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SHORT COMMUNICATION

Endophytic bacteria improve nodule function and plant nitrogen in soybean on co-inoculation with *Bradyrhizobium japonicum* MN110

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Abstract The potential of bacterial endophytes to improve symbiotic efficiency through synergistic interactions with rhizobia can help to improve nodulation and nitrogen fixation in legume plants. In the present study, we compared the effect of endophytic plant growth-promoting bacteria on nodulation and effective rhizobial symbiosis in soybean. Nodule endophyte *Bacillus megaterium* LNL6 isolated from root nodules of *Lesperdeza* sp. and plant endophyte *Methylobacterium oryzae* CBMB20 isolated from rice leaves were selected as endophytic co-inoculants. Treatment of *Bradyrhizobium japonicum* MN110 along with *B. megaterium* LNL6 and *M. oryzae* CBMB20 exhibited an increase in nodule number in pots at 35 days after sowing compared to single inoculation of MN110. Additionally, both the co-inoculation treatments showed significant increase in nodule activity which was measured in terms of nodule leghemoglobin content, nodulated root ARA and total plant nitrogen content compared to solitary inoculation of *B. japonicum* MN110. Though ACCD activity of the co-inoculated strains can be attributed to increase in nodule number, the observed increase in root nitrogenase activity and leghemoglobin content in the nodules is understood as due to plant growth promotion traits of the specific endophytic strains. High levels of IAA produced by *B. megaterium* LNL6 can be considered to have aided development of mature nodules which thereby improved the nodular nitrogen fixation. Thus, endophytic lifestyle combined with plant growth-promoting traits, such as IAA production, ACC deaminase, cellulase and nitrogenase activity by *B. megaterium* LNL6 and *M. oryzae* CBMB20, contributes to the improvement of overall symbiotic nitrogen fixation by the plant.

Keywords Co-inoculation · Nodule efficiency · *Methylobacterium* · *Bacillus* · Root nodule nitrogenase activity

Abbreviations

IAA	Indole-3-acetic acid
ACC	1-Aminocyclopropane-1-carboxylate
DAS	Days after sowing

Introduction

Several plant growth-promoting bacteria (PGPB), reported to improve plant growth on inoculation to plants, can also cause detrimental effects such as decrease in nodule number, reduction in nutrient accumulation as well as overall plant growth when used as co-inoculants along with rhizobia (Chebotar et al. 2001; Mishra et al. 2009; Masciarelli et al. 2014). This phenomenon emphasizes for a detailed investigation to obtain successful and consistent co-inoculation effect in legumes. Endophytic bacteria, which naturally colonize plant tissues and nodules, can provide effective candidates for screening as co-inoculants to be used along with rhizobia for improved nodulation, plant growth and yield.

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Root nodules of legume plants are found to be colonized by numerous endophytic bacteria (Dudeja et al. 2012). Several of these non-rhizobial endophytes have been proposed to play significant roles in enhancing overall plant growth and yield (Bai et al. 2003; Palaniappan et al. 2010; Dudeja et al. 2012; Aserse et al. 2013). These nodule endophytic isolates are not found to be capable of nodulation except for those which belong to family Rhizobiales (Palaniappan et al. 2010; Aserse et al. 2013). Such non-nodulating endophytic bacteria which naturally colonize the plants can serve as effective candidates for the selection of co-inoculants to improve nodulation and plant growth in legumes. In the present study, we selected two plant growth-promoting endophytic bacteria, *Bacillus megaterium* LNL6 isolated from root nodules of *Lespedeza* sp. and *Methylobacterium oryzae* CBMB20 from leaves of rice plants, and tested them for compatible co-inoculation with *Bradyrhizobium japonicum* MN110 to improve nodulation and nodule activity in soybean.

Materials and methods

Microorganisms and compatibility

The endophytic strains *M. oryzae* CBMB20 (AY683045) and *B. megaterium* LNL6 (GQ181059) were previously isolated and characterized in our laboratory (Madhaiyan et al. 2007; Palaniappan et al. 2010). *B. japonicum* MN110 was a gift from North Carolina State University, USA. Both *Bacillus* and *Bradyrhizobium* were maintained in congo red yeast extract mannitol agar (CRYMA), and *M. oryzae* CBMB20 was maintained in ammonium mineral salts medium (AMS). The compatibility of co-inoculant candidates and their cell-free culture supernatants with *B. japonicum* MN110 was tested according to Mishra et al. (2009) on CRYMA plates pre-seeded with *B. japonicum* MN110.

Assessment of PGP and nodule promotional characteristics

Production of IAA was quantified using nutrient broth and AMS media for respective strains (Bano and Mussarat 2003). Acetylene reduction assay was performed by growing the strains in nitrogen-free medium (Park et al. 2005). Cellulase activity of the strains was analyzed on modified carboxy methyl cellulose (CMC) congo red agar plates where 10 % soil extract was substituted with 0.05 % yeast extract (Kasana et al. 2008). Observation of halo zones around the colonies on flooding the plates with Gram's iodine was regarded as positive for cellulase activity.

Identification of *N*-fixation-related genes

The presence of partial fragment of *nodA* gene in the candidate strains was carried out by performing PCR as described earlier (Palaniappan et al. 2010). To detect the presence of *nifH* gene, fragment of the gene was amplified through nested PCR using primers pairs PolF/PolR and *nifH*For/*nifH*Rev to amplify a 360-bp fragment (Soares et al. 2006). The strains *Bradyrhizobium elkanii* LNW2 (Palaniappan et al. 2010) and laboratory strain *Massilia sp.* RK4 (unpublished) were taken as positive control for *nodA* and *nifH* gene, respectively.

Greenhouse plant experiments

Soybean seeds, *Glycine max* L. cv. Taekwang, were surface-sterilized with 2 % sodium hypochlorite containing 0.02 % Tween®20 for 5 min, followed by rinsing thrice with sterile distilled water 3 min for every wash. One hundred microliter aliquot of water from the final wash was spread on nutrient agar to ensure efficiency of surface sterilization. Seeds were germinated on sterile water agar, and 3-day-old seedlings were transferred to plastic pots of 12-cm diameter filled with vermiculite–perlite (1:1) maintained under greenhouse conditions. Thinning of seedlings was carried out after 6 days, and one seedling per pot was maintained. Bacterial inoculation of the seedlings was carried out immediately after thinning. Strains *B. japonicum* MN110, *B. megaterium* LNL6 and *M. oryzae* CBMB20 were grown until cell populations reached $\sim 10^8$ CFU mL^{-1} at 28 °C. One milliliter of inoculum mixture (500 + 500 µL) containing *B. japonicum* MN110 and tested strains was applied to all seedlings, and single inoculation treatments received 500 µL of sterile nutrient broth as the second part of inoculum. Uninoculated control received equal volumes of sterile CRYMA and nutrient broth.

Soybean plants were harvested with intact nodulated roots at 35 days after sowing (DAS). The harvested plants were studied for their root and shoot lengths, fresh and dry weights, nodule number. Additionally, leghemoglobin content of nodules, nodulated root nitrogenase activity and nitrogen content of the plants were analyzed. Leghemoglobin content in fresh nodules was estimated according to Mhadbi et al. (2009) with minor modifications. One hundred milligrams of fresh nodules was homogenized in 3 mL of Drabkin's solution and centrifuged at 5,000×*g* for 15 min. To the supernatant, 10 mL of Drabkin's solution was further added and centrifuged at 15,000×*g* for 30 min. The absorbance of the supernatant was measured at 540 nm using a Shimadzu UV1601 UV–visible spectrometer. Bovine hemoglobin was used as standard.

Acetylene reduction assay and plant nitrogen

Detached roots with intact nodules were washed carefully to remove soil debris and were blotted dry. Moisture-free roots with intact nodules were then placed in airtight glass jars with rubber stoppers and were exposed to 10 % acetylene (volume/volume of the jar) for 3 h (Ibekwe et al. 1997). Ethylene content in the headspace was measured using a gas chromatograph (GC) (DS 6200, Donam Instruments Inc, Republic of Korea). The total nitrogen content of shoots and roots of plants was assayed using Kjeltec analyzer (Kjeltec 2300 Analyzer Unit, Foss, Sweden).

Statistical analysis

Completely randomized design was used in the pot experiments. Data from the results were normalized and subjected to analysis of variance (ANOVA), and mean significant difference was compared by *t* test (LSD) at $P \leq 0.05$ using SAS package 9.1.3 service pack 4.

Results

Growth and compatibility of strains with rhizobia

Neither the endophytic strains nor their culture supernatants produced any zones of clearance on the plates preseeded with the *B. japonicum* MN110 demonstrating their compatible nature with the rhizobia.

PGP and the presence of putative nodulation-promoting characteristics

Plant growth-promoting traits of the endophytic co-inoculant candidates are summarized in Table S1. On flooding CMC congo agar plates with Gram's iodine, halo zones were observed around the colonies of *B. megaterium* LNL6 and *M. oryzae* CBMB20 indicating their cellulase activity (Online resource Fig. S1). Both the candidate strains did not show any amplification for *nodA* gene. However, they were found to harbor *N*-fixing gene *nifH* (Table S1, Online resource Fig. S2).

Greenhouse plant experiments

The co-inoculation treatments involving *B. megaterium* LNL6 and *M. oryzae* CBMB20 with *B. japonicum* MN110 improved growth and nodulation of soybean plants grown under greenhouse conditions. Significant differences were observed in root and shoot dry weights on co-inoculation of the tested endophytes with *B. japonicum* MN110 (Table 1).

Table 1 Effect of single and co-inoculations of the tested strains with *B. japonicum* MN110 at 35 DAS in pots

Treatments	35 DAS		
	Shoot length (cm)	Root length (cm)	Dry wt. (g plant^{-1})
B. japonicum MN110	61.5 ± 4.7[a]	16.0 ± 1.4[a]	0.51 ± 0.08[a]
B. japonicum MN110 + *M. oryzae* CBMB20	84.5 ± 5.2[b]	17.0 ± 2.8[a]	0.52 ± 0.07[a]
B. japonicum MN110 + *B. megaterium* LNL6	88.5 ± 1.2[b]	21.4 ± 0.5[b]	0.77 ± 0.11[b]

Each value represents mean ± standard error (n = 4). Within each column, values followed by different letters denote statistical significance according to *t* test LSD ($P \leq 0.05$)

The increase in nodule number, in both the co-inoculation treatments involving *B. megaterium* LNL6 and *M. oryzae* CBMB20 with *B. japonicum* MN110, was not found to be statistically significant. However, the nodulated root nitrogenase activities of the two co-inoculation treatments showed significant increase compared to single inoculation of *B. japonicum* MN110 (Fig. 1). Significant increase in total nitrogen contents was also observed in roots and shoots of either co-inoculated plants receiving *B. megaterium* LNL6 or *M. oryzae* CBMB20 with *B. japonicum* MN110 compared to single rhizobial inoculation. Co-inoculation treatments *B. megaterium* LNL6 + MN110 and *M. oryzae* CBMB20 + MN110 recorded an increase of 29.72 and 19.95 % plant total nitrogen compared to plants inoculated with rhizobia (Fig. 1). Leghemoglobin content in the nodules was also significantly higher in the co-inoculated treatments compared to single inoculation of MN110 (Fig. 1).

Discussion

Strains belonging to genus *Bacillus* have been reported as nodule endophytes and found to colonize nodule tissues (Bai et al. 2003; Muresu et al. 2008; Palaniappan et al. 2010). Moreover, several studies have shown *Bacillus* strains to be successful co-inoculants when used along with *Rhizobium* (Chebotar et al. 2001; Bai et al. 2003, Mishra et al. 2009). In our study, the nodule endophyte *B. megaterium* LNL6 improved nodule number and significantly increase root and shoot lengths, plant biomass. Though the increase in nodule number was not statistically significant in the co-inoculation treatment of *B. japonicum* MN110 along with *B. megaterium* LNL6, the efficiency of the nodules studied in terms of leghemoglobin content and root ARA activity was found to increase significantly. In an earlier study, endophytic *Bacillus* strains when used as co-inoculant also exhibited statistically non-significant

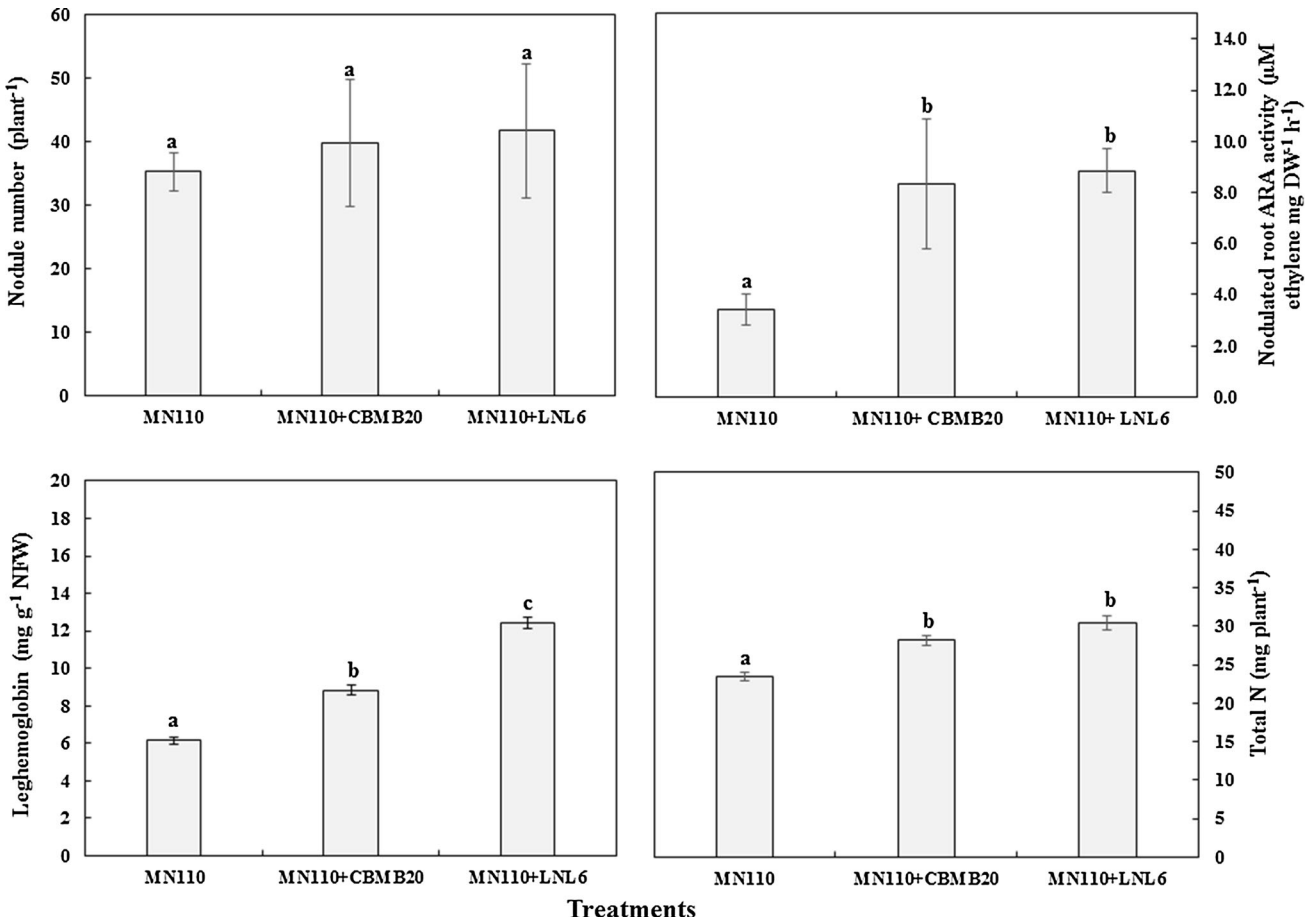

Fig. 1 Nodule number, nodule efficiency in terms of nodule leghemoglobin content, root nitrogenase activity and plant T-N at 35 DAS. *Alphabets* on the *top of error bars* are statistical grouping based on *t* test ($P \leq 0.05$) *NFW* nodule fresh weight, *DW* dry weight

increase in nodule number in pea plants on co-inoculation with *Rhizobium leguminosarum* PR1 (Mishra et al. 2009). The strain *M. oryzae* CBMB20 is a well-documented PGPB which has been reported to improve growth of several plants such of canola, red pepper, rice and tomato through synthesis of phytohormones (cytokinins and auxins) and regulation of plant ethylene levels by its enzyme ACC deaminase (Lee et al. 2006; Madhaiyan et al. 2006, 2007, 2010; Kim et al. 2010). Moreover, *M. oryzae* CBMB20 has also shown compatibility with other PGPB, such as *Azospirillum brasilense*, *Burkholderia pyrrocinia*, and mycorrhizal fungi in improving nutrient uptake of plants (Kim et al. 2010; Madhaiyan et al. 2010).

In context of nodule formation, transport of IAA to roots in legume plants has been known to be inhibited within hours of rhizobial inoculation and such condition is found to be necessary for nodulation (Praytino et al. 2006). However, at later stages, roots with mature root nodules of *Phaseolus mungo* (L.) were found to contain high levels of indole acetic acid (IAA) compared to non-nodulated roots (Ghosh and Basu 2006). Also, IAA transport inhibitors, such as 1-naphthylphthalamic acid (NPA), triiodobenzoic acid (TIBA), have been reported to reduce the number of mature nodules, thereby demonstrating the importance of IAA in maturation of nodule structures (Takanashi et al. 2011). From our results, we observe that strain *B. megaterium* LNL6 exhibits high IAA production under in vitro conditions which can be considered to have played a major role in the development of effective and mature nodules. The tryptophan pool present in the mature nodule and young roots can serve as a precursor for the IAA production by the bacterium (Ghosh and Basu 2006). In another perspective, during the process of nodulation, plant hormone ethylene and its precursor 1-aminocyclopropane-1-carboxylate (ACC) are naturally accumulated in the root. Such ethylene inhibits nodulation at its early stages by regulating threshold concentration of Nod factor required for nodule initiation (Oldroyd et al. 2001; Nascimento et al. 2012). Therefore, physiological ethylene is detrimental to plants and affects symbiotic nitrogen fixation. Such ethylene can be controlled through bacterial 1-aminocyclopropane-1-carboxylate deaminase (ACCD) (Madhaiyan et al.

2006). Both the strains used in the study were also found to produce cellulase (Online resource Fig. S1) which is essential for primary symbiotic infection of host roots by rhizobia. Therefore, degradation of the root hair tip by cellulases produced by *B. megaterium* LNL6 and *M. oryzae* CBMB20 can also be understood to be responsible for the increase in nodule number (Fig. 1).

Bacillus megaterium LNL6 and *M. oryzae* CBMB20 used in the present study, besides their ACCD activity, also showed nitrogenase, IAA, siderophore, cellulase production and phosphate solubilization. In an earlier study, single inoculation of phosphate-solubilizing *Pseudomonas, Enterobacter* and *Burkholderia* was found to improve shoot nitrogen in plants over inoculation with *Bradyrhizobium* (Fernandez et al. 2007). This phenomenon can also be observed in a recent study where single inoculation of nodule endophyte, *Bacillus amyloliquefaciens* LL2012, significantly improved overall biomass accumulation in soybean over *B. japonicum* (Masciarelli et al. 2014). The increase in nitrogenase activity of the nodulated roots and total nitrogen content of the plants observed in our results can also be attributed to the inherent N-fixing capacity of the co-inoculant strains. In the present study, we established the presence of *nifH* gene in both co-inoculant bacteria (Online resource Fig. S2). Several non-rhizobial nodule endophytes have been observed to possess nitrogenase activity, and once these bacteria are applied as co-inoculants, they are hypothesized to enter into the nodule and the ideal environment inside the nodule helps them to start fixing nitrogen (Muresu et al. 2008).

Plant growth-promoting nodule endophyte (*B. megaterium* LNL6) was found to be more effective than leaf endophytic PGPB, *M. oryzae* CBMB20. This can be explained by the fact that *B. megaterium* LNL6 naturally inhabits nodules and possesses relatively high IAA synthesis than *M. oryzae* CBMB20 (Table S1). The significant improvement of N-fixing efficiency of the nodulated roots by both the endophytic strains is well evident from the significant increase in leghemoglobin content and root nitrogenase activity. This phenomenon can be recognized as additive effect of their plant growth-promoting characteristics during synergism with *B. japonicum* MN110. Thus, plant endophytes, in particular, those harbored in the nodules of legumes can serve as excellent models to study the processes that occur in the nodule after nodulation. Further research on studying the reasons behind this specificity and effectiveness can thus help us understand the complex roles played by nodule endophytic bacteria during nodulation and plant growth.

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