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Evaluation of the Co-inoculation Effect of *Rhizobium* and Plant Growth Promoting Non-rhizobial Endophytes on *Vigna radiata*

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Abstract

A unique feature of leguminous plants is the establishment of symbiotic bacterial genera inside root or stem nodules that is being recently re-evaluated for investigating the micro-flora discrete to nitrogen fixation. The present research was carried out to evaluate non-rhizobial endophytes and *Rhizobium* from root nodules of *Vigna radiata* and ascertain their co-inoculation effect in pot and field conditions. Each strain displayed one or more plant growth-promoting behaviors in varying degrees. The ability to fix nitrogen was observed in all strains; however, a noticeable enhancement in nitrogen fixation was observed when all three strains were co-inoculated. All three strains were found to possess the *nif*H gene, which plays a key role in the nitrogen fixation process. However, only *Rhizobium* sp. AAU B3 also had the *nod*D gene present. Furthermore, combinations of all three strains produced the highest levels of phosphate solubilization, potash mobilisation, Indole Acetic Acid (IAA), and the stress-relieving enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase. Interestingly, the succession of the root nodule formation within root hairs seedlings was observed under a fluorescence microscope and two NRE were found to be located inside the root nodules, indicating that they are endophytic. Additionally, a pot and field investigation revealed that the combination of chosen *Rhizobium* and NRE strains had a favorable impact on the growth and yield characteristics of a green gram. Selected bio-inoculants can reduce the utilization of synthetic fertilizers by 75%, which might lead to the restoration of the soil's health. Therefore, these bio-inoculants might be explored commercially for sustainable agriculture production.

Introduction

Farmers frequently employ more nitrogen, phosphate, or potash fertilizers, as well as other chemical fertilizers, to boost crop production [1]. In fact, the so-called "Green Revolution" of the 1960s was brought about by the development of the technology for the manufacture of chemical fertilizers in the 1930s–1950s as well as by other scientific advancements in agriculture supported by governments and businesses [2]. Around the world, the "Green Revolution" had many benefits, but over time, it also had unfavorable effects and downsides that are still noticeable today [3]. Chemical fertilizer use has a negative impact on the environment, contributing to issues such as soil acidification or salinity, deterioration of indigenous crops or microorganisms, overexploitation of genetic resources, water eutrophication, and air pollution [3–7]. As a consequence, it is becoming increasingly critical to boost production employing sustainable techniques by avoiding or at least minimizing the usage of chemical fertilizers. The majority of the issues brought on by the use of chemical fertilizers can be resolved using "greener" fertilizers, especially biofertilizers, which are mostly based on plant growth-promoting bacteria (PGPB). Potentially beneficial bacteria for plants possess a diverse range of characteristics that can benefit the plants. They are generally involved in nutrient acquisition via nitrogen fixation, phosphate and potash solubilization as well as production of growth hormones. IAA is involved in different plant growth and development processes such as the formation of the lateral roots and root hairs as well as increases the primary root length [8]. IAA production was reported as common in plant-associated bacteria as part of a colonization strategy that involves phytostimulation and circumvention of plant defense mechanisms [9]. The rhizobium-based bioinoculants are more frequently employed for leguminous crops than other biofertilizers due to their better ability to fix nitrogen by symbiosis.

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The legume family, which includes 730 genera and more than 19,320 species, is one of the largest plant families in the world [10]. An essential pulse crop, green gram (*Vigna radiata* L.), is a member of the subfamily *papilionaceae* of the *Leguminosae* family. Pulses are well-known for the three "Fs": Fodder, food, and fertilizer. In terms of nutritional value (proteins, amino acids and fibers) among pulses, green gram has been given a higher ranking next to gram and black gram [11]. In addition to its significance for agriculture, its most significant characteristic is that it contributes to 80% of biological nitrogen fixation. This is made possible by the symbiotic association that rhizobia create with root nodule bacteria.

The signaling events between symbiotic rhizobia and leguminous plants are highly explicit. The rhizobial cells secretes specified lipopolysaccharides called nod factors at the binding sites on roots of leguminous plants thereby initiating symbiosis process systematically. Highly specific nod factors and oxygen sensitive nitrogenase enzyme promotes the growth of only narrow range of microbes inside the microaerophilic nodules [12]. Although the specificity and microaerophilic conditions present inside root nodules; it is well documented and reported that the diverse group of non-rhizobial endophytes (NRE) were present inside nodules [13]. Generally, NRE gets colonized in infection thread that initiated by host specific rhizobia by breaking the host's specificity. To study the nodule symbiosis or plant–microbe interactions use of advanced fluorescence microscopic techniques were being used by researchers world-wide. The detection of fluorescence cells of NRE isolates inside root nodule tissue confirms the accommodation of nodules by NRE bacteria strains. The genera inside root nodules may include *Bacillus, Pseudomonas, Rhizobium, Xanthomonas*, [14], *A. rhizogenes, Phyllobacterium, Stenotrophomonas, Agrobacterium tumefacien*, *Enterobacteriaceae*, *Bacillus, Bordetella, Curtobacterium*, *Pantoea* [15], *Brevibacillus brevis*, *Paenibacillus* sp., *Pantoea agglomerans*, [16] and many members of the phylum Actinobacteria including *Agromyces* and *Microbacterium* sp. [17, 18], *Curtobacterium* [19] and *Micromonospora* [20–22]. Although the invasive NRE inside the rood nodule has some phytobeneficial effects, the precise method by which this occurs is uncertain. In conjunction with *Rhizobium* sp., most NRE may help the host plants in numerous ways, including by increasing the number of nodules, encouraging aerial growth, and nutrient intake. Nonetheless, NRE does not actively contribute to the induction of root nodules.

The utilisation of PGPBs as consortia enhances not only agricultural output but also the quality of grains, fruits, and processed items from these crops [23]. Attractive from an economic and environmental standpoint, the development and deployment of single and mixed inoculants based on chosen PGPBs is now a viable option to the long-term use of synthetic fertilizers in agricultural areas. The general public has become interested in the possible application of these advantageous PGPBs in biofertilization systems. It has been demonstrated in several studies that inoculating PGPBs in consortia offers plants a variety of advantages by co-inoculating NRE and Rhizobium strains in plants [24–26]. The majority of this research, however, was conducted under controlled lab and greenhouse circumstances, while just a few studies being conducted in the field [23].

There is a paucity of information, regarding the isolation of native root endogenous endophytes, their function in nutrient bioavailability, growth stimulation, and interaction with rhizobia in green gram. Additionally, despite having so high potential, green gram is frequently produced in marginal areas with few inputs, rendering it vulnerable to numerous abiotic and biotic challenges that result in significant yield losses. Environmental factors like drought, abiotic stresses and salinity in the subsoil, which can limit root growth and hinder a plant's ability to draw moisture and nutrients from the soil.

These studies aimed to identify the key endophytic bacteria found in the root nodules of green gram plants and investigate their impact on plant nutrient acquisition. The microbiota of green gram was examined for its potential to enhance plant growth, with particular focus on utilizing nodule endophytes as a valuable resource in soil microbiology. The endophytic bacteria from the surface-sterilized root nodules of *Vigna radiata* were evaluated for their ability to promote plant growth and their interactions with different *Rhizobium* species. These NRE isolates further confirmed for their residence inside the root nodules by studying their succession of the root nodule infection thread formation within root hairs of *Vigna radiata* seedlings was observed under a fluorescence microscope after seed inoculation with tagged NRE isolates with 4′, 6-diamidino-2-phenylindole (DAPI). Lastly, the co-inoculation effect of *Rhizobium* and NRE on *Vigna radiata* growth was examined through pot and field study.

Materials and Methods

Bacterial Strains

The bacterial isolates *Rhizobium* sp. AAU B3 (GenBank® ACCN MH701891), *Bacillus* sp. AAU B6 (GenBank® ACCN MH701892) and *Bacillus* sp. AAU B12 (GenBank® ACCN MH701893) and *R. selenitireducens* AAU M1 were obtained from Department of Microbiology and Biofertilizer project, Anand Agricultural University, Anand [27].

Compatibility Study of *Rhizobium* and NRE

The *Rhizobium* sp. AAU B3, *R. selenitireducens* AAU M1 and NRE (*Bacillus* sp. AAU B6 and *Bacillus* sp. AAU B12) were cross streaked to test their compatibility with each other.

Plant Growth-Promoting Traits of Isolates

N_2 Fixation

Nitrogen fixation was determined by the quantitative Micro-Kjeldahl method described by AOAC [28] and sugar utilization was estimated using Fehling's method [29]. PCR amplification of region coding for Fe protein (component II) of the nitrogenase enzyme complex was studied as described by Dhole et al. [30]. The Fragments of *nod*D genes were amplified using two primer sets (NBA12- 5′GGATSGCAATC ATCTAYRGMRTGG3′, NBF120- 5′GGATCRAAAGCA TCCRCASTATGG3′, Y5- 5′ ATGCGKTTYARRGGMCT-NGAT 3′, and Y6- 5′ CGCAWCCANATRTTYCCNGGRTC 3′) [31, 32]. The amplification was carried out as described by Laguerre et al. [32] with Mastercycler personal (Eppendorf, Germany) with the following PCR conditions: initial denaturation at 94 °C for 2 min, denaturation at 93 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min for 35 cycles, and final extension at 72 °C for 10 min.

Phosphate and Potassium Solubilization

Phosphate solubilization activity of all isolates individually as well as in combinations with each other was determined on Sperber's agar plates (Glucose-10.0 g, $CaCO_3$-05.0 g, Yeast Extract 05.0 g, 10% K_2HPO_4—20 ml, $MgSO_4.7H_2O$—0.25 g, $CaCl_2$—0.1 g, Agar—15 g, Distilled water—1000 ml, pH—7.0) by the method described by Taurian et al. [33]. Bacterial isolates were inoculated using a sterile needle under aseptic conditions and were incubated at 30 ± 2 °C for five days with observation on colony diameter and solubilization of TCP every 24 h. A clear zone around the growing colony indicated the phosphate solubilization activity.

All the isolates were spot inoculated on Aleksandrov agar [34] containing mica for testing potassium substrate solubilization. Plates were incubated at 30 + 2 °C for five days with observation on colony diameter every 24 h. Clear zone formation around the growing colony indicated potassium solubilization activity.

Phytohormones and ACC Deaminase Production

All the isolates were grown in glucose phosphate broth containing L-tryptophan (0.005 M) for 3 days at 30 + 2 °C on a shaker at 100 rpm and then centrifuged at 3000 rpm for 20 min as described by Glickmann and Dessaux, [35]. One ml supernatant was mixed with 2 ml of Salkowaski's reagent. Un-inoculated control was kept for comparison. The intensity of pink color developed within 30 min was measured at 535 nm in UV/VIS spectrophotometer. The quantity of IAA was determined by comparison with an IAA standard curve.

Screening of bacterial isolates for ACC deaminase enzyme production was done based on their ability to use ACC as a sole nitrogen source in the minimal medium. Cultures were spot inoculated on Petri plates containing minimal medium supplemented with 3 mM ACC substrate. Plates containing minimal medium without ACC were as negative control and with $(NH_4)_2SO_4$ (2.0 g l^{-1}) as a nitrogen source served as a positive control. The plates were incubated for 3 to 4 days at 28 ± 2 °C. The growth of isolates on ACC supplemented plates was compared with positive and negative control plates. Isolates growing well on ACC plates were considered ACC deaminase enzyme producers [36].

Endophytic Nature Confirmation of NRE by DAPI Staining

The DAPI (Sigma Chemical Co., St Louis, MO, USA) 10 mg ml^{-1} stock was prepared in sterile distilled water and stored at −20 °C. Bacteria were stained according to the procedure described by Mukharjee and Ray, [37] with some modifications as follows: fresh culture (24 h after inoculation) was taken in 2 ml tubes and centrifuged at 5000 rpm for 5 min; the supernatant was discarded. The pallet was then washed with 400 µl phosphate-buffered saline (PBS) three times. Then the cells were fixed with 200 µl of 4% formaldehyde (prepared in PBS) and incubated at 28 ± 2 °C in dark for 30 min. Again, the pallet was washed with 400 µl phosphate-buffered saline (PBS) three times. Then the cells were mixed with DAPI (20 µg ml^{-1} working solution) and incubated in dark for 1 h. Slides were prepared and observed under Epifluorescence Microscope (Olympus CKX 41) with 100-time magnification. The tagged bacteria were inoculated by seed treatment in green gram in vitro and monitored for colonization from 7 days after inoculation (DAI). Roots from gnotobiotic seedlings were carefully removed; surface sterilized and thin Sections (50–100 mm) were prepared aseptically and were observed under Epifluorescence Microscope (Olympus CKX 41) under 100-time magnification for the presence of fluorescently tagged bacteria.

Co-inoculation Study of *Rhizobium* sp. and NRE in Green Gram Through Pot Study

The soil of the experimental pots was deep black loam sand with the composition of coarse sand (0.53%), fine sand

(82.20%), silt (10.55%), and clay (5.12%) and locally known as "*Goradu*", alluvial in origin and belongs to the order Alfisol [38]. The soil had pH 7.0 and electrical conductivity of 0.30 dSm^{-1} at 25 °C. The soil was well-drained and retentive of moisture having organic carbon (0.36%), available N (232.5 kg ha^{-1}), available P (40.18 kg ha^{-1}), and available K_2O (450.0 kg ha^{-1}). It responded well to irrigation as well as manuring and was reasonably suitable for green gram cultivation.

Seeds of green gram were surface sterilized with 70% ethanol solution for 30 s, followed by treatment with 0.1% $HgCl_2$ for 2 min, and then washed three times with sterile distilled water for 1 min each under aseptic conditions. Seeds were then treated with individual 24-h-old isolates grown in nutrient broth at 28 ± 2 °C containing 10^8 cells ml^{-1}, keeping untreated seeds as control. Treatment details were: T_1- control, T_2- 100% Farm Yard Manure (FYM), T_3-50% FYM, T_4- 100% Recommended Dose of Nitrogen Fertilizer (RDNF), T_5- *R. selenitireducens* AAU M1, T_6- *Rhizobium* sp. AAU B3, T_7- *Bacillus* sp. AAU B6, T_8- *Bacillus* sp. AAU B12, T_9- T_5+T_7, T_{10}- T_5+T_8, T_{11}-$T_5+T_7+T_8$, T_{12}-T_6+T_7, T_{13}-T_6+T_8, and T_{14}- $T_6+T_7+T_8$. Each treatment was repeated four times.

The percentage of germination was recorded at 10 days after sowing (DAS) and plant height after 15, 30, 45, and 60 DAS. After harvest, quantification was carried out for root and shoot length, fresh weight, dry weight, nodules per plant, bacterial count of the soil before sowing and at harvest.

The data collected on different experiments and parameters were subjected to statistical analysis using Completely Randomized Block Design (CRD). Data were subjected to analysis of variance and means and were compared by Duncan's New Multiple Range Test (DNMRT) [39].

Co-inoculation Study of *Rhizobium* sp. and NRE in Green Gram Through Field Study

The effect of co-inoculation of *Rhizobium* and NRE on green gram growth was examined through field study through seed inoculation. The green gram seeds were treated with individual 24-h-old isolates grown in nutrient broth containing approximately 10^8 cells ml^{-1}, keeping untreated seeds as control. Treatment details and observations were the same as in the pot study. Each treatment was replicated four times.

Statistical Analysis

The data collected on different experimental parameters were subjected to statistical analysis using Randomized Block Design (RBD) for field study with significance set at a probability level of 0.05. Data were subjected to analysis of variance and means compared by DNMRT [39].

Results

Plant Growth-Promoting Traits

N_2 Fixation

All the 3 isolates were capable of fixation of nitrogen and the highest 54.06 mg N g^{-1} of glucose consumed was demonstrated by the combination of all the three isolates *Rhizobium* sp. AAU B3 + *Bacillus* sp. AAU B6 + *Bacillus* sp. AAU B12, followed by the combined effect of *Rhizobium* sp. AAU B3 and *Bacillus* sp. AAU B6 (47.53 mg N g^{-1} of glucose consumed). The lowest nitrogen fixation was observed in the case of *Bacillus* sp. AAU B12 (16.86 mg N g^{-1} of glucose consumed). *Rhizobium* sp. AAU B3 individually exhibited nitrogen fixation 45.20 mg N g^{-1} of glucose consumed.

The presence of the *nif*H gene was detected by PCR amplification of region encoding for Fe-protein (component II) of the *nitrogenase* enzyme complex. The selected primer pair selectively amplified component-II coding region (ranging from 250 to 500 bp) based upon the organism and its *nif* gene sequence. All the three isolates gave a single band of size ~390 bp indicating these isolates were diazotrophs (Fig. 1a).

The *nod*D gene is a part of nod factors in *Rhizobium* which are essential for nodulation. The set of primers (NBA12 and NBF120) target the nod box regulatory elements of *nod*A and *nod*F, respectively. All the three endophytic isolates were tested for the *nod*D gene amplification and found that *Rhizobium* sp. AAU B3 gave a single band of ~980 bp size (Fig. 1b). The other two isolates (*Bacillus* sp. AAU B6 and *Bacillus* sp. AAU B12) did not show any band proving that they were unable to form root nodules.

Phosphate and Potassium Solubilization

All three isolates were tested for their phosphate solubilization capacity on Sperber media containing Tri-calcium phosphate as an insoluble source. The maximum P solubilization zone was recorded for *Bacillus* sp. AAU B6 which was NRE strain and formed a clear zone of 1.5 mm diameter with 1.10 SI on Sperber's agar plate (Table 1). However, when inoculated in combination with *Rhizobium* sp. AAUB3, both the NRE isolates demonstrated higher SI. The combination of all the three isolates, *Rhizobium* sp. AAU B3 + *Bacillus* sp. AAU B6 + *Bacillus* sp. AAU B12 proved to report higher SI 2.37 as compared to individual inoculants.

Rhizobium sp. AAU B3 and two NRE strains (*Bacillus* sp. AAU B6 and *Bacillus* sp. AAU B12) were inoculated

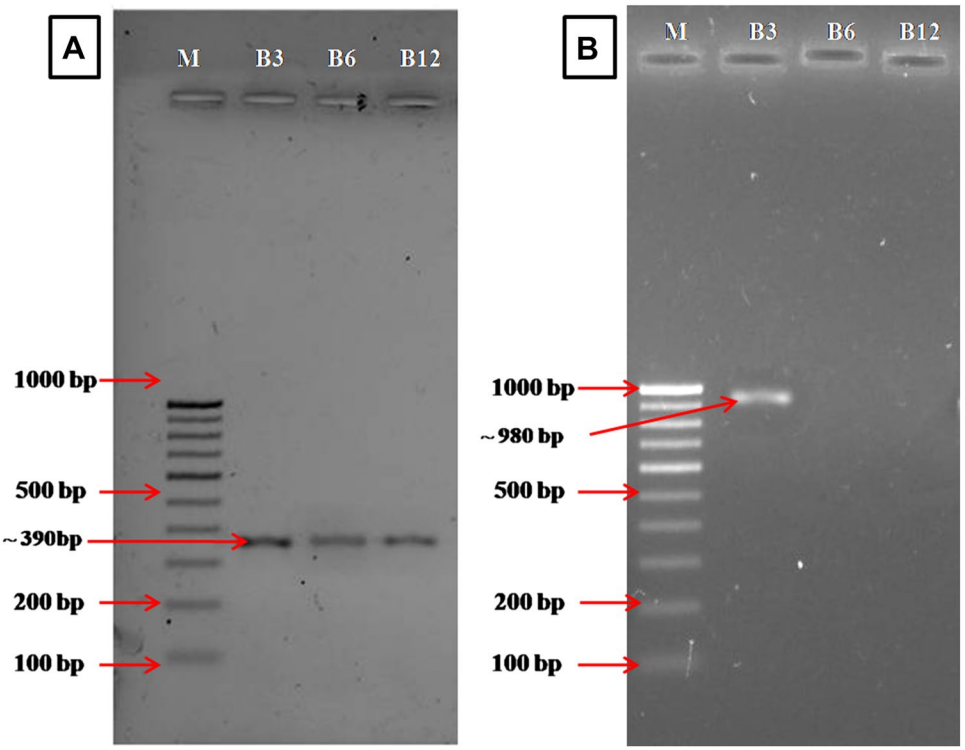


Fig. 1 PCR Amplification of *nif*H (**a**) and *nod*D gene (**b**) observed in rhizobial and non-rhizobial isolates. *nif*H amplification observed in all the three isolates whereas, only *Rhizobium* sp. can amplify the *nod*D gene. (B3-*Rhizobium* sp. AAU B3; B6- *Bacillus* sp. AAU B6; B12- *Bacillus* sp. AAU B12 and M- 100 bp Ladder)

Table 1 Nitrogen fixation, phosphate and Potassium solubilization, IAA and ACC deaminase production by root nodule endophytes

Treatment details		Nitrogen fixation mg N g^{-1} glucose consumed	Phosphate solubilization SI	Available P in liquid Sperber medium			Potassium solubilization SI	IAA production	ACC deaminase production
				1 DAI	3 DAI	5 DAI			
				($\mu g ml^{-1}$)				($\mu g\ ml^{-1}$)	
T1	*Rhizobium* sp. AAU B3	45.20	0.97	8.67	16.75	32.58	0.40	97.33	+ +
T2	*Bacillus* sp. AAU B6	18.15	1.10	36.75	76.83	99.17	1.70	115.50	+
T3	*Bacillus* sp. AAU B12	16.86	0.53	4.42	19.67	82.25	1.84	109.33	+ +
T4	*Rhizobium* sp. AA B3 + *Bacillus* sp. AAU B6	47.22	1.53	42.42	85.33	113.83	2.33	148.17	+ +
T5	*Rhizobium* sp. AAU B3 + *Bacillus* sp. AAU B12	46.53	1.47	23.83	51.75	111.67	2.15	140.17	+ +
T6	*Rhizobium* sp. AAU B3 + *Bacillus* sp. AAU B6 + *Bacillus* sp. AAU B12	54.06	2.37	52.67	94.17	123.17	0.40	165.50	+ + +
S. Em. ±		1.78	0.06	1.74	3.01	2.91	0.05	3.08	
CV%		8.10	8.49	10.73	9.07	5.37	6.36	4.12	
CD @ 5%		5.34	0.18	5.22	9.03	8.73	0.15	9.24	

Treatment means within the critical difference are not significantly different by Duncan's New Multiple Range Test at 5% ($P > 0.05$) level of significance. + + + strong, + + moderate, + present

individually and in combinations on Aleksandrov agar containing mica as an insoluble source of potassium. All three strains when inoculated together showed significant potash solubilization. However, when all the strains inoculated in the consortium was found to report the highest potassium solubilization index (2.98) as shown in Table 1.

Moreover, *Rhizobium* sp. AAU B3 has shown enhanced potassium solubilization in combination with both the NRE *Bacillus* sp.

Phytohormones and ACC Deaminase Production

The variable response was shown by strains in terms of IAA production as shown in Table 1. IAA production by the different combinations was found ranging between 97.33 and 165.50 µg ml^{-1}. The highest IAA production 165.50 µg ml^{-1} was demonstrated by a consortium containing all three strains *Rhizobium* sp. AAU B3, *Bacillus* sp. AAU B6 and *Bacillus* sp. AAU B12, followed by the combination of *Rhizobium* sp. AAU B3and *Bacillus* sp. AAU B6 (148.17 µg ml^{-1}). However, the lowest IAA production was demonstrated by *Rhizobium* sp. AAU B3 (97.33 µg ml^{-1}). From the results, it is observed that the combination of rhizobial strain with NRE increased IAA production considerably.

All the strains grew well on positive control plates containing $(NH_4)_2SO_4$ as nitrogen source and were able to grow on plates containing ACC as the sole nitrogen source and confirmed the production of ACC deaminase enzyme. However, it was observed that when all the three strains were inoculated simultaneously improved the growth on medium containing ACC as the sole nitrogen source.

Confirmation of Endophytic Nature of NRE

The succession of the infection thread formation within root hairs of green gram seedlings was observed under a fluorescence microscope (Olympus CKX 41) at 7 DAI. Figure 2A and B demonstrated the fluorescent NRE (*Bacillus* sp. AAU B6 and *Bacillus* sp. AAU B12) cells at 48 h of re-inoculation in the fresh medium after tagging with DAPI and by exciting with blue and green light. It was observed that with different wavelengths, NRE (*Bacillus* sp.) excited and clearly showed rod (bacilli)-shaped cells. These cells were used for surface sterilized seed inoculation to observe the colonization and infection thread development in green gram seedlings under gnotobiotic conditions.

The tagged NRE was only attached to the base of root hairs and root tips but failed to entre root hair in absence of *Rhizobium* sp. AAU B3. The inability of NRE (*Bacillus* sp. AAU B6 and *Bacillus* sp. AAU B12) to enter root hairs may be attributed to their inability to produce nod factors due to the absence of *nod*D gene which required attaching the tips of growing root hairs and signaling the plant to

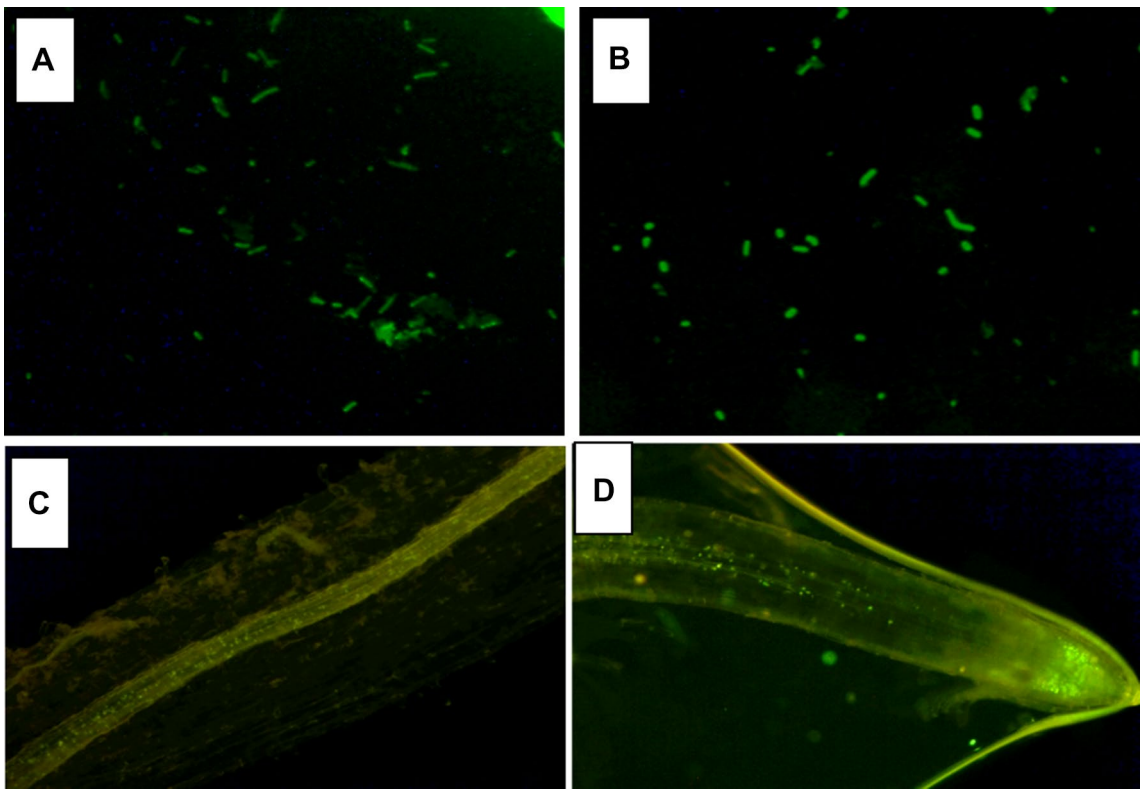


Fig. 2 Fluorescently stained cells of *Bacillus* sp. AAU B6 (**A**) and *Bacillus* sp. AAU B12 (**B**) before inoculation observed under fluorescent Microscope. Fluorescently tagged *Bacillus* sp. AAU B6 (**C**) and *Bacillus* sp. AAU B12 (**D**) observed after inoculation in *V. radiata* seedlings after 7 DAI

form nodules. *Rhizobium* sp. AAU B3 had nodulation and infection ability since it contains the *nod*D gene as shown in an earlier section. Invasion of infection thread by NRE strains is illustrated in Fig. 2C and D when co-inoculated with untagged *Rhizobium* sp. AAU B3. The colonization and occupation of tagged NRE strains inside the infection thread as well as other tissues were observed clearly. It was also observed that NRE bacteria being endophyte were not restricted to infection thread and dispersed in all adjusting tissues. The literature proved that the NRE enters root nodules only after the colonization of root nodules by *Rhizobium.* NRE isolates invaded and colonized the root nodules, accommodate inside root nodules proving the endophytic nature.

Co-inoculation Study of *Rhizobium* and NRE in Green Gram Through Pot Study

The effect of co-inoculation of *Rhizobium* sp. and NRE in green gram growth was examined through pot study and the results obtained are presented in Table 2. The germination percentage was found to be non-significant. Statistically, all the bacterial treatments increased the plant height as compared to control. The treatment T_{14} receiving all the three bacterial inoculants showed the highest plant height at 15 (24.40 cm) which was at par with T_{11}, T_4, T_{10}, $T_{12,}$ and T_{13} showing 24.20, 24.00, 22.80, 22.73, and 21.73 cm, respectively. At 30, 45, and 60 DAS treatment T_{14} receiving all the three bacterial inoculants showed significantly the highest plant height 50.13, 57.07, and 62.93 cm, respectively which was found on par with T_4 (100% RDNF), and T_{11} (*R. selenitireducens* AAU M1 + *Bacillus* sp. AAU B6 + *Bacillus* sp. AAU B12).

The highest number of root nodules were found in T_{14} (9.33) i.e. combination of two NRE and *Rhizobium* sp. strains which were at par with T_9 (8), T_{10} (8.33), T_{11} (9.00), T_{12} (8), and T_{13} (8.33). Chlorophyll content was recorded at 30 DAS and the data revealed to be non-significant. Data regarding root length, fresh and dry biomass showed that T_{14} recorded significantly the highest root length (12.60 cm), fresh (19.23 g), and dry (12.77 g) biomass which was found at par with T_4 (100% RDNF- 12.73 cm, 19.37 g, 12.87 g), and T_{11} (12.13 cm, 18.33 g, 12.63 g), respectively as compared to uninoculated control (8.30 cm, 9.90 g, 5.33 g). Data from the pot study revealed that the treatment T_4 receiving 100% RDNF showed significantly the highest seed yield showing 3.77 g per plant which was at par with T_{14} showing 3.54 g per plant followed by T_{11} (3.44 g) per plant.

Data regarding total bacterial count and *Rhizobium* population shown in Fig. 3. It was revealed that the seed inoculation with bacteria had a positive effect on the total soil bacterial and *Rhizobium* population. A higher total count was observed in treatment T_{14} showing 8.94 log cfu g^{-1} which was found at par with T_{11} showing 8.91 log cfu g^{-1}. Regarding soil *Rhizobium* population at harvest the highest population was found in T_{14} (6.04 log cfu g^{-1}) which was on par with T_2, T_5, T_6, T_9, T_{10}, T_{11}, $T_{12,}$ and T_{13} showing 4.07, 5.67, 5.73, 5.83,5.86, 6.01, 5.85, and 5.86 log cfu g^{-1}, respectively as compared to uninoculated control (3.61 log cfu g^{-1}) and initial (3.16 log cfu g^{-1}).

Co-inoculation Effect of *Rhizobium* and NRE in Green Gram Under Field Study

The effect of co-inoculation of *Rhizobium* and NRE in green gram in the field was recorded and presented in Table 3. Treatment T_{14} receiving co-inoculation of *Rhizobium* sp. AAU B3 + *Bacillus* sp. AAU B6 + *Bacillus* sp. AAU B12 showed the highest germination percentage (99.25) which was found to be at par with treatment T_5 (98.38%), T_6 (98.38%), T_9 (98.50%), T_{10} (98.75%), T_{12} (98.38%), and T_{13} (98.88%) as compared to uninoculated control (94.44%).

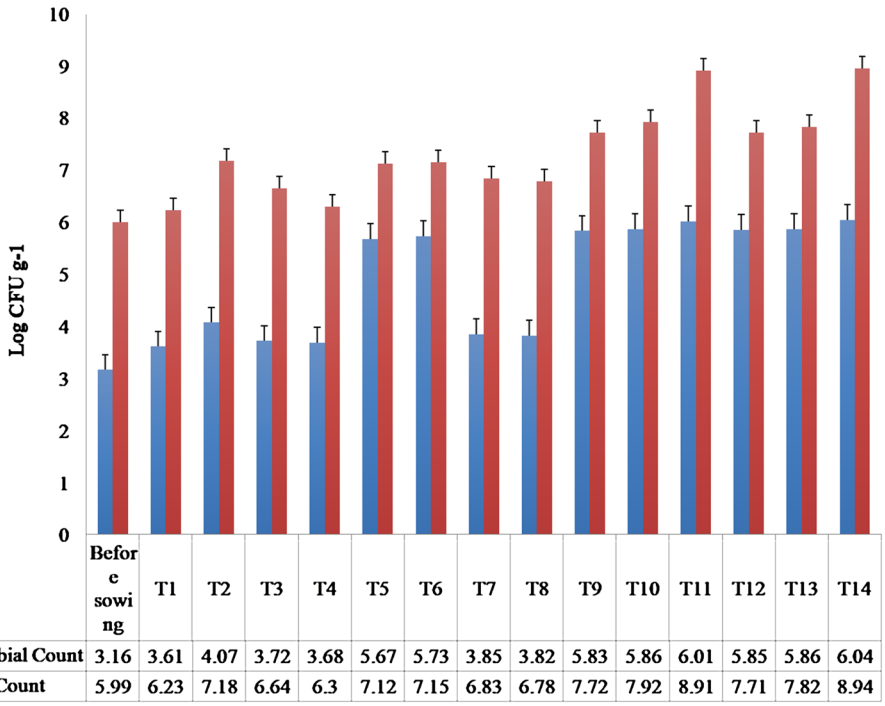
Similarly, T_{14} showed the highest plant height at 30 DAS (20.65 cm), 60 DAS (46.70 cm), and 80 DAS (57.00 cm) which was at par with T_4 (20.13, 45.60, and 55.20 cm). Data revealed that due to co-inoculation of NRE and *Rhizobium* strains, numbers of root nodules per plant increased as compared to uninoculated control. T_{14} showed significantly the highest average root nodules per plant (13.63) as compared to uninoculated control. The information recorded from the field study revealed that the consortium of *Rhizobium* sp. AAU B3, *Bacillus* sp. AAU B6 and *Bacillus* sp. AAU B12 reported significantly the highest number of pods per plant (20.55) as compared to uninoculated control (12.55). Statistics regarding root length showed that bacterial inoculants as individual and in combinations had a positive influence and increased root length. T_{14} was found statistically the best among all the treatments exhibiting an average 12 cm root length, which was at par with T_4 (100% RDNF) and T_{11} showing 11.15 and 10.60 cm root length, respectively. A record regarding fresh and dry biomass revealed that T_{14} was superior giving the highest fresh (25.45 g) and dry (16.75 g) biomass per plant, which was at par with T_4 (24.18; 15.68 g) and T_{11} (23.91; 6.83 g), respectively. Similarly, the combined application of NRE with *Rhizobium* in T_{14} was found superior regarding 100 seed weight showing 5.35 g over uninoculated control (4.50 g) followed by T_4 (100% RDNF) i.e. 5.23 g and T_{11} (standard *Rhizobium* + NRE), i.e., 5.20 g.

It was revealed that treatment receiving 100% RDNF reported the highest effect on yield of green gram showing 1584 kg ha^{-1} which was at par with by T_{14} receiving combined inoculation of *Rhizobium* sp. AAU B3, *Bacillus* sp. AAU B6 and *Bacillus* sp. AAU B12 showing 1572 kg $ha^{-1}$and T_{11} receiving *R. selenitireducens* AAU M1 + *Bacillus* sp. AAU B6 + *Bacillus* sp. AAU B12 showing 1498 kg ha^{-1}seed yield as compared to uninoculated control

Table 2 Co-inoculation effect of *Rhizobium* and NRE on plant growth parameters and yield of green gram under pot study

Treatment no	Treatment details	Germination	Plant height				Root Nodules/ plant	Root length	Fresh biomass	Dry biomass	Seed yield
			15DAS	30 DAS	45 DAS	60 DAS					
		%	cm	cm	cm	cm	No	cm	g	g	g
T_1	Absolute control	94.44	13.47^{g}	25.93^{e}	28.20^{g}	31.80^{g}	5.67^{e}	8.30^{d}	9.90^{h}	5.33^{h}	1.30^{h}
T_2	100% FYM	94.44	15.87^{efg}	33.00^{d}	40.87^{ef}	45.87^{ef}	6.33^{de}	9.47^{bc}	11.73^{fg}	6.67^{f}	1.62^{fg}
T_3	50% FYM	94.44	15.13^{fg}	32.73^{d}	39.53^{f}	44.73^{f}	6.67^{cde}	8.73^{cd}	11.07^{g}	5.90^{g}	1.36^{gh}
T_4	100% RDNF	94.44	24.20^{ab}	50.07^{a}	57.80^{a}	63.27^{a}	6.33^{de}	12.73^{a}	19.37^{a}	12.87^{a}	3.77^{a}
T_5	*R. selenitireducens* AAU M1	100.00	19.53^{cde}	38.33^{b}	43.93^{cde}	48.67^{d}	7.33^{bcd}	9.53^{bc}	13.10^{de}	8.63^{d}	2.21^{d}
T_6	*Rhizobium* sp. AAU B3	100.00	19.60^{cde}	37.53^{bc}	45.53^{bcd}	51.33^{c}	7.33^{bcd}	9.50^{bc}	13.57^{d}	8.93^{d}	2.07^{de}
T_7	*Bacillus* sp. AAU B6	100.00	17.80^{def}	33.20^{d}	43.27^{de}	46.93^{de}	6.00^{de}	8.93^{cd}	12.50^{ef}	7.40^{e}	1.83^{ef}
T_8	*Bacillus* sp. AAU B12	100.00	18.67^{def}	33.87^{cd}	43.20^{de}	47.40^{de}	667^{cde}	8.90^{cd}	12.37^{ef}	7.63^{e}	1.91^{def}
T_9	*R. selenitireducens* AAU M1 + *Bacillus* sp. AAU B6	94.44	20.33^{bcd}	40.27^{b}	47.13^{b}	54.47^{b}	8.00^{abc}	10.13^{b}	15.40^{c}	9.53^{c}	2.70^{c}
T_{10}	*R. selenitireducens* AAU M1 + *Bacillus* sp. AAU B12	94.44	21.73^{abcd}	41.33^{b}	47.73^{b}	54.33^{b}	8.33^{ab}	10.20^{b}	15.37^{c}	9.57^{c}	2.84^{c}
T_{11}	*R. selenitireducens* AAU M1 + *Bacillus* sp. AAU B6 + + *Bacillus* sp. AAU B12	100.00	24.00^{ab}	47.73^{a}	55.13^{a}	62.00^{a}	9.00^{a}	12.13^{a}	18.33^{b}	12.63^{a}	3.44^{b}
T_{12}	*Rhizobium* sp. AA B3 + *Bacillus* sp. AAU B6	94.44	22.73^{abc}	41.13^{b}	47.20^{b}	53.73^{b}	8.00^{abc}	10.03^{b}	15.60^{c}	9.83^{bc}	2.80^{c}
T_{13}	*Rhizobium* sp. AAU B3 + *Bacillus* sp. AAU B12	100.00	22.80^{abc}	40.73^{b}	46.93^{bc}	54.53^{b}	8.33^{ab}	10.20^{b}	15.67^{c}	10.13^{b}	2.81^{c}
T_{14}	*Rhizobium* sp. AAU B3 + *Bacillus* sp. AAU B6 + *Bacillus* sp. AAU B12	100.00	24.40^{a}	50.13^{a}	57.07^{a}	62.93^{a}	9.33^{a}	12.60^{a}	19.23^{ab}	12.77^{a}	3.54^{ab}
S. Em. ±		3.93	1.40	1.41	1.09	0.70	0.50	0.33	0.34	0.16	0.11
CV%		7.00	12.08	6.25	4.09	2.36	11.64	5.59	4.11	2.96	7.72

Treatment means with the letter/letters in common are not significantly different by Duncan's New Multiple Range Test at 5% ($P > 0.05$) level of significance



	Before sowing	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14
Rhizobial Count	3.16	3.61	4.07	3.72	3.68	5.67	5.73	3.85	3.82	5.83	5.86	6.01	5.85	5.86	6.04
Total Count	5.99	6.23	7.18	6.64	6.3	7.12	7.15	6.83	6.78	7.72	7.92	8.91	7.71	7.82	8.94

Fig. 3 *Rhizobium* and total bacterial count from soil collected before sowing and at harvest during pot study**.** All the samples were analyzed in triplicate using Completely Randomized Design (CRD) with Duncan's New Multiple Range Test at 5% ($P > 0.05$) level of significance

978 kg ha^{-1}. Moreover, total bacterial count and rhizobial count (Fig. 4) from the field study was found the highest in treatment T_{14} (9.07 and 6.17 log cfu g^{-1}) which was at par with T_9, T_{10}, T_{11}, $T_{12,}$ and T_{13} as compared to uninoculated control (7.19 log cfu g^{-1}).

Discussion

Traditionally, root nodules were considered as an exclusive niche for the accommodation of nitrogen-fixing rhizobial bacteria only. But recently various leguminous plants are being re-evaluated for the study of free-living non-rhizobial genera unrelated to nitrogen fixation [14, 40–42]. It has been reported that the *Vigna radiata* accommodates various types of Rhizobia [43] and NRE bacterial genera including *Inquilinus, Bosea, Rhodopseudomonas, Paracraurococcus, Phyllobacterium, Ochrobactrum, Starkeya, Sphingomonas, Pseudomonas, Agromyces, Microbacterium, Ornithinicoccus, Bacillus*, and *Paenibacillus* [10, 17, 27, 30, 44].

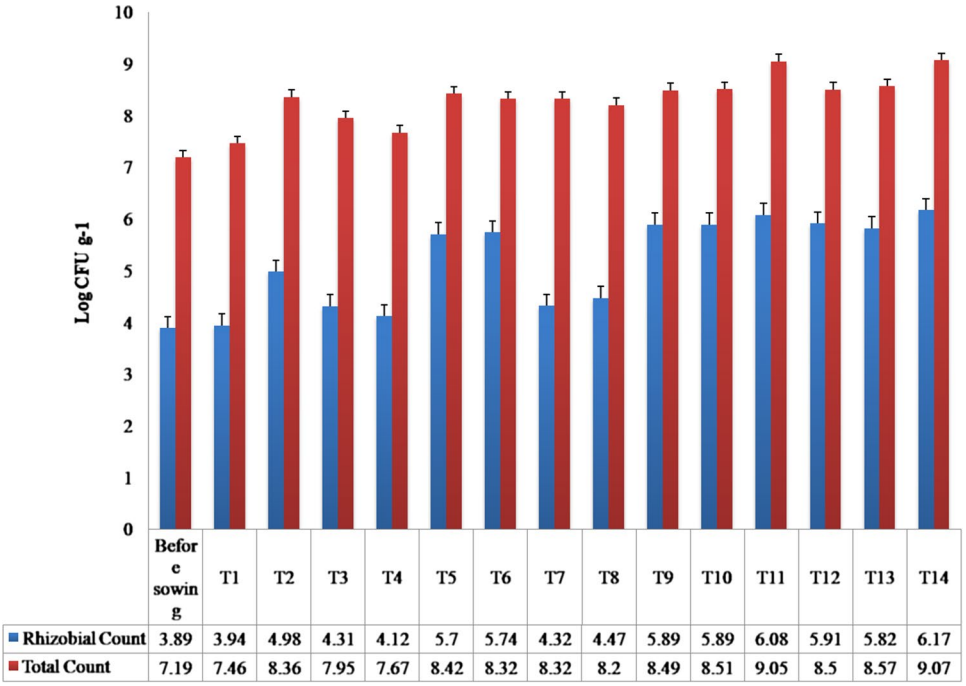
To focus on the exact role of NRE inside the root nodule the selected three isolates were evaluated separately as well as in combination with each other. The isolates must all be compatible with one another to establish a consortium or combinations of isolates. Cross streak test was used to determine whether the isolates were compatible [9, 45]. Plant growth-promoting bacteria facilitate enhanced plant nutrient acquisition, growth hormones, and bio-control activity [46]. *Rhizobium* sp. are symbiotic diazotrophs involved in the reduction of atmospheric dinitrogen to ammonia inside root nodules. In the laboratory, nitrogen fixation can be measured by different techniques like Micro-Kjeldahl [47], acetylene reduction assay [47] and Radio-labeled ^{15}N tracer technique [5]. The atmospheric nitrogen reduction involves a nitrogenase enzyme complex that contains two components (I-nitrogenase Mo-Fe protein and II- dinitrogen reductase, Fe protein). The cluster of twenty-one genes is involved in nitrogen fixation regulation at a molecular level. Among these, genes *Nif* DK codes for the component I and *Nif* H for component II. In the present investigation, the primer pairs selected to amplify the Fe–protein-coding region ranging from 250 to 500 bp. Furthermore, two sets of primers were used to amplify the nod region. The first set of primers (NBA12 and NBF120) target the nod box regulatory elements of *nod*A and *nod*F, respectively, and the amplified region included the whole of nod of 1450 bp in length. When required, a fragment internal to *nod*D was amplified using another set of primers (Y5 and Y6) which amplify a fragment of about 850 bp. All three isolates grew on a nitrogen-free medium and showed nitrogen fixation ability as well as amplification of the *Nif*H gene. While the *Nod*D region was amplified by only *Rhizobium* sp. confirming that *Rhizobium* sp. has a specific ability to develop nodules.

Next to nitrogen, phosphorus (P) and potassium (K) is the most limiting macronutrients for plant growth. Inorganic (bound, fixed, or labile) and organic (bound) forms phosphate are present in the soil. Phosphorous is constituted of nucleic acids, phospholipids and is required for plant growth and development. The bound phosphate becomes available to plants by the action of phosphate solubilizing bacteria.

Table 3 Co-inoculation effect of *Rhizobium* and NRE on plant growth parameters and yield of green gram under field study

Treatment no	Treatment details	Germination	Plan height			Root nodules	Pods	Root length	Fresh biomass	Dry biomass	100 seed weight	Seed yield
			30 DAS	60 DAS	80 DAS							
		%	cm	Cm	cm	No	No	cm	g	g	g	kg ha^{-1}
T_1	Absolute control	96.13^{e}	14.03^{i}	37.55^{e}	48.20^{f}	7.63^{d}	12.55^{e}	7.45^{f}	11.61^{g}	6.83^{i}	4.50^{f}	978^{h}
T_2	100% FYM	96.86^{de}	16.85^{gh}	39.10^{cde}	48.75^{ef}	8.75^{cd}	15.40^{d}	8.50^{ef}	13.24^{g}	8.40^{ghi}	4.58^{def}	1124^{fgh}
T_3	50% FYM	97.13^{cd}	16.60^{h}	38.35^{de}	48.80^{ef}	8.63^{d}	15.15^{d}	7.75^{def}	12.465^{g}	7.85^{hi}	4.55^{ef}	1047^{gh}
T_4	100% RDNF	98.0^{bc}	20.13^{ab}	45.60^{ab}	55.20^{ab}	7.75^{d}	20.28^{a}	11.15^{ab}	24.18^{ab}	15.68^{a}	5.23^{ab}	1584^{a}
T_5	*R. selenitireducens*AAU M1	98.38^{ab}	17.50^{fgh}	41.90^{bcd}	51.20^{cdef}	10.38^{b}	17.60^{bcd}	8.55^{def}	19.22^{de}	11.18^{ef}	4.75^{cdef}	1255^{def}
T_6	*Rhizobium sp.* AAU B3	98.38^{ab}	17.90^{efg}	41.80^{bcde}	51.50^{bcdef}	10.25^{bc}	18.20^{abc}	8.60^{def}	19.17^{de}	11.78^{de}	4.68^{cdef}	1300^{cde}
T_7	*Bacillus sp.* AAU B6	98.00^{bc}	18.40^{def}	41.60^{bcde}	48.80^{ef}	8.00^{d}	16.70^{cd}	8.00^{ef}	18.27^{ef}	9.78^{fg}	4.55^{ef}	1129^{fgh}
T_8	*Bacillus sp.* AAU B12	98.25^{b}	18.80^{cde}	42.55^{abcd}	50.20^{def}	8.50^{d}	17.60^{bcd}	7.90^{ef}	16.30^{f}	9.05^{gh}	4.65^{cdef}	1158^{efg}
T_9	*R. selenitireducens* AAU M1 + *Bacillus* sp. AAU B6	98.50^{ab}	18.85^{cde}	43.80^{ab}	53.10^{bcd}	10.75^{b}	18.30^{abc}	8.60^{def}	20.39^{d}	10.83^{ef}	4.83^{cdef}	1329^{cd}
T_{10}	*R. selenitireducens* AAU M1 + *Bacillus* sp. AAU B12	98.75^{ab}	19.70^{abc}	43.00^{abc}	53.05^{bcd}	10.50^{b}	18.95^{abc}	9.85^{bcd}	23.37^{b}	14.00^{bc}	4.98^{bc}	1310^{cde}
T_{11}	*R. selenitireducens* AAU M1 + *Bacillus* sp. AAU B6 + + *Bacillus* sp. AAU B12	98.25^{b}	20.05^{ab}	43.35^{abc}	54.35^{abc}	11.60^{b}	20.10^{ab}	10.60^{abc}	23.91^{ab}	15.40^{ab}	5.20^{ab}	1498^{ab}
T_{12}	*Rhizobium* sp. AA B3 + *Bacillus* sp. AAU B6	98.38^{ab}	19.20^{bcd}	43.10^{abc}	54.20^{abc}	10.75^{b}	20.15^{a}	9.00^{def}	21.15^{cd}	12.80^{cd}	4.85^{cde}	1425^{abc}
T_{13}	*Rhizobium* sp. AAU B3 + *Bacillus* sp. AAU B12	98.88^{ab}	19.75^{abc}	43.25^{abc}	52.45^{bcde}	10.63^{b}	19.00^{abc}	9.05^{cde}	22.80^{bc}	13.30^{cd}	4.90^{bcd}	1397^{bcd}
T_{14}	*Rhizobium* sp. AAU B3 + *Bacillus* sp. AAU B6 + *Bacillus* sp. AAU B12	99.25^{a}	20.65^{a}	46.70^{a}	57.00^{a}	13.63^{a}	20.55^{a}	12.00^{a}	25.45^{a}	16.75^{a}	5.35^{a}	1572^{a}
S. Em. ±		0.34	0.38	1.50	1.35	0.57	0.89	0.55	0.70	0.55	0.12	55.90
CV%		0.69	4.09	7.09	5.21	11.56	9.91	12.06	7.25	9.47	5.06	8.65

Treatment means with the letter/letters in common are not significantly different by Duncan's New Multiple Range Test at 5% ($P > 0.05$) level of significance



	Before sowing	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14
Rhizobial Count	3.89	3.94	4.98	4.31	4.12	5.7	5.74	4.32	4.47	5.89	5.89	6.08	5.91	5.82	6.17
Total Count	7.19	7.46	8.36	7.95	7.67	8.42	8.32	8.32	8.2	8.49	8.51	9.05	8.5	8.57	9.07

Fig. 4 *Rhizobium* and total bacterial count from soil collected before sowing and at harvest during Field study**.** All the samples were analyzed in triplicate using Randomized Block Design (RBD) with Duncan's New Multiple Range Test at 5% ($P > 0.05$) level of significance

These microorganisms solubilize phosphate with the ability to produce and release organic acids that chelate the cations bound to phosphate, converting it into soluble forms [48]. Hence, rhizobia and NRE isolates help in P release to the plants that absorb only the soluble P like monobasic ($H_2PO_4^-$) and dibasic ($H_2PO_4^{2-}$) forms. IAA phytohormone hastens the plant growth and development as it is involved in cell division, apical dominance, tissue differentiation and vascular bundle formation [49]. Phytohormone IAA, modifies a plants auxin level which ultimately leads to plant cell proliferation trough nutrient uptake and nodulation in green gram [9]. ACC deaminase is a constituent of a group of enzymes which can utilize vitamin B6 and also a part of the tryptophan synthase family. ACC deaminase converts ACC into α-ketobutyrate and ammonia [50] so serves as source of carbon and nitrogen for microorganisms. In the present study both NRE and rhizobia produced ACC deaminase, so upon inoculation positively affects the plant biochemistry and provide support to growth under stressful conditions.

DAPI, a fluorescent nuclear and chromosome counterstain stain that binds strongly to adenine–thymine rich regions nucleotide. It can enter trough cell membrane into live cell so being used broadly in fluorescence microscopy to observe the bacterial colonization in different plant tissues. In the current investigation, NRE isolates are endophytes that penetrate plant root tissues but are unable to produce rood nodules because they lack the genes needed for infection thread production and specificity. Therefore, *Rhizobium* sp. inoculation is essential when NRE are inoculated as plant growth promoting fertilizers in pot and field study.

In the present investigation, NRE were inoculated individually as well as in combination with *Rhizobium* to study their interaction with plants and *Rhizobium.* It was found that the germination, plant height, root length, number of root nodules, fresh and dry biomass were increased upon the addition of *Rhizobium* with NRE as compared to individual application. It is observed that the number of root nodules were increased in both pot and field study, because NRE plays a role in infection thread production due to the production of different cell wall degrading enzymes like cellulase, protease, and chitinase. The role of cellulase ($CelC_2$), the key cell wall-degrading enzyme, in facilitating the primary infection process is reported in *Rhizobium* sp. [13] and degradation of pectin layers by pectate lyase favors the entry of NRE. As seen in the above study, due to enhanced nutrient acquisition, IAA production, and ACC deaminase production; the *Rhizobium* sp. when applied in combination with NRE bacteria in pot and field conditions, increased plant height, number of root nodules, fresh biomass, dry biomass, rhizospheric microbial count and yield [43]. The yield was significantly increased upon application of rhizobium alone or in combination with NRE. According to the observations, applying *Rhizobium* and NRE as a bio agent in green gram can reduce the need for chemical fertilizers by 75%. Traditionally, farmers use 100% chemical fertilizers to get the highest yield of green gram but harm soil health as well as need more investments on resources. The present study showed that farmers can reduce chemical fertilizers to 75% so that soil health also preserved and the inputs also reduced by 25%.

Conclusions

Overall, it was concluded that the diverse NRE and rhizobial type bacteria reside inside the root nodules of a green gram. The selected strains *Rhizobium* sp. AAU B3, *Bacillus* sp. AAU B6 and *Bacillus* sp. AAU B12 were found compatible with each other. All three strains exhibited one or more PGP traits like nitrogen fixation, phosphate, and potash solubilization, IAA, and ACC deaminase production. Selected NRE were confirmed as endophytic by viewing successful colonization inside infection thread of green gram seedlings at 7 DAI treated with DAPI stain. In addition, the consortium of selected *Rhizobium* and NRE strains had a positive effect on the growth and yield parameters of a green gram as ascertained through pot and field study. With the use selected bio-inoculants, the use of synthetic fertilizers can be minimized by 75% as a consequence the soil health could be restored. Thus, the selected NRE (*Bacillus* sp. AAU B6, *Bacillus* sp. AAU B12) and *Rhizobium* sp. AAU B3 strains could be explored as PGP bio-inoculants to improve green gram yield for sustainable agriculture.

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Authors Contributions AMD: Research, Carried out experiment, Data analysis, Manuscript writing. HNS: Evolution, Statistical analysis, validation, Manuscript editing and reviewing. HKP: Molecular experimentation, Data analysis, Manuscript editing and reviewing. YKJ: Biochemical analysis, ARDRA analysis, Manuscript editing and reviewing.

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Data Availability All data generated or analysed during this study are included in this published article (and its supplementary information files).

Declarations

Conflict of interest The authors declare that there are no conflicts of interest associated with this publication.

Ethical Approval This research did not involve any studies with human participants or animals (vertebrates) performed by any of the authors.

Consent to Participate N/A.

Consent for Publication Authors approves consent for publication of the above work.

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