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RESEARCH ARTICLE

Does inoculation with native rhizobia enhance nitrogen fixation and yield of cowpea through legume-based intercropping in the northern mountainous areas of Vietnam?

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Abstract

In the Northern mountainous region of Vietnam, cassava–cowpea intercropping system has been widely promoted with support from the local agricultural department. However, cowpea yield is often limited because of a low Biological Nitrogen Fixation (BNF) activity due to its low natural nodulation and lack of available effective *Rhizobium* products. The aim of this study was to identify the most effective native rhizobia isolate nodulating cowpea with the potential to increase BNF and yield of cowpea. A greenhouse experiment was initially conducted with five treatments: three native rhizobia isolates (CMBP037, CMBP054, and CMBP065); a control (no inoculation and no N application); and N+ (no inoculation, application of N as KNO_3). Field inoculations were carried out and the treatments were as follows: a control (no inoculation); CMBP (037+054) – a mixture of strains from Mau Dong; CMBP065 strain from Cat Thinh. CMBP054 and CMBP065 had the highest nodulation in the greenhouse (46.4 and 60.7 nodules plant^{-1}, respectively) and were rated as effective with symbiotic efficiency (SEF) of 54.56 and 55.73%, respectively. In the field, CMBP (037+054) recorded significantly higher nodulation (19.4 nodules plant^{-1}) than the control (11.7 nodules plant^{-1}). CMBP (037+054) also increased cowpea shoot dry weight, shoot N, and yield by 28.6, 4.9, and 10.5%, respectively, compared to the uninoculated control. This effect was slope dependent (statistically significant in moderate and steep slope, not with gentle slope). Besides, the high expansion rate of intercropping with cowpea showed the high adoption level of these agroecological practices by local farmers. This study reveals the potential of native rhizobia inoculation to enhance soil fertility and sustainable agriculture in the Northern mountainous region of Vietnam and proposes enhanced efforts to promote the availability and utilization of effective inoculants for cowpea.

Keywords: Cowpea; Rhizobia; Biological Nitrogen Fixation; Intercropping; Agroecology

Introduction

The Northern mountainous region occupies about 103,000 km^2 equivalent to ~ 33% of the total land area in Vietnam, with a population of 12.5 million people, 13% of the total national population (General Statistics Office of Vietnam, 2019). In this region, over 80% of the cultivation area is on sloping land (General Statistics Office of Vietnam, 2019) and long-term cultivation on such steep slopes often leads to soil erosion. Additionally, the widespread use of monocropping systems on steep slopes and the large amounts of chemical fertilizer application, especially nitrogen (N) mineral fertilizers, results in low soil fertility and imbalanced nutrient content. Cassava, an important cash crop for local smallholders, is commonly grown as a monocrop in this region, and we established that during the period between 2016 and 2017, local farmers were applying 100–120 kg N ha^{-1} $year^{-1}$, well above the recommended dose from the local agricultural department of 80–100 kg N ha^{-1} $year^{-1}$. Excessive application of mineral N fertilizers increases greenhouse gas emission (N_2O) (Nyoki and Ndakidemi, 2016) and also affects soil and water by causing soil acidification and toxification, resulting in water contamination, negatively impacting fish and aquatic animals (Bashir *et al.*, 2013; Boman *et al.*, 2002; Compton *et al.*, 2011).

Agroecological practices, especially intercropping, can improve resilience and sustainable productivity to rural smallholders by mitigating the decline in soil fertility in upland areas (De Schutter, 2010; Tittonell, 2014). Numerous studies have indicated that intercropped systems provide advantages such as better use of land and labor, food security, increasing soil nutrients and moisture, decreasing soil erosion, natural pest control, and income benefits for smallholders (Agegnehu *et al.*, 2008; Duchene *et al.*, 2017; Dwivedi *et al.*, 2015; Knörzer *et al.*, 2009; Weerarathne *et al.*, 2017). According to Bedoussac *et al.* (2017), intercropping systems, especially with legume crops, help to cover the soil surface, reducing soil erosion, improve soil moisture and soil nutrients. In a study on cassava–peanut intercropping system, the amount of eroded soil was significantly reduced compared to the traditional monocropping (Trung *et al.*, 2013).

One of the most important benefits of legume-based intercropping systems is their unique role of fixing atmospheric N through the process of Biological Nitrogen Fixation (BNF), in symbiosis with soil bacteria known as rhizobia (Nyfeler *et al.*, 2009). Studies have reported the N contribution of legume crops in intercropping systems to be equivalent to about 55–96 kg of N fertilizer ha^{-1} $season^{-1}$ (Cong *et al.*, 2015; Mandimba, 1995). According to Herridge *et al.* (2008), symbiotically fixed N_2 in legumes ranged from 100 to 380 kg N ha^{-1} $year^{-1}$, but other studies also reported exceptionally large amounts of more than 500 kg N ha^{-1} $year^{-1}$. Herridge (2002) revealed that a combination of *Rhizobium* inoculants and N fertilizer at doses of 30–40 kg ha^{-1} resulted in similar groundnut yield compared to N fertilizer doses of 60–90 kg ha^{-1}. In comparison with chemical fertilizer application, inoculation of soybean with rhizobia products translated to significantly higher economic benefits of about US$ 135.5 ha^{-1} (Boonkerd, 2002). Cowpea (*Vigna unguiculata* var. cylindrica) is one of the most important, widely cultivated legumes that can fix atmospheric N ranging from 9 to 120 kg N ha^{-1} (Awonaike *et al.*, 1990; Boddey *et al.*, 1990; Toomsan *et al.*, 1995). Cowpea also shows high tolerance to drought and high temperatures, and can thrive in infertile acidic soils (Watanabe *et al.*, 1997). While cowpea needs phosphorus (P) and can partly self-support its N requirements through BNF, cassava requires high amounts of potassium (K) for storage root formation and N for leaf production (Howeler, 1991), showing the advantages in nutrient demands of the two crops in an intercropping system. Cowpea is also highly suitable for cassava in terms of growth patterns and canopy development (Howeler and Hershey, 2002). Notably, in the Northern mountainous areas of Vietnam, cowpea can effectively increase smallholders' income due to its high sale price at local markets.

The local agricultural department in Yen Bai province has been encouraging cassava–cowpea intercropping system through farmer associations to mitigate soil degradation and improve soil health. Despite this, we found from our preliminary investigation that the natural nodulation of cowpea is very low (< 10 nodules $plant^{-1}$ on average) across farms in this province (Supplementary Figure S1). Factors such as the absence of compatible native rhizobia, low population of rhizobia, or ineffective/low effective native rhizobia, may inhibit the symbiosis and BNF in cowpea (Date, 2000; Ojo *et al.*, 2015; Vanlauwe and Giller, 2006). This situation could be improved by inoculating cowpea seeds with effective rhizobia strains (Bala *et al.*, 2010; Koskey *et al.*, 2017). Several authors indicated the importance of utilizing effective native rhizobia strains in increasing cowpea production (Mathu *et al.*, 2012; Ngeno, 2018; Yohane, 2016). Ampomah *et al.* (2008) isolated five native cowpea rhizobia, assessed their symbiotic effectiveness and competitiveness, and reported that the strain All-5-2 was the most effective inoculant for improving cowpea yield in N-deficient regions of Ghana.

However, currently, no available rhizobia products for cowpea are available in the Vietnam market. Promotion of cowpea needs to be combined with identifying, formulating, and scaling up effective rhizobia inoculants to enhance and sustain BNF. Thus, the aims of this study were: (1) to screen the native rhizobia nodulating cowpea and identify potential elite inoculum strains under greenhouse conditions for further field experiments and (2) to evaluate the effective isolates under intercropped field condition and scale-up cowpea intercropping through farmer associations.

Materials and Methods

Greenhouse screening experiment

Cowpea nodules were collected from 12 different farms in Yen Bai province, North Vietnam (5 farms in Mau Dong commune, Van Yen district, and 7 farms in Cat Thinh commune, Van Chan district) where only 1 local cowpea variety *Dau Den Xanh Long* is cultivated. During the mid-flowering stage, at each farm, we determined the sampling lines based on the water flows and the number of plant samples in each line (a distance of 1 m between plant samples). From each cowpea plant, all the effective nodules were removed from the roots and surface sterilized in 70% ethanol. The nodules were then stored in labeled McCartney bottles containing 10 ml of 40% glycerol. All the bottles were kept in a cool box with ice and transferred to the laboratory for further analyses.

The isolation of native rhizobia from cowpea nodules was performed at the Common Microbial Biotechnology Platform (CMBP) at The Alliance of Bioversity and CIAT, Asia Hub, Hanoi, Vietnam. The rhizobia isolates were purified by phenotypic characterization (colony morphology) and by gram staining. Purified cultures of isolated colonies were sent to the Institute of Genome Research (Hanoi, Vietnam) for DNA extraction, PCR, and 16S rRNA sequencing. The sequence data was then submitted for comparison with the National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Three native rhizobia strains (CMBP037, CMBP054, and CMBP065) sequenced from cowpea nodules are shown in Table 1. To screen the performance of three native rhizobia strains isolated from cowpea nodules, a pot experiment was established in a greenhouse at the Vietnam National University of Agriculture (VNUA), Hanoi, Vietnam. The five treatments were: uninoculated plant without N application (negative control), uninoculated plant with applied N using KNO_3 at a rate of 480 mg N pot^{-1} (positive control or N+), and separate inoculation treatments with strains CMBP037, CMBP054, and CMBP065. The experiment was arranged in a completely randomized design (CRD) with five replicates.

After culturing on Yeast Extract Mannitol Agar (YEMA – 0.5 g l^{-1} KH_2PO_4, 0.2 g l^{-1} $MgSO_4$, 0.1 g l^{-1} NaCl, 1 g l^{-1} Yeast Extract, 10 g l^{-1} Mannitol, 15 g l^{-1} Agar) plates, single purified colonies of the individual strains were selected to prepare rhizobia inoculant cultures. Each colony was transferred into 50 ml YEM broth in 200 ml Erlenmeyer flasks and incubated at 28 °C on a rotary shaker at 200 rpm, for 2 days for *Rhizobium* species and 4 days for *Bradyrhizobium* species. Before applying to the pots, direct cell count for each inoculum was done using the spread plate method (SOP-MI10 LH-V01) to ensure that at least 10^6 rhizobia cells ml^{-1} was applied per plant.

Cowpea seeds were surface sterilized by soaking in 3.3% NaOCl solution for 5 min and rinsed thoroughly several times with sterile distilled water. Surface-sterilized seeds were immersed in water for 1 h to imbibe, transferred to Petri dishes with moistened sterile cotton wool, and then placed in a growth chamber at 28°C to germinate in the dark for 24 h. Three preselected healthy seeds of uniform size were chosen and sown in plastic pots (12 cm diameter and 16 cm in length). Each pot was

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Table 1. Description of the treatments in the greenhouse experiment

No.	Treatment	Strain	% ID	District	Commune	Accession number
1	Negative control	No inoculation	-	-	-	-
2	Positive control (N+)	No inoculation	-	-	-	-
3	CMBP037	*Rhizobium freirei*	99%	VY	MD	KY292476.1
4	CMBP054	*Bradyrhizobium elkanii*	99%	VY	MD	KT900890.1
5	CMBP065	*Bradyrhizobium elkanii*	99%	VC	CT	KT900890.1

% ID: Sequence similarity (%) of 16S rRNA gene with identical sequences identified by using the National Center for Biotechnology Information (NCBI) database.
CT, Cat Thinh; MD, Mau Dong; VC, Van Chan; VY, Van Yen.

sterilized with 70% ethanol, filled with 1.3 kg of sterilized sand, and irrigated with 150 ml of distilled water in preparation for sowing. Five drainage holes were made at the bottom of each pot.

Four days after sowing (DAS), 3 ml of each inoculant were added to the base of seedlings (1 ml per seedling) in the pots. Plants were thinned to two healthy plants per pot at 7 DAS. Essential nutrients with the exception of N were added to each pot every 2 days, as nutrient solution [K_2SO_4 0.5 M, KOH, KH_2PO_4 1 M, $CaCl_2$ 2 M, $MgSO_4$ 0.5 M, $MnSO_4$ 0.002 M, $ZnSO_4$ 0.001 M, $CuSO_4$ 0.0004 M, $CoSO_4$ 0.0002 M, H_3BO_4 0.004 M, $NaMoO_4$ 0.0002 M, $FeSO_4$ 0.08 M, and EDTA ($C_{10}H_{16}N_2O_8$) 0.08 M] modified from Broughton and Dilworth (1971). The plants were alternatively watered with 150 ml of distilled water or with 150 ml of the nutrient solution.

Cowpea plants were harvested at the flowering stage (6 weeks after sowing). Chlorophyll content of the youngest fully developed cowpea leaves was measured using a SPAD-502 chlorophyll meter (Minolta corporation, Ltd., Osaka, Japan). Shoots were cut at 1 cm above the surface using a clean, sharp knife. The roots were gently washed and the nodules were separated from the roots. Number of nodules per plant, shoot fresh weight, and root fresh weight were recorded. Shoots, roots, and nodules were oven-dried at 60 °C for 2 days before measuring dry weights. Total N content (%) of the oven-dried shoot was determined by Kjeldahl method (Bremner, 1996). Symbiotic efficiency (SEF) was also calculated using the formulae by Lalande *et al.* (1990):

SEF (%) = (shoot dry weight of inoculated plant/shoot dry weight of positive control plant) * 100.

SEF was classified as: very effective (> 80%); effective (51–80%); less effective (35–50%) or ineffective (<35%).

Field inoculation experiment

In March 2018, based on the results from the greenhouse experiment, an on-farm experiment was conducted to evaluate the effectiveness of native strains inoculated with cowpea in an intercropping system with cassava under field conditions in Mau Dong commune, Van Yen district, Yen Bai province. The field location is shown in Supplementary Figure S2. In order to assess the interaction effect of different native rhizobia from the same location on cowpea production, CMBP037 and CMBP054 were mixed together before inoculation. This experiment was composed of nine treatments resulting from a factorial combination of three inoculation treatments and three field slope categories (steep, moderate, and gentle) commonly encountered in the area. The slope was classified as gentle slope (< 5 °C); moderate slope (5–15 °C); steep slope (> 15 °C) (Jahn *et al.*, 2006). Supplementary Table S1 shows the soil characteristics of the field site from each slope category. Inoculation treatments included (i) non-inoculated control (Non_I), (ii) a mixed inoculant containing *Rhizobium* strains CMBP037 and CMBP054 isolated from Mau Dong commune, in a ratio of 1:1 in volume (CMBP (037+054), and (iii) *Rhizobium* strain CMBP065 isolated from Cat Thinh commune. The treatments were each applied to 6 randomly selected representative

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farmers' fields, but the total number was reduced to 24 farms after removing mismanaged farms from the study.

Inoculant preparation and seed sterilization were done as described above for the greenhouse experiment. Inoculants were transported to the field in cooler boxes and applied at a rate of 50 ml kg^{-1} of seeds. Seed inoculation was done just before sowing in the shade to maintain the viability of bacterial cells. Seeds were allowed to air dry for about 30 min before sowing and were immediately covered by soil after sowing. Cowpea was intercropped with cassava whereby one row of cowpea was planted between two rows of cassava at a density of 10,000 cowpea plants ha^{-1}.

At the mid-flowering stage (7 weeks after sowing), 10 cowpea plants positioned on 2 diagonal lines in each farm were harvested. Each plant sample was considered as one replicate. Shoots were cut at 1 cm above the soil surface using a clean, sharp knife. Roots were gently washed and nodules were separated from the roots and the number of nodules per plant was recorded. Shoots and roots were oven-dried at 60 °C for 2 days to measure dry weights. Oven-dried shoots were analyzed for total N content (%) as described above. At the maturity stage (9 weeks after sowing), three random areas of 5 m^2 from each farm were harvested and cowpea seed yield (kg ha^{-1}) was calculated.

Investigation in the expansion of cassava–cowpea intercropping system in 2017–2018 in Van Yen district, Yen Bai province

Muoi village in Mau Dong commune is one of the most productive cassava areas of Van Yen district, Yen Bai province, and the smallholder farmers in this village have participated in this conservation agricultural practice since the beginning of the project started in 2016. Muoi village has a total land area of over 200 ha and 95 households, with an average household owning 0.5 ha of land. In order to identify the adoption and expansion of such a cropping system, we assessed the cassava cropping systems in the area during the period between 2017 and 2018. From all the farms in Muoi village, we surveyed the total number and area of cassava farms and the number and area of cassava–cowpea intercropping farms, as well as their correlative percentages.

Statistical analysis

A two-way analysis of variance (ANOVA) was performed using R version 3.4.2 to assess the effects of slope category, inoculation treatments, and their interaction on nodulation, shoot and root biomasses, shoot N, and yield of cowpea. Significance of difference was evaluated at $p < 0.05$. Data screening was performed to test whether ANOVA's assumptions of homoscedasticity and normality were violated. Cowpea nodulation and root biomass have skewed distributions; thus, natural log transformation was applied. Adjusted LS means of treatments were calculated and Tukey's test was used for multiple comparisons. Simple correlation analysis was used to determine the association between nodule dry weight and shoot dry weight of cowpea.

Results

Response of cowpea to native rhizobia inoculation under greenhouse screening experiment

As shown in Table 2, inoculation with strain CMBP037 recorded the lowest number of nodules (7.7 nodules $plant^{-1}$). The highest rate of nodulation was found in CMBP054 and CMBP065 inoculants (46.4 and 60.7 nodules per plant, respectively). CMBP065 also recorded the highest nodule dry weight (0.17 g $plant^{-1}$) while there was no significant difference in nodule dry weight between CMBP037 and CMBP054 treatments.

Rhizobia inoculation significantly affected cowpea biomass (Table 2). The highest shoot and root dry biomasses were recorded in the positive control N+. There was no significant difference in shoot and root dry biomasses among CMBP037, CMBP054, and CMBP065. As shown in

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Table 2. Response of cowpea to native rhizobia inoculation in the greenhouse screening experiment

Treatment	Nodules per plant	Nodule dry weight (g plant^{-1})	Shoot dry weight (g)	Root dry weight (g)	SEF (%)	Shoot N content (%)	SPAD value
p-value	<0.0001	<0.0001	<0.0001	<0.0001	0.0012	0.0007	<0.0001
Control	-	-	0.47 ± 0.05^{c}	0.12 ± 0.02^{b}	-	2.18 ± 0.52^{c}	15.6 ± 1.8^{b}
N+	-	-	4.52 ± 0.36^{a}	0.76 ± 0.06^{a}	100.00 ± 7.92^{a}	3.70 ± 0.75^{bc}	33.3 ± 2.6^{a}
CMBP037	7.7 ± 2.4^{b}	0.07 ± 0.02^{b}	0.87 ± 0.13^{bc}	0.27 ± 0.03^{b}	19.27 ± 2.77^{c}	5.21 ± 0.86^{ab}	30.0 ± 3.5^{a}
CMBP054	46.4 ± 4.6^{a}	0.09 ± 0.01^{b}	2.47 ± 0.20^{b}	0.23 ± 0.03^{b}	54.56 ± 4.35^{b}	6.53 ± 0.52^{a}	35.7 ± 2.1^{a}
CMBP065	60.7 ± 8.3^{a}	0.17 ± 0.04^{a}	2.52 ± 0.29^{b}	0.33 ± 0.08^{b}	55.73 ± 6.37^{b}	5.48 ± 0.31^{ab}	38.4 ± 2.0^{a}

Means followed by different letters within the same column are significantly different at $p < 0.05$ according to Tukey's HSD test.
SEF, symbiotic efficiency; SPAD value, index value displayed by Konica Minolta Chlorophyll meters and having a correlation to chlorophyll density.

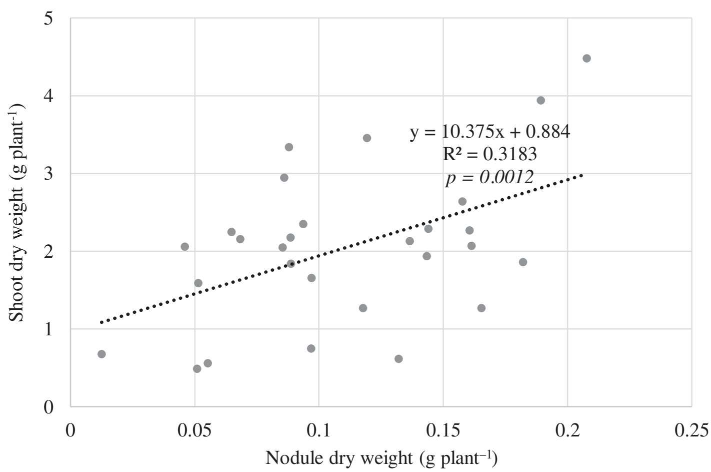

Figure 1. Correlation analysis between nodule dry weight and shoot dry weight in the greenhouse experiment.

Figure 1, there was a significant positive correlation between nodule dry weight and shoot dry weight of cowpea.

All the inoculated treatments produced nodules, therefore, they were considered for SEF determination. There was a significant difference in SEF among native rhizobia isolates (Table 2). CMBP037 had the lowest SEF of 19.27%. The SEF values did not differ significantly between CMBP054 (54.56%) and CMBP065 (55.73%).

Regarding total shoot N analysis, the highest shoot N content was reported in CMBP054 inoculation (6.53%), but this was not significantly different from CMBP037 and CMBP065 inoculation (Table 2). The negative control had the lowest shoot N content (2.18%).

SPAD value determines the relative amount of chlorophyll, which will increase in proportion to the amount of N in a leaf. Therefore, a high SPAD value shows a healthy plant. The highest and lowest SPAD values were recorded in CMBP065 inoculation and control (38.4 and 15.6), respectively, (Table 2). SPAD values differed significantly between rhizobia inoculation and control, but not between rhizobia inoculation and N+ treatment.

Response of cowpea to native rhizobia inoculation under field condition

Table 3 shows responses of cowpea nodulation, shoot, and root dry weight to different rhizobia inoculants and slope categories. Rhizobia inoculation and the interaction effect of inoculation-slope significantly affected cowpea nodulation, shoot dry weight, total shoot N, and yield, while slope

Table 3. Response of cowpea nodulation, shoot, and root dry weight to native rhizobia inoculation and slope in the field experiment

	Slope								
	Nodules per plant			Shoot dry weight (g)			Root dry weight (g)		
Inoculation	Gentle	Moderate	Steep	Gentle	Moderate	Steep	Gentle	Moderate	Steep
Non_I	10.2 ± 0.1[a]	10.9 ± 0.1[b]	5.8 ± 0.1[b]	18.18 ± 1.07[a]	13.55 ± 1.09[b]	13.10 ± 1.06[b]	2.05 ± 0.14[a]	1.64 ± 0.18[a]	1.57 ± 0.12[a]
CMBP (037+054)	9.4 ± 0.1[a]	32.3 ± 0.3[a]	23.7 ± 0.3[a]	18.40 ± 1.09[a]	28.07 ± 1.17[a]	20.09 ± 1.17[a]	1.80 ± 0.18[a]	2.23 ± 0.21[a]	1.41 ± 0.21[a]
CMBP065	5.4 ± 0.3[a]	7.8 ± 0.3[b]	8.7 ± 0.1[b]	14.76 ± 1.17[a]	15.66 ± 1.17[b]	14.77 ± 1.09[ab]	1.52 ± 0.21[a]	1.18 ± 0.21[a]	1.50 ± 0.18[a]

Means followed by different letters within the same column are significantly different at P<0.05 according to Tukey's HSD test.

Table 4. Response of shoot total N content and yield of cowpea to native rhizobia inoculation and slope in the field experiment

	Slope					
	Shoot total N content (%)			Yield (kg ha^{-1})		
Inoculation	Gentle	Moderate	Steep	Gentle	Moderate	Steep
Non_I	2.88 ± 0.05[a]	2.80 ± 0.06[b]	2.92 ± 0.04[b]	385.8 ± 9.3[a]	381.4 ± 10.1[b]	384.3 ± 11.7[c]
CMBP (037+054)	2.99 ± 0.06[a]	3.03 ± 0.10[a]	3.12 ± 0.10[a]	385.5 ± 10.7[a]	436.4 ± 14.1[a]	428.6 ± 8.8[a]
CMBP065	2.51 ± 0.10[b]	3.45 ± 0.10[a]	2.77 ± 0.06[c]	392.7 ± 11.6[a]	375.6 ± 16.7[b]	403.8 ± 17.0[b]

Means followed by different letters within the same column are significantly different at P<0.05 according to Tukey's HSD test.

significantly affected nodulation, total shoot N, and yield of cowpea. On moderate and steep slopes, the mixture of CMBP (037+054) had the highest nodulation (32.2 and 23.7 nodules plant^{-1}, respectively), while there was no significant difference between the uninoculated control and CMBP065. On gentle slopes, there was no significant difference in the number of nodules per plant for all treatments.

There were no significant differences in shoot dry weight among all treatments on gentle slope. On moderate and steep slopes, the highest shoot dry weight was found in CMBP (037+054) (28.07 and 20.09 g plant^{-1}, respectively), while there was no significant difference between the uninoculated control and CMBP065. Root dry weight of cowpea ranged from 1.4 to 2.2 g plant^{-1}, and showed no significant differences among rhizobia inoculants or slope categories in the field experiment (Table 3).

Total shoot N content and yield of cowpea affected by different rhizobia inoculants are shown in Table 4. On gentle slope, total shoot N was lowest in CMBP065 (2.51%) and there was no significant difference between the uninoculated control and CMBP (037+054) (2.88 and 2.99%, respectively). On moderate slope, total shoot N of cowpea was the same for CMBP (037+054) and CMBP065, while the uninoculated control recorded the lowest shoot N content (2.80%). On steep slope, total shoot N was highest in CMBP (037+054) (3.12%), followed by uninoculated (2.92%), and was lowest in CMBP065 treatment (2.77%). On gentle slope, there was no significant difference in total shoot N among all treatments. Regarding cowpea yield, on moderate and steep slopes, the mixture CMBP (037+054) recorded the highest yield (436.4 and 428.6 kg ha^{-1}, respectively) (Table 4). There was no significant difference between the yield of uninoculated control and CMBP065 on moderate slope, while cowpea yield in CMBP065 was higher (403.8 kg ha^{-1}) than the uninoculated control (384.3 kg ha^{-1}) on steep slope.

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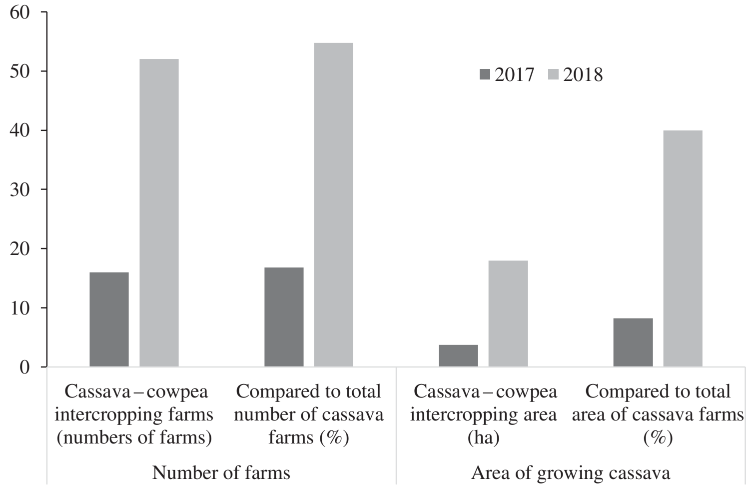

Figure 2. Number of farms and areas practicing cassava–cowpea intercropping system at Mau Dong commune, Van Yen district, Yen Bai province, Vietnam during 2017–2018.

The expansion of cassava–cowpea intercropping system in 2017–2018 in Van Yen district, Yen Bai province

The adoption level of agricultural practices can be assessed by the expansion rate of such practices through the number of farms using it and also the surface of land dedicated to cassava monocropping versus cassava intercropping with cowpea (Figure 2). The number of farmers practicing cassava–cowpea intercropping in 2018 (52 farmers, or 54.74% of the total cassava farms) had more than tripled since 2017 (only 16 farmers, or 16.84%). Similarly, the area of intercropped fields in 2018 (18.0 ha, or 40 % of the total area) was 4.8 times higher than in 2017 (3.7 ha, or 8.22% of the total area).

Discussion

The results from the greenhouse experiment showed that rhizobia inoculation with CMBP054 and CMBP065 strains significantly increased cowpea nodulation and shoot N accumulation as compared to the non-inoculation treatments (Table 2). The superior performance obtained from these inoculants can be attributed to their ability to infect, form nodules, and fix N with cowpea. These results concur with previous studies (Ampomah *et al.*, 2008; Gómez Padilla *et al.*, 2016; Yohane, 2016), which showed the competitive potential of native isolates nodulating cowpea when compared to the non-inoculated treatment. Gómez Padilla *et al.* (2016) reported that the isolated strain VIBA-1 (*Bradyrhizobium liaoningense*) highly competed against other native strains in the soil. Yohane (2016) concluded that the native rhizobia strains isolated from the fields had higher symbiotic effectiveness than the strain (MG5013) used in inoculant products. The result from our greenhouse experiment also showed a significant correlation between shoot dry weight and nodule dry weight of cowpea (Figure 1), which is consistent with previous studies (Kawaka *et al.*, 2014; Koskey *et al.*, 2017). As N-fixing capacity can be assessed by shoot dry weight of legumes (Beck *et al.*, 1993; Gibson, 1987), this finding revealed that inoculation with native rhizobia strains enhanced nodulation of cowpea, which consequently improved shoot biomass and symbiotic N fixation.

SEF plays an important role in evaluating the response of legumes to inoculation and choosing effective isolates for inoculant production (Fening and Danso, 2002). It is well known that diverse rhizobia strains show wide variation in their SEF on host plants and in this study, there were significant differences with respect to SEF among the inoculation treatments in the greenhouse

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(Table 2). Based on the SEF classification by Lalande *et al.* (1990), CMBP037 was rated as ineffective, as this strain could not deliver any functional advantage as compared to the negative controls. Whereas, CMBP054 and CMBP065 were rated as effective isolates (> 50%), and thus potential native strains for enhancing cowpea N fixation needs to be evaluated under further intercropped field condition. Of these two effective strains, CMBP065 showed significantly higher nodule dry matter under greenhouse conditions. According to Beck *et al.* (1993), high nodule dry weight can lead to higher efficiency in BNF and higher shoot biomass.

In the field experiment, inoculation with the treatment CMBP (037+054) significantly increased cowpea nodulation, shoot dry weight, shoot total N, and yield on moderate and steep slopes, showing that this mixture is the most effective inoculant for moderate and steep sloping fields at this location. These findings are supported by numerous studies, which showed that inoculation with *Bradyrhizobium* strains resulted in a significant increase in cowpea nodulation and yield (Nyoki and Ndakidemi, 2013; Ulzen *et al.*, 2016; Yoseph *et al.*, 2017).

The superior performance of the combination of native rhizobia isolates from Mau Dong commune may be attributed to the ability to outcompete other native strains in the soil, nodule infection competitiveness, as well as SEF. Numerous studies have shown the positive effects of native rhizobia strains nodulating cowpea in comparison to the uninoculated controls under field conditions (Danso and Owiredu, 1988; Gómez Padilla *et al.*, 2016; Yoseph *et al.*, 2017).

Although strain CMBP065 was more effective that strain CMBP054 in the greenhouse experiment, CMBP065 was less effective than the mixture CMBP (037+054) in the field conditions. This suggests that strain CMBP037, which had the lowest SEF in the greenhouse experiment, may enhance the effectiveness of CMBP054 strain when co-inoculated. The two strains CMBP037 and CMBP054 were isolated from Mau Dong commune and the advantage of using both strains could be attributed to their better adaption to the local soil and climatic conditions, as well as the relationship with other rhizospheric microorganisms (Koskey *et al.*, 2017; Meghvansi *et al.*, 2010; Svenning *et al.*, 2001). One other possible explanation of this is the synergism between these two native rhizobia strains. This finding is on the contrary to several reports, which showed that inoculation with multiple strains is less effective as compared to the single rhizobia strain (Danso and Owiredu, 1988; Martinez-Romero, 2003; Nkot *et al.*, 2015), or different strains may compete with each other in the same inoculant (Raposeiras *et al.*, 2006). Synergistic interaction between rhizobia and plant growth-promoting rhizobacteria (PGPR) or phosphate-solubilizing bacteria (PSB) has been shown (Korir *et al.*, 2017; Veena and Poonam, 2011), but little is known of the synergism between different rhizobia strains.

Degree of slope, one of the important geographical factors, could affect the soil characteristics, plant physiological processes, and soil microorganisms. The combination of CMBP (037+054) significantly improved cowpea nodulation, shoot dry weight, total shoot N, and yield on moderate and steep slopes, whereas, there was no significant effect of rhizobia inoculations on cowpea on gentle slope. This finding indicates the significant interaction between rhizobia inoculation and the physical slope factor. The influence on local climate, erosion process, soil characteristics, and plant communities, soil slope has been shown to indirectly or directly affect bacterial diversity, composition, and activities (Haiyan *et al.*, 2016; Orwin *et al.*, 2006). It is still unclear how sloping land affects rhizobia BNF efficiency, particularly in the Northern mountainous areas of Vietnam. Therefore, further studies should be conducted in order to identify the mechanism of the interaction between slope and rhizobia inoculation.

The results from our investigation about cassava–cowpea intercropping expansion revealed the high adoption level of local farmers with the inclusion of cassava–cowpea intercropping system. However, due to the low natural nodulation of cowpea (as shown in Figure S1), resulting in low BNF in cowpea, it showed the urgent need to improve cowpea yield in such intercropping system by inoculation with effective native rhizobia.

Conclusion

This study revealed the potential of the mixture of native rhizobia strains from Mau Dong [CMBP (037+054)] as an effective inoculant for improving cowpea N fixation and yield, especially on moderate and steep slopes. While the native isolate CMBP065 showed the highest SEF under the greenhouse conditions, the mixture of native isolates CMBP (037+054) displayed superior performance and adaptability under the intercropped field conditions. The results suggest that it would make sense to isolate and screen more native rhizobia in order to get more effective and competitive strains. Further studies should also be conducted to better characterize the populations of rhizobia for cowpea at the experimental sites. We have shown that it is possible to promote the utilization of native rhizobia strains and provide cheap and effective inoculants for cowpea to local smallholders. By the inclusion of cassava–cowpea intercropping system, farmers can significantly reduce the utilization of mineral fertilizers, sustain the production, and consequently increase economic benefits.

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References

Agegnehu G., Ghizaw A. and Sinebo W. (2008). Yield potential and land-use efficiency of wheat and faba bean mixed intercropping. *Agronomy for Sustainable Development* **28**, 257–263.

Ampomah O. Y., Ofori-Ayeh E., Solheim B. and Svenning M. M. (2008). Host range, symbiotic effectiveness and nodulation competitiveness of some indigenous cowpea bradyrhizobia isolates from the transitional savanna zone of Ghana. *African Journal of Biotechnology* **7**(8), 988–996.

Awonaike K., Kumarasinghe K. and Danso S. (1990). Nitrogen fixation and yield of cowpea (*Vigna unguiculata*) as influenced by cultivar and *Bradyrhizobium* strain. *Field Crops Research* **24**, 163–171.

Bala A., Abaidoo R. and Woomer P. (2010). Rhizobia strain isolation and characterisation protocol. Available at www.N2Africa.org (accessed 10 December 2010).

Bashir M. T., Ali S., Ghauri M., Adris A. and Harun R. (2013). Impact of excessive nitrogen fertilizers on the environment and associated mitigation strategies. *Asian Journal of Microbiology, Biotechnology and Environmental Sciences* **15**, 213–221.

Beck, D. P., Materon, L. A. and Afandi, F. (1993). *Practical Rhizobium-legume technology manual.* Aleppo: International Center for Agricultural Research in the Dry Areas (ICARDA).

Bedoussac L., Journet E., Hauggaard-Nielsen H., Naudin C., Corre-Hellou G., Jensen E. S. and Justes E. (2017). Ensuring sustainable grain legume-cereal cropping systems. In *Achieving Sustainable Cultivation of Grain Legumes*, Cambridge: Burleigh Dodds Science publishing.

Boddey R. M., Urquiaga S., Neves M. C. P. and Peres J. (1990). Quantification of the contribution of N_2 fixation to field-grown grain legumes—a strategy for the practical application of the 15N isotope dilution technique. *Soil Biology and Biochemistry* **22**, 649–655.

Boman B. J., Wilson P. C. and Ontermaa E. A. (2002). Understanding water quality parameters for citrus irrigation and drainage systems. *Univ. of Florida IFAS Extension, Circ* 1406.

Boonkerd N. (2002). Development of inoculant production and utilisation in Thailand. *Inoculants and nitrogen fixation of legumes in Vietnam*, 95–104.

Bremner J. (1996). Nitrogen-total. *Methods of soil analysis part 3—chemical methods*, 1085–1121.

Broughton W. and Dilworth M. (1971). Control of leghaemoglobin synthesis in snake beans. *Biochemical Journal* **125**, 1075–1080.

Compton J. E., Harrison J. A., Dennis R. L., Greaver T. L., Hill B. H., Jordan S. J., Walker H. and Campbell H. V. (2011). Ecosystem services altered by human changes in the nitrogen cycle: a new perspective for US decision making. *Ecology Letters* **14**, 804–815.

Cong W. F., Hoffland E., Li L., Six J., Sun J. H., Bao X. G., Zhang F. S. and Van Der Werf W. (2015). Intercropping enhances soil carbon and nitrogen. *Global Change Biology* **21**, 1715–1726.

Danso S. and Owiredu J. (1988). Competitiveness of introduced and indigenous cowpea *Bradyrhizobium* strains for nodule formation on cowpeas [*Vigna unguiculata* (L.) Walp.] in three soils. *Soil Biology and Biochemistry* **20**, 305–310.

Date R. (2000). Inoculated legumes in cropping systems of the tropics. *Field Crops Research* **65**, 123–136.

De Schutter O. (2010). Agroecology and the right to food. *United Nations. December.*

Duchene O., Vian J.-F. and Celette F. (2017). Intercropping with legume for agroecological cropping systems: Complementarity and facilitation processes and the importance of soil microorganisms. A review. *Agriculture, Ecosystems & Environment* **240**, 148–161.

Dwivedi A., Dev I., Kumar V., Yadav R. S., Yadav M., Gupta D., Singh A. and Tomar S. (2015). Potential role of maize-legume intercropping systems to improve soil fertility status under smallholder farming systems for sustainable agriculture in India. *International Journal of Life Sciences Biotechnology and Pharma Research* **4**, 145.

Fening J. and Danso S. (2002). Variation in symbiotic effectiveness of cowpea bradyrhizobia indigenous to Ghanaian soils. *Applied Soil Ecology* **21**, 23–29.

General Statistics Office of Vietnam. (2019). Result of rural, agricultural and fishery census 2018. *The situation of agricultural, forestry and fishery production*. Hanoi: Statistical Publishing House.

Gibson A. (1987). Evaluation of nitrogen fixation by legumes in the greenhouse and growth chamber.

Gómez Padilla E., Ruiz-Díez B., Fernández-Pascual M., López Sánchez R., Bloem E. and Eichler-Löbermann B. (2016). Inoculation with native Bradyrhizobia strains improved growth of cowpea plants cultivated on a saline soil. *Communications in Soil Science and Plant Analysis* **47**, 2218–2224.

Haiyan C., Xiang X., Jian Y., Adams J. M., Zhang K., Yuntao L. and Yu S. (2016). Effects of slope aspects on soil bacterial and arbuscular fungal communities in a boreal forest in China. *Pedosphere* **26**, 226–234.

Herridge D. (2002). Legume N and inoculants: Global and Vietnamese perspectives. *Inoculants and nitrogen fixation of legumes in Vietnam*, 7–9.

Herridge D. F., Peoples M. B. and Boddey R. M. (2008). Global inputs of biological nitrogen fixation in agricultural systems. *Plant and Soil* **311**, 1–18.

Howeler R. H. (1991). Long-term effect of cassava cultivation on soil productivity. *Field Crops Research* **26**, 1–18.

Howeler R. H. and Hershey C. H. (2002). Cassava in Asia: Research and development to increase its potential use in food, feed and industry: a Thai example.

Jahn R., Blume H., Asio V., Spaargaren O. and Schad P. (2006). *Guidelines for soil description.* Rome: FAO.

Kawaka F., Dida M. M., Opala P. A., Ombori O., Maingi J., Osoro N., Muthini M., Amoding A., Mukaminega D. and Muoma J. (2014). Symbiotic efficiency of native rhizobia nodulating common bean (*Phaseolus vulgaris* L.) in soils of Western Kenya. *International Scholarly Research Notices*, 2014.

Knörzer H., Graeff-Hönninger S., Guo B., Wang P. and Claupein W. (2009). The rediscovery of intercropping in China: a traditional cropping system for future Chinese agriculture–a review. In *Climate change, intercropping, pest control and beneficial microorganisms,* 13–44l: Springer.

Korir H., Mungai N. W., Thuita M., Hamba Y. and Masso C. (2017). Co-inoculation effect of rhizobia and plant growth promoting rhizobacteria on common bean growth in a low phosphorus soil. *Frontiers in Plant Science* **8**, 141.

Koskey G., Mburu S. W., Njeru E. M., Kimiti J. M., Ombori O. and Maingi J. M. (2017). Potential of native rhizobia in enhancing nitrogen fixation and yields of climbing beans (*Phaseolus vulgaris* L.) in contrasting environments of Eastern Kenya. *Frontiers in Plant Science* **8**, 443.

Lalande R., Bigwaneza P. and Antoun H. (1990). Symbiotic effectiveness of strains of *Rhizobium leguminosarum* biovar phaseoli isolated from soils of Rwanda. *Plant and Soil* **121**, 41–46.

Mandimba G. (1995). Contribution of nodulated legumes of the growth of *Zea mays* L. under various cropping systems. *Symbiosis* **19**, 213–222.

Martinez-Romero E. (2003). Diversity of *Rhizobium-Phaseolus vulgaris* symbiosis: overview and perspectives. *Plant and Soil* **252**, 11–23.

Mathu S., Herrmann L., Pypers P., Matiru V., Mwirichia R. and Lesueur D. (2012). Potential of indigenous bradyrhizobia versus commercial inoculants to improve cowpea (*Vigna unguiculata* L. walp.) and green gram (*Vigna radiata* L. wilczek.) yields in Kenya. *Soil Science and Plant Nutrition* **58**, 750–763.

Meghvansi M., Prasad K. and Mahna S. (2010). Symbiotic potential, competitiveness and compatibility of indigenous *Bradyrhizobium japonicum* isolates to three soybean genotypes of two distinct agro-climatic regions of Rajasthan, India. *Saudi Journal of Biological Sciences* **17**, 303–310.

Ngeno J. K. (2018). *Abundance, genetic diversity and symbiotic efficiency of cowpea (Vigna unguiculata L.) rhizobia in soils of South Western Kenya*. Doctoral of Philosophy, University of Nairobi.

Nkot L. N., Fankem H., Adamou S., Ngakou A., Nwaga D. and Etoa F.-X. (2015). Abundance of legume nodulating bacteria in soils of diverse land use systems in Cameroon. *Universal Journal of Plant Science* **3**, 97–108.

Nyfeler D., Huguenin-Elie O., Suter M., Frossard E., Connolly J. and Lüscher A. (2009). Strong mixture effects among four species in fertilized agricultural grassland led to persistent and consistent transgressive overyielding. *Journal of Applied Ecology* **46**, 683–691.

Nyoki D. and Ndakidemi P. (2013). Economic benefits of *Bradyrhizobium japonicum* inoculation and phosphorus supplementation in cowpea (*Vigna unguiculata* (L) Walp) grown in northern Tanzania. *American Journal of Research Communication* **1**, 173–189.

Nyoki D. and Ndakidemi P. A. (2016). Intercropping system, rhizobia inoculation, phosphorus and potassium fertilization: A strategy of soil replenishment for improved crop yield. *International Journal of Current Microbiology and Applied Sciences* **5**, 504–522.

Ojo A., Dare M. O., Fagbola O. and Babalola O. (2015). Variations in infectivity of indigenous rhizobial isolates of some soils in the rainforest zone of Nigeria. *Archives of Agronomy and Soil Science* **61**, 371–380.

Orwin K. H., Wardle D. A. and Greenfield L. G. (2006). Ecological consequences of carbon substrate identity and diversity in a laboratory study. *Ecology* **87**, 580–593.

Raposeiras R., Marriel I. E., Muzzi M. R. S., Paiva E., Pereira Filho I. A., Carvalhais L. C., Passos R. V. M., Pinto P. P. and Sá N. M. H. d. (2006). *Rhizobium* strains competitiveness on bean nodulation in Cerrado soils. *Pesquisa Agropecuária Brasileira* **41**, 439–447.

Svenning M., Gudmundsson J., Fagerli I.-L. and Leinonen P. (2001). Competition for nodule occupancy between introduced strains of *Rhizobium leguminosarum* biovar trifolii and its influence on plant production. *Annals of Botany* **88**, 781–787.

Tittonell P. (2014). Food security and ecosystem services in a changing world: it is time for agroecology.

Toomsan B., McDonagh J., Limpinuntana V. and Giller K. (1995). Nitrogen fixation by groundnut and soyabean and residual nitrogen benefits to rice in farmers' fields in Northeast Thailand. *Plant and Soil* **175**, 45–56.

Trung N. T., Nakasathien S. and Vichukit V. (2013). Enhancing sustainable cassava production in hilly areas of Van Yen, Yen Bai province, Vietnam. *Kasertsart Journal (National Science)* **47**, 492–505.

Ulzen J., Abaidoo R. C., Mensah N. E., Masso C. and AbdelGadir, A. H. (2016). *Bradyrhizobium* inoculants enhance grain yields of soybean and cowpea in Northern Ghana. *Frontiers in Plant Science* **7**, 1770.

Vanlauwe B. and Giller K. E. (2006). Popular myths around soil fertility management in sub-Saharan Africa. *Agriculture, Ecosystems & Environment* **116**, 34–46.

Veena K. and Poonam S. (2011). Potential for enhancing lentil (*Lens culinaris*) productivity by co-inoculation with PSB, plant growth-promoting rhizobacteria and *Rhizobium*. *Indian Journal of Agricultural Sciences* **81**, 932–934.

Watanabe I., Hakoyama S., Terao T. and Singh B. (1997). Evaluation methods for drought tolerance of cowpea. *Advances in cowpea research*, 87–98.

Weerarathne L., Marambe B. and Chauhan B. S. (2017). Does intercropping play a role in alleviating weeds in cassava as a non-chemical tool of weed management?–A review. *Crop Protection* **95**, 81–88.

Yohane E. N. (2016). *Assessment of the abundance and effectiveness of cowpea [Vigna unguiculata (L) Walp] rhizobia in soils from different fields in Chiwosya extension planning area, Mchinji district*. Master, University of Malawi Bunda.

Yoseph T., Baraso B. and Ayalew T. (2017). Influence of Bradyrhizobia inoculation on growth, nodulation and yield performance of cowpea varieties. *African Journal of Agricultural Research* **12**, 1906–1913.

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