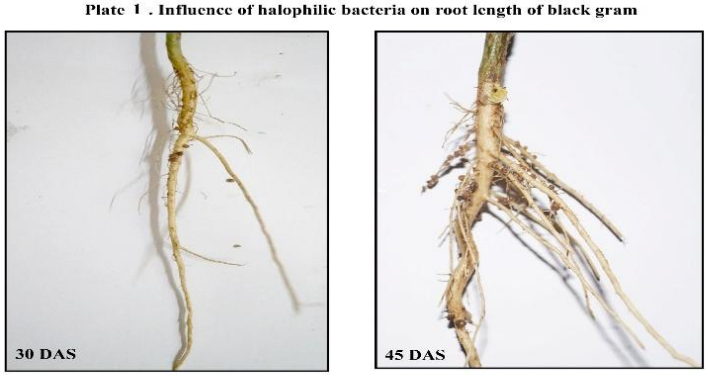

Plate 2. Influence of halophilic bacteria inoculation on number of nodules at 45 das

Plate 3. Influence of halophilic bacteria on number of pods, pod length, number of seeds of black gram

4. DISCUSSION

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 alumino silicate 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 doesn't showed significant damage of salt stress. In addition, plants uptake of Nitrogen and potassium doesn't affected 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 [16-18]. In the present study consortium treatment increased the plant height by 13.58% 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 [19]. Significant reduction in plant height was reported control (75% RDF). Similarly, reduced shoot length was observed in uninoculated control by Paranychianakis and Chartzoulakis [20]; Rodríguez et al. [21]; Hasan et al. [22].

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 has 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 especially black gram [23]. Impact of salinity stress on black gram was gradually decreased with the inoculation of 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 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 [24]. Salinity increases the Na and Cl concentration in leaves of black gram [4]. The difference between consortium treated plants and non-treated plants aroused due to protection of plant roots from salinity stress by halophilic bacteria [25]. The reduction in number of branches in non-consortium (75% RDF treatment) treated plants was up to 42.03% and 35.21% in 100% RDF treatment. Decrease in the number of branches in the control was noticed in black gram [26].

Fresh and dry weight of the inoculated plants were 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 recorded in consortium treatment (T_8) than control (100% RDF). Clear stunting and reduced dry weight of black gram was reported under salinity stress by Parida and Das [25]. Number of flowers were maximum at 30 DAS, fewer number of flowers were recorded after 45 days of sowing. Number of pods at harvest was recorded highest in consortium treatment (T_8), which is 41.77% higher than control (100% RDF). Number of pods was significantly reduced at 90 mM NaCl concentration [22]. Number of pods reduced as salt concentration increased [27], might be due to the reduced nutrient availability and decreased photosynthetic activity as reported by Furkan, [17].

Number of effective nodules were significantly higher (40.48%) in T_8 at 45 DAS. Enhanced nitrogen and phosphorous uptake was reported in co-inoculated treatment over the mono-inoculated and un-inoculated control treatments. Application of insoluble phosphorous along with the phosphate solubilizing bacteria enhanced the phosphorous uptake [28]. In this study we used zinc, phosphorous solubilizing and potassium releasing halophilic bacteria hence increased nutrient absorption by plants was reported. Increase in nitrogen and phosphorus uptake in black gram was reported after inoculation with PSB and *Rhizobium* [29]. Land configuration has tremendous effect on the nitrogen and phosphorous uptake, ridge and furrow method in saline soils enhanced the uptake [30]. Micronutrient availability also plays a major role in the uptake of nutrients specially N and P [31]. Corresponding yield increase in consortium treated plants also reported in this study. The reduction in yield in control was due to salt stress, slight reduction in the grain yield at optimal salt concentrations was reported by Raptan [26]. Under higher salinity reduced food material transport to the grains/pods results in the reduction in the number of seeds per pod [26]. Halophilic bacteria helped black gram plants to adopt to nearly 4 dS m^{-1} salinity by producing ACC deaminase and nutrient compensation. Our observations are typical examples of yield enhancing halophilic bacteria.

5. CONCLUSIONS

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. The black gram plants were aided by halophilic bacteria under salinity stress by supplying nutrients and relieving salinity stress through ACC deaminase production. Increased nutrient uptake was reported in the 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. Environmental friendly-microbial based scientific principles are not promulgated and are not encouraged owing to their unobtrusive and repercussive nature. Our study promotes sustainable management of saline soils and crop production. However, this study is limited by not covering the molecular mechanisms underlying these changes plants, which potentially influences the biology of plants and in turn their survival.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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