THESIS

CASSAVA-COWPEA INTERCROPPING IN UPLAND NORTHERN VIETNAM: NODULATION AND MYCORRHIZATION, CASSAVA AND COWPEA YIELDS, AND CONTROL OF SOIL EROSION

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GRADUATE SCHOOL, KASETSART UNIVERSITY

2020

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Tropical Agriculture)
Graduate School, Kasetsart University
2020

Nguyen Thanh Trung 2020: Cassava-Cowpea Intercropping in Upland Northern Vietnam: Nodulation and Mycorrhization, Cassava and Cowpea Yields, and Control of Soil Erosion. Doctor of Philosophy (Tropical Agriculture), Major Field: Tropical Agriculture, Faculty of Agriculture. Thesis Advisor: Associate Professor Sutkhet Nakasathien, Ph.D. 172 pages.

In upland Northern Vietnam, the local agricultural departments are very supportive for scaling up the cassava-cowpea intercropping system in order to mitigate soil degradation, improve soil health and increase farmer's income. However, in 2017, our survey found that the natural nodulation of cowpea was very low regardless of soil characteristics, slope degrees or seasons, showing the urgent need to improve cowpea production by the inoculation of effective native rhizobia while no available rhizobia inoculant was found on the markets in Vietnam.

Two experiments were conducted in Yen Bai province, Vietnam during 2017-2018. There were screening experiments of isolated native rhizobia under the greenhouse and field conditions using 21 native rhizobia strains isolated from cowpea nodules in three study sites (11 *Rhizobium* strains and 10 *Bradyrhizobium* strains). An on-farm erosion experiment was also carried out in 2018, including cassava monocropping and cassava-cowpea intercropping systems on different slope categories (gentle and steep slope). In the field screening experiment, the mixture of native isolates CMBP037+054 showed its superior performance and adaptability by significantly increasing nodulation of cowpea (19.4 nodules per plant, or 65.8%) and resulting in highest cowpea dry biomass, shoot total N content and yield (24.88 g plant^{-1}, 3.02%, and 424.7 kg ha^{-1}, respectively). CMBP037+054 inoculation showed the improvement of cowpea biomass (26.81%), shoot N content (4.86%) and yield (10.54%). In the greenhouse experiment, strains CMBP054, CMBP063, CMBP065 and CMBP066 significantly increased cowpea nodulation, shoot total N content and cowpea biomasses. These strains were rated as effective strains (with symbiotic efficiency percentages of 54.56%, 58.77%, 55.73% and 51.64%, respectively) inferring that they are potential native strains for enhancing cowpea N fixation and could be evaluated under further field condition for producing effective inoculant products. In the on-farm erosion experiment, on both gentle and steep slopes, the results showed that the inclusion of cowpea in cassava cropping system effectively improved soil covering level (2.2 and 1.7 out of 10, respectively), reduced soil erosion (40.0% and 58.2%, respectively) and nutrients losses. Moreover, cassava-cowpea intercropping did not affect cassava yield, significantly increased the incomes of local farmers (718 and 771 US$ ha^{-1}) and B:C ratio (22.3% and 37.5%, respectively) in both gentle and steep sloping fields. With the great potential in soil conservation, stable productivity and economic enhancement, the inclusion of this intercropping system shows great potential as an appropriate strategy or a climate-smart agricultural practice for the sustainable agricultural production of the local farmers in Northern mountainous region of Vietnam.

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ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Associate Professor Dr. Sutkhet Nakasathien, who has been my advisor since the beginning of my graduate study, for the warm encouragement, understanding, precious guidance and suggestions given throughout my work at Faculty of Agriculture (FOA), Kasetsart University (KU). I am very proud to be his advisee and his kindness and willingness will always be remembered by me. I am deeply grateful to my co-advisor, Associate Professor Dr. Ed Sarobol, for his supervision and valuable advice during my Ph.D. study. Great appreciation is also given to my co-advisor, Dr. Didier Lesueur, for his direction, encouragement, valuable recommendations, and support of the opportunity to do my Ph.D. research at CIAT-Asia, Hanoi, Vietnam.

I would like to especially thank Associate Professor Dr. Vichan Vichukit, Associate Professor Dr. Wanchai Chanprasert and Assistant Professor Dr. Pasajee Kongsila for the valuable recommendations during the comprehensive and final examination. I would like to express my appreciation to FOA staff, Tropical Agriculture International Program office, and Crop Physio-Molecular Biology (CPMB) lab mates, especially Assistant Professor Dr. Pattavipha Songkumarn, Dr. Arunee Wongkaew, Ms. Benjamaporn Panrat, Ms. Orawan Kumdee and Ms. Kulwadee Kanavittaya for their kind help, support, and scientific suggestions during my study at KU.

I am very grateful to my colleagues from CMBP (CIAT-Asia, Hanoi, Vietnam), VNUA and NOMAFSI, especially Dr. Mary Atieno, Dr. Laetitia Hermann, Dr. Bui Le Vinh, Dr. Nguyen The Binh, Mr. Nguyen Tri Kien and Mr. Nguyen Tuan Cuong, for their kind help, great cooperation, suggestion and comments throughout my work in both the field and laboratory work, and in writing up my Thesis publications.

I would like to sincerely thank the Southeast Asian Regional Center for Graduate Study and Research in Agriculture (SEARCA) for granting the scholarship to my study. I gratefully thank Dr. Maria Cristeta Cuaresma and Ms. Zacyl Jalotjot for their kind consideration and help.

Last, but not the least, my deepest gratitude is due to my parents, my sister, my beloved wife and daughter for their inspiration, understanding and love. This degree could not be earned without their encouragement.

Nguyen Thanh Trung

June, 2020

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LIST OF ABBREVIATIONS

ADN	=	Deoxyribonucleic Acid
ANOVA	=	Analysis of Variance
AMF	=	Arbuscular Mycorrhiza Fungus
ARN	=	Ribonucleic Acid
B:C	=	Benefit: Cost
BLAST	=	Basic Local Alignment Search Tool
BNF	=	Biological Nitrogen Fixation
C	=	Carbon
CFU	=	Colony Forming Unit
CIAT	=	The International Center for Tropical Agriculture
CMBP	=	The Common Microbial Biotechnology Platform
CRD	=	Completely Randomized Design
CT	=	Cat Thinh
DAS	=	Days After Sowing
E	=	Effective
FYM	=	Farmyard Manure
ICIPE	=	The International Centre of Insect Physiology and Ecology
IE	=	Ineffective
IG	=	Cassava Intercropping on Gentle slope
IGS	=	Intergenic Spacer
IS	=	Cassava Intercropping on Steep slope
ISFM	=	Integrated Soil Fertility Management
K	=	Potassium
LE	=	Low Effective
MD	=	Mau Dong
MG	=	Cassava Monocropping on Gentle slope
MS	=	Cassava Monocropping on Steep slope
N	=	Nitrogen
NCBI	=	The National Center for Biotechnology Information
NMR	=	Northern Mountainous Region

LIST OF ABBREVIATIONS (Continued)

NS	=	Not Significant
OC	=	Organic Content
P	=	Phosphorus
PCR	=	Polymerase Chain Reaction
PGPR	=	Plant Growth-Promoting Rhizobacteria
PSB	=	Phosphate Solubilizing Bacteria
RDA	=	Redundancy Analysis
RFLP	=	Restriction Fragment Length Polymorphism
SCL	=	Soil Covering Level
SEA	=	South East Asia
SEF	=	Symbiotic Efficiency
SOC	=	Soil Organic Carbon
SOM	=	Soil Organic Matter
SOP	=	Standard Operating Procedure
SPAD	=	Soil Plant Analysis Development
ST	=	Son Thinh
VNUA	=	Vietnam National University of Agriculture
YEMA	=	Yeast Extract Manitol Agar

CASSAVA-COWPEA INTERCROPPING IN UPLAND NORTHERN VIETNAM: NODULATION AND MYCORRHIZATION, CASSAVA AND COWPEA YIELDS, AND CONTROL OF SOIL EROSION

INTRODUCTION

The Northern Mountainous Region (NMR) is one of the main regions of Vietnam leading to cassava area and production (Kim *et al.*, 2017). In this zone, over one-third (35.2%) of the communes (707 communes) are at an altitude of over 600 m, and 783 communes (39.0%) are at an altitude between 200 and 600 m (Vien, 2003). About 22% of the total cultivation areas are on a slope degree of less than 8^{o}, which are mainly used for agriculture or agroforestry production. Whereas, the areas with slope degree of 8-15^{o} occupy around 16% of arable land, and the rest are steep sloping lands with slope degree more than 15^{o} (about 62% of the total cultivation areas) (General Statistics Office of Vietnam, 2018). Since historical times, mountain dwellers have been using sloping lands to support their livelihoods. Over time, due to an increase in population density and economic demand, agricultural land use was bound to extend on sloping lands when the increasing demands were not met from low land intensification (Clemens *et al.*, 2010; Lippe *et al.*, 2011). With the intensive cultural practices and the heavy rainfall patterns in such areas, short cultivation cycles have been continually practiced thus leading to a dramatic increase in the rate of soil erosion (Doanh and Tuan, 2004). Moreover, Tuan *et al.* (2014) revealed that the widespread practice of monocropping systems on steep slopes in northern Vietnam had led to severe erosion up to 174 tons ha^{-1}, as well as an imbalance of nutrients in the soil. Whereas, using ^{137}Cs measurement, Häring *et al.* (2014) reported that the rates of bulk soil erosion were ranged from 12 to 89 tons ha^{-1} in the NMR of Vietnam. In such region, cassava, one of the major cash crops for local poor farmers, is commonly planted as a monocrop because of its tolerance to drought and infertile acidic soils, and minimal requirement for nutrients and land preparation (Howeler and Hershey, 2002; Leihner *et al.*, 1996). However, Howeler *et al.* (2001) indicated that the continuous growth of

mono-cropped cassava had caused severe soil erosion on sloping lands. This is mainly due to the wide planting spaces and the slow growth of cassava at the initial stage, resulting in detached soil particles and hence an increase in topsoil erosion by raindrops and runoff. This risk is even more serious in tropical countries where there are heavy rainfalls in the early period of cassava growth (Putthacharoen *et al.*, 1998). According to Isabirye *et al.* (2007), another possible cause is due to the harvest process, i.e. pulling cassava storage roots out of the ground, that disturbs topsoil structure and facilitates faster erosion. After studying the intensity of soil loss by erosion under different cropping systems in upland areas, Phien and Vinh (2002) also showed that soil loss was highest for cassava monocropping system (98.6 tons ha^{-1} $year^{-1}$) and could result in nutrient loss of up to 14 times higher for nitrogen (N), 22 times for phosphorus (P), and 10 times for potassium (K) without good soil management, as compared with that of most other crops. According to Podwojewski *et al.* (2008), the cassava monocropping system produced both an increase in soil loss and a decrease in earthworms' population. Upland soils have more constraints for crop growth in which low organic matter, soil acidity and low levels of nutrients were the main characteristics (Baritz *et al.*, 2018). Several studies (Shrestha *et al.*, 2014; Six *et al.*, 2000) have indicated that the reduction of soil organic matter reduces soil aggregation, consequently increases soil erosion, especially in the uplands. Phien and Vinh (2002) and Wezel *et al.* (2002b) found that the decline in soil fertility usually resulted in serious negative impacts on the productivity and sustainability of the sloping lands in Northern Vietnam. Moreover, soil and nutrient losses resulted in the decline in cassava production of 31% in Northern Vietnam, or equivalent to the production cost of 200-700 US$ ha^{-1} (Howeler, 1996; Wezel *et al.*, 2002a). Besides, according to our previous investigation during 2016-2017, for growing cassava, the local farmers are still applying a high amount of 100 to 120 kg N ha^{-1} $year^{-1}$. The excessive application of mineral N fertilizers causes greenhouse gas emission (N_2O) and could make negative consequences for global agriculture (Nyoki and Ndakidemi, 2016). Moreover, superfluous level of N released into the environment can also affect soil and water by causing soil acidification and toxification, death of fish and aquatic organisms, and water contamination (Bashir *et al.*, 2013; Boman *et al.*, 2002; Compton *et al.*, 2011).

Soil erosion, decline in soil fertility as well as difficulties in crop production in the uplands can be mitigated by agroecological practices, especially intercropping, which can bring resilience and sustainable productivity to local smallholders and provide important ecosystem services across the landscape. Intercropping, which is commonly practiced in many tropical countries around the world, is one of the most effective agroecological practices for enhancing soil fertility and sustainable production (Dung and Preston, 2007; Latati *et al.*, 2017). Intercropping is a type of agricultural system of growing two or more crops simultaneously in the same field during a growing season (Baritz *et al.*, 2018). There are numerous socio-economic, biological and ecological advantages of intercropping practice as compared with the traditional monocropping practices. Bedoussac and Justes (2011) reported that the intercrop of durum wheat-winter pea consistently had higher yield and shoot total nitrogen than the sole pea crop. In a study on Integrated Soil Fertility Management (ISFM), Sanginga and Woomer (2009) showed that maize-bean intercropping, which is practiced broadly in Africa, induced a quicker germination rate and a better establishment of maize, shade-tolerant understory growth, edible leaves, green pods and seeds, and fast-maturing of beans. Intercropping systems allow more than one harvest per year so farmers can maximize land usage (Agegnehu *et al.*, 2008) and reduce the risk of crop failure while being better able to cope with price variability (Knörzer *et al.*, 2009). Intercropping is described as an eco-functional exercise, which is widely used to boost crop production (Dwivedi *et al.*, 2015; Mureithi *et al.*, 2005; Niggli *et al.*, 2009) and it also reduces pests and weeds (Corre-Hellou *et al.*, 2011; Weerarathne *et al.*, 2017; Zimdahl, 2007). Also, intercropping systems might be useful in emitting a lower amount of greenhouse gases compared with sole cropping systems (Bayer *et al.*, 2016; Jensen *et al.*, 2012; Latati *et al.*, 2017). Oelhermann *et al.* (2009) reported that the N_2O production rate in the maize-soybean intercropping system was significantly lower (17.48%) than in the sole crops, while the soil in maize-soybean intercropping resulted in significant higher CH_4 sink comparing with monocropping systems. According to Evers *et al.* (2010), tree-based intercropping systems had the potential to reduce 1.2 kg ha^{-1} $year^{-1}$ of N_2O emissions as well as to increase soil organic carbon by 77% comparing with the nearby agricultural monocropping system. Intercropping accelerates the restoration of degraded soils to make them more fertile and more

sustainable for food production (Adjei-Nsiah, 2012; Mapfumo *et al.*, 2001). Muli *et al.* (2015) indicated that soil moisture was considerably greater in both sorghum and sweet potato-based intercropping systems compared with sole crops. Moreover, intercropping also enables the build-up of a wider range of soil microorganisms which will stimulate activities of earthworms and soil microbes (Duchene *et al.*, 2017; Garland *et al.*, 2017).

Nowadays, due to the intensive use of land and the shortening of the fallow period, nutrient management must be improved by legume-based intercropping systems. One of the most important benefits of legume-based intercropping systems is the unique ability of legumes in fixing atmospheric N through the process of Biological Nitrogen Fixation (BNF) in symbiosis with soil bacteria known as rhizobia (Nyfeler *et al.*, 2009). Several studies have reported the N contribution of legume crops in intercropping systems to be equivalent to around 110-190 kg of N fertilizer ha^{-1} $year^{-1}$ (Cong *et al.*, 2015; Mandimba, 1995). According to Herridge *et al.*, 2008, symbiotically fixed N_2 in legumes ranged from 100 to 380 kg of N ha^{-1} $year^{-1}$. Moreover, Herridge (2002) revealed that the combination of rhizobium inoculants-N fertilizer doses of 30-40 kg ha^{-1} provided the same groundnut yield compared with the N fertilizer doses of 60-90 kg ha^{-1}. Additionally, in comparison with chemical fertilizer application, inoculation of legumes with rhizobial products showed significantly higher economic benefits in both wet and dry soybean seasons of about US$126.7 ha^{-1} and US$144.2 ha^{-1}, respectively (Boonkerd, 2002). The substitution of N mineral fertilizers by improved exploitation of BNF would be an important contribution to resource-efficient agricultural systems and sustainable productions (Gruber and Galloway, 2008). Cowpea (*Vigna unguiculata* var. cylindrica) is one of the important widely cultivated legumes which can fix atmospheric N ranging from 9-120 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). This legume needs mainly phosphorus (P) and can self-support part of their N requirements through BNF, while cassava needs 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 the intercropping system. Besides, cowpea is highly suitable for cassava in terms of growth patterns and canopy

development (Howeler and Hershey, 2002). Moreover, in Northern mountainous areas of Vietnam, cowpea can effectively increase smallholders' income because it is easy to sell cowpea grain on local markets with stable higher prices (around 2.5 US$ kg^{-1}) compared with the other local legumes (soybean, peanut, mung bean, etc.). That is the reason why the agricultural department in Yen Bai province is very supportive for scaling up the cassava-cowpea intercropping system through farmer associations in order to mitigate soil degradation and improve soil health, instead of applying much of mineral fertilizers. However, currently, no available rhizobia products for cowpea have been found in the Vietnam market. The promotion of cowpea needs to be incorporated with identifying and scaling up effective rhizobia inoculants to enhance and sustain BNF.

Intercropping of main crops and cover crops increases the percentage of soil cover, thus inhibits the direct impact of raindrops and sunshine on the soil, consequently maintaining soil fertility, improves soil moisture, and reduces soil erosion and nutrient losses (Blanco and Lal, 2008; Korkanç, 2018; Podwojewski *et al.*, 2008). Several studies have reported that cassava-cowpea intercropping has the highest land-use efficiency (Aye and Howeler, 2012). Trung *et al.* (2013) reported that cassava-peanut intercropping decreased the amount of eroded soil by 63.2%, as compared to traditional monocropping. Dalton *et al.* (2007) also reported that intercropping cassava with peanut, mung bean and soybean produced soil loss amount of 25.7 tons ha^{-1} yr^{-1}, while cassava monocropping produced 53.2 tons ha^{-1} yr^{-1} of soil loss. Moreover, intercropping with legumes, which are known to have the ability to fix atmospheric nitrogen (N), could bring the additional benefit of N contribution to the main crops (Herridge *et al.*, 2008). Peoples *et al.* (2009) implied that the contribution of cowpea to main crops was about 20 kg N ha^{-1} $season^{-1}$. Additionally, intercropping systems enhanced the organic matter content and the nutrient input to the soils due to biomass residues of legume crops (Ghosh *et al.*, 2006; Mulumba and Lal, 2008). Intercropping systems can also boost crop production (Dwivedi *et al.*, 2015) and increase the economic benefit of smallholders (Hy, 1998; Sharma *et al.*, 2017). Besides, legume-based intercropping systems also have other advantages of controlling pests and weeds, decreasing N mineral fertilizer input and utilizing resources efficiently, thus reducing

the use of chemical input by smallholders and indirectly translate to higher economic returns (Wang *et al.*, 2014; Weerarathne *et al.*, 2017).

Little information exists about the effectiveness of the cassava-cowpea intercropping system in the Northern mountainous region of Vietnam. Therefore, the aim of this study was to evaluate the effectiveness of cassava-cowpea intercropping on sustainable crop productivity, soil erosion, and economic benefits to the local farmers. Besides, this study would find out the way to improve the efficiency of such intercropping system through the usage of native rhizobia inoculants.

OBJECTIVES

1. To survey the expansion of cassava-cowpea intercropping system from 2017 to 2018 and assess the natural nodulation by native rhizobia nodulating cowpea at different locations in upland Northern Vietnam.

2. To isolate the native rhizobia strains from collected cowpea nodules and screen under the greenhouse and field conditions to identify the potential native strains for effective rhizobia inoculants and the scaling-up production through farmer associations.

3. To estimate the changes in soil and nutrient losses from the cassava-cowpea intercropping system and assess the effects of the cassava-cowpea intercropping system in terms of crop yields and economic returns to smallholders.

LITERATURE REVIEW

1. Cassava production in Northern mountainous region of Vietnam

Cassava in Vietnam is not only among the four most important food crops but also is the main crop in the strategy of national food security. There are 6 main regions for cassava production as shown in Figure 1. Total cassava production of Vietnam was about 10.67 million tons in 2015, up from only 1.99 million tons in 2000. This was the result of both area expansions, from 237,600 ha to 566,500 ha, and markedly increases in yield, from 8.36 t ha^{-1} in 2000 to 18.47 t ha^{-1} in 2015 (FAO, 2015). Vietnam is now the second-largest exporting country of cassava products while animal feed factories also contribute significantly to the increasing demand for cassava roots. Kim *et al.* (2013) reported that cassava yield and production in several provinces have more than doubled due to the planting of new high-yielding cassava varieties and the adoption of more sustainable production practices. According to Howeler (2002), in Vietnam about 66% of cassava is grown on Ultisols, 17% on Inceptisols, 7% on Oxisols, 4% on Alfisols, 2% on Entisols and 3% on Vertisols. The soil pH generally varies from 4.5 to 6.0. In the Northern of Vietnam, cassava is grown mainly in hilly topography and about 68% of the cassava growing area has rocky soil while 12% have sandy soils, respectively. In Southern Vietnam, cassava is grown mainly on sandy-grey soils, which tend to be flat and poor in nutrients (Bien *et al.*, 1996).

In Northern Vietnam, prior to 2002, since cassava processing had not developed, cassava was used mainly for animal husbandry, drying and manual processing. New cassava varieties had developed slowly, since processing area of families was just approximately 1,000 m^2. Since 2002, together with the development of the national cassava processing, numerous cassava starch processing factories and manual cassava processors have been established, leading to the development of cassava supplying areas (Howeler, 2002). In 2009, numerous of new varieties, mainly KM94, KM140, KM98-1, SM937-26, KM98-7, were grown in more than 500,000 ha. This corresponds to more than 90% of the total cassava area in the country (Kim *et al.*, 2008). Kim *et al.* (2017) reported that two new promising cassava varieties, KM98-7

and SA21-12, are being disseminated to a large number of households in the Northern mountainous areas. Currently, in this region, cassava is planted on around 117,000 hectares, of about 20% total cassava area in Vietnam, with the production of about 1,485,500 tons.

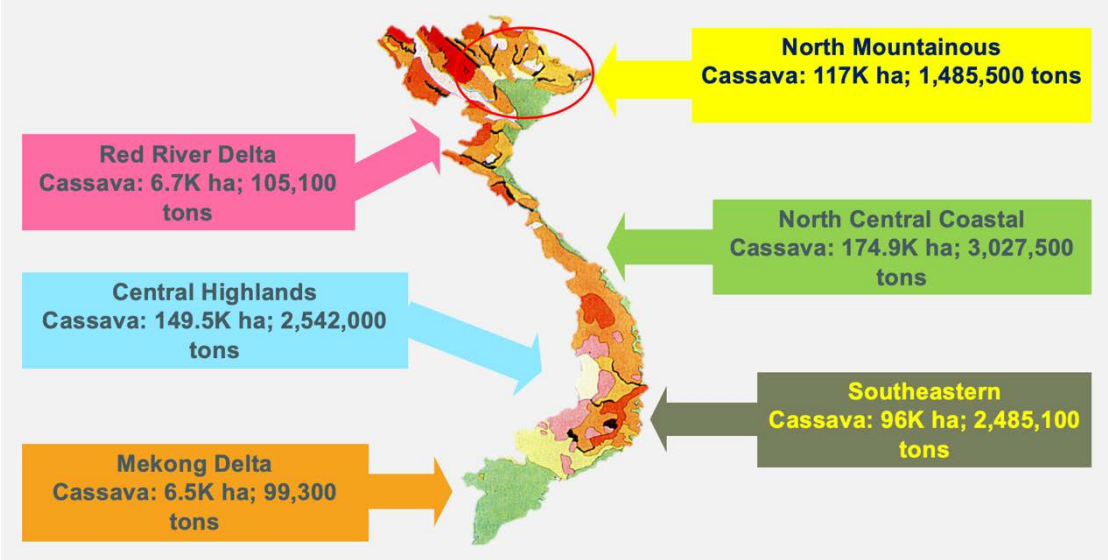

Figure 1 Main regions for cassava production in Vietnam.

Source: General Statistics Office of Vietnam (2018).

2. Soil erosion in cassava cropping systems

Soil, a key natural resource, plays a vital role in producing food, feed, fibre, fuel, and living environment for human well-being (Palm *et al.*, 2007). All the soil components, including soil micro-climate, physical, chemical and biological characteristics, have profited humankind for several centuries (Alcamo, 2003). Nevertheless, according to a recent publication by Stavi and Lal (2015), about 23% of the world soils are greatly degraded and the rate of 5-10 million ha is increased annually due to both human activities (mainly from intensive agricultural production) and natural processes mainly erosion or other causes such as salinization, acidification, pollution, nutrient depletion. Currently, soil erosion has been indicated as one of the major soil

threats worldwide (Liu *et al.*, 2017; Ochoa *et al.*, 2016; Wei *et al.*, 2014). Soil erosion is a natural geomorphic process that detaches and removes the high fertile topsoil from the surface by water, wind or cultivation practices (Lupia-Palmieri, 2004; Thomas *et al.*, 1999). This process could carry away 75 billion tons of fertile topsoil yearly, significantly decrease 50% crop yields, highly increase fertilizer input expenses, greatly reduce biodiversity and soil fertility, or result in a huge economic loss of about 10 billion euros annually (Lal, 2001; Lippe *et al.*, 2014; Pimentel and Burgess, 2013). After the 2-years erosion experiment in Thailand, Pansak *et al.* (2008) predicted that the identical soil loss could reach 21.2 tons ha^{-1} a^{-1}. Moreover, soil erosion also has negative effects on the environment and human/animal health such as soil and water pollution, waterlogging and water scarcity, energy loss, infrastructure damages, and landscape deterioration (El Kateb *et al.*, 2013; Palm *et al.*, 2007).

On sloping lands, cassava is known as one of the food crops resulting in severe soil erosion (Daellenbach *et al.*, 2005; Putthacharoen *et al.*, 1998). The reasons can be due to the frequent loss of topsoil during land preparation, weeding, harvesting, as well as the limited of soil cover percentage in the early stages (Howeler *et al.*, 2001; Isabirye *et al.*, 2007). According to Howeler (2014), the results from many cassava erosion trials showed that soil erosion can be a serious problem in Asia due to extreme population pressure on land, relative steep slopes, high intensity rainfalls and erodible soils. In Thailand, after a four years experiment on 7% slope on a sandy loam soil, Putthacharoen *et al.* (1998) revealed that the soil loss from cassava production was 71.4 tons ha^{-1} $year^{-1}$, which was 2-3 times higher than those of maize, peanut and mung-bean cropping systems, and 2-6 times higher than those caused by sugarcane and pineapple cropping systems. In Hainan island of China, the average dry soil loss due to erosion from cassava fields was about 128-154 tons ha^{-1} $year^{-1}$ (Howeler, 1993). Whereas, the dry eroded soils of cassava erosion trials in Indonesia, Malaysia, and the Philippines were 36, 10, and 54 tons ha^{-1} $year^{-1}$, respectively (Howeler, 1993). In Vietnam, about 60-66% of cassava farms were cultivated in monocropping system, which could lead to severe soil losses due to erosion process (Bien *et al.*, 1996). Howeler (1993) also showed that the average soil loss from cassava erosion trials on 15% slope on a sandy clay loam soil was 105 tons ha^{-1} $year^{-1}$ in Thai Nguyen province, Vietnam.

3. Intercropping practice and its advantages and disadvantages

Intercropping is defined as a multiple cropping system, in which two or more crops are planted together concurrently on the same area (Andrews and Kassam, 1976), but there is no need to sow or harvest at the same time (Malézieux *et al.*, 2009). In this type of cropping system, there is commonly one primary crop for economic or food production purposes and one or more extra crops (Dwivedi *et al.*, 2015). Intercrops are common in wild plant communities (Bedoussac and Justes, 2011) but with the intensification of agriculture in the last few decades, intercropping has been long practiced worldwide (Francis, 1986). Traditional tropical agriculture has frequently integrated different forms of intercropping (as shown in Table 1), where many crops are grown in association with one another and shared environmental factors (such as water, light, temperature) and soil nutrients.

Table 1 Different intercropping systems commonly used by the farmers in tropical agriculture in some Asian countries.

No.	Country	Particular region	Intercropping systems	References
1	China	- South subtropical and tropical zones	Legume-cereal (e.g. wheat/maize, soybean/sugarcane, soybean/cassava…)	Jensen (1996); Li *et al.* (2016); Zhang and Li (2003)
		- Southern China	Peanut/traditional medicinal plants (especially *Atractylodes lancea*)	Dai *et al.* (2013); Li *et al.* (2018); Li *et al.* (2014)
2	India	- Kerala, Tamil Nadu and Karnataka	Jungle rubber-based agroforestry; Multistrata agroforestry	Francis (1986); Li *et al.* (2013); Vandermeer (1989)
		- Southeastern Uttar Pradesh, Vidarbha	Legume-cereal (e.g. maize/soybean, maize/faba bean, pigeon pea/sorghum…)	Ghosh (2004); Ghosh *et al.* (2009); Sharma and Banik (2013); Zomer *et al.* (2009)
		- Western Ghats, Eastern Ghats, North Eastern regions	Arecanut/medicinal and aromatic plants	Balasimha (2009); Sujatha and Bhat (2010); Sujatha *et al.* (2011)
		- Kerala, Tamil Nadu and Andhra Pradesh	Cassava/legumes; Cassava/vegetables; Cassava/banana; Cassava/coconut; Cassava/rubber	Edison *et al.* (2006); Howeler *et al.* (2013); Nayar *et al.* (2002); Weerarathne *et al.* (2017)

Table 1 (Continued)

No.	Country	Particular region	Intercropping systems	References
3	Indonesia	- North and South Sumatra, Riau, Lampung and Java	Jungle rubber-based agroforestry; Multi-strata agroforestry	Francis (1986); Li *et al.* (2013); Vandermeer (1989)
		Mountainous areas	Cassava/upland rice/maize/legumes	Howeler *et al.* (2013)
		- Uplands of Java, Sumatra and Sulawsi	Pigeon pea/maize	Karsono and Surmano (1987)
4	Myanmar	- Coastal region	Rubber is intercropped with maize, melon, pepper, southern pea and vegetables; Oil palm is intercropped with upland rice, melon, pineapple	Mar *et al.* (2007)
		- Delta region	Oil seeds/cereal; Oil seeds/legumes; Legumes/legumes	Mar *et al.* (2007)
		- Hilly region	Maize/pigeon pea; Maize/castor	Mar *et al.* (2007)
		- Semi-arid wet zone	Sugarcane/tomato; Sugarcane/lablab bean; Maize/castor/pigeon pea	Mar *et al.* (2007)
		- Dry zone	Pigeon pea is intercropped with sesame, groundnut, short staple cotton	Mar *et al.* (2007); Wallis *et al.* (1988)

Table 1 (Continued)

No.	Country	Particular region	Intercropping systems	References
5	Thailand	- South Thailand	Rubber is intercropped with champak, iron wood, fruit trees and pigeon pea	Romyen *et al.* (2018); Wallis *et al.* (1988)
		- Northeast Thailand	Cassava/cowpea; Cassava/pigeon pea	Howeler *et al.* (2013); Wongwiwatchai *et al.* (2002)
6	Vietnam	- Northern mountainous areas, Southern Vietnam	Cassava is intercropped with groundnut, mung bean, cowpea and peanut	Dung and Sam (2002); Howeler (1996); Trung *et al.* (2013); Van De *et al.* (2008)
		- Northern mountainous areas, Central Highland-Central Coast Upland	Maize/legumes; Maize/*Mucuna Pruriens*; Maize/*Brachiaria ruziziensis*	Dinh Thao *et al.* (2004); Husson *et al.* (2000); Trung *et al.* (2013)
		- Central Highland, Southeast-Mekong Delta	Maize/legumes/coffee; Sweet orange/Tieu mandarin; pomelo/lime or a mixture of these	Dinh Thao *et al.* (2004); Van De *et al.* (2008); Van Mele and Van Chien (2004)

There are numerous socio-economic, biological and ecological advantages of intercropping practice as compared to the traditional mono-cropping practices. Bedoussac and Justes (2011) reported that the intercrop of durum wheat-winter pea consistently had higher yield and shoot total nitrogen than the sole pea crop. Leihner (1983) revealed that cassava-climbing bean intercropping system significantly increased cassava yield (31.4 tons ha^{-1}) compared to cassava monoculture (28.2 tons ha^{-1}). Sanginga and Woomer (2009) showed that maize-bean intercropping, which is practiced broadly in Africa, induced a quicker germination rate and a better establishment of maize, shade-tolerant understory growth, edible leaves, green pods and seeds, and fast-maturing of beans. Intercropping systems allow more than one harvest per year so farmers can maximize land usage and reduce the risk of crop failure (Agegnehu *et al.*, 2008) while being better able to cope with price variability (Knörzer *et al.*, 2009). Intercropping is described as an eco-functional exercise, which is widely used to boost crop production (Mureithi *et al.*, 2005; Niggli *et al.*, 2009) and it also reduces pests and weeds (Lichtfouse, 2009; Weerarathne *et al.*, 2017; Zimdahl, 2007). Also, intercropping systems might be useful in emitting a lower amount of greenhouse gases compared to sole cropping systems (Jensen *et al.*, 2012; Latati *et al.*, 2017). Oelhermann *et al.* (2009) reported that the N_2O production rate in maize-soybean intercropping system was significantly lower (17.48%) than in the sole crops, while the soil in the maize-soybean intercropping resulted in significant higher CH_4 sink comparing to monocropping systems. According to Evers *et al.* (2010), tree-based intercropping systems had the potential to reduce 1.2 kg ha^{-1} y^{-1} of N_2O emissions as well as to increase soil organic carbon by 77% comparing to the nearby agricultural monocropping system. Intercropping accelerates the restoration of degraded soils to make them more fertile and more sustainable for food production (Adjei-Nsiah, 2012; Mapfumo *et al.*, 2001; Smith *et al.*, 2016). Muli *et al.* (2015) indicated that soil moisture was considerably greater in both sorghum and sweet potato-based intercropping systems compared to sole crops. Moreover, intercropping with a diversity of plant species will reduce the impact of the heavy tropical rains on soil surfaces as the increased root biomass holding the soils together. It also enables the build-up of a wider range of soil microorganisms which will stimulate activities of earthworms and soil microbes (Duchene *et al.*, 2017; Garland *et al.*, 2017; Taschen *et al.*, 2017). In addition,

many studies have shown that intercropping systems would bring higher economic benefits for farmers (Knörzer *et al.*, 2009; Sharma *et al.*, 2017; Whitmore *et al.*, 2000). In cassava intercropping systems, especially with the inclusion of legume species, farmers can get the benefits from additional food or cash from selling intercrop products before harvesting cassava (Howeler, 1993). In Northern Vietnam, according to Loan *et al.* (2008), the intercropping system of cassava with one or two rows of peanut usually observed the highest economic returns of 300-500 US$ ha^{-1}.

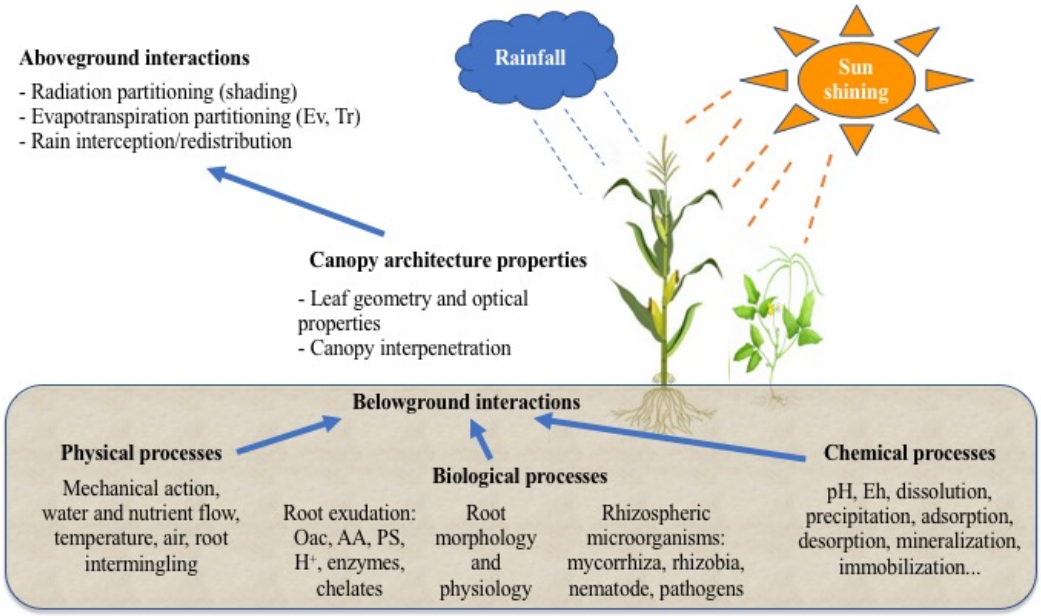

Figure 2 Above and below ground competition for resources in multispecies systems [Adapted from Malézieux *et al.* (2009); Zhang *et al.* (2004)].

In intercropping systems, different species interact resulting in a set of properties including not only facilitation but also above and below-ground competition for space, light, water and nutrients, as described in Figure 2. Consequently, these could also cause negative impacts to crop production and farmer's management practices. Fukai and Trenbath (1993) indicated that in intercropping systems, the modification of the growth environment and the competition with other component crops might alter the phenological development of the crop. As a consequence, one crop commonly grows better than the other intercrop. Moreover, the crops may not be well-suited

together and may lead to poor yields of both intercropped plants and main crops (Chui and Shibles, 1984; Natarajan and Willey, 1980).

The above-ground competition, which mainly involves light and space, may lead to limited possibilities for production, mechanization and harvesting, low grain yields (Shumba *et al.*, 1990), and a reduction of cash crops. According to Kumwenda *et al.* (1996), low-growing legume species are often covered by taller cereals, especially under farmer practices with the low-input condition, so the poor or late emergence and slow growth of the intercropped legumes are common (Chang and Shibles, 1985; Dalal, 1974). Hence, this can limit the nitrogen and organic matter supply from the legumes to soils (Kumwenda *et al.*, 1995).

Contrary to above-ground matter, below-ground competition appears when main crops compete with intercropped plants for soil growth resources (for instance water, mineral nutrients) thus decrease the growth, survival or fecundity of intercropped plants (Casper and Jackson, 1997; Strydhorst *et al.*, 2008; Tofinga *et al.*, 1993). Previous studies revealed that, in cereal-legume intercropping systems, belowground species interactions had more significant effects on intercrop productivity than aboveground (Ghosh *et al.*, 2009; Lv *et al.*, 2014). Mariotti *et al.* (2009) indicated that crops having finer roots can have a higher belowground competitive ability than legumes, which leads to the more efficient exploration in soil volume and higher nutrient uptake capacity. The spatial use of soil moisture by crops of similar demand or root extraction zones may lead to high competition between primary crops and intercrops (Midmore, 1993; Wu *et al.*, 2012). Thus, root system characteristics, including their position in time and space, root biomass, root surface or length, and proportion of water and nutrient uptake, are very important for designing agroecosystems in order to improving crop productivity and sustainability.

4. Common intercropping systems used by farmers in tropical agriculture

4.1 Commonly used intercropping systems in tropical agriculture

Currently, intercropping is a very popular practice in many regions of the world, especially in the tropical region such as South East Asia (SEA), East Asia, Africa, and Central America (Altieri, 1999; Leihner, 1983; Zomer *et al.*, 2009). According to Dwivedi *et al.* (2015), numerous models of intercropping practice were utilized in ancient Greece approximately 300 BC, where wheat, barley, millet and certain pulses were grown at different times during the cultivating season often combined with vines and olives, thus demonstrating the early intercropping systems. Several historians also reported that intercropping probably occurred early in agriculture's evolution (Anders *et al.*, 1995), even though it has been argued that intercropping systems first emerged in areas where root crop agriculture was predominant (Johannessen *et al.*, 1970; Sauer, 1969). In Latin America, it is estimated that almost 40% of cassava is intercropped and the Mayas practiced an early crop establishment of cassava with maize (Leihner, 1983). Farmers often grow 80% of field beans intercropped with maize, potatoes, or other crops (Papanastasis *et al.*, 2004). Leihner (1983) showed that intercropping of cassava with common beans or cowpeas are also common in South America, but used predominantly in Central America, Colombia, and Brazil. In tropical areas of West Africa, yellow guinea yam, white guinea yam, kafir potato, yam pea and paisa are widely intercropped (Anders *et al.*, 1995). In most tropical Africa, relay intercropping is practiced, beginning with other crops (for instance cowpea, taro, beans, groundnut) and inter-planting cassava when the earlier crops are about to be harvested (Leihner, 1983). Maize-cowpea relay intercropping is one of the most popular types of cereal and legume intercropping practiced (Mpangane *et al.*, 2004; Van Kessel and Roskoski, 1988). In tropical Asian countries, according to Viswanathan and Shivakoti (2008), rubber tree intercropping has occurred as a resilient farming system in the traditional rubber tree growing countries such as China, Indonesia, Malaysia and Thailand. In China, rubber is frequently intercropped with tea, recognized as a beneficial strategy in reducing soil

erosion (Guo *et al.*, 2006). Kassam (1976) also reported that groundnut is usually intercropped with maize in SEA.

4.2 Common cassava intercropping systems in Asia

Table 2 Commonly used cassava intercropping systems in some Asian countries.

No.	Country	Associated crops
1	Cambodia	Upland rice, maize, cashew nut, rubber
2	China	Maize, watermelon, sweet potato, peanut, rubber
3	East Timor	Maize, peanut, vegetables, banana
4	India	Maize, cowpea, vegetables, coconut
5	Indonesia	Upland rice, maize, soybean , cowpea, mung-bean, peanut, coconut, rubber
6	Lao PDR	Upland rice, maize, Job's tear, peanut
7	Myanmar	Maize, peanut, common bean, banana
8	Philippines	Maize, peanut, sweet potato
9	Thailand	Maize, rubber, coconut, cashew nut
10	Vietnam	Maize, upland rice, peanut, black bean, rubber, cashew nut, coffee, tea

Source: Aye and Howeler (2012).

In many countries in Asia, particularly in smallholder farms, intercropping cassava with other crop species is of great importance to contributing to enhanced human nutrition (Leihner, 1983). Relay cropping of cassava planted 30 days after groundnuts are also common. As shown in Table 2, in Indonesia, dryland cassava is the third crop after upland rice and maize. They are planted simultaneously and cassava is intercropped 30 to 40 days later (Suryatna *et al.*, 1979). In Thailand, cassava is rarely intercropped, but an occasional association with maize is found (Howeler *et al.*, 2013). In India, Malaysia and the Philippines, cassava is also intercropped with perennial crops such as coconut, oil palm, rubber, mango and banana (Kumar and Hrishi, 1978). In

Guangxi province of China, the popular associated crops with cassava in intercropping systems are maize, peanut, sweet potato or watermelon, while in Hainan province, cassava is often intercropped with young rubber trees or bananas. On the other hand, in the mountainous regions of Vietnam, intercropping systems of legumes and cassava can bring many benefits such as high economic profit, improved soil fertility, and reduced soil loss (Doanh and Tuan, 2004). Whereas, in South Vietnam, cassava is often intercropped with maize or planted among young rubber or cashew trees.

5. Biological nitrogen fixation in legume-based intercropping systems

Legumes' unique ability, biological nitrogen fixation (BNF) (Figure 3), plays a crucial role in sustaining crop yields in the tropics, where soil N can be depleted by about 20 kg ha^{-1} annually (Smaling *et al.*, 1997) and chemical N fertilizers are not readily available or their costs are prohibitive for smallholders (Jama *et al.*, 2000; Mugwe *et al.*, 2011). Cereal-legume intercropping systems have a great capacity to replenish soil mineral N as well as support crop production (Dakora and Keya, 1997; Dwivedi *et al.*, 2015). Legumes can fix N from the atmosphere through BNF in the symbiosis with soil rhizobia, with fixed N ranging from 100 to 380 kg atmosphere N ha^{-1}, though higher amounts of more than 500 kg of N ha^{-1} $year^{-1}$ have also been stated recorded (Herridge *et al.*, 2008). The additional benefit of legume-based intercropping systems is the direct transfer of N to main crops (Chen *et al.*, 2004). For instance, in the maize-cowpea intercropping system, about 24.9% of N fixed by cowpea was transferred to maize (Wang *et al.*, 2017). Mandimba (1995) revealed that the contribution of groundnut to maize in intercropping systems is approximately 96 kg of N fertilizer ha^{-1}.

Ofori and Stern (1987) identified that the amount of BNF in cereal-legume intercropping systems depends on species, plant morphology and density of each crop, root characteristics, agricultural practices, and crop competitive ability. A number of nutrients, genetic and environmental factors can inhibit the extent of legume nodulation and BNF. In Sahelian Africa, degraded soils with low moisture content and extremes of temperature (too high or too low) can decrease nodule function characterized by

collapsing of lenticels (Schomberg and Weaver, 1992; Whitehead and Sutcliffe, 1995), reducing respiratory capacity of bacteroids, and declining in nitrogenase activity and leghemoglobin content of nodules (Guérin *et al.*, 1991). An excessive amount of N fertilizer application can also significantly decrease BNF (Nambiar *et al.*, 1983; Ofori and Stern, 1987). Fujita *et al.* (1992) reported that component crop densities, which determine the space between crops and legume growth stages, affect the associated non-leguminous crop in intercropping systems. Additionally, soil mineral nutrients affect BNF in legumes by constraining both nodule formation and nitrogenase activity (Weisany *et al.*, 2013).

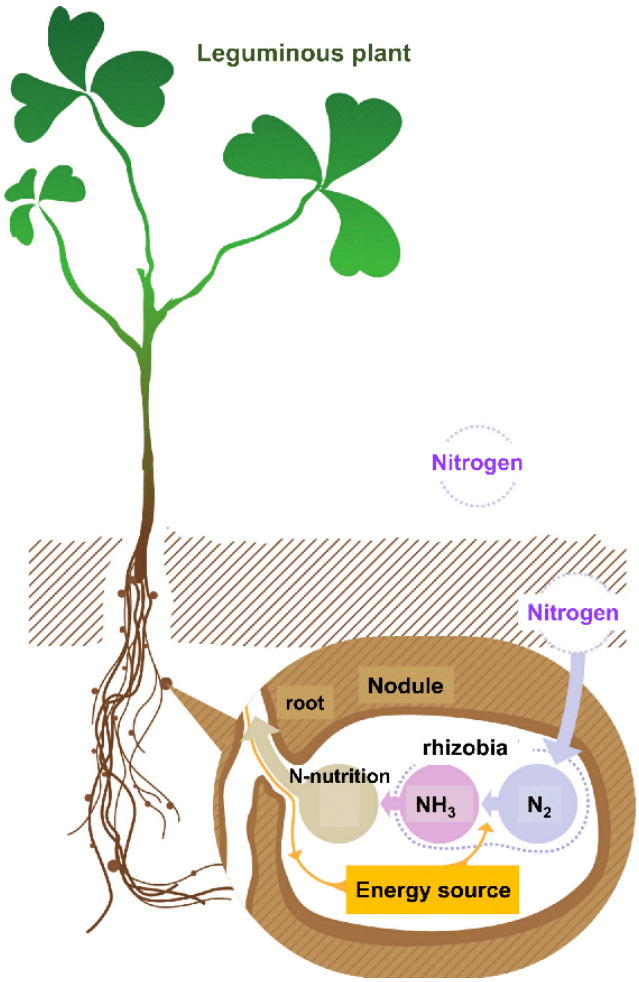

Figure 3 Symbiotic nitrogen fixation established by legumes and rhizobia.

Source: https://grad.eng.kagoshima-u.ac.jp/researcher_e/uchiumi-toshiki/.

6. The potential of legume-based intercropping on maintaining soil fertility

6.1 Increasing soil organic carbon stock

As one of the potential strategies for intensification of agroecological agriculture, legume-based intercropping systems could enhance climate change mitigation through increasing soil organic carbon (SOC) stock (Bommarco *et al.*, 2013). For both legumes and cereals, numerous studies showed that the C input to the soil through biomass residues of intercropping was greater than in monocropping systems (Cong *et al.*, 2015; Dimassi *et al.*, 2014; Tang *et al.*, 2014; Yang *et al.*, 2010). After 11 years of practicing quick-stick plant-maize intercropping, Makumba *et al.* (2006) reported that the topsoil organic carbon content was 3 kg higher than in the maize monocropping system. Similarly, large amounts of organic C and N were introduced to the soil when applying legume green-manures, cover-crops, or high vegetative residue legume species (Jensen *et al.*, 2012; Nesper *et al.*, 2015). Cong *et al.* (2015) also showed that strip intercrops resulted in higher levels of SOC than monocrops after 7 years of cultivation. Organic carbon content at the topsoil (0-20 cm depth) was 12.1 ± 0.13 in mono-cropping and 12.5 ± 0.15 g kg^{-1} in intercrop systems (a 4%-difference), and 10.2 ± 0.12 comparing to 10.7 ± 0.11 g kg^{-1} at the depth of 40 cm (a 3%-difference). After a long-term investigation, Bright *et al.* (2017) confirmed that continuous camel's foot tree-based intercropping drastically increased C stock in a sandy soil after 11 years. Tang *et al.* (2014) also revealed that the C content of the rhizosphere microbial biomass was significantly higher in intercropping than in monocropping. Several studies also indicated that, through biomass residues, intercropping systems enhance greater C input into the soil compared to monocropping (Ghosh *et al.*, 2006; Li *et al.*, 2011), thus intercropping can support the accumulation of organic matter and mitigating atmospheric CO_2 rise. Cong *et al.* (2015) explained that the concurrent increases of C and N in the soil suggested the probability of positive relationship whereby N sequestration and C sequestration enhance one another. The organic N will be remobilized regularly and contributed to greater biomass yield, resulting in higher C sequestration and N retention.

6.2 Enhancing nitrogen supply in crop production

Nitrogen plays a vital role in every agricultural system. Various authors have confirmed that N cycling can be enhanced to effectively decrease field losses through intercropping practice and increase soil N content in later cultivating seasons (Coombs *et al.*, 2017; Delgado *et al.*, 2010; Spehn *et al.*, 2002). After a 7-year experiment, Cong *et al.* (2015) found that intercropping systems achieved 11% ± 1% greater N stock in the topsoil, equivalent to a difference of 45 ± 10 kg N ha^{-1} $year^{-1}$ as compared to monocropping. According to numerous authors, legume species are found to allocate significant amounts of N to following crops, which may make them the ideal choice for N supply purpose (Gaudin *et al.*, 2013; Ketterings *et al.*, 2015; Thilakarathna *et al.*, 2015). Kumwenda *et al.* (1996) indicated that legumes in intercropping systems could contribute considerable amounts of N to the subsequent crop through residual biomass of the leaf fall, even if the seeds are harvested. Similar results were reported by Lunnan (1989) and Smith *et al.* (2016). Further, Rowe *et al.* (2005) reported that the organic residue N input from above-ground parts to the soil in intercropping systems was 15-20 times higher than in sole cropping. Legume species can enrich soils by BNF, converting it to forms that are available for plant uptake, thus replacing N fertilization wholly or in part (Dwivedi *et al.*, 2015). BNF is the key source of N in legume-cereal systems when N fertilizers are limited or their prices are high comparing to the economic status of smallholders (Fustec *et al.*, 2011). After a 3-year study of sorghum-pigeon pea intercropping, Adu-Gyamfi *et al.* (1997) reported that there is a high potential for boosting BNF without necessarily increasing the amount of fertilizer N use. Similarly, alfalfa is recognized as a most prevalent forage crop that may decrease N fertilizer input demands of the subsequent crop (Entz *et al.*, 2002; Hoeppner *et al.*, 2006), and increase N stock by over 120 kg N ha^{-1} (Kelner and Vessey, 1995). According to Ghosh *et al.* (2007), legumes can contribute considerably to N nutrition of maize in intercropping systems, whereby intercropped maize required only 60 kg N ha^{-1} from fertilizers while maize monocrop required up to 120 kg N ha^{-1}. A number of studies have also shown that, together with the movement of N from legumes to cereals, intercropped legumes facilitate the absorption of soil mineral N by cereals (Bedoussac *et al.*, 2017; Stern, 1993; Xiao *et al.*, 2004). Legume-cereal intercropping cultivated in

wide rows can even reduce NO_3 leaching, thus supporting the ability of soil N supply (Corre-Hellou *et al.*, 2011; Crews and Peoples, 2004).

6.3 Increasing crop P nutrition

P is one of the most important nutrients for plants, which plays important role in several key plant structure compounds, numerous biochemical reactions, and many primary functions. However, the total P stock of topsoil is generally quite low and soil available P is decreased easily by P fixation, soil erosion, and crop removal. Numerous studies have shown the enhancement of P nutrition for crops in legume-based intercropping (Hallama *et al.*, 2018; Hassan *et al.*, 2013; Rose *et al.*, 2011). Legume residues in intercropping systems can be mineralized and consequently available P can be transferred to main crops (Horst *et al.*, 2001). The exploration of legume root systems (e.g., *Lupinus* or *Mucuna* species) allows main crops to acquire available P from a deeper and larger soil volume (Dube *et al.*, 2014; Lambers *et al.*, 2006). Pypers *et al.* (2007) reported that incorporation of velvet bean residues considerably increased soil P availability, hence, significantly increased maize yield as compared to maize monocropping system. Moreover, some legumes (e.g., white lupin or pigeon pea) are known for their ability to mobilize and mineralize P by releasing large amounts of carboxylates, phosphatase enzymes, or organic acids by their root exudates (Hassan *et al.*, 2013; Hinsinger, 2001). According to Hallama *et al.* (2018), the P acquiring mechanisms of legumes may be supported by the interactions with different soil microorganisms. Gaind and Nain (2015) indicated that a higher number of phytate-mineralizing and P-solubilizing fungi were found in the rhizospheric soil of legume species comparing to cereal crops. Some studies also showed that the P concentration and dry biomass of legumes were significantly higher with mycorrhizal inoculation (Li *et al.*, 1991; Turk *et al.*, 2006). In addition, legumes may enhance the soil microbial activities in the main crop stages, thus increasing the capacity of the main crops in mining P from the soils (Hallama *et al.*, 2018; Lehman *et al.*, 2012).

6.4 Reducing soil erosion and drought stress and increasing soil moisture

Legume-based intercropping is an excellent way to reduce soil erosion, increase soil moisture and drought tolerance, which efficiently improves soil quality for agricultural sustainability (Bedoussac *et al.*, 2017). Such cropping systems that leave the soil bare and prone to be washed away by heavy rains result in the erosion of the fertile topsoil. The shallow roots can penetrate further to absorb water and nutrients and help to decrease soil loss by binding soil particles at the surface (Dwivedi *et al.*, 2015). Intercropping can also reduce soil erosion by protecting bare soil from raindrops that result in increased surface runoff (Seran and Brintha, 2010; Siddoway and Barnett, 1976). Soil erosion control is one of the clearest benefits of conservation agriculture. Numerous studies have shown that the use of legumes as ground cover effectively decreased runoff and soil losses in cassava cropping system (Amanullah *et al.*, 2007; Duchene *et al.*, 2017; El-Swaify *et al.*, 1988). Trung *et al.* (2013) showed that when cassava was intercropped with peanut, the amount of eroded soil was reduced by 63.2 to 80.2% compared to the farmer's traditional practice of monocropping. Kariaga (2004) concluded that while soil loss under maize sole cropping is 35% of that of bare ground, under maize intercropped with cowpeas soil loss is reduced 2.5-fold. According to Zougmore *et al.* (2000), sorghum-cowpea intercropping can lessen water runoff by 20-30% and 45-55% compared to sorghum and cowpea monocrop, respectively, while eroded soil was decreased in intercropping system at least 50% comparing to sole crops. Regarding soil water status, several authors indicated that when aerating the deeper layers of soils, the shallow roots in multi-cropping systems help to improve the water holding capacity, as well as decrease soil surface exposure that lessens soil moisture loss (Locke and Bryson, 1997; Mungai *et al.*, 2016). For instance, Makumba *et al.* (2006) revealed that soil moisture was significantly greater in quick stick–maize system than in maize sole crop at 0–120 cm soil layers. After a 3-year experiment, Sun *et al.* (2018) reported that more water was consumed by both crops causing the water use efficiency of intercropping systems to be 7% lower than that of the monocropping systems.

6.5 Enriching useful soil microorganisms

Intercropping of legume species with other crops can promote biodiversity by delivering numerous beneficial insects and soil microorganisms that would not exist in mono-crop systems (Duchene *et al.*, 2017). Stable agroecosystems must be diverse, containing more soil biota and activities by having various kinds of plants, arthropods, mammals, birds, and microorganisms (Christoffoleti *et al.*, 2007; Roscher *et al.*, 2013). Some studies reported that legumes can promote arbuscular mycorrhizae fungi (AMF) activities or enhance AMF abundance and diversity (Pivato *et al.*, 2007; Siqueira *et al.*, 1991). Alvey *et al.* (2003) revealed that millet/cowpea and maize/groundnut intercropping systems had significant effects in increasing the total number of unique bacterial groups per plant species than in mono-crop systems. A number of intercropping studies have achieved similar results, in which microbial biomasses and activities varied from those in monocropping (Latati *et al.*, 2014; Song *et al.*, 2007; Tang *et al.*, 2014). According to Schelud'ko *et al.* (2009), those results can be explained by the formation and release of lectins by legume species that enhance root colonization and the Phyto-beneficial activity of Plant Growth-Promoting Rhizobacteria (PGPR). Likewise, it is theorized that intercropping systems build favorable environments for belowground interactions and that legume species can secrete numerous flavonoids, which attract and stimulate the activity of beneficial microorganisms (Marschner *et al.*, 2001; Morris *et al.*, 1998; Teplitski *et al.*, 2000). The legume rhizospheres also support microorganism activities through increased soil organic matter (SOM) from legume residues, which encourages the activities of bacteria communities (Blagodatskaya and Kuzyakov, 2008; Fontaine *et al.*, 2003; Malézieux *et al.*, 2009). This implies that intercropping systems can significantly boost bacterial SOM mineralization for the profits of both species.

Numerous authors have indicated that the diversification of legume-based intercropping systems positively affects the diversity of soil fauna (Birkhofer *et al.*, 2011; Elba *et al.*, 2014; Liu *et al.*, 2016). Blanchart *et al.* (2006) discovered that maize-Mucuna intercropping enhanced the growth of different species such as earthworms, millipedes, centipedes, Diptera larvae and Isopoda. This enhancement might be from

the accumulation of organic matter, which is the basic environment for fauna society, especially for the ecosystem engineers and the litter transformers. According to Kautz *et al.* (2006) and Sileshi and Mafongoya (2006), improved soil cover by legumes can also lead to better protection for soil fauna from water stress and high temperature. Intercropping practices also add more leaf and litter biomass to the soil, which can favour the growth of earthworms, millipedes and termites (Sileshi *et al.*, 2008). The carbon to nitrogen ratio plays an important role and can influence the macrofauna abundance. Intercropping with pigeon pea, which has a slow decomposition rate, provided a greater amount of food for the detritivores than that in maize sole cropping. Moreover, the modification of the vegetation structure influences the composition of the soil community because the plant's diversity can supply food for a large diversity of consumers (Li *et al.*, 2014).

In general, intercropping increases the species richness without increasing the species abundance because it promotes pest biological controls (Elba *et al.*, 2014). Some studies also highlighted the suppressive effect on diseases of the soil biodiversity increased by intercropping practice. For instance, the existence of Mucuna can limit the growth of phytophagous nematodes that have adverse impact on crops (Blanchart *et al.*, 2006). In South Africa, a reduction of sugar cane root infestation by nematodes such as *Meloigogyne javanica* was reported in intercropping fields with sugar bean (Berry *et al.*, 2009). The study of the nematode communities by Siddiqui and Alam (1987) showed that where vegetables are intercropped with marigold plants, a decrease in plant parasitic nematodes has been observed. In China, when wheat was planted in inter-rows with jujube trees, the percentage of herbivore nematodes was decreased (Liu *et al.*, 2016). Such practices lead to a more balanced nematode community often dominated by less damaging species. A meta-analysis by Tonhasca Jr (1993) showed that in 52-70% of cases, crop diversification conferred a decrease in pest density. This is because diversified field confused the insects' ability to find their host plants in particular if the herbivore species were oligophages. The International Centre of Insect Physiology and Ecology (ICIPE) in Kenya established the "Push-Pull" technique for mitigating the negative impact of stem borers. According to Khan *et al.* (2009), the "Push-Pull" platform technology includes two plant components, the "Pull" and the "Push" plants.

The “Pull” plants are established in the border around the main crop fields in order to emit chemicals to attract invading adult moths and the borers’ natural enemies. Whereas, the “Push” ones are planted between the rows of the main crop so they will release the substances called kairomones which can keep stemborer moths away from the main crop. The “Pull” plants are often low-growing plants so they do not compete with the growth of the main crop and have some advantages of reducing runoff, improving soil organic matter content and nitrogen fixation. In maize-based systems intercropped with the legume Desmodium (*Desmodium uncinatum*) and Nappier grass, known to have stem borer’s attractive ability, Desmodium wades off the female’s moths (push) which will then be attracted by the Nappier grass (pull). Desmodium changes the microclimate by improving the soil fertility through nitrogen fixation, enhance soil arthropods, increase the organic matter and make the environment less favourable for the stem borers to eventually improve grain yield (Khan *et al.*, 2009; Midega *et al.*, 2006). Even if it is hard to quantify the response of soil fauna to human management, connecting biotic interaction with agricultural techniques seems to be promising. Soil fauna should be considered in the soil management policy to promote a sustainable agriculture through intercropping.

MATERIALS AND METHODS

Materials

1. Plant materials

1.1 Cassava variety: SA21-12.

Cassava variety SA21-12 and cowpea local variety (*Dau Den Xanh Long*) were used in this study. In 2012, Root Crop Research and Development Center released the promising line SM2354-4 from the national trial sets and named SA21-12. This variety has good plant architecture, high root yield (30-35 tons ha^{-1}), high starch content (about 28%), high dry biomass (about 39%) and low cyanogenic percentage (Kim *et al.*, 2017).

1.2 Cowpea: local variety (*Dau Den Xanh Long*).

Dau Den Xanh Long is a local cowpea variety that was selected many years ago in Vietnam and had been used in the study location since 2005. The seeds can be harvested in about 100–120 days from the planting date. This cowpea variety has a main stem height of about 50–100 cm, purple flower, black coat and green entrails. The average seed yield is 300–400 kg ha^{-1} (Aye and Howeler, 2012).

2. Experimental materials and equipment

- Organic fertilizer (Farmyard manure – FYM); NPK+S fertilizer (5:10:3:8); Urea (46% N); Potassium fertilizer (60% K_2O).
- Balance, oven, wire tags, plastic and paper bags, nylon mesh bags, spades, scissors, GPS devices, SPAD-502 chlorophyll meters, 1.5 ml Eppendorf tubes, Fill McCartney bottles, cooling boxes, sterile plastic pestles, plastic plates.
- Harvesting and yield data collection materials.
- Soil and plant sampling and analyzing materials.

3. Chemicals

3.1 General chemicals

Sterile distilled water
70% ethanol
3.3% NaOCl
40% glycerol
K_2HPO_4
$MgSO_4.7H_2O$
NaCl
Yeast extract
Mannitol
Agar
KNO_3

3.2 Chemicals for nutrient solution [modified from Broughton and Dilworth (1971)]

K_2SO_4 0.5 M
KOH
KH_2PO_4 1M
$CaCl_2$ 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
EDTA ($C_{10}H_{16}N_2O_8$) 0.08 M

3.3 Chemicals for mycorrhizal infection analysis

Black ink (brand Sheaffer)

3% H_2O_2

KOH

25% NH_4OH

White vinegar (5% acetic acid)

Climatic Data

Climatic data for the period of 8 years (2011–2018) were collected at the location where experiment was carried out. The average values of monthly precipitation and temperature were shown in Figure 4 below.

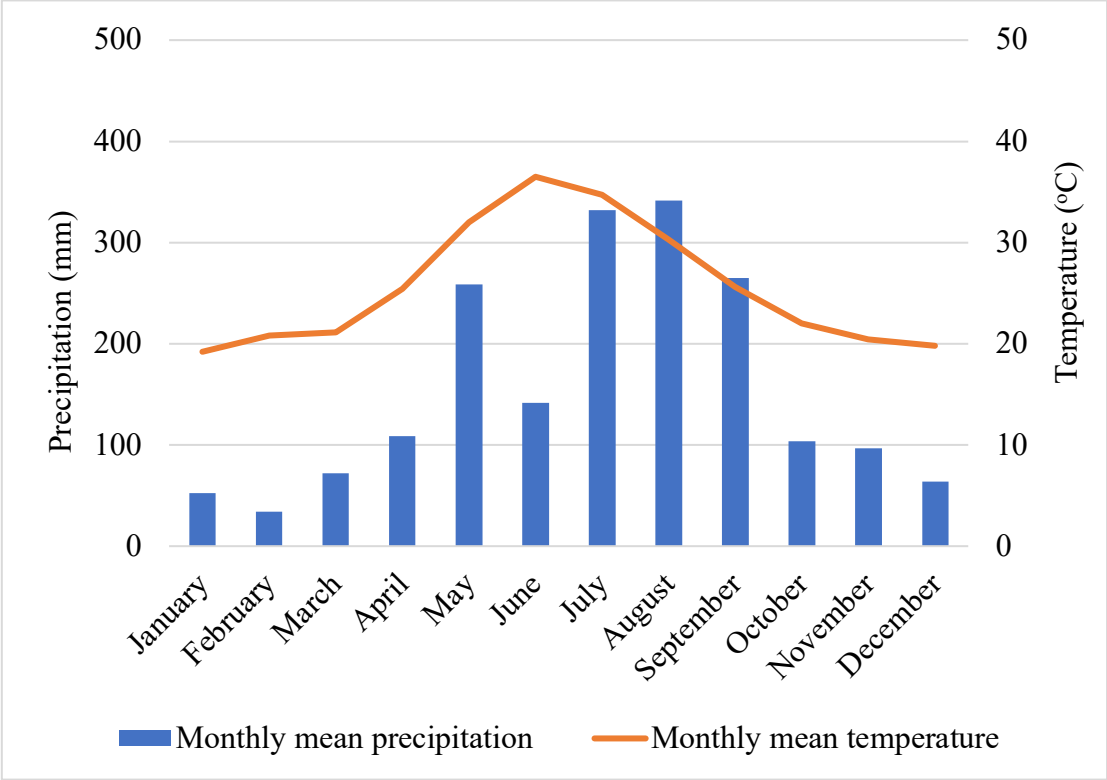

Figure 4 Mean of monthly precipitation and temperature of the study site during 2011-2018.

Methods

1. Survey the expansion of cassava-cowpea intercropping system from 2017 to 2018 and assess the natural nodulation of cowpea at different locations in Van Yen and Van Chan districts, Yen Bai province

In 2017, to assess the natural nodulation of cowpea and collect cowpea nodules for isolation of native rhizobia, an investigation was conducted at two locations, Van Yen and Van Chan districts, Yen Bai province, Vietnam. There was a cassava-cowpea intercropping system in Van Yen district and a maize-cowpea intercropping system in Van Chan district. In Mau Dong commune, Van Yen district, there were 5 selected farms (MD1 to MD5) with a total area of 3.7 hectares. In Son Thinh and Cat Thinh commune, Van Chan district, there were 7 farms (4 in Son Thinh and 3 in Cat Thinh, respectively) and the total area is 1.5 hectares. The natural nodulation of cowpea intercropping with maize in Son Thinh and Cat Thinh was investigated during 2 seasons of maize.

Nodule samples were collected from each farm in both Van Yen and Van Chan district. At each farm, after determining the sampling lines and points (Figure 5A, 5B and 5C), the GPS coordinates and other information (date, name of farmer, number of samples, slope degree and characterization) were collected. At the mid-flowering stage, cowpea plants were dug up by the spades, the root parts were carefully removed the soil around and then smoothly cleaned in 2-3 basins of water. The effective nodules (with reddish/pink color) of each cowpea plant were counted. After that, the nodules were detached from cowpea roots by the small scissors and dip in 70% ethanol solution for 10 seconds. The nodules from each farm were put into a labelled Fill McCartney bottle occupied with 10ml of 40% glycerol. All the bottles were kept in the cool box with ice and transferred to the laboratory for further analyses.

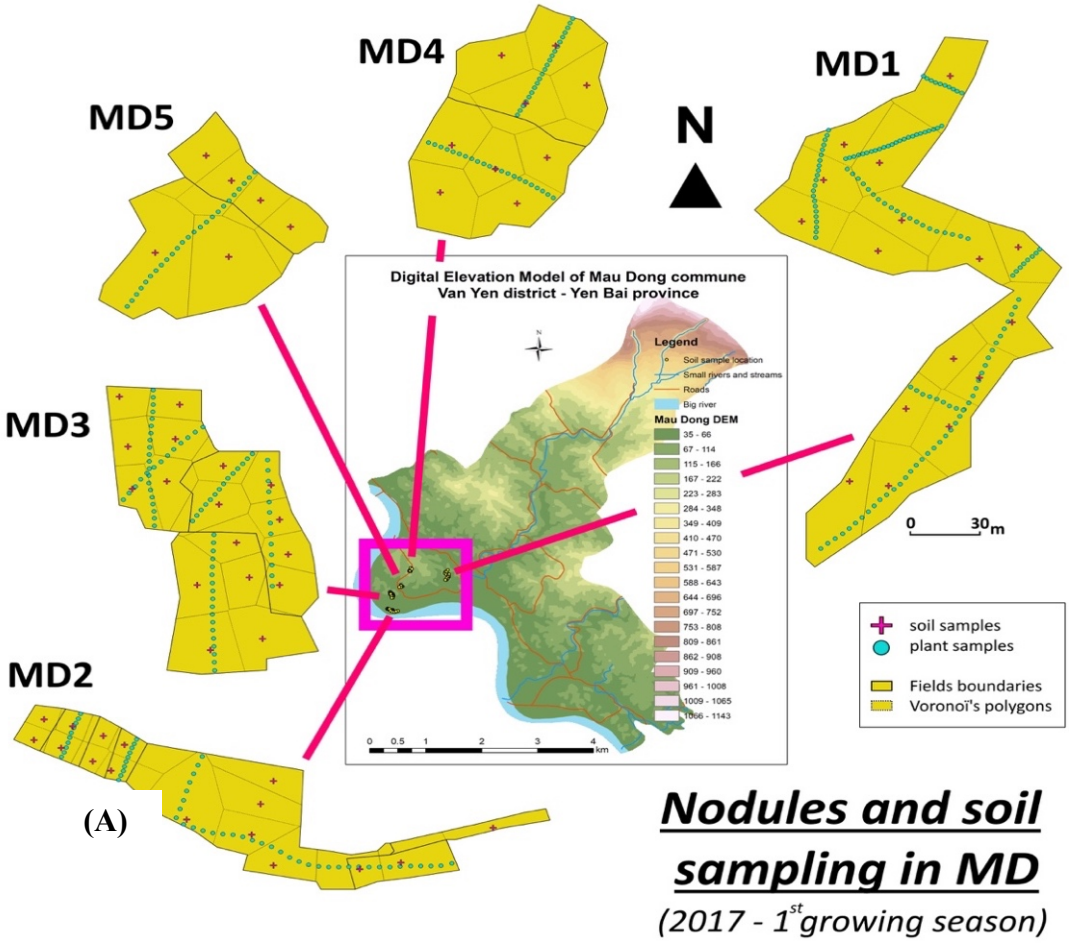

Figure 5 A) Sampling map at Mau Dong commune, Van Yen district, Yen Bai province in 2017. MD1 – MD5: Mau Dong farm 1 to farm 5.

B) Sampling maps at Son Thinh commune, Van Chan district, Yen Bai province in 2017. ST1 – ST4: Son Thinh farm 1 to farm 4.

C) Sampling map at Cat Thinh commune, Van Chan district, Yen Bai province in 2017. CT1 – CT3: Cat Thinh farm 1 to farm 3.

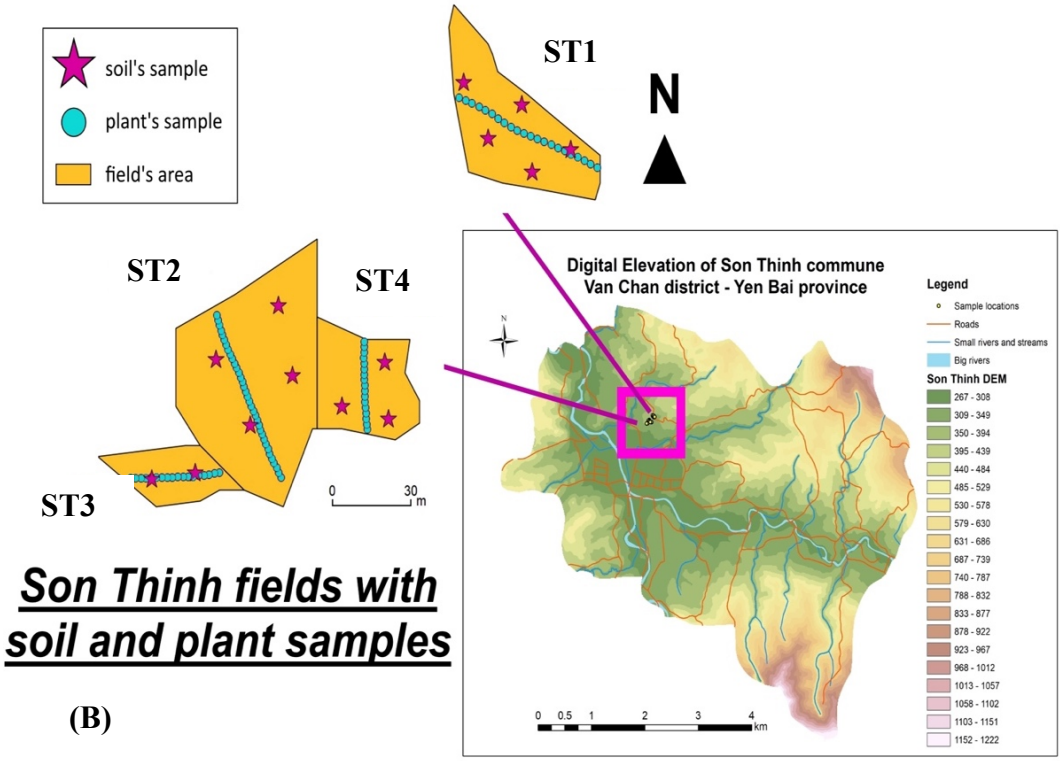

Figure 5 (Continued)

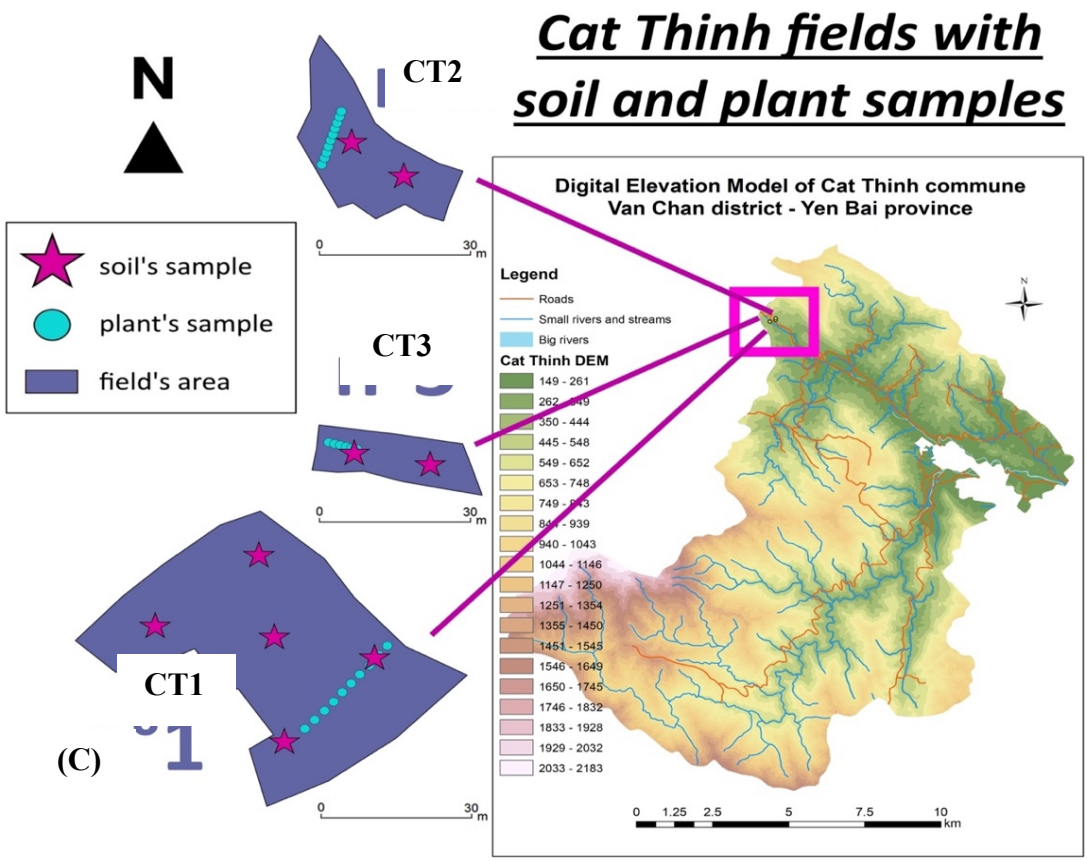

Figure 5 (Continued)

In Van Yen district, Yen Bai province, intercropping systems of cassava with legumes was started practicing a long time ago. However, the inclusion of cassava-cowpea intercropping has just been widely promoted since 2016 by the projects from CIAT-Asia. Muoi village in Mau Dong commune is one of the most productive cassava areas of Van Yen district and the smallholder farmers in this village have participated in this conservation agricultural practice since the beginning of the projects. Muoi village has a total land area of over 200 ha and 95 households. The land capacity is about 5,000 square meters per household. In order to identify the expansion and importance of such a cropping system, we carried out the investigation in regard to the cassava cropping systems being practiced by the local farmers from 2017 through 2018. From all the farms in Muoi village, the total number and area of cassava farms, the number and area of cassava-cowpea intercropping farms, and their correlative percentages were collected.

2. Experiment I - Isolation and screening of native rhizobia strains nodulating cowpea under field conditions and greenhouse condition

2.1 Isolation of native rhizobia strains from cowpea nodules

Isolation of native rhizobia was done at The Common Microbial Biotechnology Platform (CMBP), the International Center for Tropical Agriculture (CIAT-Asia), Hanoi, Vietnam. Ten nodules from each farm were randomly selected from the nodule bottles previously collected in the fields. Nodules were surface disinfected with 70% ethanol for 30 sec and 3.3 % $Ca(OCl)_2$ solution for 2 min. Then the nodules were rinsed three times with sterile distilled water. Each nodule was put in a sterile 1.5 ml Eppendorf tube, 150 µl of sterile distilled water was added and the nodule was crashed using a sterile plastic pestle. After that, nodule suspensions were streaked onto yeast extract mannitol agar (YEMA - 0.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4.7H_2O$, 0.1 g/l NaCl, 1 g/l Yeast extract, 10 g/l Mannitol, 15 g/l Agar) (SOP-MI03/LH-V01). YEMA cultures were placed in an incubator at 28^oC which is the optimized temperature for rhizobia growth. The isolated colonies on YEMA were assessed by macroscopic observation (SOP-MI06/LH-V01) and purified three times on the same media. Pure rhizobia cultures were finally cultivated in liquid media (SOP-MI04/LH-V01) and stored in 20% glycerol at -80^oC for subsequent analyses.

Purified cultures of isolated colonies were sent to Institute of Genome Research (Hanoi, Vietnam) for DNA extraction, PCR and 16S rADN sequencing. The primers used were 27F and 1492R (Guimarães *et al.*, 2012; Lane, 1991). The sequence data was then submitted for comparison with the National Center for Biotechnology Information (NCBI) database using BLAST (Basic Local Alignment Search Tool) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Based on the sequencing analyses of 16S rADN gene, species richness and diversity indices by Shannon and Simpson were determined in each study location (Magurran, 2013).

Shannon's diversity index (H'): $H' = -\sum (Pi \times \ln(Pi))$.

Where Pi is the proportion of individuals belonging to species i; ln is the natural logarithm.

Simpson's index of diversity (D): $D = 1 - \frac{\sum (n \times (n-1))}{N \times (N-1)}$

Where n is the number of individuals of each species; N is the total number of individuals of all species.

2.2 Inoculation experiment of native rhizobia strains under field conditions

a. Experimental design

Based on the number of native rhizobia strains had been isolated from cowpea nodules, in March 2018, an on-farm experiment was conducted to evaluate the effectiveness of native strains inoculated with cowpea in the intercropping system with cassava under field conditions in Mau Dong commune, Van Yen district, Yen Bai province. The field location is shown in Figure 6. In order to assess the interaction effect of different native rhizobia from the same location (Mau Dong commune) on cowpea production, CMBP037 and CMBP054 was mixed together for inoculation. This experiment was composed of three treatments as follows: non-inoculated control (referred to as Non_I) (14 farms), inoculation of a mixed inoculant containing rhizobium strains CMBP037 and CMBP054 in the mix ratio of 1:1 in volume (referred to as CMBP037+054) (5 farms), and inoculation with rhizobium strain CMBP065 isolated from Cat Thinh commune (5 farms). Each farm was considered as one replication according to Mutsaers (1997). The slope degree of each farm was also considered and classified as: gentle slope ($\leq 5^o$); moderate slope ($> 5 - < 15^o$); steep slope ($\geq 15^o$) (Jahn *et al.*, 2006).

Figure 6 Field experiment location in Van Yen district, Yen Bai province.

Rhizobia inoculant cultures were prepared by picking single purified colonies of the individual strains from plates 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) (SOP-MI03/LH-V01). Each colony was transferred into 50 ml of fresh YEM broth in 200 ml Erlenmeyer flasks and incubated at 28°C at a speed of 200 rpm, for 2 days for *Rhizobium* species and 4 days for *Bradyrhizobium* species. Before inoculating in the field, direct cell count for each inoculum was done using spread plate method (SOP-MI10/LH-V01) to ensure that at least 10^6 rhizobia cells ml^{-1} was reached. Inoculants were transported to the fields in cooler boxes and applied at a rate of 50 ml per kg of seeds. Before inoculating, cowpea seeds were surface sterilized by soaking in 3.3% NaOCl solution for 5 minutes and rinsed thoroughly several times with sterile distilled water. All inoculation was done just before sowing under the shade to maintain the viability of bacterial cells. Seeds were allowed to air dry for about 30 minutes and then planted. Seeds were immediately covered by soil after sowing. Cowpea was intercropped with cassava in which one row of cowpea was planted between two rows of cassava at a density of 10,000 cowpea plants ha^{-1}.

b. Sampling procedure and data collection

(1) Soil sampling

Just before conducting the experiment, soil samples at 0-20 cm depth were collected from ten random points in each plot using an auger. Soil samples from each plot were pooled into a composite sample of approximately 0.5 kg and analysed for nutrient composition (pH H_2O, organic content (OC), total N, available P_2O_5, available K_2O and soil texture) at Soils and Fertilizers Research Institute (Hanoi, Vietnam).

(2) Cowpea nodulation

At the mid-flowering stage (7 weeks after sowing), 10 cowpea plants positioned on the 2 diagonal lines of each farm were harvested after collecting

their GPS coordinates. Cowpea roots were washed gently 2-3 times by water and the number of nodules per plant was recorded.

(3) Cowpea dry biomass

Shoots were cut at 1 cm above soil surface using a clean, sharp knife and put into labelled paper bags. Roots were kept separately in labelled paper bags. Shoots and roots were oven dried at 60^{o}C for 2 days to measure shoot and root dry weights (g plant^{-1}).

(4) Shoot total N

Oven-dried shoots were analysed for total N content (%) according to Kjeldahl method (Bremner, 1996) at Soils and Fertilizers Research Institute (Hanoi, Vietnam).

(5) Mycorrhizal infection rate of cassava and cowpea roots

Oven-dried roots of cassava and cowpea were analysed and assessed for arbuscular mycorrhizal infection rate (%) (SOP-MI16/LH-V01). The roots from each plant were put in test tubes filled with tap water and stored at room temperature. After 24 hours, the water was removed and 10% KOH was added to cover the roots. The tubes were placed in the breaker and incubated at about 90^{o}C for 30 minutes. After that, the KOH solution was removed from the tubes and a fresh 10% KOH solution was added. The tubes were incubated again at 90^{o}C. After 30 minutes, the tubes were emptied and rinsed with tap water 3 times. After the last rinse, the clearing solution (1 NH_4OH:9 H_2O_2 in volume) was added to cover the roots and the tubes were incubated at room temperature. After 30 minutes, the tubes were emptied and rinsed with tap water 3 times. After the last rinse, the black ink solution was added to cover the roots and the tubes were incubated again in the breaker at 90^{o}C. After 30 minutes, the tubes were emptied and rinsed with pure white vinegar (5% acetic acid). The white vinegar was removed about 90% from the tubes and the tap water was added

to rinse the roots the 2nd time. The solution in the tubes was removed about 90% again and the tap water as added to rinse the roots the 3rd time. After the last rinse, the tubes were added with tap water and kept at 4°C overnight for further assessment.

For assessment of mycorrhizal infection rate, the roots were put in a petri dish. fifteen fragments (about 1 cm long) of the roots were cut and placed on the slide. The root fragments were gently squeezed between the slide and the coverslip using the forceps and the presence of AMF was recorded by a microscope. The intensity of infection was scored from 1 to 5 following Figure 7 below.

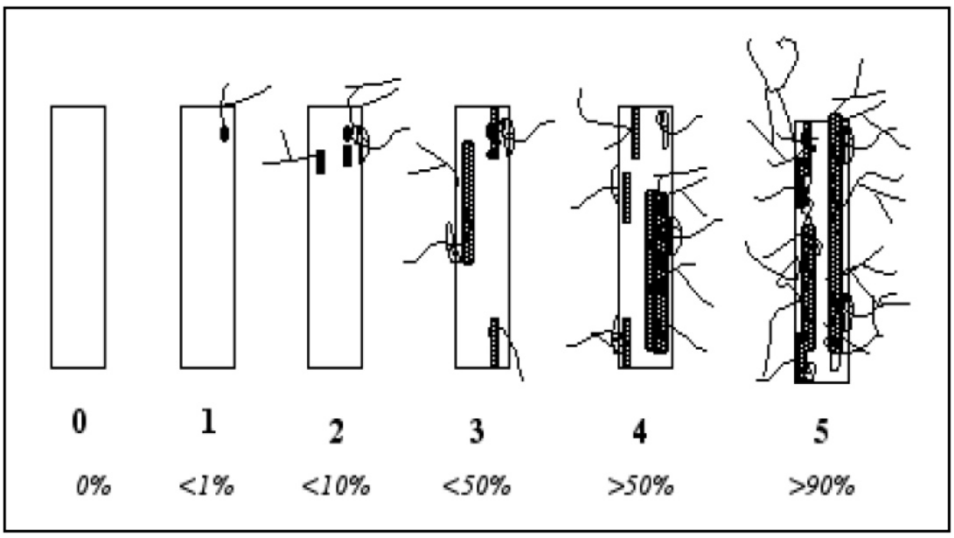

Figure 7 Mycorrhizal infection scoring guideline.

Source: SOP-MI16/LH-V01.

The intensity of the mycorrhizal colonization in the root system (M %) was calculated using the equation as follows:

M % = (95 x number of fragments scored "5" + 70 x number of fragments scored "4" + 30 x number of fragments scored "3" + 5 x number of fragments scored "2" + 1 x number of fragments scored "1") / (total number of fragments)

(6) Cowpea yield

At the maturity stage (9 weeks after sowing), 3 random areas of 5 m^2 from each farm were harvested and total seed yield (kg ha^{-1}) was relatively calculated.

(7) Cowpea nodule occupancy analysis by RFLP

A total of 173 nodules from the field inoculation experiment were analysed. Nodules were surface sterilised with 70% ethanol and 3.3% NaOCl solution then rinsed thrice with sterile distilled water. Each nodule was crushed in a sterile 1.5 mL centrifuge tube with 150 µL of sterile micro pure water using a sterile plastic pestle. The nodule suspension was then used for DNA extraction using FastDNA™ Spin Kit (MP Biomedicals, USA) (SOP-BM04/LH-V01).

A fragment of the intergenic region between the 16S and 23S rDNA [930—1050 base pairs (bp)] was amplified by polymerase chain reaction (PCR) using two primers: FGPS 1490-72; 5'-TGCGGCTGGATCCCCTCCTT-3' (Normand *et al.*, 1996), and FGPL 132-38; 5' CCG GGTTTCCCCATTCGG-3' (Ponsonnet and Nesme, 1994). PCR amplification was performed using the following program: initial denaturation for 5 min at 94°C, 35 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 58°C, extension for 30 sec at 72°C, and final extension for 7 min at 72°C (SOP-BM05/LH-V01). PCR amplification was carried out in a 25 µL reaction volume containing 2 µL of total DNA extract, 1.0 µL of each primer from a solution at 10 pmol μL^{-1}, 8.5 µL of sterile distilled water, and 12.5 µL of commercial master mix. PCR amplified DNA was visualized after electrophoresis on a 1.5% pre-stained agarose gel (w/v) (SOP-BM06/LH-V01). *MspI* was the only restriction enzyme used and it provided the required resolution for this study. Aliquots (10 µL) of PCR products were added to the total mixture volume of 21 µL including 1 µL of a 5 U *MspI* restriction endonuclease, 2 µL restriction buffer, and 18 µL of sterile distilled water and incubated for 2 h at 37°C (SOP-BM07/LH-V01). The restriction fragment length polymorphism (RFLP) profiles were then visualised by gel electrophoresis on a 3% pre-stained agarose

gel (w/v). The gels were run at 100 V for 3 h then visualized under UV transillumination and photographed using gel documentation system. Strains with identical restriction fragment profiles (in individual fragment size and number) were classified into the same intergenic spacer (IGS) group.

2.3 Screening of native rhizobia strains under greenhouse condition

a. Experimental design

To screen the performance of the isolated native rhizobia strains nodulating cowpea, a pot experiment was established in a greenhouse at Vietnam National University of Agriculture (VNUA), Hanoi, Vietnam during June-August of 2018. In this experiment, there were a total of 23 treatments: uninoculated plant without N application (referred to as negative control), uninoculated plant with applied N using KNO_3 at a rate of 480 mg N pot^{-1} (referred to as positive control or N+), and inoculation separately with 21 native rhizobia strains. The experiment was arranged in a completely randomized design (CRD) with 5 replications. The isolated native rhizobia strains are shown in Table 6.

Rhizobia inoculant cultures were prepared by picking single purified colonies of the individual strains from plates 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). Each colony was transferred into 50 ml of fresh YEM broth in 200 ml Erlenmeyer flasks and incubated at 28^{o}C at a speed of 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 spread plate method (SOP-MI10 LH-V01) to ensure that at least 10^6 rhizobia cells ml^{-1} was reached before inoculation in the greenhouse.

Cowpea seeds were surface sterilized by soaking in 3.3% NaOCl solution for 5 minutes and rinsed thoroughly several times with sterile distilled water. Surface sterilized seeds were immersed in water for 1 h to initiate germination, and

afterwards placed in petri dishes with moistened sterile cotton wool for germination (in a growth chamber at 28°C in the dark for 24 h). Three pre-selected healthy seeds of uniform size were chosen and sown in plastic pots (12 cm diameter and 16 cm length). Each pot was sterilized with 70% Ethanol and 1.3 kg of sterilized sand was added followed by addition of 150 ml of distilled water in preparation of sowing. Five drainage holes were made in the bottom of each pot.

Four days after sowing (DAS), 3 ml of the inoculant was added at the base of each seedling (1 ml per seedling) for each pot. 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 two 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). 140 ml of distilled water was added in alternating days with the nutrient solution.

b. Sampling procedure and data collection

(1) Chlorophyll content

Cowpea plants were harvested at the flowering stage (6 weeks after sowing). Before harvesting, chlorophyll content of the youngest fully developed cowpea leaves was measured by Soil Plant Analysis Development (SPAD) index using SPAD-502 chlorophyll meter (Minolta corporation, Ltd., Osaka, Japan).

(2) Nodulation and nodule dry weight

Cowpea roots were washed gently and the number of nodules per plant was counted. The nodules from each plant were separated from the roots and kept in labelled paper bags. The nodules were then transferred to the laboratory and oven dried at 60°C for 2 days before measuring nodule dry weight (g plant^{-1}).

(3) Cowpea fresh and dry biomass

Cowpea shoots were cut at 1 cm above the surface using a clean, sharp knife and measured for shoot fresh weights (g plant^{-1}). Root fresh weights (g plant^{-1}) were also measured. Shoots and roots were kept separately in labelled paper bags and oven dried at 60^{o}C for 2 days before measuring dry weights (g plant^{-1}).

(4) Symbiotic efficiency

Symbiotic efficiency (%) of different native rhizobia strains were calculated following Aynalem *et al.* (2018):

SEF (%) = (shoot dry weight of inoculated plants/shoot dry weight of positive control plants) x 100.

SEF was classified as: highly effective > 80%; effective = 50 to 80%; low effective = 35 to 50%; ineffective < 35%.

(5) Shoot total N content

Shoot total N content (%) of oven-dried shoots were determined by Kjeldahl method (Bremner, 1996).

3. Experiment II – On-farm soil erosion experiment

3.1 Experimental site and design

Experiment II was carried out in Mau Dong commune, Van Yen district, Yen Bai province in March 2018. The study location is shown in Figure 8. This site is located at a latitude from 21° 50′ to 22^{o} 12’ N and a longitude from 104° 23′ to 104^{o} 39’ E. The topography is mostly hilly areas, with a mean altitude of 150 m above sea level. At this study site, the mean annual temperature was 25.5 °C, the mean annual

rainfall was 1,860 mm and the average air humidity of 81–86% during the period of 2011–2018 (Fig. 2). The total area of the district is 138,884 ha, of which 10,890 ha is agricultural land. Cassava is cultivated in about 6,500 ha, mostly on steep sloping land ranging from 15–30^{o}. Before setting up the trial, at each plot, core soil samples were collected from 10 random points at a depth of 20 cm, then completely mixed to make a composite sample of about 0.5 kg. Soil samples were subsequently analyzed for the following properties: texture (Bouyoucos, 1927); soil organic content (OC) (Walkley and Black, 1934); pH H_2O (Jackson, 1973); available N (Subbaiah, 1956); available P by Bray II (Bray and Kurtz, 1945); and NH_4OAc-K (Jackson, 1973). Soil samples were also collected using a cylinder (100 cm^3) and analyzed for soil bulk density (Blake and Hartge, 1986). The result of soil characteristics analysis is shown in Table 3.

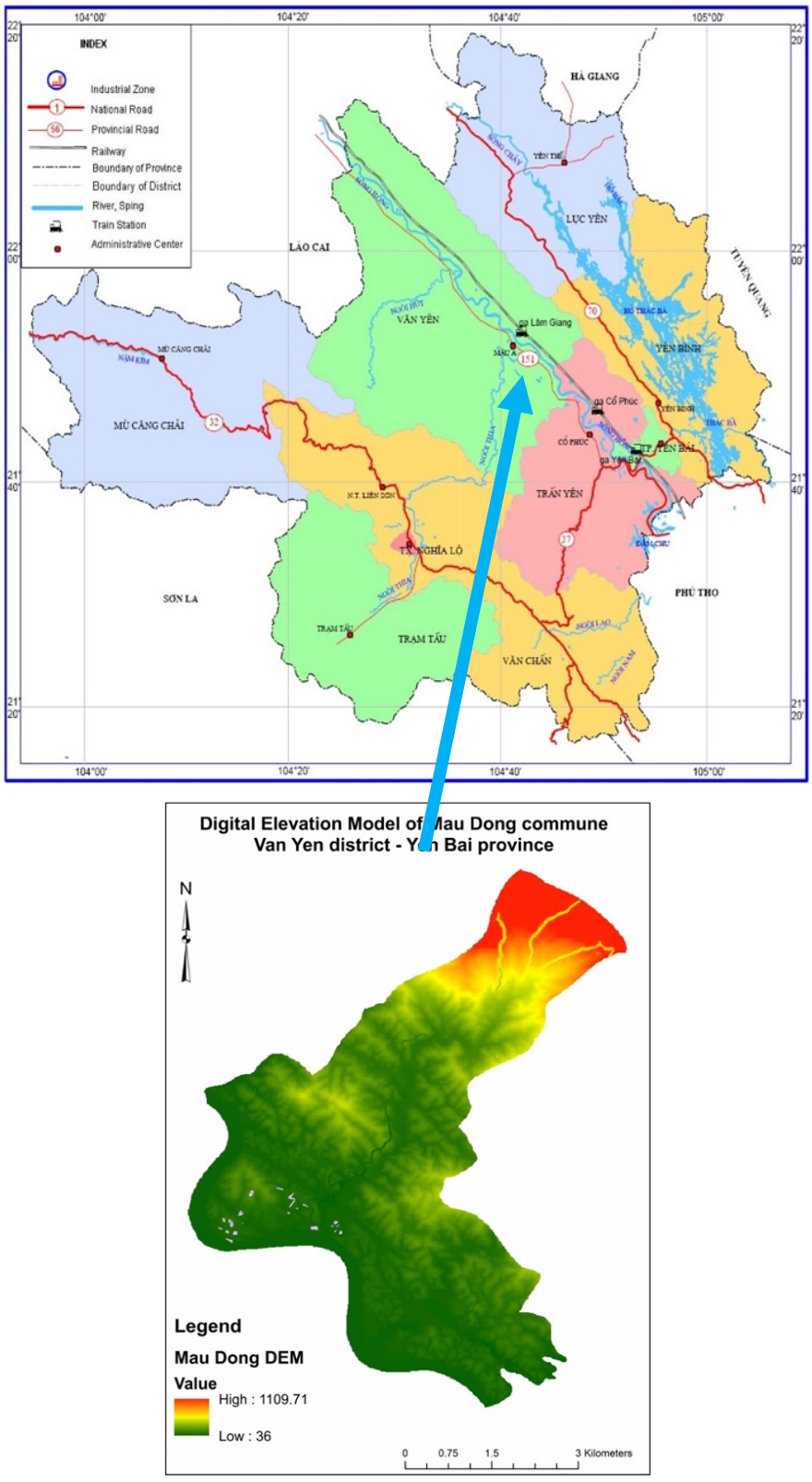

Figure 8 On-farm erosion experiment location at Mau Dong commune, Van Yen district, Yen Bai province, Vietnam in 2018.

Table 3 Initial soil characteristics (at the depth of 20 cm) of the study site.

Soil characteristics	Unit	Mean	Standard deviation
pH H_2O	-	4.09	0.14
Organic matter	%	1.43	0.13
Available N	kg ha^{-1}	240.7	4.3
Available P	kg ha^{-1}	107.4	5.7
Available K	kg ha^{-1}	112.8	2.4
Sand	%	52.05	3.28
Silt	%	23.53	1.74
Clay	%	24.42	1.55
Bulk density	(g cm^{-3})	1.45	0.03

Table 4 Treatment description of on-farm erosion trial.

Treatments	Cropping system	Slope category	Number of replications
MG	Cassava monocropping	Gentle slope	3
MS	Cassava monocropping	Steep slope	3
IG	Cassava-cowpea intercropping	Gentle slope	3
IS	Cassava-cowpea intercropping	Steep slope	3

The experiment comprised of four treatments: cassava monocropping on gentle slope (≤ 5°) (referred to as MG); cassava monocropping on steep slope (≥ 15°) (referred to as MS) (Jahn *et al.*, 2006); cassava-cowpea intercropping on gentle slope (referred to as IG); cassava-cowpea intercropping on steep slope (referred to as IS) (Table 4). The experiment was laid out in a completely randomized design (CRD) with three replications and each plot area was 80 m^2 (4.0 x 20 m). On each plot, an erosion trap

was dug with the dimension of 4.0 x 0.5 x 0.6 m (length x width x depth). Cassava density was 10,000 plants ha^{-1} (1.0 x 1.0 m). After planting cassava, 1 row of cowpea was sown between 2 rows of cassava in intercropping plot treatments. The recommended dose of fertilizer mixture (kg ha^{-1}): 100 N, 60 P, and 100 K in the form of NPK+S (5:10:3:8) was applied to all the plots.

3.2 Sampling procedure and data collection

(1) Soil covering level

On each plot, 40 days after sowing (DAS) cowpea, soil covering level (SCL) was assessed at 3 random points of 5 m^2 following the visual method described by Mansuy *et al.* (2018). Scoring was done on a grading scale of 1 to 9 based on the percentage of soil cover from 1% to 100% as shown in Table 5.

Table 5 Assessment guideline for soil covering level.

Grade	Percentage	Observation
1	1	A few residues can be seen
2	7	Less than 1 crop residue by m^2
3	15	At least 1 crop residue by m^2
4	30	30 % of soil cover
5	50	50 % of soil cover
6	70	70 % of soil cover
7	85	At least 1 hole of soil / m
8	93	It is only possible to see a very few portions of soil
9	100	Soil is fully covered by mulch

Source: Mansuy *et al.* (2018).

(2) Soil and nutrient losses

For measuring the amount of soil loss from the plot surface, an erosion trap was built along the lower edge of each plot and the eroded soil was directly weighed event-based (after every rainfall that produced runoff) in the field. The subsamples were collected and dried at 60^{o}C until constant weight to measure the percentage of moisture content. Then the annual dry soil loss (ton ha^{-1}) was calculated from the measured soil loss in the field, moisture content and the contributing area. From each erosion trap, after each time of measuring eroded soil weight, one soil sample (about 0.5 kg) was collected from a mix of five points within the trap. After the last sampling, all the individual soil samples were pooled into one composite soil sample of about 0.5 kg and the composite sample was analyzed for available N (Subbaiah, 1956); available P by Bray II (Bray and Kurtz, 1945); and NH_4OAc-K (Jackson, 1973).

(3) Crop yields

On intercropping fields, cowpea was harvested at 65 DAS. Cowpea seeds were harvested and weighed from all the cowpea plants of each plot, then seed yield (kg ha^{-1}) was relatively calculated. Cowpea biomass residues was left in the fields. At the harvesting time (10 months after planting), ten cassava plants were randomly selected from each plot, total tuber weight of each plant was weighed and cassava tuber yield (ton ha^{-1}) were measured.

(4) Economic returns

Economic returns were calculated based on the differences of input and output prices at the study location. The cost of cultivation (US$ ha^{-1}) of each treatment was accounted for fertilizers, pesticides, herbicides, cowpea seeds (intercropping fields) and the hiring charges of human labor. Gross return (US$ ha^{-1}) included the returns from selling cassava and cowpea products (cassava tubers and cowpea seeds).

Net return (US$ ha^{-1}) = Gross return - Cost of cultivation.

Benefit: Cost (B:C) ratio = Gross return/Cost of cultivation.

Statistical Analysis

Analysis of variance (ANOVA) was done using Microsoft Excel for MacOS 2016 and R version 3.4.2 (2017-09-28). The effects of treatments on the variables were compared by a Tukey's HSD test at $p < 0.05$. The correlation between different parameters were also computed at the significance of $p < 0.05$. Natural logarithm transformation was applied where necessary to protect against violation of homoscedasticity and normality of the ANOVA.

RESULTS AND DISCUSSION

1. The expansion of the cassava-cowpea intercropping system during 2017-2018 and the natural nodulation of cowpea in Van Yen district, Yen Bai province

1.1 The expansion of the cassava-cowpea intercropping system during 2017-2018 in Van Yen district, Yen Bai province

Figure 9 Numbers of farms and their areas practicing cassava-cowpea intercropping system at Mau Dong commune, Van Yen district, Yen Bai province, Vietnam during 2017-2018.

In Yen Bai province, intercropping systems of cassava with legume crops was started practicing long time ago. However, the inclusion of cassava-cowpea intercropping has just been widely promoted since 2016 by the projects of the International Center for Tropical Agriculture (CIAT-Asia, Hanoi, Vietnam). The impact of promoting an agricultural practice could be identified by the expansion rate of such practice in terms of number of fields and areas. The number and area of cassava monocropping and intercropping with cowpea is shown in Figure 9. The number of farmers practicing cassava-cowpea intercropping in 2018 (52 farmers, or 54.74% of the total cassava farms) had more than tripled to 2017 (only 16 farmers, or 16.84%). Similarly, the area of intercropping fields in 2018 (18.0 ha, or 40.00% of the total area) was 4.8 times higher than in 2017 (3.7 ha, or 8.22% of the total area). Such results revealed the high adoption level of local farmers with the inclusion of cassava-cowpea intercropping system.

1.2 The natural nodulation of cowpea in Van Yen and Van Chan district, Yen Bai province in 2017

The natural nodulation of cowpea, one of the most important parameters for field measurement of legume N fixation capacity, is shown in Figure 10. This preliminary investigation showed that cowpea natural nodulation was very low regardless of study locations, soil characteristics, or seasons. Among all the farms, the highest average nodulation was only 18 nodules per plant. Conditions such as absence of compatible native rhizobia, limited population of rhizobia, ineffective/low effective native rhizobia, may inhibit the symbiosis and BNF of cowpea (Date, 2000; Ojo *et al.*, 2015; Vanlauwe and Giller, 2006). This situation is expected to be solved by inoculating cowpea seeds with the high effective rhizobia strains (Bala *et al.*, 2010; Koskey *et al.*, 2017). Currently, there are no available inoculant products on the markets in Vietnam. Thus, the low natural nodulation displayed the low capacity of cowpea in BNF and showed the urgent need to improve cowpea yield in such intercropping system by the inoculation of effective native rhizobia.

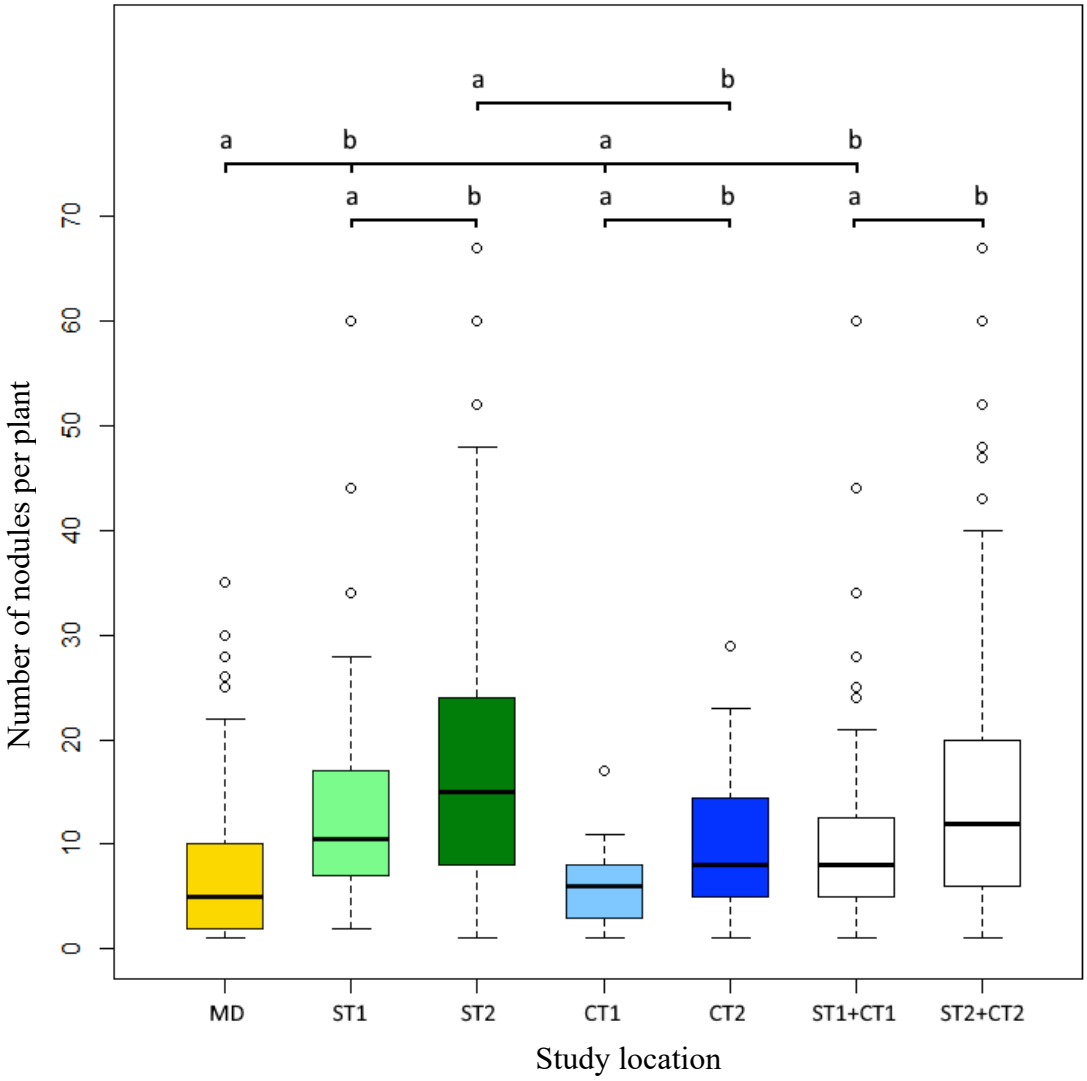

Figure 10 The natural nodulation of cowpea in Van Yen and Van Chan district, Yen Bai province, Vietnam in 2017. MD: Mau Dong; ST1–ST2: Son Thinh season 1 and 2; CT1–CT2: Cat Thinh season 1 and 2. Means followed by the same letter are not significantly different according to Tukey's HSD test at $p < 0.05$.

2. Isolation of native rhizobia strains and responses of cowpea to native rhizobia inoculation under field conditions and greenhouse condition

2.1 Isolation of native rhizobia strains

The total number of native rhizobia strains isolated from cowpea nodules in the three study locations of Yen Bai province was 21 (Table 6). Based on the sequencing analysis, these strains were closely related to known bacteria distributed in genera *Rhizobium* (11 strains) and *Bradyrhizobium* (10 strains). Several studies have found that cowpea is usually nodulated by the species within genera *Bradyrhizobium* (Appunu *et al.*, 2009; Bejarano *et al.*, 2014; Mathu *et al.*, 2018a; Sarr *et al.*, 2011), which has its main distribution area in tropical soils with slightly-highly acidic and fluctuation temperature. Nevertheless, the current study showed that the isolates from cowpea nodules across different areas were approximately equal between the genus *Rhizobium* and *Bradyrhizobium* (11 and 10 strains, respectively). There is a need to collect, isolate and test more effective native rhizobia strains for cowpea in these locations.

Table 7 shows the species distribution, richness and diversity indices of native rhizobia assessed in the 3 study locations, based on 16S rARN sequencing analysis and BLAST results from NCBI gene bank. Generally, the isolates had close similarity to 7 species of rhizobia, but 4 of them (or 57.14%) were isolated only in specific regions. *Rhizobium freirei* and *Rhizobium tropici* were found only in Mau Dong commune, Van Yen district. *Rhizobium leguminosarum bv. trifolii* and *Rhizobium pusense* were isolated only in Son Thinh commune, Van Chan district. Mau Dong had highest species richness (11) and diversity compared with the two other locations on both Shannon's and Simpson's indices (1.47 and 0.82, respectively), followed by Son Thinh with species richness of 7, Shannon's and Simpson's indices of 1.28 and 0.81, respectively. Cat Thinh had the lowest species richness and diversity indices of 3, 0.64 and 0.67, respectively. The results can be possibly explained by the history of cowpea cultivation in the three sites. Cowpea was introduced to Mau Dong since 2005, while it was introduced to Son Thinh and Cat Thinh only since 2016.

Table 6 List of isolated native rhizobia strains.

No.	Isolate code	Strain	% ID	District	Commune	Accession number
1	CMBP013	*Rhizobium tropici*	97%	Van Yen	Mau Dong	KY292434.1
2	CMBP016	*Rhizobium tropici*	96%	Van Yen	Mau Dong	KY292434.1
3	CMBP022	*Rhizobium sp.*	99%	Van Chan	Son Thinh	MG836228.1
4	CMBP028	*Rhizobium sp.*	96%	Van Yen	Mau Dong	KF008233.1
5	CMBP032	*Rhizobium leguminosarum bv. trifolii*	98%	Van Chan	Son Thinh	MH553182.1
6	CMBP037	*Rhizobium freirei*	99%	Van Yen	Mau Dong	KY292476.1
7	CMBP038	*Rhizobium tropici*	99%	Van Yen	Mau Dong	KY412843.1
8	CMBP043	*Rhizobium sp.*	99%	Van Chan	Son Thinh	JQ697683.1
9	CMBP044	*Rhizobium pusense*	98%	Van Chan	Son Thinh	MK542912.1
10	CMBP050	*Rhizobium sp.*	98%	Van Chan	Son Thinh	MG836228.1
11	CMBP052	*Bradyrhizobium elkanii*	97%	Van Chan	Cat Thinh	MK228880.1
12	CMBP054	*Bradyrhizobium elkanii*	99%	Van Yen	Mau Dong	KT900890.1
13	CMBP055	*Rhizobium pusense*	97%	Van Chan	Son Thinh	MG997082.1

Table 6 (Continued)

No.	Isolate code	Strain	% ID	District	Commune	Accession number
14	CMBP056	*Bradyrhizobium sp.*	98%	Van Yen	Mau Dong	MH213326.1
15	CMBP057	*Bradyrhizobium elkanii*	98%	Van Yen	Mau Dong	MN733003.1
16	CMBP059	*Bradyrhizobium sp.*	97%	Van Yen	Mau Dong	MH213320.1
17	CMBP062	*Bradyrhizobium elkanii*	99%	Van Yen	Mau Dong	MK228880.1
18	CMBP063	*Bradyrhizobium elkanii*	99%	Van Chan	Son Thinh	MN733003.1
19	CMBP065	*Bradyrhizobium elkanii*	99%	Van Chan	Cat Thinh	KT900890.1
20	CMBP066	*Bradyrhizobium elkanii*	97%	Van Yen	Mau Dong	KU058256.1
21	CMBP067	*Bradyrhizobium sp.*	97%	Van Chan	Cat Thinh	MK183850.1

% ID: Sequence similarity (%) of 16S rADN gene with identical sequences identified by using The National Center for Biotechnology Information (NCBI) database.

Table 7 Species distribution, species richness and diversity indices of isolated native rhizobia from cowpea nodules sampled from three study locations in Yen Bai province.

Species	Study location		
	Mau Dong commune, Van Yen district	Cat Thinh commune, Van Chan district	Son Thinh commune, Van Chan district
Bradyrhizobium elkanii	4	2	1
Bradyrhizobium sp.	2	1	0
Rhizobium freirei	1	0	0
Rhizobium leguminosarum bv. trifolii	0	0	1
Rhizobium pusense	0	0	2
Rhizobium sp.	1	0	3
Rhizobium tropici	3	0	0
Species richness	11	3	7
Shannon diversity index	1.47	0.64	1.28
Simpson's index of diversity	0.82	0.67	0.81

2.2 Responses of cowpea to native rhizobia inoculation under field conditions

2.2.1 Soil characteristics of the experimental sites

Composite field-soil samples were collected from each experimental site and used to determine the chemical characteristics. It was shown that soil chemical characteristics varied across farms (Table 8). The experimental sites had different soil texture including sandy loam, loam, sandy clay loam and sandy clay. Most of the soil samples were acidic with pH ranging from 4.26 to 5.03. Soil organic content ranged from 0.64 to 2.23%. Soil total N ranged from 0.04 to 0.18%. There were no significant differences in soil OC, total N, and available K among all the experimental sites. The

highest available P was observed in the sites inoculated with CMBP037+054 treatment, while there was no significant difference between the non-inoculated control and CMBP065 treatment.

2.2.2 Cowpea nodulation, biomass, shoot N accumulation and yield

Table 9 shows responses of cowpea to rhizobial inoculation in the field experiment. It was observed that rhizobial inoculants significantly affected nodulation in cowpea. The mixture of CMBP037+054 had highest nodulation (19.4 nodules plant^{-1}), while the lowest nodulation was observed in Non_I treatment (11.7 nodules plant^{-1}). Inoculation with the treatment CMBP037+054 treatment significantly increased nodulation of cowpea plants (65.8%) compared to Non_I treatment. There was no significant difference in the number of nodules per plant between CMBP037+054 and CMBP065 treatments. Slope degree, one of the important geographical factors, could affect the soil characteristics, plant physiological processes and soil microorganisms. Hence, this study considered the effects of different slope degrees on the efficiency of native rhizobial inoculation. Generally, slope degrees can be classified into three categories (gentle slope, moderate slope and steep slope) as mentioned above. There were no significant differences with regards to nodulation among all slope categories. However, there was a significant interaction effect observed on cowpea nodulation between inoculation treatment and slope category. CMBP037+054 treatment showed the highest nodulation (28 nodules plant^{-1}) on moderate slope, while Non_I produced the lowest nodulation (9.5 nodules plant^{-1}) on steep slope (Figure 11). This result therefore reveals the varied efficiencies of native rhizobia inoculants on different slope categories. Taking in to account how the impact of different slope categories interacted with different inoculation treatments, the combination of CMBP037+054 showed their significant higher nodulation on moderate slope than Non_I treatment on gentle slope. This finding is an evidence that indicated the significant interaction between rhizobial inoculation and the geographical slope factor. Different slope degrees, which affect soil microorganisms by complicated physical and biological processes, result in the actual benefits for rhizobial inoculation. By 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 on how sloping lands affect rhizobial inoculation efficiency, particularly in Northern mountainous areas of Vietnam. Therefore, further studies should be conducted in order to clarify the mechanism of the interaction between each sloping category and specific rhizobial inoculants.

Cowpea biomass, shoot total N content and yield affected by rhizobial inoculation in the field experiment is shown in Table 9. CMBP037+054 observed highest cowpea dry biomass, shoot N content and yield (24.88 g plant^{-1}, 3.02%, and 424.7 kg ha^{-1}, respectively), even though there were no significant differences among the treatments. The lowest cowpea yield was observed in the Non_I treatment (384.2 kg ha^{-1}). This study also considered the impacts of slope degrees on cowpea biomass, shoot N content and yield. Different slope categories did not significantly affect cowpea biomass, shoot N content, as well as yield of cowpea. No significant interaction effects between inoculation treatment and slope degree on cowpea biomass, shoot total N, and cowpea yield were observed. Although no significances were reported, the combination CMBP037+054 inoculation showed the improvement of cowpea biomass (26.81%), shoot N content (4.86%) and yield (10.54%). These findings are supported by numerous studies which affirmed that inoculation with *Bradyrhizobium* strains resulted in 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. The inoculated native strains with highly competitive nodule infection and N fixing capacity could result in a greater inoculation response in the presence of native rhizobia population in the soil. There is evidence from the previous reports which showed the positive effectiveness of native rhizobia strains inoculating with cowpea in comparison to the non-inoculated controls under field conditions (Danso and Owiredu, 1988; Gómez Padilla *et al.*, 2016; Yoseph *et al.*, 2017). This study therefore reveals the presence of effective rhizobia at

that research location and their potential as efficient inoculants for the scaling-up production in the fields.

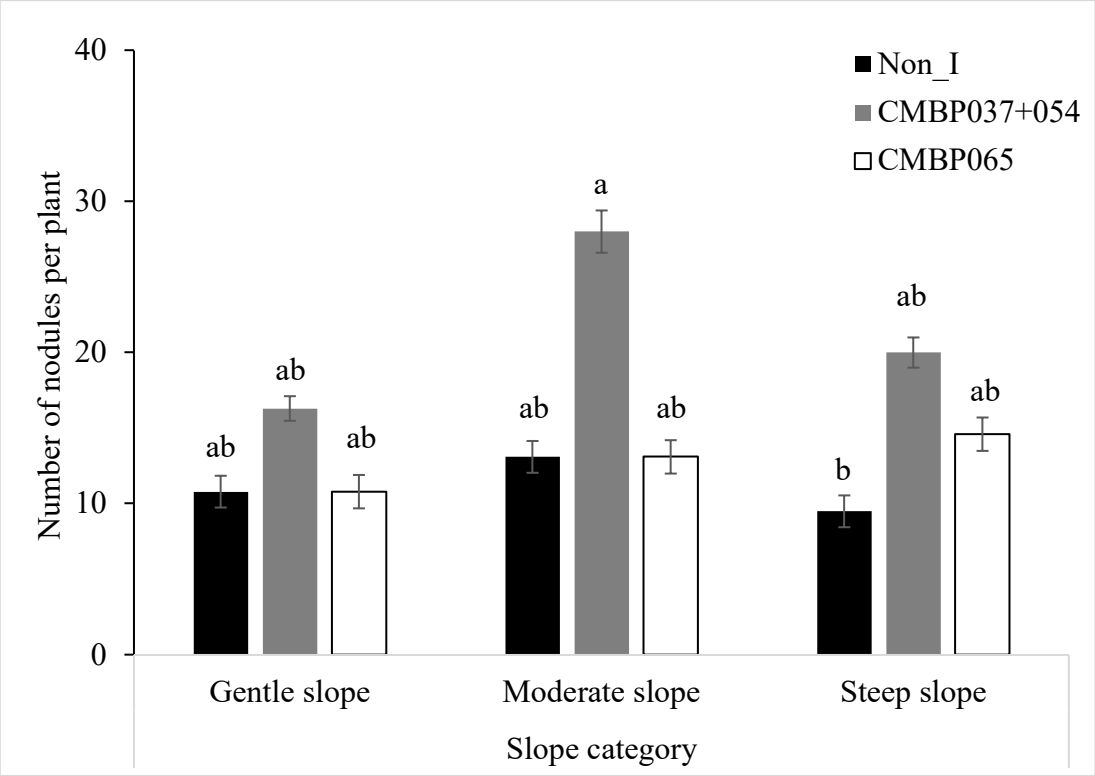

Figure 11 Interactive effects of inoculation treatment with slope category on nodulation of cowpea in the field experiment Bars followed by the same letter are not significantly different according to Tukey's HSD test at *P<0.05*.

Table 8 Soil characteristics of experimental sites (before planting).

Treatment	Number of replicates	Sand (%)	Silt (%)	Clay (%)	pH H_2O	OC (%)	Total N (%)	Available P (mg P_2O_5/100g of soil)	Available K (mg K_2O/100g of soil)
p-value		0.5688	0.0784	0.0788	0.0908	0.5876	0.1732	0.0416	0.5752
Non_I	14	47.43[a]	19.96[a]	32.61[a]	4.40[a]	1.46[a]	0.12[a]	9.97[b]	8.24[a]
CMBP037+054	5	48.10[a]	29.20[a]	22.70[a]	5.03[a]	1.50[a]	0.11[a]	22.87[a]	9.88[a]
CMBP065	5	51.90[a]	11.08[a]	37.02[a]	4.26[a]	1.68[a]	0.15[a]	7.83[b]	7.18[a]

Means followed by different letters are significantly different at *P<0.05* according to Tukey's HSD test.

Table 9 Response of cowpea to native rhizobia inoculation under field conditions.

Treatments	Number of replications	Nodulation (number of nodules per plant)	Shoot dry weight (g plant^{-1})	Root dry weight (g plant^{-1})	Shoot total N (%)	Yield (kg ha^{-1})
Rhizobia inoculant						
Non_I	14	11.7±1.3^{b}	17.96±1.48a	1.66±0.10^{a}	2.88±0.11^{a}	384.2±12.5^{a}
CMBP037+054	5	19.4±2.8^{a}	23.10±2.73^{a}	1.78±0.15^{a}	3.02±0.07^{a}	424.7±11.8^{a}
CMBP065	5	13.5±1.8^{ab}	15.86±0.81a	1.52±0.11^{a}	2.85±0.17^{a}	407.6±24.1^{a}
Slope category						
Gentle slope	9	14.1±1.7^{a}	19.37±1.58^{a}	1.76±0.11^{a}	2.88±0.16^{a}	411.6±17.1^{a}
Moderate slope	5	16.1±3.6^{a}	20.70±4.21^{a}	1.64±0.24^{a}	2.98±0.15^{a}	378.0±11.4^{a}
Steep slope	10	12.1±1.5^{a}	16.85±1.13^{a}	1.57±0.06^{a}	2.90±0.10^{a}	394.6±15.7^{a}
P-values of the main factors and their interactions						
Rhizobia inoculant		*	NS	NS	NS	NS
Slope category		NS	NS	NS	NS	NS
Rhizobia inoculant x Slope category		*	NS	NS	NS	NS

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

* significant at $P<0.05$; NS = not significant at $P<0.05$.

The strain CMBP065 was less effective than the mixture CMBP037+054 in the field conditions. This result suggests that the strain CMBP037 may enhance the effectiveness of CMBP054 strain in their mixture. As the two strains CMBP037 and CMBP054 was isolated from Mau Dong commune, such advantage of the mixture 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 an increased rhizobia diversification does not have good efficacy as compared to the single rhizobia strain (Danso and Owiredu, 1988; Martinez-Romero, 2003; Nkot *et al.*, 2015), or different strains may highly compete each other in the same inoculant (Raposeiras *et al.*, 2006).

2.2.3 Mycorrhizal infection rate of cowpea and cassava

Besides N fixing rhizobia, cowpea is regularly associated with AMF which improve its uptake capacity of low mobility nutrients, especially P acquisition (Püschel *et al.*, 2017). P is clearly known as one of the most important nutrients which may affect plant growth and physiological processes, especially BNF (Tairo and Ndakidemi, 2013). P is the necessary element for the conversion of N_2 to NH_4 (Dakora and Keya, 1997) which requires high amounts of energy (Schulze *et al.*, 2006). Moreover, P plays a crucial role in improving legume nodulation and enhancing rhizobia density in the soil (Bashir *et al.*, 2011; Sa and Israel, 1991). Responses of cowpea mycorrhizal infection rate to rhizobial inoculation and slope category in the field experiment is shown in Figure 12. Rhizobial inoculation did not significantly affect mycorrhizal infection rate in cowpea. The mycorrhizal infection rate of cowpea roots was quite high (84.04-86.76%) in all treatments. Likewise, cassava mycorrhizal infection rate was similar in different treatments (60.45-67.52%). Even though there was no significant effect of rhizobial inoculation on mycorrhizal infection rate of cowpea and cassava, but the high rates of mycorrhizal infection (> 80% and >60%, respectively) was found. This implies that the natural mycorrhizal infection rate of cowpea and cassava is fairly high at this research location, regardless of the rhizobial

inoculation or slope degrees. Thus, this study suggests that intercropping with cowpea at this site could encourage cassava production by effectively absorbing poorly mobile nutrients in the soils (Moyer-Henry *et al.*, 2006), as well as improve BNF of cowpea. This finding is in line with the previous report which specified that the symbiosis with both AMF and N fixing rhizobia efficiently increased cowpea growth and production (Stancheva *et al.*, 2017).

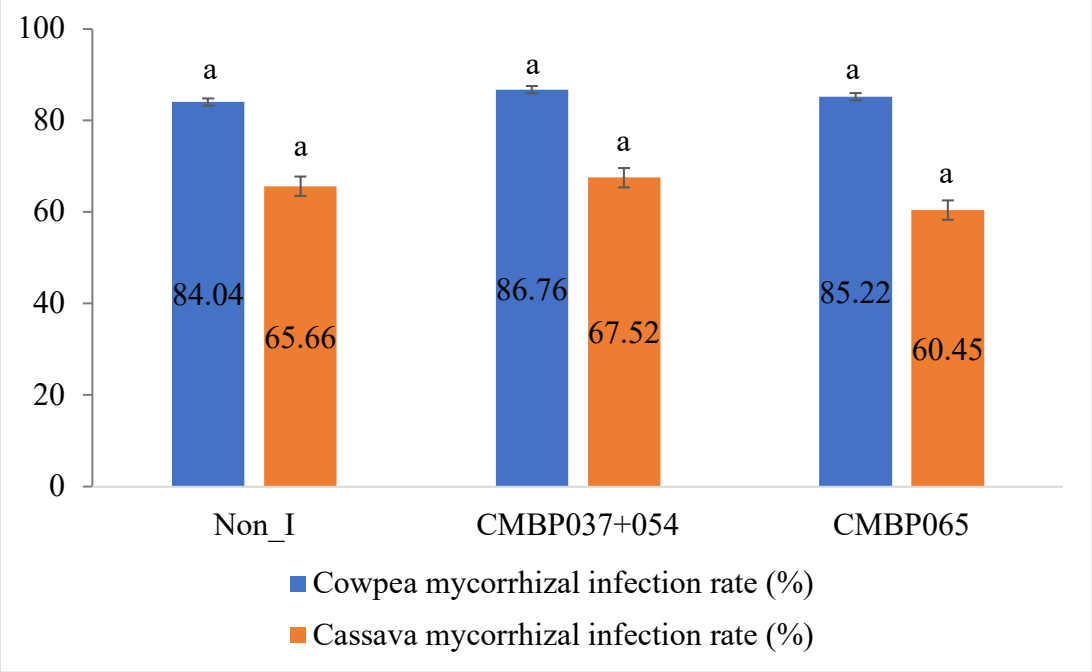

Figure 12 Mycorrhizal infection rate of cowpea and cassava under different native rhizobia inoculants.

2.2.4 Nodule occupancy assessment

A total of 173 nodules were analysed from 24 farms and 4 profile groups were obtained as follows: IGS1 from CMBP065 strain, IGS2 from native strain (experimental fields), IGS3 from CMBP054 and IGS4 from CMBP037 strain. The results showed that IGS3 profile was only found in the control treatment with very low percentage (3.57%), so the strain CMBP054 was the very poor survival (Figure 13). Regarding to IGS2 profile, there were different percentages among all treatments but

they were not significant. Whereas, there were significant differences when comparing profile IGS1 percentages among all the treatments. IGS1 was dominant in treatment CMBP037+054 while no difference was observed between CMBP065 and the non-inoculated control. Even though IGS1 occupied the highest percentage of nodules from treatment CMBP037+054 and IGS2 was higher in treatment CMBP065, there was no significant difference between profile IGS1 and IGS2 at each treatment.

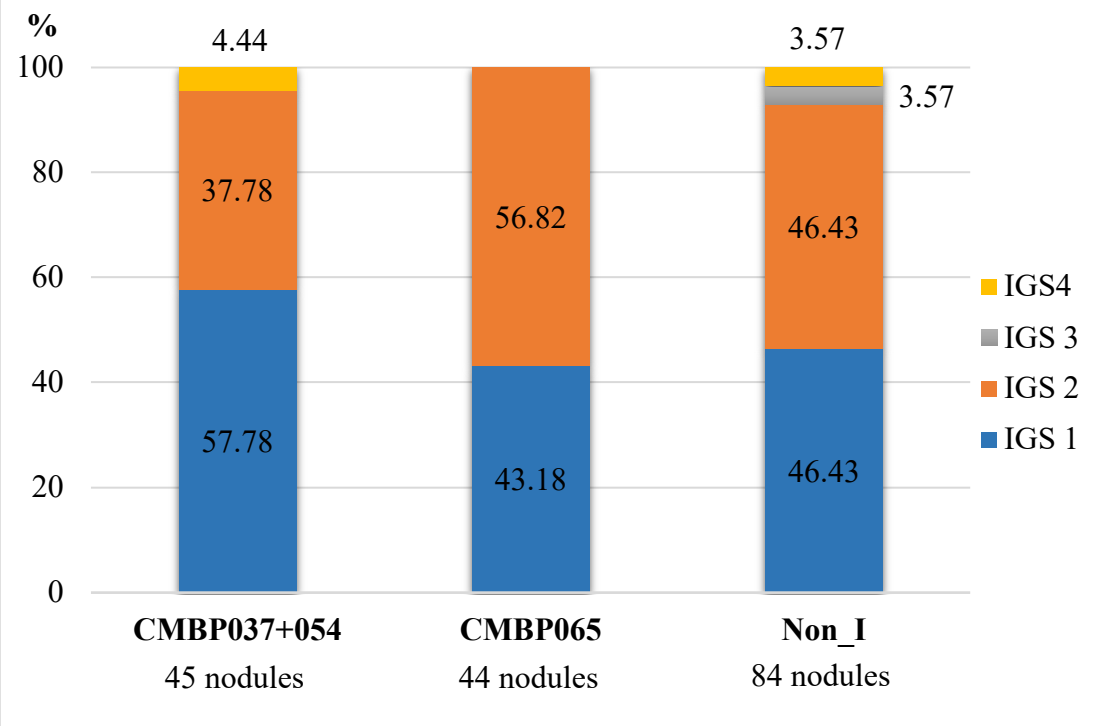

Figure 13 Nodule occupancy of different rhizobial strains.

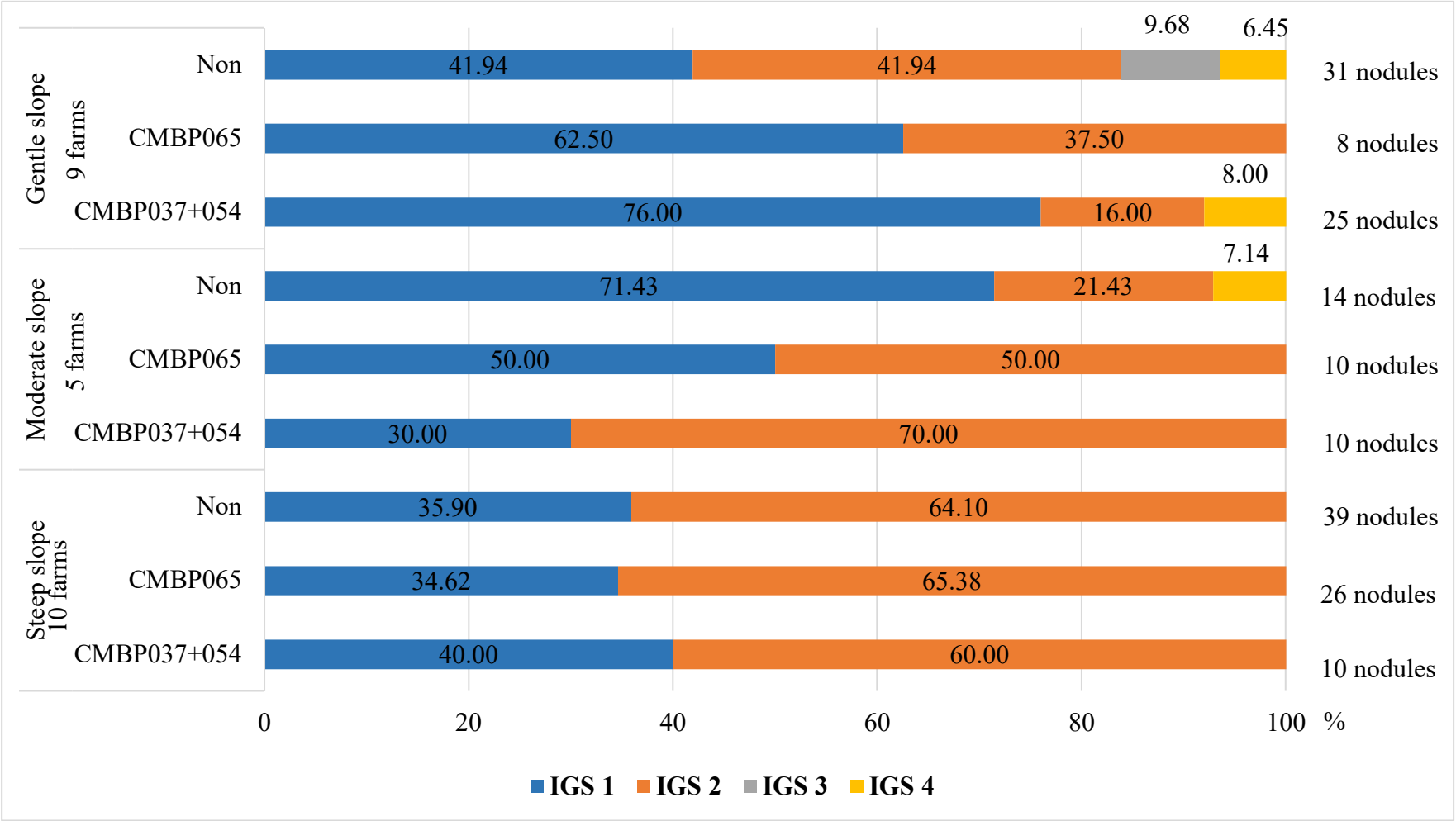

Figure 14 Nodule occupancy of different rhizobial strains classified by slope category.

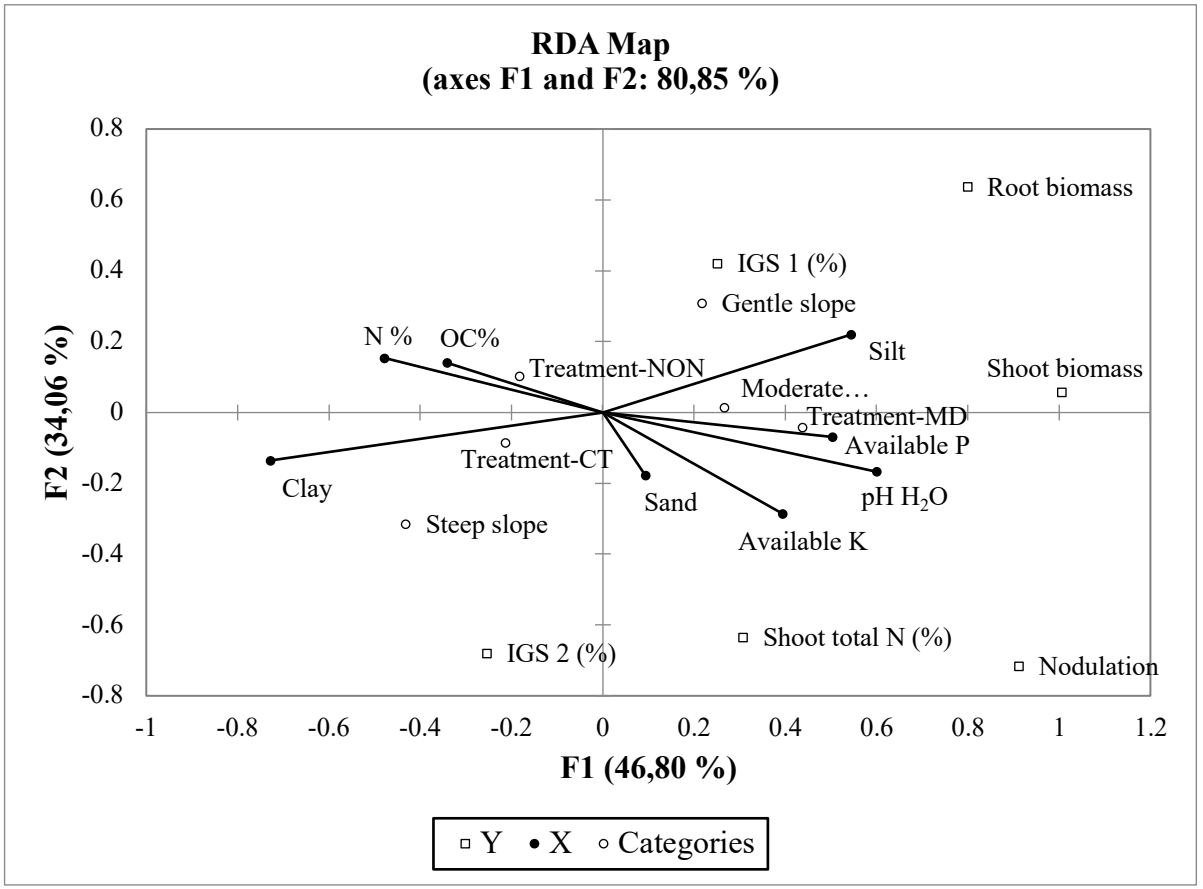

Figure 15 Redundancy analysis (RDA) between the two profiles (IGS1 and IGS2) of inoculated strains, different slope categories and other parameters.

Figure 14 shows the impact of different slope categories on nodule occupancy. At the gentle sloping farms, IGS1 profile was highest. On the contrary, IGS2 profile was dominant at the steep sloping farms. Comparing the percentage of each inoculated strain among all slope categories, the IGS2 profile was highest on steep slope and there was no significant difference between moderate slope and gentle slope. The IGS1 profile was dominant on gentle slope. The redundancy analysis (RDA) result also showed the same trend, IGS1 was dominant at the gentle sloping farms, while IGS2 occupied higher percentage at the steep sloping farms (Figure 15). The nodule occupancy had no significant effect on other parameters (nodulation, biomass, shoot N content, and cowpea yield).

Commonly, there are several rhizobia strains competing for infection and occupancy on plant roots and nodules in the field (Mathu *et al.*, 2012; Mathu *et al.*, 2018b). The low nodule occupancy of inoculated strains from the field result can be attributed to several reasons. First, high abundance of native rhizobia, or the presence of high competition but ineffective native strains, may inhibit root infection or nodule formation of introduced strains (Leite *et al.*, 2009), even though the concentration of inoculated strains in our study was quite high ($>10^8$ CFU ml^{-1}) (data not shown). This is in agreement with the results from several authors (Cheminingâ *et al.*, 2011; Mathu *et al.*, 2012; Mathu *et al.*, 2018b) which showed the low nodule occupancy of inoculated rhizobia strains. McInnes and Haq (2007) indicated that the high population of soil rhizobia limits the nodule occupancy of inoculation strains, consequently decreasing symbiotic N fixation capacity. Secondly, the difficulties in inoculation under tropical climatic conditions and poor practical technique by local farmers may contribute to low nodule occupancy of inoculated strains (Bantilan and Johansen, 1995; Mathu *et al.*, 2018b). Somasegaran *et al.* (1984) indicated that high temperature may decrease the viability of rhizobia strains in inoculants, especially during the inoculation process in summer season. This matter was confirmed by the studies from Deaker *et al.* (2004) and Law *et al.* (2007). There are several solutions that can enhance inoculant competitiveness including the use of highly competitive introduced strains and the re-inoculation in several seasons (Hungria *et al.*, 2006; Mathu *et al.*, 2018b; Zhang *et al.*, 2014).

2.3 Responses of cowpea to native rhizobia inoculation under greenhouse condition

2.3.1 Cowpea nodulation

Table 10 shows the effects of different native rhizobia inoculants on cowpea nodulation, shoot dry weight, shoot total N content and symbiotic efficiency under the greenhouse condition. There were significant differences in the mentioned parameters between the different native rhizobia inoculants in this experiment. No nodule was formed on roots of the control and N+ treatment without inoculation. Nodulation varied in response to inoculation by different rhizobia strains, ranged from 0 to 63.7 nodules per plant in plants inoculated with strains CMBP044 and CMBP056, respectively. There was no significant difference in the number of nodules per plant between inoculation with strain CMBP056 and CMBP052, CMBP054, CMBP057, CMBP062, CMBP063, CMBP065, CMBP066 and CMBP067. Nodule dry weight ranged from 0 to 0.17 g per plant in CMBP044 and CMBP065 treatments. There was no significant difference between inoculation with strain CMBP065 and other strains except strains CMBP013, CMBP016, CMBP028, CMBP032, CMBP037, CMBP038, CMBP050, CMBP055 and CMBP057 that had low nodule dry weight of 0.06, 0.03, 0.02, 0.01, 0.07, 0.08, 0.01, 0.03 and 0.07 g per plant, respectively. According to Beck *et al.* (1993), high nodule dry weight could result to higher efficiency in BNF and higher shoot biomass. Thus, our results showed that there are effective rhizobia strains from the study locations that can be tested and selected for effective inoculants under the further field experimental conditions.

2.3.2 Shoot dry weight, shoot N content and symbiotic efficiency of cowpea

Shoot dry weight was used to assess symbiotic efficiency of cowpea in response to different native rhizobia inoculants. Shoot dry weight ranged from 0.35 to 4.52 g per plant in CMBP050 and N+ treatment (Table 10). The positive control N+ observed the highest shoot dry weight and there were significant differences between N+ and other inoculated treatments.

SEF plays an important role in evaluating the response of legume to inoculation and choosing effective isolates for inoculant production (Fening and Danso, 2002). It is well known that diverse rhizobia strains show widely variation in their SEF on host plants. In this study, all the inoculated treatments producing nodules were considered for SEF assessment. There were significant differences in respect to SEF among the inoculation treatments in the greenhouse (Table 10). SEF ranged from 7.79% to 58.77% in CMBP050 and CMBP063 treatments. This finding is in agreement with Mathu *et al.* (2018b) who revealed that the native rhizobia isolates varied in their symbiotic effectiveness on cowpea. There was no significant difference in SEF between CMBP063 and CMBP054 (54.56%), CMBP065 (55.73%) and CMBP066 (51.64%). According to the SEF classification indicated by Aynalem *et al.* (2018), these strains were rated as effective strains inferring that they are potential native strains for enhancing cowpea N fixation and could be evaluated under further field condition. Whereas, the ineffective strains could not deliver any functional advantage as compared to the uninoculated controls.

Table 10 Responses of cowpea nodulation, shoot dry weight, shoot N content and symbiotic effectiveness to different native rhizobia inoculants under greenhouse condition.

No.	Treatments	Nodules per plant	Nodule dry weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	Shoot total N (%)	SEF	
						%	Rate
1	Control	0	0	0.47 ^{fg}	2.18 ^{fg}	-	-
2	N+	0	0	4.52 ^{a}	3.70 ^{bcdefg}	100 ^{a}	-
3	CMBP013	2.3 ^{e}	0.06 ^{bcd}	0.69 ^{efg}	3.35 ^{defg}	15.32 ^{fgh}	IE
4	CMBP016	1.7 ^{e}	0.03 ^{cd}	0.87 ^{defg}	2.55 ^{efg}	19.15 ^{defgh}	IE
5	CMBP022	5.3 ^{e}	0.08 ^{abcd}	0.81 ^{efg}	4.28 ^{abcdefg}	17.99 ^{efgh}	IE
6	CMBP028	1.3 ^{e}	0.02 ^{cd}	0.56 ^{efg}	3.53 ^{cdefg}	12.36 ^{fgh}	IE
7	CMBP032	0.6 ^{e}	0.01 ^{d}	0.69 ^{efg}	1.91 ^{g}	15.2 ^{fgh}	IE
8	CMBP037	7.7 ^{de}	0.07 ^{bcd}	0.87 ^{defg}	5.21 ^{abcde}	19.27 ^{defgh}	IE
9	CMBP038	10.7 ^{cde}	0.08 ^{bcd}	1.76 ^{bcdef}	6.58 ^{ab}	38.92 ^{bcdefg}	LE
10	CMBP043	11.8 ^{cde}	0.09 ^{abcd}	1.11 ^{cdefg}	5.88 ^{abcd}	24.48 ^{cdefgh}	IE
11	CMBP044	0 ^{e}	0 ^{d}	0.38 ^{g}	2.06 ^{fg}	8.38 ^{h}	IE
12	CMBP050	0.2 ^{e}	0.01 ^{d}	0.35 ^{g}	1.88 ^{g}	7.79 ^{h}	IE

Table 10 (Continued)

No.	Treatments	Nodules per plant	Nodule dry weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	Shoot total N (%)	SEF	
						%	Rate
13	CMBP052	48.3 ^{ab}	0.13 ^{ab}	1.84 ^{bcde}	5.35 ^{abcde}	40.75 ^{bcdef}	LE
14	CMBP054	46.4 ^{ab}	0.09 ^{abcd}	2.47 ^{b}	6.53 ^{ab}	54.56 ^{b}	E
15	CMBP055	4.0 ^{e}	0.03 ^{bcd}	0.44 ^{fg}	3.01 ^{defg}	9.64 ^{gh}	IE
16	CMBP056	63.7 ^{a}	0.11 ^{abc}	2.19 ^{bc}	4.43 ^{abcdefg}	48.35 ^{bc}	LE
17	CMBP057	38.1 ^{abc}	0.07 ^{bcd}	1.79 ^{bcdef}	6.39 ^{abc}	39.51 ^{bcdefg}	LE
18	CMBP059	34.9 ^{bcd}	0.09 ^{abcd}	2.19 ^{bc}	6.99 ^{a}	48.38 ^{bc}	LE
19	CMBP062	53.9 ^{ab}	0.11 ^{abc}	2.13 ^{bcd}	5.67 ^{abcd}	47.07 ^{bcd}	LE
20	CMBP063	36.1 ^{abc}	0.08 ^{abcd}	2.66 ^{b}	6.81 ^{a}	58.77 ^{b}	E
21	CMBP065	60.7 ^{ab}	0.17 ^{a}	2.52 ^{b}	5.48 ^{abcd}	55.73 ^{b}	E
22	CMBP066	60.8 ^{ab}	0.11 ^{abc}	2.33 ^{bc}	5.31 ^{abcde}	51.64 ^{bc}	E
23	CMBP067	62.3 ^{ab}	0.13 ^{ab}	2.06 ^{bcd}	4.77 ^{abcdef}	45.60 ^{bcde}	LE

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. IE: ineffective; LE: low effective; E: effective.

As shown in Table 10, shoot total N content ranged from 1.88% to 6.99% in CMBP050 and CMBP059 treatments. There was no significant difference in shoot total N content between CMBP059 and other strains except the control, N+, CMBP013, CMBP016, CMBP028, CMBP032, CMBP044 and CMBP055. The results from the greenhouse experiment showed that inoculation with native effective rhizobia strains significantly increased cowpea nodulation and shoot N content as compared to the non-inoculation treatments. The superior performance obtained from these inoculants could be attributed to their capability to infect, form nodules and fix N with cowpea. These results concur with the 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 results of simple linear regression between nodule dry weight and shoot dry weight and shoot total N content is shown in Figure 16 and Figure 17. There was a significant positive correlation between nodule dry weight and shoot dry weight of cowpea ($R^2 = 0.3183$, $p = 0.0012$) (Figure 16). This result is consistent with the previous studies (Kawaka *et al.*, 2014; Koskey *et al.*, 2017) which also showed the strong positive correlation between nodule dry weight and shoot dry weigh. As N-fixing capacity could be relatively 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. The correlation between nodule dry weight and shoot total N content was also investigated. As shown in Figure 17, a significant positive relationship between nodule dry weight and shoot total N content was found ($R^2= 0.4240$, $p=0.0019$). This finding is in agreement with the studies from Koskey *et al.* (2017) and Stajković *et al.* (2010) who revealed that there is a direct relationship between nodule dry weight and shoot total N content in legumes. This study, therefore, confirmed that

the inoculation of native rhizobia enhanced cowpea nodulation and N fixation, consequently improved shoot biomass and N content.

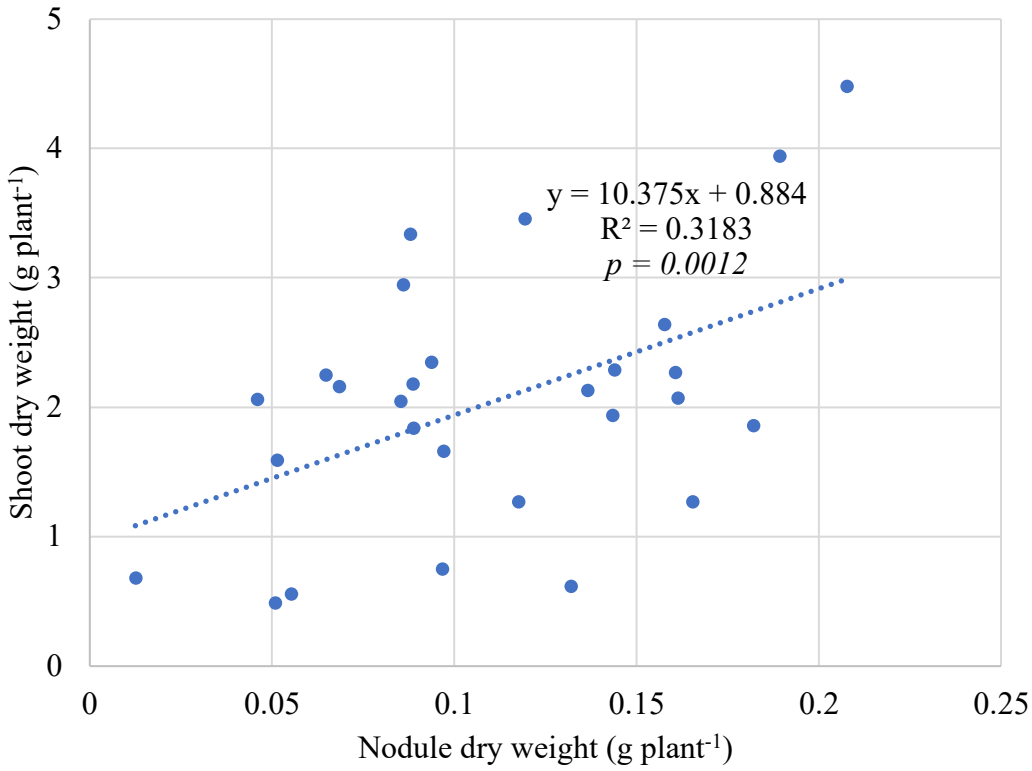

Figure 16 Simple linear regression between nodule dry weight and shoot dry weight in the greenhouse experiment.

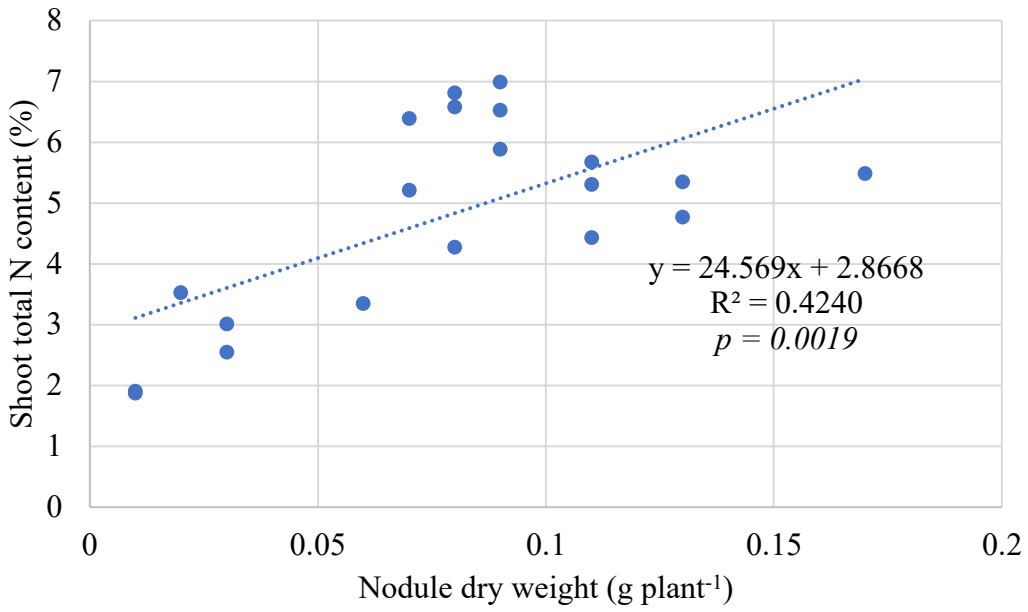

Figure 17 Simple linear regression between nodule dry weight and shoot total N content in the greenhouse experiment.

2.3.3 Shoot and root biomass and SPAD value

Shoot fresh weight and root fresh and dry weight of cowpea responded to different native rhizobia inoculants under the greenhouse condition is shown in Figure 18, 19 and 20. There were significant differences between different native rhizobia strains in the mentioned parameters. As shown in Figure 18, shoot fresh weight ranged from 2.19 to 17.92 g per plant in CMBP050 and N+ treatment. There was no significant difference in shoot fresh weight between N+ treatment and CMBP063, CMBP062 and CMBP066 that had shoot fresh weight of 14.28, 13.00 and 12.83 g per plant, respectively. Root fresh weight of cowpea ranged from 0.83 to 6.83 g per plant in the uninoculated control and N+ treatment, respectively (Figure 19). Treatment N+ observed root fresh weight significantly higher than other inoculated treatments. Regarding root dry weight, the same trend was observed while there were significant differences between N+ treatment and the inoculated treatments (Figure 20). Root dry weight ranged from 0.12 to 0.76 g per plant in the uninoculated control and N+ treatment. There was no significant among CMBP054, CMBP063, CMBP065 and

CMBP066 which had root dry weight of 0.23, 0.45, 0.33 and 0.38 g per plant, respectively.

SPAD index 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 particular plant. The response of cowpea SPAD index to different native rhizobia inoculants under the greenhouse condition is shown in Figure 21. The highest and lowest SPAD was observed in CMBP057 and CMBP032 treatments (44.76 and 13.79, respectively). There was also no significant difference among CMBP054, CMBP063, CMBP065 and CMBP066 which had SPAD index of 35.72, 41.98, 38.35 and 38.07, respectively.

Generally, the findings from this study agree with the previous studies which affirmed that the native rhizobia strains varied in their symbiotic efficiency on cowpea under the greenhouse condition (Ampomah *et al.*, 2008; Fening and Danso, 2002; Mathu *et al.*, 2012). The best performing strains (CMBP054, CMBP063, CMBP065 and CMBP066) can be selected for further inoculation experiments under field conditions to make available effective inoculant products for cowpea in the Northern mountainous regions of Vietnam.

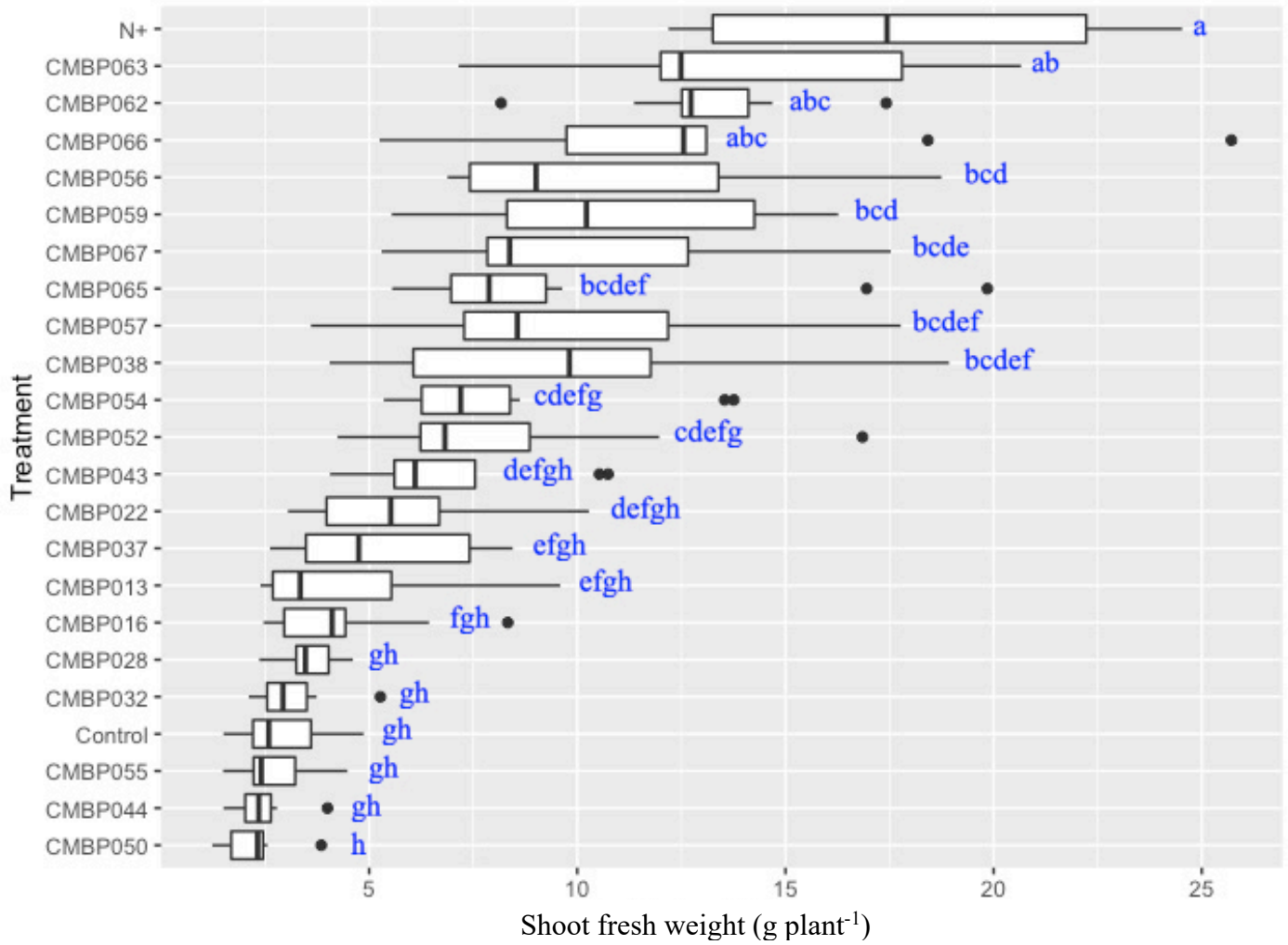

Figure 18 Responses of cowpea shoot fresh weight to different native rhizobia inoculants under greenhouse condition.

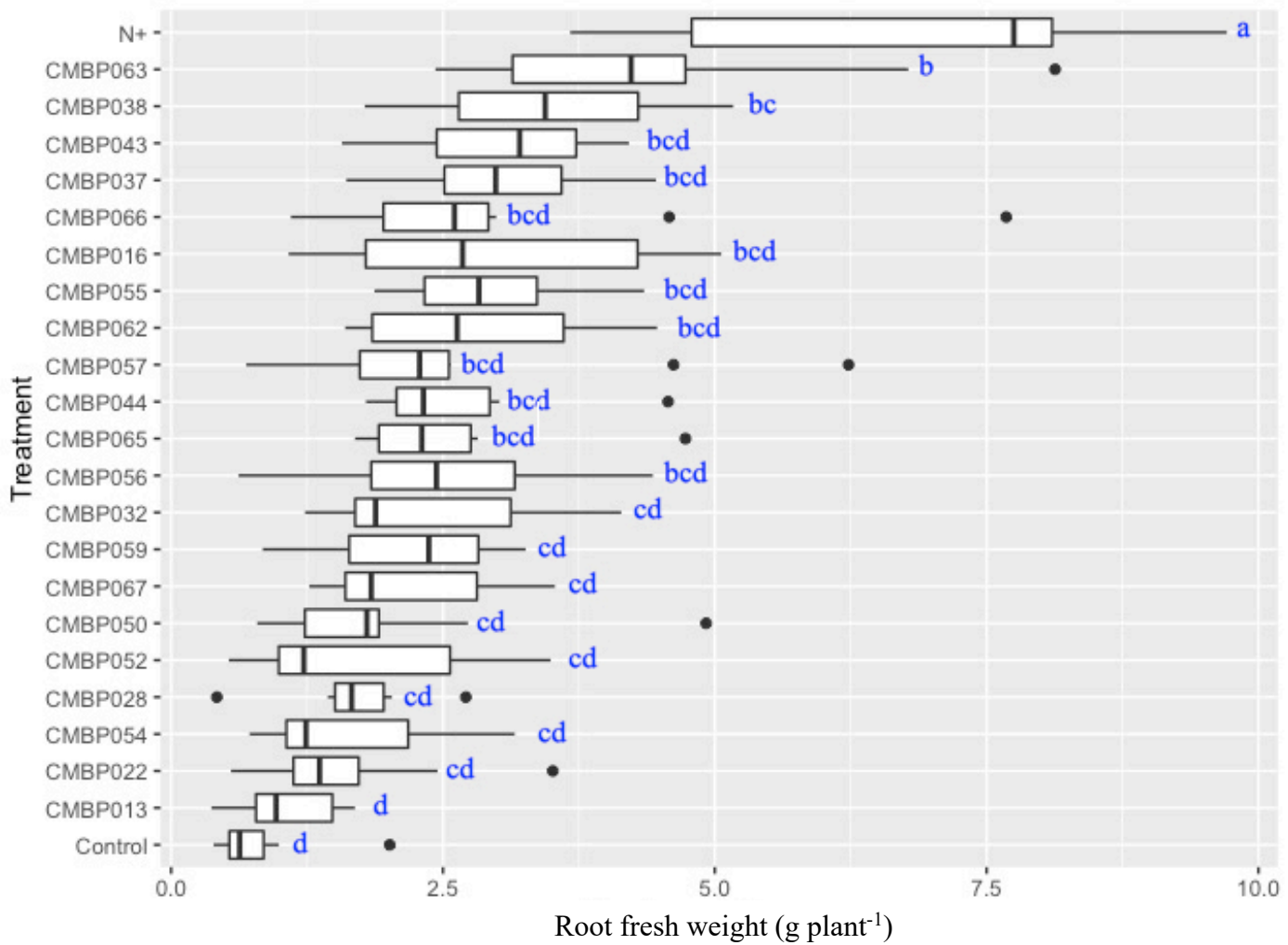

Figure 19 Responses of cowpea root fresh weight to different native rhizobia inoculants under greenhouse condition.

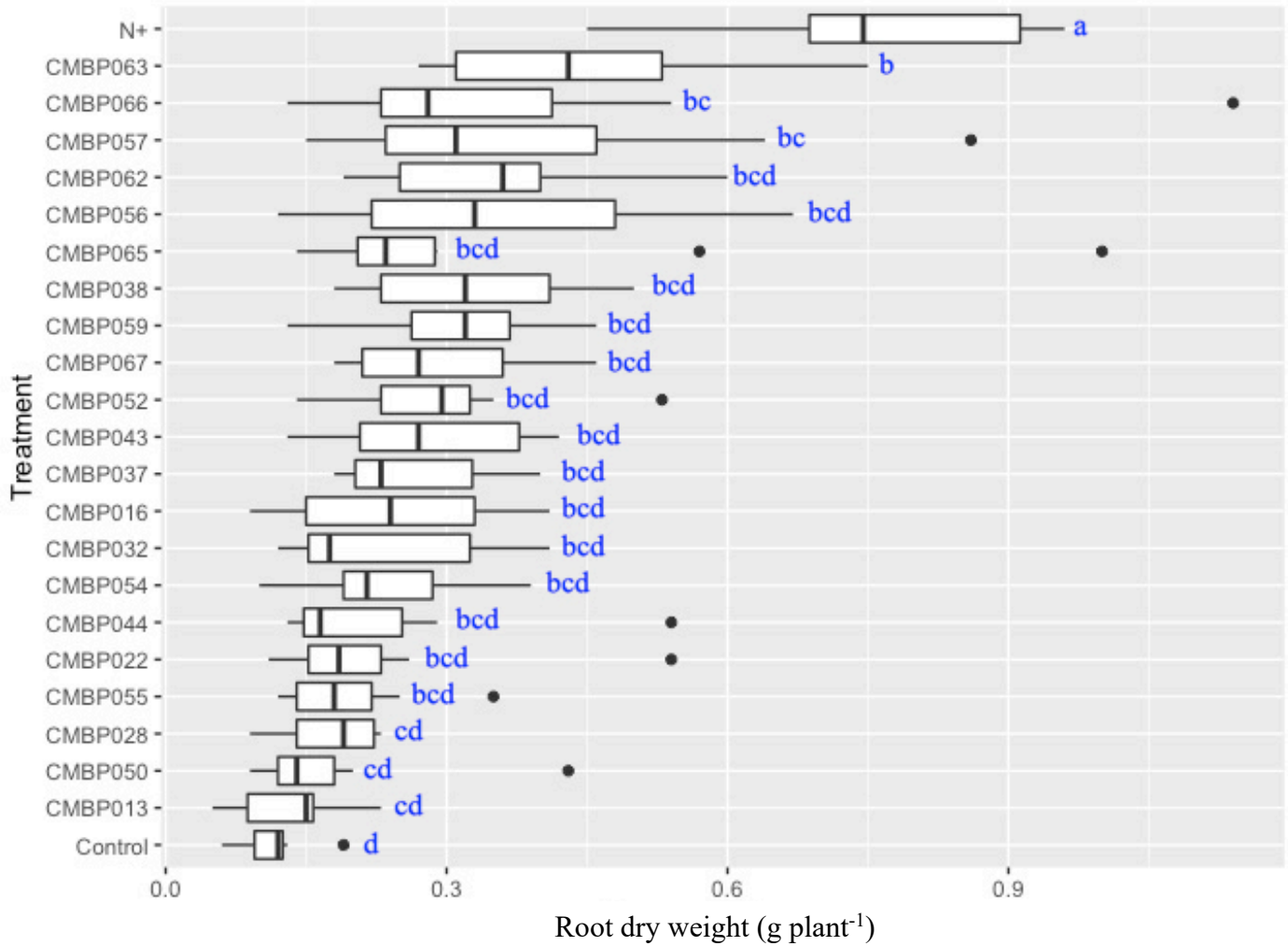

Figure 20 Responses of cowpea root dry weight to different native rhizobia inoculants under greenhouse condition.

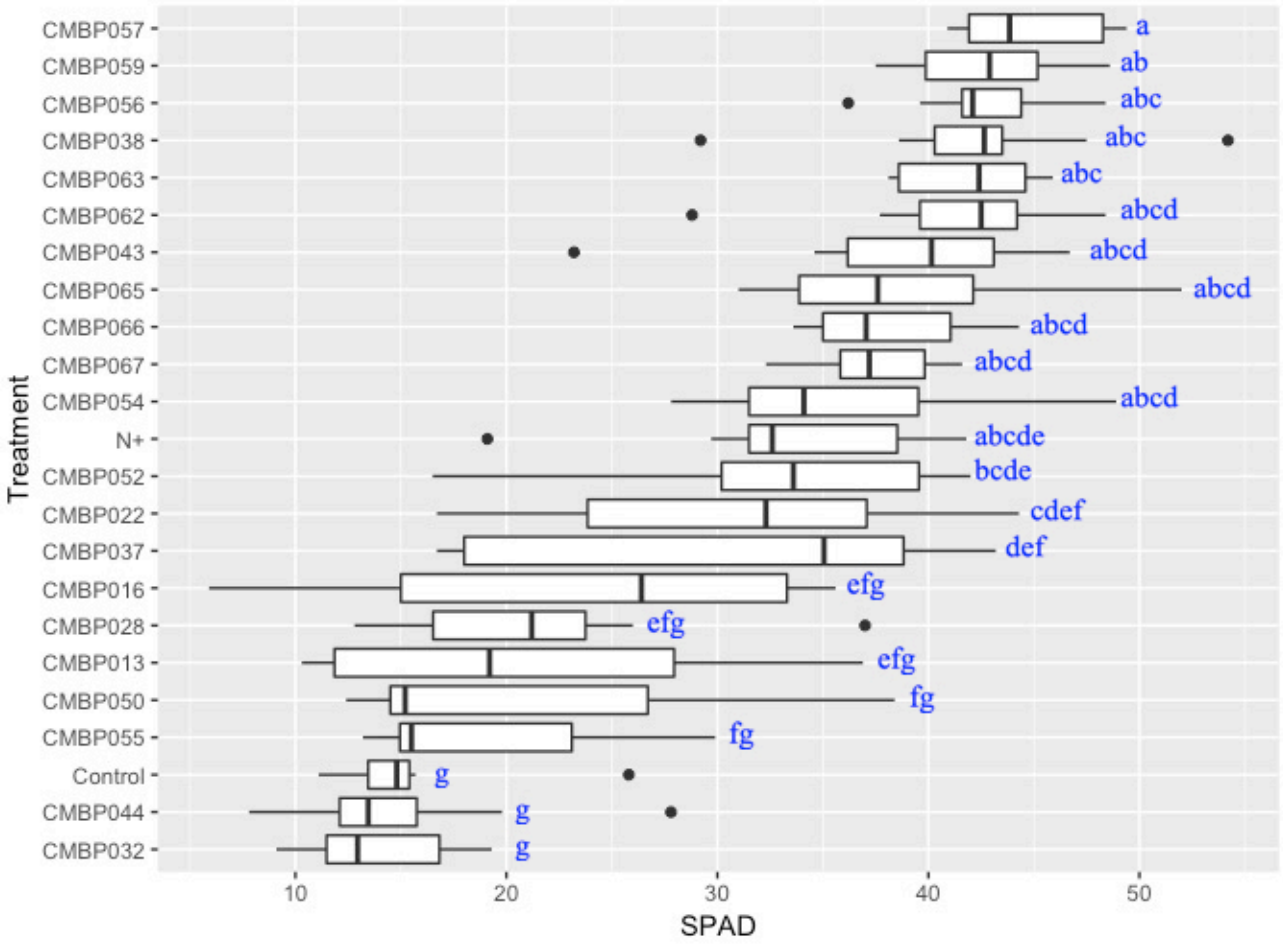

Figure 21 Responses of Soil Plant Analysis Development (SPAD) index of cowpea to different native rhizobia inoculants under greenhouse condition.

3. Effects of cassava-cowpea intercropping on soil erosion and economic returns of smallholders on sloping lands

3.1 Soil covering level and crop productivity

As shown in Table 11, there were significant effects of different treatments on SCL at 40 DAS. Treatment IG had highest SCL (8.3 out of 10), followed by treatment IS (7.1). There was no significant difference between treatment MG and MS in regard to SCL (6.1 and 5.4, respectively). This result revealed the potential of intercropping with cowpea in improving canopy cover percentage, consequently decreasing the influence of rain drops and overland flow on sloping lands. These findings are consistent with the studies from Zougmore *et al.* (2000) and Kariaga (2004) who reported that intercropping with cowpea maintained higher crop canopy cover than monocropping systems, thus significantly reduced soil erosion rate. The higher SCL value from intercropping treatments compared to monocropping confirms the benefit of intercropping in term of soil conservation regardless of slope degrees.

Table 11 Effects of different treatments on soil covering level, cassava and cowpea yields.

Treatments	Soil covering level	Cassava yield (ton ha^{-1})	Cowpea yield (kg ha^{-1})
MG	6.1 ± 0.3^{c}	21.5 ± 0.6^{ab}	-
MS	5.4 ± 0.2^{c}	20.3 ± 0.4^{b}	-
IG	8.3 ± 0.2^{a}	23.0 ± 0.6^{a}	412.5 ± 12.5^{a}
IS	7.1 ± 0.3^{b}	21.0 ± 0.6^{ab}	429.2 ± 11.0^{a}

MG-cassava monocropping on gentle slope; MS-cassava monocropping on steep slope; IG-cassava-cowpea intercropping on gentle slope; IS-cassava-cowpea intercropping on steep slope.

Means followed by same lowercase letter(s) within the same column of each factor are not significantly different at $p < 0.05$ according to Tukey's HSD test.

Significant differences were observed in cassava yield as influenced by different cropping systems (Table 11). Cassava yield was highest in treatment IG (23.0 tons ha^{-1}), and lowest in MS (20.3 tons ha^{-1}). No significant difference was observed between MG and IS treatments (21.5 and 21.0 tons ha^{-1}, respectively). Interestingly, intercropping of cassava with cowpea did not affect cassava yield, however it reported the higher productivity as compared to other monocropping treatments. This might be due to the growing characteristics of the two crops which do not result in the competition for growth and natural resources. According to Aye and Howeler (2012), cowpea has different root architectures and growth habits and duration, which makes it well-suited for cassava-based intercropping system. Cassava has a slow initial growth, while cowpea has a fast growth and short growth duration (Aye and Howeler, 2012). Cowpea is harvested early (about 65 DAS) thus cassava will have the advantages of both space and time in utilizing the resources, resulting in a higher total yield (Howeler, 1996). Moreover, cowpea, a legume crop with biological N fixation (BNF) ability, has various advantages in improving soil fertility, soil moisture and microbial communities (Sharma *et al.*, 2017; Wang *et al.*, 2014). Besides the contribution from BNF, legumes could also benefit cassava crop by its biomass residues left on the ground for mulching process (Makinde *et al.*, 2007). Thus, cassava can benefit from this legume and result in higher productivity. Such finding is on the contrary with several studies which showed that cassava yield under monocropping is higher than under legume-based intercropping (Adekunle *et al.*, 2014; Daellenbach *et al.*, 2005; Sikirou and Wydra, 2008). The possible explanation can be the competition between cassava and unsuitable legume species used in those intercropping systems regarding to natural resource requirements or growth cycle (Howeler *et al.*, 2001; Hy, 1998). Cassava yield on steep sloping lands tends to be lower than that on gentle slope. This might be due to the low soil fertility and nutrient losses on steep sloping fields, especially when the soil is left bare (Tuan *et al.*, 2014).

On intercropping fields, no significant effect was reported in cowpea yield between IG and IS treatments. As shown in Table 11, high yield of cowpea was

recorded on both gentle and steep slopes, of 412.5 and 429.2 kg ha^{-1}, respectively, thus increasing the total crop productivity and income from intercropping systems.

3.2 Soil and nutrient losses

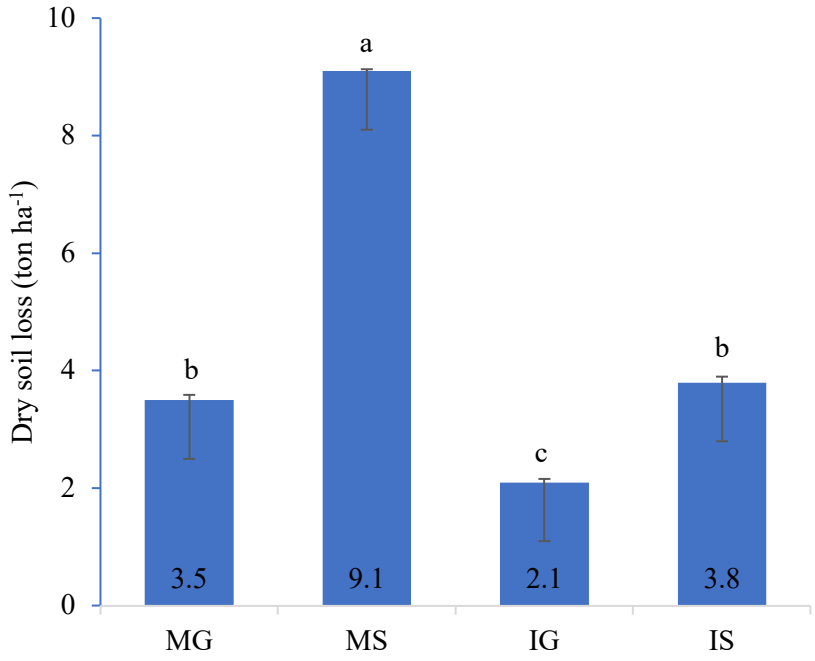

Figure 22 Impacts of different treatments on the loss of dry soil. Different letters on standard error bars indicate significant difference at $p<0.05$ between different treatments according to Tukey's HSD test. MG: cassava monocropping on gentle slope; MS: cassava monocropping on steep slope; IG: cassava-cowpea intercropping on gentle slope; IS: cassava-cowpea intercropping on steep slope.

Maximum dry soil loss (9.1 tons ha^{-1}) was observed in cassava monocropping on steep slope (MS) while minimum soil loss (2.1 tons ha^{-1}) was recorded in cassava-cowpea intercropping on gentle slope (IG) (Figure 22). There was no significant difference in dry eroded soil between the MG and IS treatments (3.5 and 3.8 tons ha^{-1}, respectively). This revealed that by intercropping one row of cowpea in between two rows of cassava on gentle and steep slopes, dry soil loss was reduced by 1.4 tons ha^{-1} (or 40.0%) and 5.3 tons ha^{-1} (or 58.2%) 40.0% and 58.2%, respectively.

Besides, cassava monocrop on gentle slope also reduced soil loss by 5.6 tons ha^{-1} (or 61.5%) compared to cassava monocrop on steep sloping lands. This finding is in line with previous studies which concluded that cassava monocropping often causes severe erosion when grown on sloping lands as compared to other common crops such as maize, wheat, or potato (Daellenbach *et al.*, 2005; Leihner *et al.*, 1996; Putthacharoen *et al.*, 1998). It is clearly evident that the inclusion of intercropping, one of the most effective agroecological practices, can considerably reduce runoff and soil loss (Garland *et al.*, 2017; Liu *et al.*, 2017; Trung *et al.*, 2013; Tuan *et al.*, 2014).

This study well confirmed the effectiveness of intercropping practices in reduction of runoff and soil loss by demonstrating a significant negative correlation (R^2 = 0.63; $p = 0.002$) between dry soil loss and soil covering level (Figure 23). An improvement of crop canopy and greater root structures could decrease the impacts of raindrops and surface water flux which result in the destruction of soil particles and the loss of surface fertile soils (Chen *et al.*, 2018; Chen *et al.*, 2003; Gebru, 2015; Gyssels *et al.*, 2005). At the study location, during the high rainfall period at the end of April or beginning of May, cassava development is still at the early stage and the canopy is not yet closed, therefore the inclusion of cover crop canopy is extremely valuable in reducing runoff and soil erosion. Moreover, according to Sharma *et al.* (2017), the root systems of cover crops improve infiltration of water into the soil and reduce the speed of surface water flow, thus result in induced water infiltration, decreased surface erosion and increased soil moisture. Yu *et al.* (1998) indicated that well-developed cassava canopy at later stages can only protect the topsoil from raindrop impact, but it cannot effectively inhibit the overland and down-slope water flow, which is more injurious to the topsoil on steep slopes. In addition, after harvesting cowpea, the residue mulches of cowpea left on the ground can also contribute to control soil erosion by improving soil aggregation and water infiltration, decreasing flow velocity and reducing runoff (Jordán *et al.*, 2010; Nzeyimana *et al.*, 2017; Rees *et al.*, 2002). Regarding to the impact of different slope degrees on soil loss, this study is in agreement with Koulouri and Giourga (2007) and El Kateb *et al.* (2013), who reported that soil loss was higher at steep slopes than at gentle slopes. This finding again affirmed that cassava-cowpea intercropping system is the most appropriate agricultural

form towards substantial reduction of soil erosion for the NMR of Vietnam. However, there is still a study indicated that whether slope degree is nonlinearly related to soil loss amount (Zhang *et al.*, 2018). He revealed that the soil loss rate only shows an increasing trend when slope length increases. This might be due to the complicated relationship between soil erosion rate and soil moisture, soil surface roughness, rock outcrops and climatic characteristics (Assouline and Ben-Hur, 2006; Kimaro *et al.*, 2008; Sadeghi *et al.*, 2013).

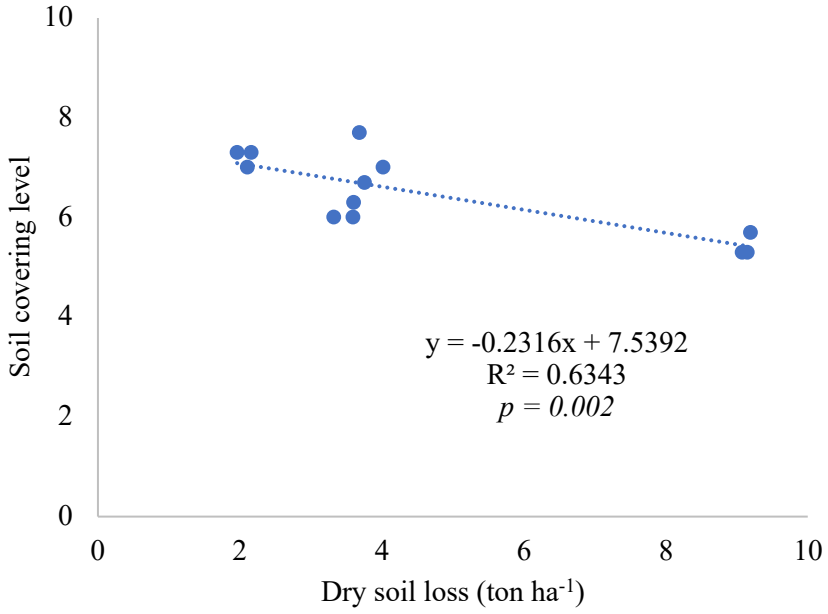

Figure 23 Simple linear regression between dry soil loss and soil covering level.

The impacts of rainfall and overland flow not only lead to thinning fertile topsoil, but also to the decrease in soil organic matter, beneficial microorganisms and essential nutrients. Regarding the nutrient losses through soil erosion, the highest N loss was recorded in treatment MS (35.4 kg ha^{-1}), followed by IS (22.8 kg ha^{-1}) (Figure 24). There was no significant difference between MG and IG treatments (15.1 and 12.7 kg ha^{-1}, respectively). Among all the treatments, a similar P loss was observed (Figure 25). K loss varied across different treatments (Figure 26) with the maximum K loss observed in MS (24.3 kg ha^{-1}) and the minimum recorded in IG (13.1 kg ha^{-1}). In IS, K loss amount (20.7 kg ha^{-1}) was higher than in MG (16.9 kg ha^{-1}). This result implied that there is a uniform trend between eroded soil and N and K losses from eroded soil across

the treatments. The N and K losses as well as soil loss tend to be highest in cassava monocropping, whereas, such nutrient losses are lowest in cassava-cowpea intercropping system on gentle slope, where the eroded soils are lowest. This was confirmed by the positive correlation between eroded soil and N and K losses (Figure 27 and 29). Such finding is consistent with previous studies showing that increased soil loss leads to the higher nutrient loss (Pereira *et al.*, 2017; Sharma *et al.*, 2017). Even though there was no significant difference in P loss among all the treatments, there was a significant correlation ($R^2 = 0.53$; $p = 0.007$) between eroded soil and P loss (Figure 28). This result concurs with the positive correlation between other nutrients and dry soil loss as mentioned above. Thus, this study suggests that by controlling runoff and soil loss using cassava-cowpea intercropping, we can effectively reduce nutrient losses.

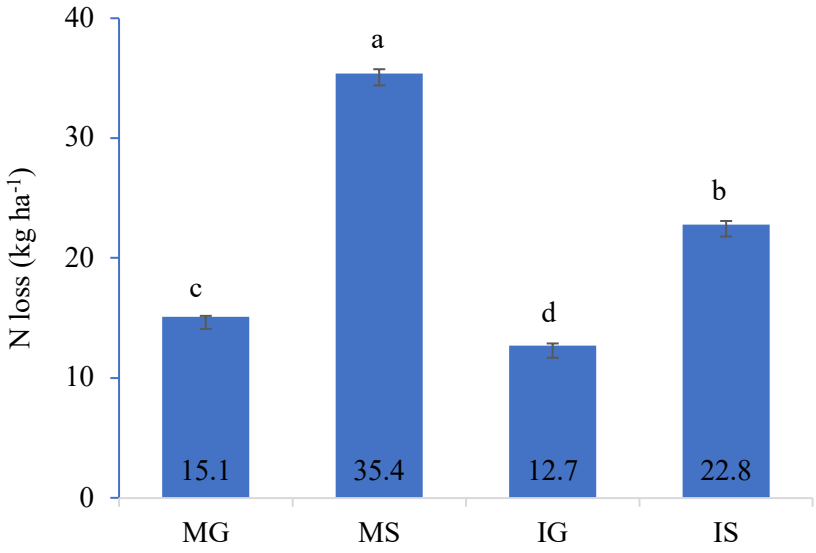

Figure 24 Impacts of different treatments on the loss of soil nitrogen (N). Different letters on standard error bars indicate significant difference at $p<0.05$ between different treatments according to Tukey's HSD test. MG: cassava monocropping on gentle slope; MS: cassava monocropping on steep slope; IG: cassava-cowpea intercropping on gentle slope; IS: cassava-cowpea intercropping on steep slope.

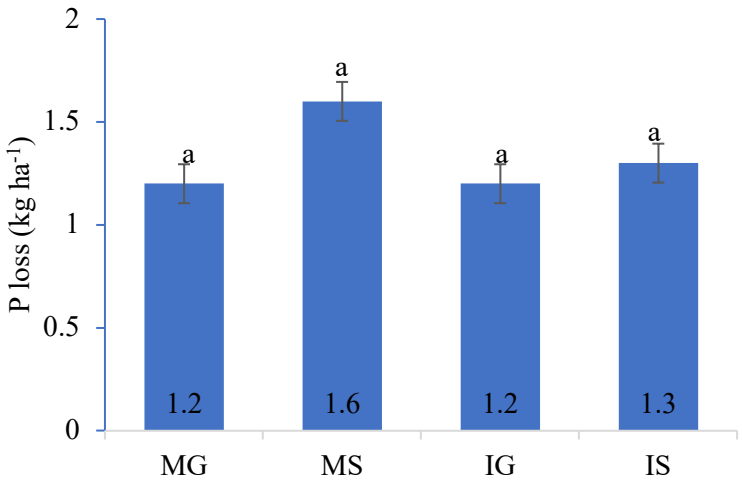

Figure 25 Impacts of different treatments on the loss of soil phosphorus (P). Different letters on standard error bars indicate significant difference at $p<0.05$ between different treatments according to Tukey's HSD test. MG: cassava monocropping on gentle slope; MS: cassava monocropping on steep slope; IG: cassava-cowpea intercropping on gentle slope; IS: cassava-cowpea intercropping on steep slope.

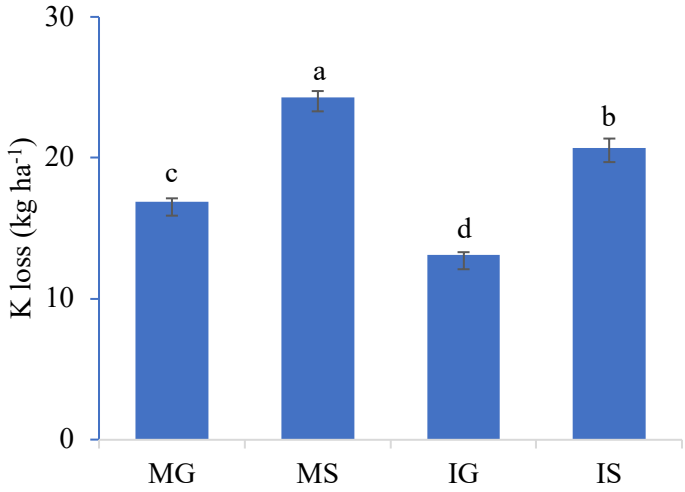

Figure 26 Impacts of different treatments on the loss of soil potassium (K). Different letters on standard error bars indicate significant difference at $p<0.05$ between different treatments according to Tukey's HSD test. MG: cassava monocropping on gentle slope; MS: cassava monocropping on steep slope; IG: cassava-cowpea intercropping on gentle slope; IS: cassava-cowpea intercropping on steep slope.

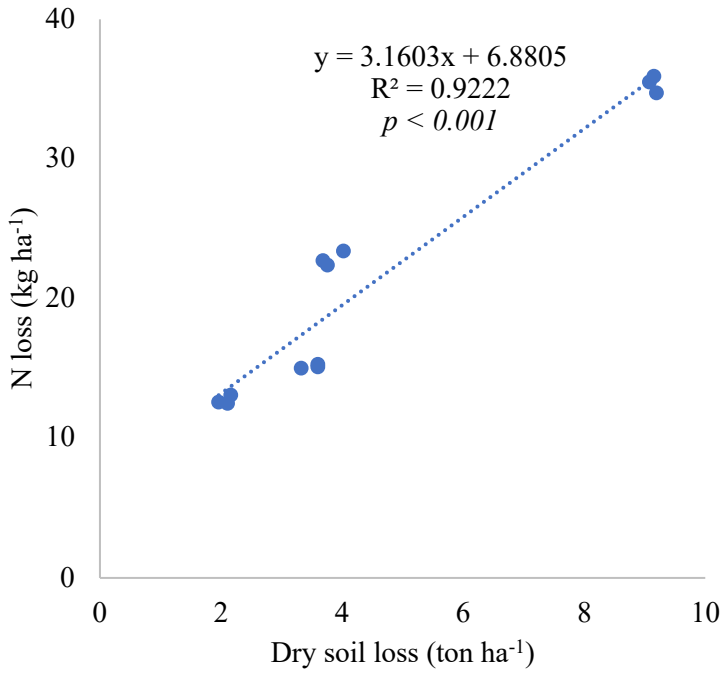

Figure 27 Simple linear regression between dry soil loss and soil nitrogen (N) loss.

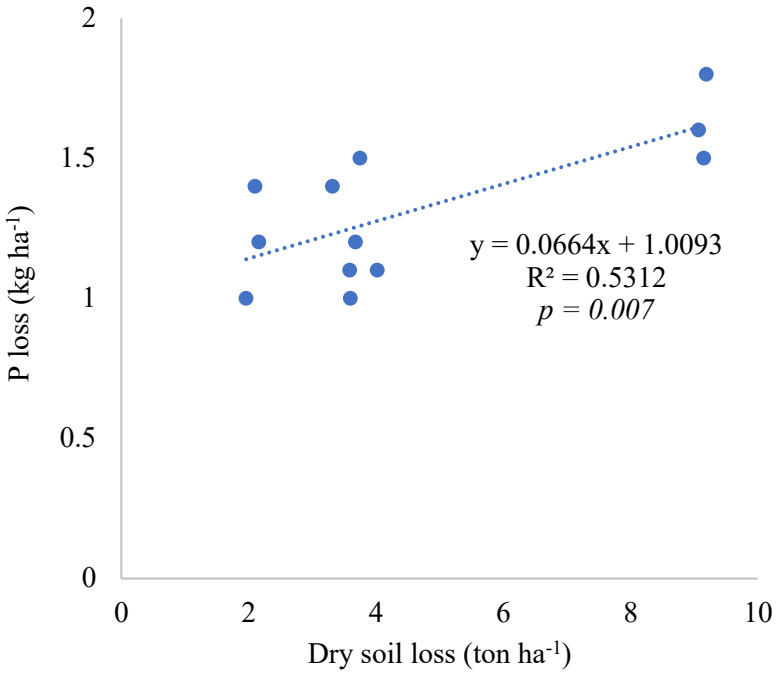

Figure 28 Simple linear regression between dry soil loss and soil phosphorus (P) loss.

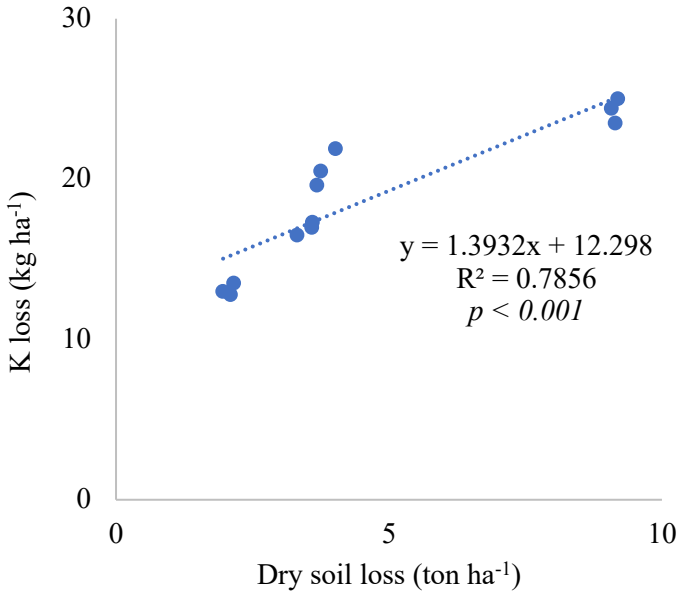

Figure 29 Simple linear regression between dry soil loss and soil potassium (K) loss.

3.3 Economic benefits

Cassava-cowpea intercropping on steep slope recorded the highest cost of cultivation (997 US$ ha^{-1}), while the least cost of cultivation was acquired by cassava monocrop on gentle slope (696 US$ ha^{-1}) (Table 12). The main element contributed to this high cost is the hiring charges of human labours for planting, sowing, weeding and harvesting on steep slope which required more human labours. Moreover, cassava-cowpea intercropping on gentle slope got higher cost of cultivation (970 US$ ha^{-1}) than cassava monocrop on steep slope (789 US$ ha^{-1}). Regarding the gross return, the intercropping treatments acquired higher gross returns (2397 and 2305 US$ ha^{-1}, respectively) than the monocropping treatments (1405 and 1326 US$ ha^{-1}, respectively). There were no significant differences in gross returns between either the two intercropping treatments or the two monocropping treatments. The same trend was observed in regard to net returns. Higher net return was found in the intercropping treatments: IG and IS (1427 and 1308 US$ ha^{-1}, respectively), while lower net return was achieved in the monocropping treatments: MG and MS (709 and 537 US$ ha^{-1}, respectively). The cassava-cowpea intercropping treatments IG and IS also acquired maximum B:C ratio (2.47 and 2.31, respectively) however there was no significant

difference between the two treatments. Cassava monocropping on gentle slope got the B:C ratio of 2.02, while the least B:C ratio was achieved in cassava monocropping on steep slope of 1.68. The result showed that the inclusion of intercropping of cassava with cowpea on either gentle slope or steep slope got higher net returns (718 and 771 US$ ha^{-1}, respectively) and B:C ratio (0.45 and 0.63, respectively). Such higher benefit was mainly from cowpea seeds in intercropping systems, which was 897 US$ ha^{-1} (or 37.4% of the total gross return) and 933 US$ ha^{-1} (or 40.5% of the total gross return) on gentle and steep sloping fields, respectively. Thus, the local farmers can almost double their income by practicing the intercropping system of cassava and cowpea.

At the study location, cowpea is easily sold to the local markets and has the higher market value (about 2.5 US$ kg^{-1}) as compared to the other legumes (peanut, soybean mung bean, etc.). Cowpea production could also bring additional income to the local farmers before cassava is harvested. These advantages of cowpea make it becomes the promised legume crop for local farmers to get the higher and stable income, while the price of cassava has fluctuated around only about 0.07 US$ kg^{-1}. Another reason contributed to the higher economic benefits of the intercropping systems is the greater net returns from increased cassava tuber yield. On gentle sloping fields, cassava yield in intercropping system was higher (of 1.5 tonnes ha^{-1}) than that in monocropping system, resulting in an increased net return of 98 US$ ha^{-1}. Whereas, on steep sloping fields, the inclusion of cassava-cowpea intercropping system resulted in the increased cassava yield of 0.7 tonnes ha^{-1}, or an increased net return of 46 US$ ha^{-1}. This might be due to the reduced soil and nutrient losses, or improved soil fertility in intercropping systems. This finding is consistent with previous studies which showed the economic benefits of legume-based intercropping systems (Howeler, 2015; Hy, 1998; Sharma *et al.*, 2017). Thus, this study proves the cassava-cowpea intercropping system in the mountainous region is the potential cropping system to increase the smallholder farmers' economic return.

Table 12 Cost of cultivation, gross and net income and Benefit: Cost ratio as influenced by cassava-cowpea intercropping system.

Treatments	Cost of cultivation (US$ ha^{-1})	Gross return (US$ ha^{-1})	Net return (US$ ha^{-1})	B:C ratio
MG	696 ^{d}	1405 ^{b}	709 ^{b}	2.02 ^{b}
MS	789 ^{c}	1326 ^{b}	537 ^{b}	1.68 ^{c}
IG	970 ^{b}	2397 ^{a}	1427 ^{a}	2.47 ^{a}
IS	997 ^{a}	2305 ^{a}	1308 ^{a}	2.31 ^{a}

Means followed by same lowercase letter(s) within the same column of each factor are not significantly different at $p < 0.05$ according to Tukey's HSD test.

MG: cassava monocropping on gentle slope; MS: cassava monocropping on steep slope; IG: cassava-cowpea intercropping on gentle slope; IS: cassava-cowpea intercropping on steep slope.

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

In upland Northern Vietnam, the local agricultural departments are very supportive for scaling up the cassava-cowpea intercropping system in order to mitigate soil degradation, improve soil health and increase farmer's income. From our survey, the percentage of farmers practicing cassava-cowpea intercropping in 2018 (54.74% of the total cassava farms) had more than tripled to 2017 (16.84% of the total cassava farms) and the area of intercropping farms in 2018 (18.0 ha) was 4.8 times higher than in 2017 (3.7 ha). This expansion revealed the high adoption level of local farmers with the inclusion of cassava-cowpea intercropping system at the study location in terms of crop production and economic benefits. In 2017, the natural nodulation of cowpea was very low regardless of soil characteristics, slope degrees or seasons, showing the urgent need to improve cowpea production by the inoculation of effective native rhizobia while no available rhizobia inoculant was found on the markets in Vietnam.

From collected cowpea nodules, based on the isolation, 16S rADN analysis and BLAST results from NCBI gene bank, there was a total of 21 native rhizobia strains isolated and sequenced from cowpea nodules in three study sites (11 *Rhizobium* strains and 10 *Bradyrhizobium* strains). Mau Dong commune had highest species richness (11), Shannon's and Simpson's diversity indices (1.47 and 0.82, respectively), followed by Son Thinh (7, 1.28 and 0.81, respectively) and Cat Thinh (3, 0.64 and 0.67, respectively). This result showed the possibility of using the native rhizobia strains as inoculant products for cowpea at these locations.

In the field screening experiment, the mixture of CMBP037+054 significantly increased nodulation of cowpea (19.4 nodules per plant, or 65.8%) compared to the uninoculated treatment. CMBP037+054 also resulted in high cowpea dry biomass, shoot total N content and yield (24.88 g plant^{-1}, 3.02%, and 424.7 kg ha^{-1}, respectively). CMBP037+054 inoculation showed the improvement of cowpea biomass (26.81%), shoot N content (4.86%) and yield (10.54%). Taking in to account how the impact of

different slope categories interacted with different inoculation treatments, the combination of CMBP037+054 showed their significant higher nodulation on moderate slope than Non_I treatment on gentle slope. This finding indicated the significant interaction between rhizobial inoculation and the geographical slope factor. The superior performance of the mixture CMBP037+054 revealed the presence of effective rhizobia at that research location and their potential as efficient inoculants for the further scaling-up production. The high rates of cowpea and cassava mycorrhizal infection (> 80% and >60%, respectively) implies that intercropping with cowpea at this site could encourage crop production by effectively absorbing poorly mobile nutrients in the soils and improve BNF of cowpea.

In the greenhouse screening experiment, the strains CMBP054, CMBP063, CMBP065 and CMBP066 significantly increased cowpea nodulation, shoot total N content and cowpea biomasses. These strains were rated as effective strains (with SEF of 54.56%, 58.77%, 55.73% and 51.64%, respectively) inferring that they are potential native strains for enhancing cowpea N fixation and could be evaluated under further field condition for producing effective inoculant products. There were significant correlations between nodule dry weight and shoot dry weight ($R^2 = 0.3183$, $p = 0.0012$), nodule dry weight and shoot total N content ($R^2 = 0.4240$, $p = 0.0019$). This study, therefore, confirmed that the inoculation of native rhizobia enhanced cowpea nodulation and N fixation, consequently improved shoot biomass and N content.

In the on-farm erosion experiment, on both gentle and steep slopes, the results showed that the inclusion of cowpea in cassava cropping system effectively improved soil covering level (2.2 and 1.7 out of 10, respectively), reduced soil erosion (40.0% and 58.2%, respectively) and nutrients losses. Moreover, the cassava-cowpea intercropping maintained cassava yield and significantly improved the incomes of local farmers (718 and 771 US$ ha^{-1}) and B:C ratio (22.3% and 37.5%, respectively). Regression analysis revealed that dry eroded soil showed significant correlation with percentage of land cover and nutrients losses. Thus, the inclusion of cassava-cowpea intercropping system significantly reduced the utilization of mineral fertilizers, sustained the production and consequently increased economic benefits to upland

smallholder farmers. With the great potential in soil conservation, stable productivity and economic enhancement, the inclusion of this intercropping system showed great potential as an appropriate strategy or a climate-smart agricultural practice for the sustainable agricultural production of the local farmers in Northern mountainous region of Vietnam.

Recommendations

This study suggests that it would make sense to isolate and screen more native rhizobia in order to better characterize the populations of rhizobia for cowpea at the experimental sites and get more effective and competitive strains. Further studies should be conducted to clarify the mechanism of the interaction between sloping categories and rhizobia inoculation efficiency. It is possible to promote the utilization of native rhizobia strains and provide cheap and effective inoculants for cowpea to the upland smallholder farmers. Further studies should also be conducted to find out the specific interaction between different native rhizobia strains in the same inoculant while there are only studies showed the synergistic interaction between rhizobia and plant growth promoting rhizobacteria (PGPR) or phosphate solubilizing bacteria (PSB).

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APPENDIX

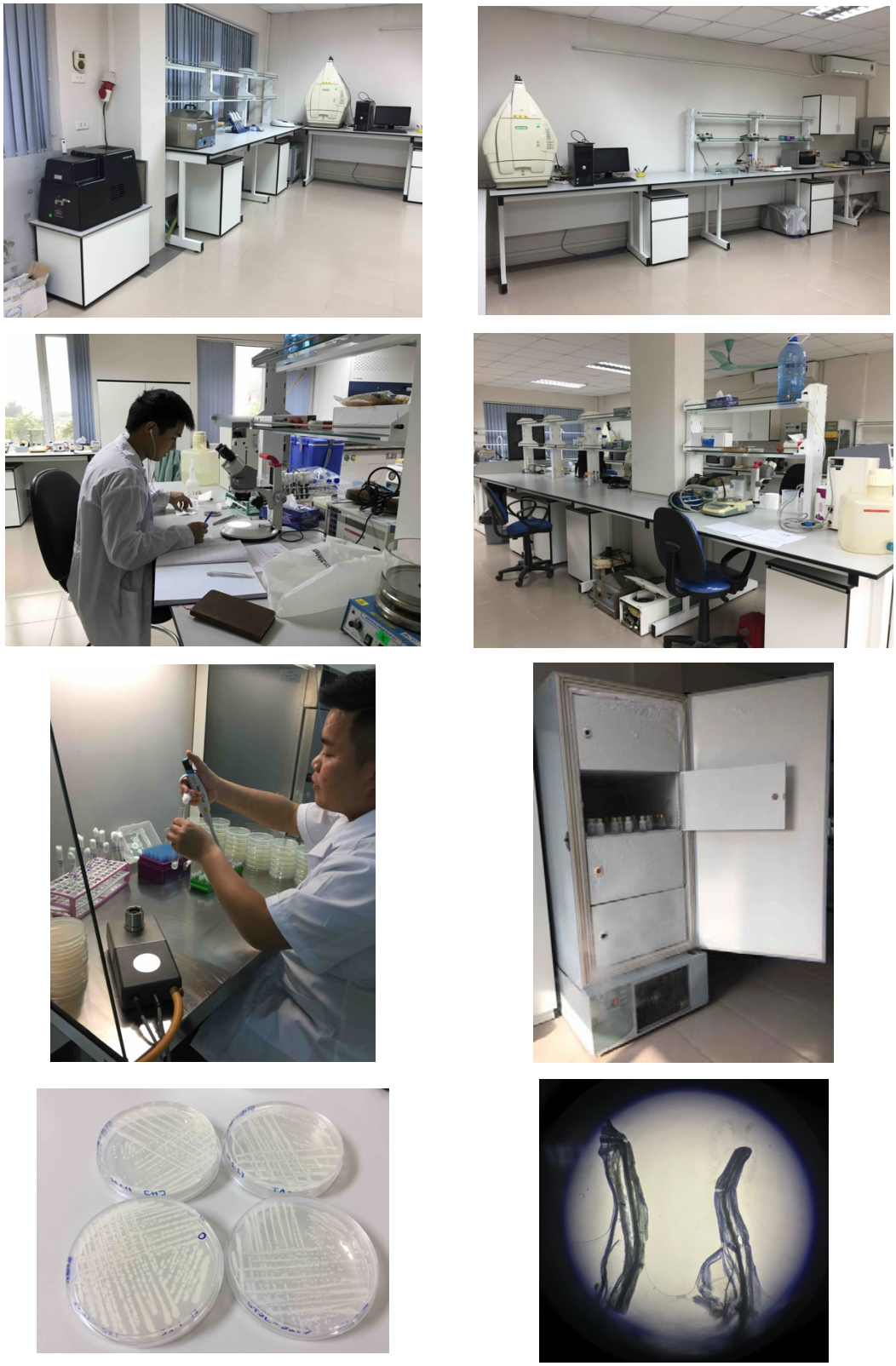





Appendix Figure 1 Preliminary investigation of natural cowpea nodulation and collection of cowpea nodules at different locations in 2017.

Appendix Figure 2 Inoculation experiment under field conditions at Mau Dong commune, Van Yen district, Yen Bai province in 2018.

Appendix Figure 3 Some photos from the on-farm erosion experiment at Mau Dong commune, Van Yen district, Yen Bai province, and the screening experiment under greenhouse condition at VNUA, Hanoi in 2018.



Appendix Figure 4 The Common Microbial Biotechnology Platform (CMBP) at CIAT-Asia, Hanoi, Vietnam.

SOP-MI06 LH-V01: Macroscopic observation of a bacteria culture (liquid and solid media

I. Objective

In a liquid media, the bacteria disperse freely, and the growth leads to the turbulence of the media. On a solid media, the bacteria are dropped on the surface of the gel and multiply to form clusters, which are visible with the naked eyes. These are called « colonies ».

Observation of the colonies formed on a solid media or the aspect of the tubes after growth can be a tool for identification of the bacteria. This procedure describes how to observe the different criteria which can be useful for the identification process.
Also read the procedure "SOP-MI01/V01: General information in a Microbiology laboratory" before starting to work in the microbiology laboratory.

II. Definitions

- Aseptic conditions/Aseptically: Environment where no microorganism is present. This can be obtained using a Bunsen burner (the aseptic zone is the spherical area around the flame with a diameter of approximately 15 cm) or a laminar flow hood (sterile air is continuously produced and present in the hood and the difference of pressures between the inside and outside of the hood prevents the air outside to come inside and contaminate the environment under the hood).

- Contaminated by microorganisms: Every plate, tube, pipette, or other instruments (glassware, pestles, Eppendorf tube...) which has been in contact with microorganisms and cannot be sterilized by the flame of a Bunsen burner is considered as contaminated.

- Contaminated by toxic chemicals: Every tube, flask, pipette or other instruments (weighing boats, glassware…) which has been in contact with toxic chemicals is considered as contaminated.

- Good Laboratory Practices (GLP): The Principles of Good Laboratory Practice (GLP) have been developed to promote the quality and validity of results and of the analysis conducted in a laboratory. It is a managerial concept covering the organization and the conditions under which laboratory studies are planned, performed, monitored, recorded and reported. Its principles also include the protection of man and the environment.

- Mother tube/plate/product: Tube/plate/product which the bacteria are picked from. The result of the growth of this inoculation is considered as the daughter which can become the mother for the next inoculation...

III. Abbreviations

- °C: Celsius degree
- GLP: Good Laboratory Practices
- GO: Ordinary Gelose
- mm: millimetre

IV. Procedure

A. Materials, furniture, reagents

1) Chemicals

- Plates/tubes (mother)

2) Material

- Bunsen burner
- Laminar hood

B. Description of analysis

1) Liquid culture

- The observation of several characteristics of a liquid culture may be a tool for identifying a bacterial species.
- It can be done in three steps:

1) Before agitation:
   - Surface of the liquid media: incline gently the tube and observe the presence of a floating piece of culture (as a trouble or a shadow) or a ring sticking on the tube sides.
   - Media: presence or absence of turbulence, thick or faint, uniform or heterogeneous.
   - Bottom of the tube: presence or absence of deposit, colour, texture…

2) After agitation: describe the turbulence and the deposit: sticky, homogeneous, heterogeneous…

3) Smell of the culture: Some bacteria have a specific smell, such as *Pseudomonas aeruginosa* which smells like honey. Open the tube in aseptic conditions (under the hood or near the Bunsen burner) and don't breathe too close to the tube.

2) Colony on solid media

- Select an isolated colony, as big as possible, and assess the different characteristics:

- Shape:
  - round, circular 
  - irregular 
  - stringy 
  - elongated 
  - ...

- Outlines
  - regular 
  - crenel 
  - wavy 
  - foiled 
  - stringy 
  - curly 
  - ...

- Size
  - Very small: almost not visible
  - small: < 2 mm (diameter)
  - medium: between 2 and 5 mm (diameter)
  - big: >5 mm (diameter)

- Pigment: colour, diffusion in the media

- Opacity: transparent, translucent, opaque
- Height:

- flat
- rounded 
- high centre 
- high 
- convex 
- ...

- Surface: smooth and brilliant (type S) or rough and wrinkled (type R)
- Texture: mucous (honey), greasy, creamy, dry
- Smell: if present, try to describe it.

C. Safety

- Biological hazards: Manipulating microorganisms poses a risk not only to the one who is working, but also to other people in the lab and potentially to the environment in case of dissemination. The rules of safety have to be well understood and respected in order to avoid any contamination of the staff and/or environment (Read the "Hygiene and Security rules in a laboratory" document for more details). In case of accidental contamination (broken test tubes, suspension spilled on the bench, direct contact with microorganisms, wounds with contaminated material...), clean and disinfect properly before the activities can be restarted. Dispose contaminated waste as indicated in the section E. For more details, consult also the "Hygiene and Safety rules in a laboratory" document.
- Bunsen burner: The risk of fire can be minimised by following a few simple rules. If the hood is being used, turn on the fire only when it is needed, don't cross your arms, don't pass your arm on the burner... Also see the safety rules about fire in the "Hygiene and Safety rules in a laboratory" document.
- Laminar flow hood: If the hood is not working properly, it can lead to a fire risk. The maintenance has to be done regularly and the results recorded in a specific file (Maintenance file, available in the office).

D. Quality control management

- The name and origin of the samples, name of the plates, results of the observation, the date of observation and any other relevant information are recorded in the lab book.

- A data base can be done to follow the plates with time.
- The plates are observed every day, at least once a day.

- Equipment maintenance: All the equipment are regularly checked in regard to the specific specifications. The results are recorded and in case of repairs, the details about the intervention are recorded. Details about maintenance services and repairs are compiled in the Maintenance file, available in the office.
- Hood maintenance: In addition to the general maintenance, simple tests can be run to assess the good functioning of the hood: Petri plates of GO (or other media depending on the targeted microorganisms) are left open for some time under the hood while on and then incubated. If the plates are highly contaminated, then the hood is not working properly. Bacteriological control can also be performed, as explained in the SOP-GA02/V01 "Cleaning plan".
- The general Good Laboratory Practices: They must be respected by everyone in the lab. The "Hygiene and Safety rules in a laboratory" document describes the general rules to be observed in the laboratories.
- In case of accident: Every accident must be reported to the lab manager and the staff if needed. Lab manager must put necessary measures in place to avoid the accident to occur again. For details on what to do in case of accident, read the "Hygiene and Safety rules in a laboratory" document.
- Use of the equipment: For specific equipment, a form has to be filled when used. This may include the date and time of use (start and end), the name of the user, the notification of any deviations or problems and any relevant information about the equipment. These forms are also used to estimate the needs and the frequency of the maintenance. Books are available near the specific equipment and should not be taken away.

E. Waste and decontamination

- Non-contaminated waste is eliminated in the normal bin.
- Anything contaminated by microorganisms should be decontaminated before appropriate elimination/cleaning. Waste are put in a special autoclave bag and autoclaved for 20 min at 121°C. The autoclave bag can then be disposed off as non-contaminated waste. Re used material (glassware, small tools as sieves, pestles, ...) are autoclaved and then cleaned as non-contaminated items. Not reused glass instruments (pipettes, slides, cover glasses, broken glassware...) are put in a beaker containing Sodium hypochlorite solution for decontamination before being eliminated as non-contaminated glass waste.
- Solid (including broken glass) and liquid waste contaminated by toxic chemicals are placed in separate containers (labelled with the mention: "Toxic waste, Danger"). Contaminated glassware is properly rinsed with tap water and the water is collected in a specific container for toxic liquid waste. The glassware can then be cleaned as non-contaminated items. Consult the MSDS of the product for more information since non-compatible products should not be put in the same container.

F. Cleaning

A complete Cleaning Plan is available for details. Consult SOP-GA02/V01 “Cleaning plan”.

- The rooms are cleaned on a daily basis.
- Before starting and after manipulation, the hood and/or the bench is/are cleaned with a disinfectant and/or with 70% Ethanol.
- In case of contamination of the bench, floor, user, ..., it has to be cleaned and disinfected before the work can be continued (cf. “Hygiene and Safety rules in a laboratory” document).
- Non-contaminated or decontaminated items are cleaned with soap, rinsed with water and eventually rinsed with distilled water.
- Equipment: cf the SOP-GA02/V01 “Cleaning plan” for details.

SOP-MI10 LH-V01: Counting bacteria from a suspension-solid, using poured plates method and Malassez slide

I. Objective

To assess the bacterial concentration of a suspension, the suspension must be diluted many times until the last dilutions, spread on a solid media, make possible countable isolated colonies. With the number of colonies, the spread volume and the dilution, it is possible to estimate the bacterial concentration of the initial suspension.

In accordance with the GPL, this procedure describes how to prepare the dilutions, how to pour them, how to read the results and make the calculations for both methods.
Also read the procedure "SOP-MI01/V01: General information in a Microbiology laboratory" before starting working in the microbiology laboratory.

II. Definitions

- Aseptic conditions/Aseptically: Environment where no microorganism is present. This can be obtained using a Bunsen burner (the aseptic zone is the spherical area around the flame, with a diameter of approximately 15 cm), or a laminar flow hood (sterile air is continuously produced and present in the hood and the difference of pressures between the inside and outside of the hood prevents the air outside to come inside and contaminate the environment under the hood).

- Contaminated by microorganisms: Every plate, tube, pipette, or other instruments (glassware, pestles, Eppendorf tube...) which has been in contact with microorganisms and cannot be sterilized by the flame of a Bunsen burner is considered as contaminated.

- Contaminated by toxic chemicals: Every tube, flask, pipette or other instruments (weighing boats, glassware…) which has been in contact with toxic chemicals is considered as contaminated.

- Good Laboratory Practices (GLP): The Principles of Good Laboratory Practice (GLP) have been developed to promote the quality and validity of results and of the analysis conducted in a laboratory. It is a managerial concept covering the organization and the conditions under which laboratory studies are planned, performed, monitored, recorded and reported. Its principles also include the protection of man and the environment.

- Mother tube/plate/product: Tube/plate/product which the bacteria are picked from. The result of the growth of this inoculation is considered as the daughter which can become the mother for the next inoculation...

III. Abbreviations

- °C: Celsius degree
- cm: centimetre
- g: gram
- g/l: gram per litre
- GLP: Good Laboratory Practices
- GO: Ordinary Gelose
- ml: millilitre
- MSDS: Material Safety Data Sheet
- NaCl: Sodium Chloride
- O_2: Dioxygen
- sec: second
- UFC/ml: Unit Forming Colony per millilitre

IV. Procedures

A. Materials, furniture, reagents

1) Chemicals

- 70% Ethanol
- 9 ml physiological water in tubes (NaCl, 9 g/l)
- Hypochlorite solution
- Mother suspension/product (to be counted)
- Solid media (in plates)

2) Material

- Bunsen burner
- Incubators and Rotative incubator
- Laminar hood
- Microscope
- Vortex

3) Instruments

- Dropper
- Malassez slide + coverslip
- Mother plates, tubes or products
- Plastic box for incubation
- Racks for test tubes
- Soft tissue
- Sterile Pasteur pipettes; Sterile volumetric pipettes

B. Description of analysis

1) Poured plates

- Dilutions

- A suspension can be very rich in bacteria, for example 2 900 000 bacteria per ml. For example, if 0.1 ml of this suspension is spread on a solid media, 290 000 isolated colonies may be expected. This is obviously not possible to get: colonies will not be isolated and will just form a homogenous growth.
- If the initial suspension is diluted by 1000 (10^{-3} dilution), the concentration will be around 2900 bacteria per ml. For instance, after pouring 0.1 ml on the plate, 290 isolated colonies may be expected, which is much easier to count on a plate.

- Dilutions are generally done with a 10 factor. That means that the proportion between the initial and final suspension is 1 for 10.
- Usually, the first dilution is done using a bigger quantity to get a representative sample of the initial product to test. The quantity depends on the nature of the sample and the feasibility, but 10 ml or 10 g are generally used (mixed with 90 ml of physiological water).
- To calculate the bacterial concentration of a solid product (food, soil…), a suspension must be made because the solid product can obviously not be pipetted. The sample and the thinner are mixed so that the bacteria from the product are transferred into the liquid suspension. As explained before, the proportions of product and thinner are calculated to get a 1/10 suspension (to get the « 10^{-1} dilution »): 10 g of product are mixed with 90 ml of thinner.

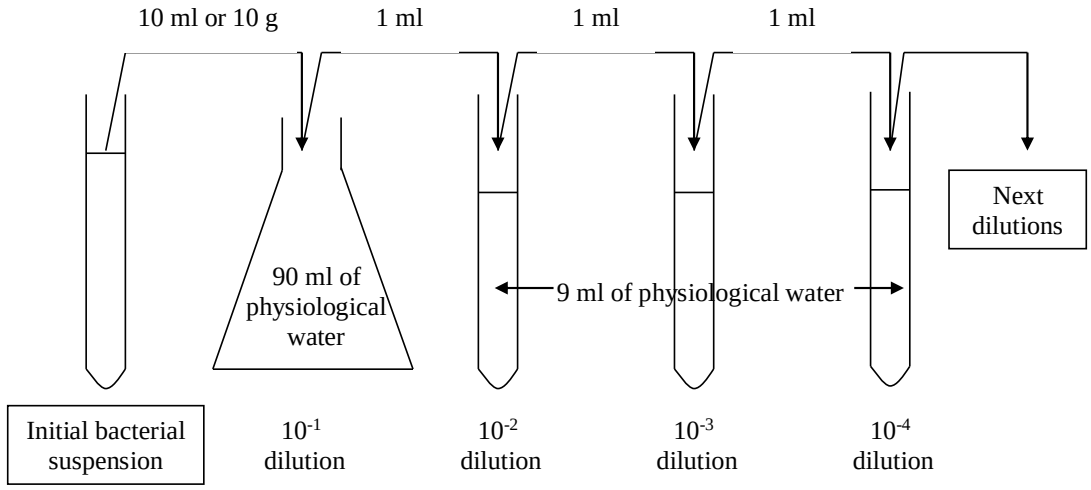
- The subsequent dilutions are made from this suspension.
- Several thinners may be used to dilute liquid culture:
    - Sterile physiological water (NaCl, 9 $g.l^{-1}$): It is the most commonly used and the one used in the microbiology laboratory.
    - Tryptone or peptone solution (peptone or tryptone, 1 g/l)
    - Sterile distilled water can be used if dilutions are immediately spread. Otherwise the bacteria will die because of osmotic pressure.

- Procedure

- Pipetted volumes must be very accurate so sterile calibrated pipettes are used to make all the dilutions.
- Prepare 9 ml tubes and 90 ml flasks of sterile physiological water and label them.
- Take 10 ml or 10 g of the product to analyse and under aseptic conditions pour in the 90 ml of physiological water.
- Shake the mother suspension or mix the product to make it very well homogenised. Best is to put the flask under agitation in a rotative incubator for some time to get a better suspension. That represents the 10^{-1} dilution.
- Open the flask, pass the top through the flame and pipette exactly 1 ml of the suspension.

- Keep the pipette vertically, pass the top of the tube through the flame, close the tube and put it back on the rack. Take the 10^{-2} dilution tube, open it, pass it through the flame, and add the 1 ml of the initial suspension. Pass the tube through the flame, close it and put the pipette into a bleach solution. Don't pass the pipette through the fire to avoid emissions of micro drops of bacteria suspension.
- Shake the tube manually by rotations (don't allow the liquid to touch the cap or the cotton wool) or better with a vortex for at least 10 sec. This tube is the 10^{-2} dilution.
- For the 10^{-3} suspension, pipette 1ml of 10^{-2} dilution and put it into the next tube (9 ml of sterile physiological water).
- Repeat this protocol until all the needed dilutions are prepared.

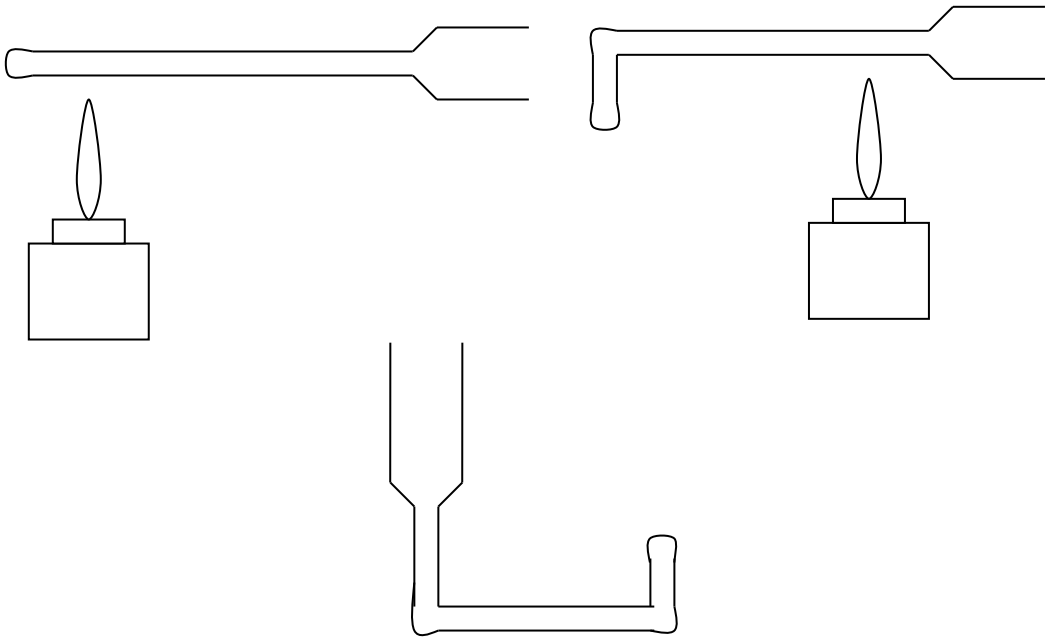
- To know approximately how many dilutions must be prepared, one needs to have an idea of the initial bacteria concentration of the mother suspension/product. For solid products, literature and previous analyses may give some information. For a suspension, the more the media has turbulence, the more the concentration is important, so the more it will have to be diluted. It is assumed that at the maximum growth (when the plateau is reached), the concentration of bacteria is about 10^8 UFC/ml.



- <u>Plating</u>

0.1 ml of every dilution is spread on a plate and the colonies formed by bacteria present in the volume are counted. The volume must be spread uniformly on all the surface of the plate.

- With a sterile Pasteur pipette, make a tool as described below:



- Put the end of the pipette (1 cm approximately) in the blue part of the flame and wait until it makes a bend. In the same way, do it at the top of the thin part of the pipette (1 cm or so).
- With a pipette, put 0.1 ml of the most diluted suspension on a plate, and use the tool to spread it. Either a volumetric pipette or a Pasteur pipette can be used. It is assumed that three drops from a Pasteur pipette are equal to 0.1 ml. If the dilutions are put from the lowest to the highest, the same pipette and the same tool can be used to pour and spread the different dilutions. Be careful, the parts which are in contact with the bacteria suspensions or the plates mustn't touch the bench or the sides at any time. If the same pipette is used to pour all the dilutions, don't let the drops dry before spreading.
- To improve the quality of the results, three plates (replicates) are usually done for every dilution. Put all the plates in a plastic box and incubate at optimized conditions (time, temperature).

- For bacteria growing in a small concentration of O_2 (semi anaerobic bacteria for example), the spreading can be done with a different method called depth counting. This technique is also used to avoid the growth of invasive bacteria in pluribacterial suspension and to improve the growth of the one which must be selected.
- The dilution method is the same, but the plates are prepared differently. Pour 1 ml in an empty plate and pour a thin stratum of media on it. Shake gently and once it is solidified, add a second thin stratum of media. Let it solidify without any movement and incubate as previously indicated.

- Calculations

- Plates must have isolated colonies only. If not, the dilution is not taken into account.

- If the colonies are too many on a plate (>300), even if they are isolated, the plate is not selected. Same thing if they are too few (<30). So only the plates which 30 to 300 isolated colonies can be counted are selected.
- Depending on the type of experiment, only the typical colonies are counted (for example, counting of *E. coli* in a water sample containing a high diversity of bacteria).
- The concentration of bacteria in the initial suspension is calculated as follows:

$$N = \frac{n}{C \times V}$$

N: concentration of the initial suspension (unit = UFC (Unit Forming Colony) per ml)
n: number of isolated colonies counted on the plate (no unit)
C: dilution (no unit)
V: Spread volume (unit = ml; 0.1 ml or 1 ml depending on the method)

For example: 250 isolated colonies are counted on a plate from 10^{-3} dilution. Initial suspension concentration is:

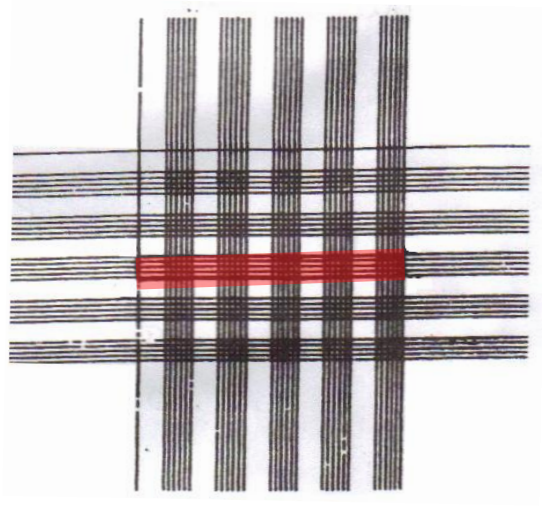
$N = 250 / (10^{-3} \times 0.1) = 2500000 = 2.5 \times 10^6$ UFC/ml

- If one or more replicates for the same dilution is/are not selected (too many or too few colonies), the rest of the replicates mustn't be selected as well.
- Normally, just one or two dilutions are supposed to be selected. If several dilutions may be used, just calculate the concentration for each and then calculate the average of the results.
- The results are always presented with 2 significant numbers only.

2) Malassez slide

- Principle

Malassez slide has an engraved lines network. This network is formed by 100 rectangles, grouped into 10 main lines. Every rectangle is composed by 20 small squares (smallest division). A drop of the suspension is put on the network, and is covered with a specific lamella which is flat. The sides of the slide are slightly upswept, so the lamella supports on them, and between the slide and the lamella, the total volume of the network is exactly 1 mm^3. The counting is usually done on a line (0.1 mm^3), as seen on the schema, but it is better to repeat the counting on two or three lines.



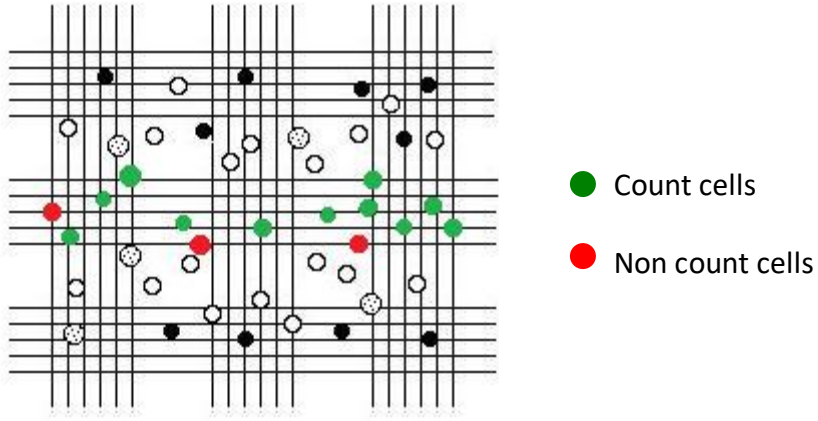
- Limits of the method:

  - If the cellular concentration is too low, the counting is not efficient. It is possible to repeat many counting to confirm the results.
  - A fixative agent should be added if the cells are moving too fast because of their mobility. The dilution created by the agent has to be taken into consideration.
  - If the cells are too small, the zoom lens x40 is not sufficient, and it is not easy to count with the x100 lens.
  - Dead and alive cells are counted without any distinction, so are different species if the sample is a mix of different bacteria.
  - Suspensions must be diluted if more than 5 cells are counted in a small square.

- Procedure

- Clean the slide with 70% Ethanol and soft tissue.
- Slightly wet the sides of the slide (where the coverslip will be supported) with water and add the coverslip. If it slippers, there is too much water.
- Pipette a small volume of the dilution to analyse, touch the side of the coverslip and completely fill the space between the slide and the coverslip. If the coverslip falls, there was too much liquid.
- Place under the microscope and focus on the grid. See the SOP-MI07/V01 “Using a microscope” for details.
- Count the bacteria on one line and replicate the counting.
- To be sure not to count twice the same cell when they are on the lines, only the cells on the top and on the right borders are counted. Cells between the lines will be counted normally.

For example, if the counting is done on the middle line, the green cell will be counted, and the red ones will not.



- Calculations

- The concentration of bacteria in the initial suspension is calculated as follows:

$$N = \frac{n \times 10^3 \times C}{v}$$

N: cellular concentration (number of cells per mL)
n: total number of cells counted
v: studied volume in mm^3 (number of lines where the cells are counted x 0.1)
C: dilution factor if the suspension is diluted

- The results are always presented with 2 significant numbers only.

C. Safety

- Autoclave: The autoclave can pose a great danger if not used correctly because of the high pressure and temperature. Before using, visually check the general aspect (no corrosion, no leak), the quantity of water and the settings. Strictly follow the instructions to start it. During the heating phase, the pressure increases. Be sure that the door is correctly closed so that there is no leakage. Don't try to force the autoclave to open. The autoclave has a safety device and will refuse to open if the temperature is still high or if the pressure is not back to the atmospheric pressure. The maintenance must be done regularly, and the results recorded in a specific file (Maintenance file, available in the office).
- Biological hazards: Manipulating microorganisms poses a risk not only to the one who is working, but also to other people in the lab and potentially to the environment in case of dissemination. The rules of safety must be well understood and respected to avoid any contamination of the staff and/or environment (Read the "Hygiene and Security rules in a laboratory" document for more details). In case of accidental contamination (broken test tubes, suspension spilled on the bench, direct

contact with microorganisms, wounds with contaminated material...), clean and disinfect properly before the activities can be restarted. Dispose contaminated waste as indicated in the section E. For more details, consult also the "Hygiene and Safety rules in a laboratory" document.

- Bunsen burner: The risk of fire can be minimised by following a few simple rules. If the hood is being used, turn on the fire only when it is needed, don't cross your arms, don't pass your arm on the burner... Also see the safety rules about fire in the "Hygiene and Safety rules in a laboratory" document.
- Chemicals: Before using a new chemical, the information about the toxicity, conditions of use, risks and safety phrases... must be understood and observed. Use the equipment for protection if needed (gloves, masks, hood...). The MSDS (Material Safety Data Sheet) of the different chemicals are also available to get more details about the products. MSDS files are available in the Preparation Room and in the office.
  Special caution for:
  - Sodium hypochlorite: Contact with acids liberates toxic gas. Causes burns.
- Laminar flow hood: If the hood is not working properly, it can lead to a fire risk. The maintenance must be done regularly, and the results recorded in a specific file (Maintenance file, available in the office).

D. Quality control management

- The protocol, date of analysis and counting, name and origin of the samples, name of the plates, number of dilutions prepared and poured, calculations and any other relevant information are recorded in the lab book.
- Before inoculation, the tubes of physiological water and the plates of media are visually checked. The plates and the tubes which are contaminated or wet (plates) are discarded.
- After incubation, when counting the colonies, every dilution should contain about 10 times less UFC than the previous dilution (more diluted, less concentrated). If not, the experiment should be repeated because that can highlight a problem during the dilution process.
- The results obtained for the replicates of the same dilution should be approximately the same. If it is not the same, the entire dilution is discarded.

- All the equipment are regularly checked in regard to the specific specifications. The results are recorded and in case of repairs, the details about the intervention are recorded. Details about maintenance services and repairs are compiled in the Maintenance file, available in the office.
- Hood maintenance: In addition to the general maintenance, simple tests can be run to assess the good functioning of the hood: Petri plates of GO (or other media depending on the targeted microorganisms) are left open for some time under the hood while on and then incubated. If the plates are highly contaminated, then the hood is not working properly. Bacteriological control can also be performed, as explained in the SOP-GA02/V01 "Cleaning plan".

- The general Good Laboratory Practices: They must be respected by everyone in the lab. The "Hygiene and Safety rules in a laboratory" document describes the general rules to be observed in the laboratories.
- In case of accident: Every accident must be reported to the lab manager and the staff if needed. Lab manager must put necessary measures in place to avoid the accident to occur again. For details on what to do in case of accident, read the "Hygiene and Safety rules in a laboratory" document.
- Use of the equipment: For specific equipment, a form has to be filled when used. This may include the date and time of use (start and end), the name of the user, the notification of any deviations or problems and any relevant information about the equipment. These forms are also used to estimate the needs and the frequency of the maintenance. Books are available near the specific equipment and should not be taken away.

E. Waste and decontamination

- Non-contaminated waste is eliminated in the normal bin.
- Non-contaminated glass waste (Pasteur pipette, slides, broken glassware...) are put in a separate container labelled with the mention: "Broken glass".
- Anything contaminated by microorganisms should be decontaminated before appropriate elimination/cleaning. Waste are put in a special autoclave bag and autoclaved for 20 min at 121°C. The autoclave bag can then be disposed off as non-contaminated waste. Re used material (glassware, small tools as sieves, pestles, ...) are autoclaved and then cleaned as non-contaminated items. Not reused glass instruments (pipettes, slides, cover glasses, broken glassware...) are put in a beaker containing Sodium hypochlorite solution for decontamination before being eliminated as non-contaminated glass waste.
- Solid (including broken glass) and liquid waste contaminated by toxic chemicals are placed in separate containers (labelled with the mention: "Toxic waste, Danger"). Contaminated glassware is properly rinsed with tap water and the water is collected in a specific container for toxic liquid waste. The glassware can then be cleaned as non-contaminated items. Consult the MSDS of the product for more information since non-compatible products should not be put in the same container.

F. Cleaning

A complete Cleaning Plan is available for details. Consult SOP-GA02/V01 "Cleaning plan".

- The rooms are cleaned on a daily basis.
- Before starting and after manipulation, the hood and/or the bench is/are cleaned with a disinfectant and/or with 70% Ethanol.
- In case of contamination of the bench, floor, user, ..., it has to be cleaned and disinfected before the work can be continued (cf. "Hygiene and Safety rules in a laboratory" document).

- Non-contaminated or decontaminated items are cleaned with soap, rinsed with water and eventually rinsed with distilled water.
- Equipment: cf the SOP-GA02/V01 “Cleaning plan” for details.

SOP-MI16 LH-V01: AMF infection assessment

I. Objective

The objective of this protocol is to stain the AMF contained in roots, and to assess the intensity of the mycorrhizal infection.

II. Abbreviations

- °C: Celsius degree
- g/l: gram per litre
- min: minute
- ml: millilitre
- MSDS: Material Safety Data Sheet

III. Procedures

A. Materials, furnitures, reagents

1) Chemicals

- Black ink (brand Sheaffer)
- H_2O_2 (3%)
- KOH
- NH_4OH (~25%)
- White vinegar (5% acetic acid)
- 70% Ethanol

2) Materials

- Fume hood
- Heating plate / water bath
- Microscope or binocular microscope

3) Instruments

- Coverslip 22x50mm
- Microscope slides
- Petri dish
- Pipette + Dropper
- Forceps

- Scalpel
- Glass tubes and rack
- Beaker (in glass)
- Spatulas/spoons
- Plastic beakers (size doesn't matter)
- Soft tissue
- Gloves

B. Description of analysis

1) KOH 10% solution preparation

- For 24 tubes, approximately 750 ml of KOH 10% is required.
- To prepare 1 litre, add 100 g of KOH pellets in approximately 700 ml of water.
- CAUTION: NEVER add the water on the pellets but ALWAYS the pellets in the water.
- The dissolving pellets will produce heat, so add them slowly and shake slowly to reduce the heat production.
- Once all the pellets have been dissolved, top up to 1 litre with water.

2) Clearing solution preparation

- The clearing solution must be prepared just before the experiment. DO NOT prepare it in advance.
- For 24 tubes, approximately 200 ml of clearing solution is required.
- To prepare 200 ml: add 20 ml of NH_4OH solution to 180 ml of H_2O_2 under a chemical hood. The solution has to turn yellow.
- CAUTION: the NH_4OH solution is highly toxic, so it must stay under the chemical hood at all times.

3) Black ink solution

- To prepare 250 ml of black ink solution: add 20 ml of black ink to 230 ml of white vinegar (5% acetic acid)

4) Root preparation

- Separate the roots, take only the thinnest roots.
- Label the tubes with tape and a marker pen.
- Put the roots in test tubes: avoid breaking them if they are dry.

- If the roots were dried before analysis, fill the tubes with tap water and store at room temperature for 24-48 hours. You can change the water after 24 hours if required.

5) Root staining

- Place the heating plate or the water bath under a chemical hood.
- If using a heating plate, fill a glass beaker with water and place it on the plate.
- Heat the water until it boils (or at least 90°C).
- Remove the water from the test tubes and add the KOH solution to cover the roots.
- Place the test tubes in the beaker or in the water bath and incubate at >90°C for 30 min.
- Remove the KOH solution from the tubes and replace it with a fresh KOH solution.
- Incubate again for 30 min at >90°C.
- Empty the tubes and rinse the roots with tap water 3 times.
- After the last rinse, add the clearing solution to cover the roots.
- Incubate for 30 min at room temperature.
- Empty the tubes and rinse the roots with tap water 3 times.
- After the last rinse, add the black ink solution to cover the roots.
- Incubate for 30 min at >90°C in the water bath or on the heating plate.
- Empty the tubes and rinse the roots with pure white vinegar (5% acetic acid).
- Fill the bottle of vinegar with water (top up) and use this solution to rinse the roots the second time.
- Fill the bottle of vinegar again with water and rinse the roots a 3rd time.
- DO NOT empty the tubes after the last rinse.
- Keep the tubes containing the stained roots + the vinegar/water solution at 4°C overnight.

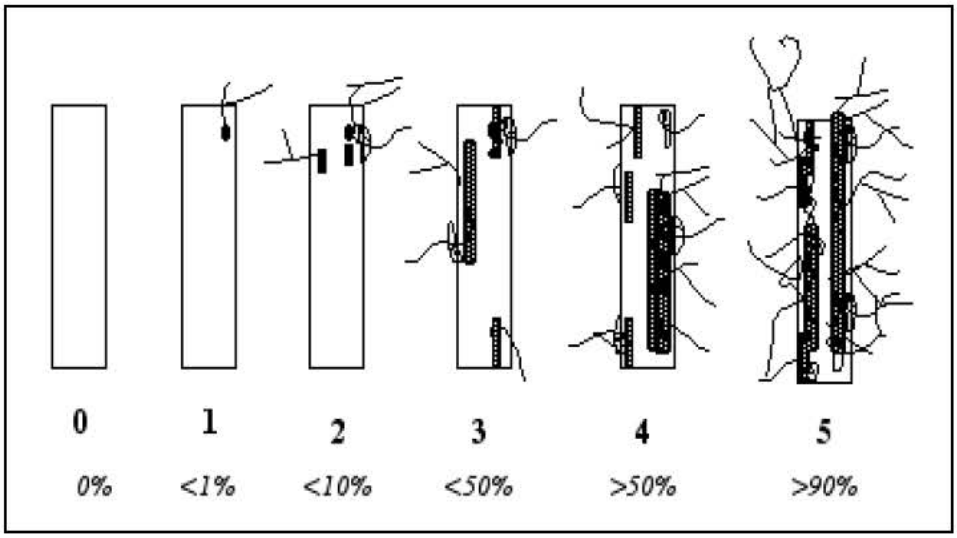
6) Preparation of the slide for AMF scoring

- Put the water and the roots in a petri dish.
- Clean a microscope slide with 70% ethanol.
- Cut 15 fragments (1 cm long) of the roots and place them on the slide. If needed, you can prepare 2 slides instead of 1 to have a better separation of the root fragments.
- Place a coverslip over the fragment and gently squeeze the root fragments between the slide and the coverslip with the back of the forceps.

7) Slide reading

- The results can be read with a microscope or a binocular microscope.
- For each fragment, the presence of AMF (stained in blue) is recorded.

- If infection is detected, the intensity of infection is scored from 1 to 5, following this intensity guideline:



8) AMF colonization calculation

- The frequency of mycorrhizae in the root system or **F%:**

F% = (number of colonized fragments / total number of fragments) x 100

- The intensity of the mycorrhizal colonization in the root system or **M%**:

M% = (95 x number of fragments scored « 5 » + 70 x number of fragments scored « 4 » + 30 x number of fragments scored « 3 » + 5 x number of fragments scored « 2 » + 1 x number of fragments scored « 1 ») / (total number of fragments)

- The Intensity of the mycorrhizal colonization in the root fragments **m%:**

m% = M% / F x 100

C. Safety

- Chemicals: Before using a new chemical, the information about its toxicity, conditions of use, risks and safety phrases... have to be understood and observed. Use the equipments for protection if needed (gloves, masks, hood...). The MSDS (Material Safety Data Sheet) of the different chemicals can also be read to get more details about the product.
- Special caution for:
  - Acetic acid: Flammable. Causes severe burns.

- H_2O_2: Toxic by inhalation and ingestion. Oxidizer, corrosive, and carcinogen
- KOH: Causes severe burns. Harmful if swallowed.
- NH4OH: Causes burns. Very toxic to aquatic organisms.
- Ethanol: highly flammable.

- Fume hood: If the hood is not working properly, it can lead to a fire risk or contamination of the room by toxic chemicals. The maintenance has to be done regularly.

D. Waste

- KOH solution can be disposed in the sink after dilution.
- NH4OH solution should be disposed as toxic chemicals.
- Solid wastes contaminated by NH4OH (pipettes, tissue paper…) should be disposed as toxic waste.

SOP-BM04 LH-V01: DNA extraction from nodules and liquid cultures using MP kit

I. Objective

This method is based on the binding of the DNA on a liquid matrix, allowing the efficient DNA extraction from many types of samples such as plant and animal tissue, bacteria, yeast, algae and fungi. It is very easy to use and avoids the use of hazardous chemical products sometimes used for DNA extraction (i.e. phenol and chloroform for instance). The cells are lysed using specific beads and undesirable components (i.e. proteins, intracellular components…) are eliminated while DNA is bound to the matrix. This kit allows the recovery of high yields of good quality DNA, in a short time (few hours).

Also read the procedure "SOP-BM01 LH-V01: General information in a Molecular Biology laboratory" before starting to work in the laboratory.

II. Definitions

- Contaminated by toxic chemicals: Every tube, flask, pipette or other instruments (weighing boats, glassware…) which has been in contact with toxic chemicals is considered as contaminated.

- Good Laboratory Practices (GLP): The principles of Good Laboratory Practice (GLP) have been developed to promote the quality and validity of results and of the analysis conducted in a laboratory. It is a managerial concept covering the organization and the conditions under which laboratory studies are planned, performed, monitored, recorded and reported. Its principles also include the protection of man and the environment.

III. Abbreviations

- °C: Celsius degree
- g: gram
- GLP: Good Laboratory Practices
- M: molar
- min: minute
- ml: millilitre
- m/s: meter per second
- MSDS: Material Safety Data Sheet
- rpm: rotation per minute
- sec: second
- µl: microlitre
- µg/ml: microgram per millilitre

IV. Procedures

A. Materials, furniture, reagents

1) Chemicals

- Absolute Ethanol
- Concentrated SEWS-M
- DES
- DNA Binding Matrix
- Lysing Matrix A
- CLS-VF 90 ml
- PPS
- CLS-TC
- CLS-Y

2) Instruments

- Balance
- Cooling centrifuge
- Freezer
- Fridge
- Rotative shaker
- Vortex
- Water bath
- Bead beater

3) Materials

- Eppendorf tubes (1.5 ml and 2 ml)
- Pipettes and tips
- Racks
- Tubes containing beads (supplied with the kit)
- Spin modules
- Catch tubes

4) Preparation of reagents

-Before using SEWS-M solution, add 100 ml of 100% ethanol and mark on the bottle label the date ethanol was added. Ensure that the bottle is securely closed to prevent evaporation, and store at room temperature.

B. Description of analysis

- Before starting, switch on the centrifuge and set it at 4°C for the next steps. Switch on the water bath (add distilled water if needed) and set it at 55°C.

- Add 200 µl of crushed nodule suspension to Lysing Matrix A tube. For bacteria, centrifuge a sufficient volume of culture to provide a pellet size of 50-100 mg wet weight or up to 10^9 bacteria. Resuspend pellets in 200 µl physiological water to give a maximum suspension volume of 200 µl and transfer to Lysing Matrix A tube.

NOTE: Label your samples on the side of the tubes because the bead beater and reagents can erase the cap of the tubes.

- Add appropriate Cell Lysis Solution (CLS) to the sample tubes. For nodule samples add 800 µl CLS-VF and 200 µl PPS. For bacteria cultures add 1.0 ml CLS-TC. Invert to mix.
- Place the tubes in the bead beater rack and do a first run for 40 secs.
- Centrifuge for 10 min at 14000 rpm at 4°C.

- Transfer the supernatant into a 2 ml tube (the catch tubes supplied with the kit may be used) and dispose the tube containing the beads.
- Add an equal amount of Binding Matrix and invert to mix. The binding matrix suspension must be well shaken before adding to each sample, to avoid precipitation.
- Homogenize with gentle agitation for 10 min at room temperature using a rotative shaker, or shake by hand.

- Transfer half (approximately 800 µl) of the suspension to a Spin Filter (supplied with the kit). Tubes AND filters must be labelled (write on the edge of the filter to avoid contact with Ethanol in the following steps).
- Centrifuge for 1 min at 14000 rpm 4°C.
- Empty the catch tube then add the remaining suspension to the Spin Filter. Centrifuge for 1 min at 14000 rpm 4°C. Empty the catch tube again.

- Resuspend the matrix with 500 µl SEWS-M (previously diluted in Absolute Ethanol).
- Centrifuge for 1 min at 14000 rpm 4°C. Discard the contents of the catch tube.
- Without addition of liquid, centrifuge a second time at 14,000 rpm for 1 minute to ensure all ethanol has been eluted. Replace the catch tube with a new, clean tube.

- Elute DNA by gently resuspending the Binding Matrix above the Spin Filter in 100 µl of DES. - Incubate for 5 minutes at 55˚C in a heat block or water bath.
- Centrifuge for 1 min at 14 000 rpm at 4°C to bring eluted DA into the clean catch tube.
- Discard the Spin Filter and store DNA samples at -20°C for downstream applications.

C. Safety

- Centrifuge: Before starting the centrifuge, make sure that the tubes are well equilibrated in the rotor. A non-equilibrated centrifuge can cause great damages to the centrifuge and can also lead to a "flying" centrifuge.
- Chemicals: Before using a new chemical, the information about its toxicity, conditions of use, risks and safety phrases... have to be understood and observed. Use the equipment for protection if needed (gloves, masks, hood...). The MSDS (Material Safety Data Sheet) of the different chemicals are also available in specifics files in the laboratory to get more details about the products.

D. Quality control management

- The protocol, date of preparation, quantity prepared, calculations and any other relevant information are recorded in the lab book.
- The stock solutions are labelled with the date of reception, name or initials of the person who received it, the number of the container (x of n), date of opening, name or initial of the person who opened it.
- The reagents are labelled with the name of the contents, date of preparation, name or initials of the person who prepared them and any other relevant information.
- If the DNA is stored at -20ºC before analysis, the name of the samples, origin, date of storage, name or initial of the person who analysed and stored them and any other relevant information are recorded in a database that should be available to all the staff in the laboratory. An example of a database is given in Appendix 1. This database must be updated every time sample are added or removed from the freezer.

- Equipment maintenance: All the equipment should be regularly checked in regard to their specifications. The results should be recorded and in case of repairs, the details about the intervention should be recorded. Details about maintenance services and repairs should be compiled in the Maintenance file, available in the office or in the laboratory.
- The general Good Laboratory Practices: They must be respected by everyone in the lab. The procedure "SOP-GA03/LH-V01: Hygiene and Safety rules in a laboratory" describes the general rules to be observed in the laboratory.
- In case of accident: Every accident must be reported to the lab manager and the staff if needed. Lab manager must put necessary measures in place to avoid the accident to occur again. For details on what to do in case of accident, read the procedure "SOP-GA03/LH-V01: Hygiene and Safety rules in a laboratory".
- Use of the equipment: For specific equipment, a form has to be filled when used. This may include the date and time of use (start and end), the name of the user, the notification of any deviations or problems and any relevant information about the equipment. These forms are also used to estimate the needs and the frequency of the maintenance. Books are available near the specific equipment and should not be taken away.

E. Waste and decontamination

- Non-contaminated waste is eliminated in the normal bin.
- Solid (including broken glass) waste contaminated by toxic chemicals is placed in separate containers (labelled with the mention: “Danger” and the nature of the waste ie “Guanidine thiocyanate waste” for example). Consult the MSDS of the product for more information since non-compatible products should not be put in the same container. A private company comes regularly to collect waste for elimination.
- Contaminated liquids are stocked in specific containers (labelled with the mention: “Danger”.
- Contaminated glassware is properly rinsed with tap water and the water is collected in a specific container for toxic liquid waste. The glassware can then be cleaned as non-contaminated items.

F. Cleaning

- The rooms are cleaned on a daily basis.
- Before starting and after manipulation, the hood and/or the bench is/are cleaned with a disinfectant.
- In case of contamination of the bench, floor, user..., it has to be cleaned and disinfected if needed before the work can be continued (see the procedure “SOP-GA03/LH-V01: Hygiene and Safety rules in a laboratory” for details).

Non-contaminated or decontaminated items are cleaned with soap, rinsed with water and eventually rinsed with distilled water.

SOP-BM05 LH-V01: Polymerase Chain Reaction (PCR)

I. Objective

PCR is a technique which allows the amplification of a targeted sequence of DNA, in order to obtain many copies in a very short time. After amplification, the quantity of DNA is enough to run other analyses such as RFLP... As it uses DNA, PCR is a quite sensitive method and the quality of the amplification determines the results of the subsequent tests.

This procedure describes how to prepare and run a PCR, avoiding contamination and denaturation of the template, to ensure a good quality of the amplified products.
Also read the procedure "SOP-BM01/LH-V01: General information in a Molecular Biology laboratory" before starting to work in the laboratory.

II. Definitions

- Good Laboratory Practices (GLP): The principles of Good Laboratory Practice (GLP) have been developed to promote the quality and validity of results and of the analysis conducted in a laboratory. It is a managerial concept covering the organization and the conditions under which laboratory studies are planned, performed, monitored, recorded and reported. Its principles also include the protection of man and the environment.

- PCR master mix: It contains all the components which are essential for the amplification to take place, except the template. Commercial master mix is available and contain the dNTP, enzyme (Taq polymerase), $MgCl_2$… at optimal concentrations. Primers are mixed with it to get a complete PCR master mix.

- PCR product: the PCR amplification of one single copy of a double strand DNA fragment results in many copies of the same fragment as the template. After amplification in the PCR tube, the Taq polymerase is deactivated, the primers and the bases are in very low concentration and the DNA quantity is high. This is called PCR product and is used for subsequent analysis (such as RFLP).

III. Abbreviations

- °C: Celsius degree
- DNA: Deoxyribose Nucleic Acid
- dNTP: mix of the 4 deoxyribonucleotides: dATP, dTTP, dGTP, dCTP
- g/l: gram per litre
- IGS: Inter Genic Spacer
- M: molar

- min: minute
- ml: millilitre
- mM: millimolar
- MSDS: Material Safety Data Sheet
- PCR: Polymerase Chain Reaction
- pmol: picomol
- pmol/µl: picomole per microlitre
- rDNA: ribosomal Deoxyribose Nucleic Acid
- sec: second
- Sterile distilled water, or better, sterile micro-pure water
- UV: Ultraviolet
- µl: microlitre

IV. Procedures

A. Materials, furniture, reagents

1) Chemicals

- Commercial master mix
- Primers (Forward and Reverse)
- Sterile distilled water or better, sterile micro-pure water (might also be commercially purchased)

2) Instruments

- Bench centrifuge
- PCR hood
- PCR thermocycler
- Vortex

3) Materials

- Cool box and ice
- Eppendorf tubes
- PCR tubes and racks
- Pipette and tips

B. Description of analysis

- Before starting, switch on the PCR hood and clean the bench with disinfectant.
- Turn on the UV light for at least 30 min.

- Put some ice in a cool box, and let the primers, the samples, the sterile distilled water and the master mix thaw slowly. Gently homogenize.
- Label the PCR tubes (0.2 ml tubes) with the name of the samples, date of analysis and any other relevant information.
- In an Eppendorf tube, prepare the PCR mix including for one extra sample to cover up for pipetting errors.
- The composition of the mix for one sample (final volume 25 µl) using commercial master mix is as following:
    - Commercial master mix: 12.5 µl
    - Sterile distilled water: 8.5 µl
    - Reverse primer (from a solution at 10 pmol/µl): 1 µl
    - Forward primer (from a solution at 10 pmol/µl): 1 µl
    - NB: the composition of the mix can be modified depending on the concentration of primers. Usually, a final quantity of 10 pmol of each primer is used.

- Mix gently using a vortex or by pipetting.
- Put 23 µl of the PCR mix in each PCR tube.
- Add 2 µl of the DNA template (sample). This DNA can be diluted depending on the quality and quantity obtained during extraction. Generally, a dilution of 1/100 in sterile micro-pure water is suitable.

NB: the quantity of template can be modified depending on the quality and concentration of the DNA. If so, the volume of water put in the mix is adjusted accordingly to obtain a final volume of 25 µl.

- Always include at least one negative control in the samples: 2 µl of sterile water are added instead of the DNA template.

- Mix the PCR tubes gently using a vortex.
- Centrifuge the PCR tubes using a bench centrifuge to eliminate bubbles and drops on tube sides.
- Put the PCR tubes in the thermocycler, set the machine as required and start the program.
- After amplification, store the PCR products at 4°C.
- The most commonly used primers and programs for PCR are given in the Appendix 1.

- Confirmation of the PCR products:

- The presence and the quality of the PCR amplification is checked by loading 3 µl of the product on a 1.5% agarose gel. The gel is then viewed under UV trans-illumination and photographed using Bio Rad Gel Doc XR+. Read the procedure “SOP-BM07/LH-V01: Electrophoresis (agarose gel)” for details.

C. Safety

- Chemicals: Before using a new chemical, the information about its toxicity, conditions of use, risks and safety phrases... have to be understood and observed. Use the equipment for protection if needed (gloves, masks, hood...). The MSDS (Material Safety Data Sheet) of the different chemicals are also available in specifics files in the laboratory to get more details about the products.
- PCR hood: If the hood is not working properly, it can lead to a fire risk. The maintenance has to be done regularly and the results recorded in a specific file (Maintenance file, available in the office or in the laboratory).

D. Quality control management

- The protocol, date of preparation, quantity prepared, calculations and any other relevant information are recorded in the lab book.
- The stock solutions of the primers are labelled with the date of reception, name or initials of the person who received it, the number of the container (x of n), date of resuspension, name or initial of the person who opened it, the concentration after resuspension and any other relevant information.
- If the PCR products are stored at 4°C before analysis, the rack has to be labelled with the name of the samples, origin, date of storage, name or initial of the person who analysed and stored them, and any other relevant information.

- Equipment maintenance: All the equipment should be regularly checked in regard to their specifications. The results are recorded and in case of repairs, the details about the intervention are recorded. Details about maintenance services and repairs should be compiled in the Maintenance file, available in the office or in the laboratory.
- The general Good Laboratory Practices: They must be respected by everyone in the lab. The procedure "SOP-GA03/LH-V01: Hygiene and Safety rules in a laboratory" describes the general rules to be observed in the laboratory.
- In case of accident: Every accident must be reported to the lab manager and the staff if needed. Lab manager must put necessary measures in place to avoid the accident to occur again. For details on what to do in case of accident, read the procedure "SOP-GA03/LH-V01: Hygiene and Safety rules in a laboratory".
- Use of the equipment: For specific equipment, a form has to be filled when used. This may include the date and time of use (start and end), the name of the user, the notification of any deviations or problems and any relevant information about the equipment. These forms are also used to estimate the needs and the frequency of the maintenance.

E. Waste

- PCR waste is considered as non-contaminated waste and should be eliminated as normal waste.

F. Cleaning

- The rooms are cleaned on a daily basis.
- Before starting and after manipulation, the hood and/or the bench is/are cleaned with a disinfectant.
- In case of contamination of the bench, floor, user..., it has to be cleaned and disinfected if needed before the work can be continued (see the procedure "SOP-GA03/LH-V01: Hygiene and Safety rules in a laboratory").
- Non-contaminated items are cleaned with soap, rinsed with water and eventually rinsed with distilled water.

V. Bibliography

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<u>Program and primers for different types of amplification:</u>

➢ <u>Bacteria rDNA 16S (for sequencing)</u>

Primers: 27f (5'-AGAGTTTGATCCTGGCTCAG-3'),
1492r (5'-TACGGTTACCTTGTTACGACTT-3')

Program: 16S Sequencing (Techne TC-4000)

- Pre-denaturation: 94°C for 5 min
- Cycles (×35): Denaturation 94°C for 1 min
  Annealing: 55°C for 1 min

Elongation: 72°C for 2 min
- Final extension 72°C for 15 min.

➢ Bacteria rDNA 16S-23S (IGS)

Primers: FGPS 1490-72 (5'-TGCGGCTGGATCCCCTCCTT-3'),
FGPL 132-38 (5'-CCGGGTTTCCCCATT CGG-3')
Program: 'IGS RFLP FGPS FGPL' (Techne TC-4000)
- Pre-denaturation 94°C for 5 min
- Cycles (×35): Denaturation: 94°C for 30 secs
  Annealing: 58°C for 30 secs
  Elongation: 72°C for 30 secs
- Final extension: 72°C for 7 min.

➢ Soil Bacteria rDNA 16S

Primer: 341F (5'- CCTACGGGNGGCWGCAG -3'
785R (5'- GACTACHVGGGTATCTAATCC -3')
Program: '341 785 16S' (Techne TC-4000)
- Pre-denaturation 94°C for 5 min
- Cycles (×35): Denaturation: 94°C for 1 min
  Annealing: 53°C for 1 min
  Elongation: 72°C for 1 min
- Final extension: 72°C for 10 min.

➢ Bacteria rDNA 16S (for sequencing, PSB)

Primers: fD1 (5'-AGAGTTTGATCCTGGCTCAG-3'),
rD1 (5'-AAGGAGGTGATCCAGCC-3')
Program: 16S PSB (Techne TC-4000)
- Pre-denaturation: 94°C for 3 min
- Cycles (×35): Denaturation 94°C for 1 min
  Annealing: 55°C for 1 min
  Elongation: 72°C for 2 min
- Final extension 72°C for 3 min.

SOP-BM06 LH-V01: Electrophoresis (Agarose gel)

I. Objective

Electrophoresis is the motion of dispersed particles (which have an electric surface charge) relative to a fluid, under the influence of a spatially uniform electric field. An agarose gel is used as a matrix for horizontal electrophoresis. It acts as a net which allows the separation of the particles depending on their size. The higher the agarose concentration, the tighter the net.

This procedure describes how to prepare an agarose gel, load it, and how to photograph it using the Bio Rad Gel Doc XR+.
Also read the procedure "SOP-BM01/LH-V01: General information in a Molecular Biology laboratory" before starting to work in the laboratory.

II. Definitions

- Good Laboratory Practices (GLP): The principles of Good Laboratory Practice (GLP) have been developed to promote the quality and validity of results and of the analysis conducted in a laboratory. It is a managerial concept covering the organization and the conditions under which laboratory studies are planned, performed, monitored, recorded and reported. Its principles also include the protection of man and the environment.

- PCR product: the PCR amplification of one single copy of a double strand DNA fragment results in many copies of the same fragment as the template. After amplification in the PCR tube, the Taq polymerase is deactivated, the primers and the bases are in very low concentration and the DNA quantity is high. This is called PCR product and is used for subsequent analysis (such as sequencing, RFLP).

III. Abbreviations

- µl: microlitre
- g/l: gram per litre
- GLP: Good Laboratory Practices
- h: hour
- M: molar
- min: minute
- ml: millilitre
- MSDS: Material Safety Data Sheet
- PCR: Polymerase Chain Reaction
- rpm: rotation per minute
- TBE: Tris base, Boric acid, EDTA

- V: Volt
- w/v: weight per volume

IV. Procedures

A. Materials, furniture, reagents

1) Chemicals

- Agarose
- GelRed solution (10000X or RedSafe (20000X)
- Loading dye (Bromophenol blue, 0.05% (w/v); Sucrose, 40%; EDTA pH = 8, 0.1 M)
- TBE 1X (Tris Base, 10,8 g/l; Boric acid, 5,5 g/l; EDTA pH=8, 0.002 M)

2) Instruments

- Balance
- Bio Rad Gel Doc XR+
- Electrophoresis tank
- Microwave
- Power pack

3) Materials

- Conical flask
- Gel support, rack and combs
- Pipettes and tips
- Spatula and weighing boats (or foil)

B. Description of analysis

1) Casting the gels (pre-stained)

- Prepare the gel support(s) on the rack and adjust the screws to make it tight and put it on a flat surface.
- Weigh the agarose (the quantity depends on the concentration and volume of gel that is prepared) and put it in a conical flask. Usually, a concentration of 1-3% is suitable. The volume the gel is determined by the number and nature of samples.
- Measure the corresponding volume of TBE 1X and add into the conical flask.
- Warm in the microwave until the agarose is completely dissolved. Be careful not to leave the agarose to boil too much or it will pour out of the flask. Shake gently to avoid projection (use a heat-proof glove), no visible crystals of agarose are left in solution.
- Put the support on a flat surface.

- Let the agarose cool down (or cool the flask with water above sink but be careful don't put water in the agarose solution) until you can handle it with your hand (the temperature should be less than 60°C).
- Add the corresponding volume of GelRed/ RedSafe solution to obtain a final concentration of 2.5%. Homogenise well while avoiding formation of bubbles. The GelRed/RedSafe can be added even if the agarose is still hot.
- When cool enough, gently pour the agarose in the support(s). Remove the bubbles with a tip before the gel sets, place the comb(s) and let it set until the gel is completely solid.
- Remove the combs (gently).
- Remove the support from the rack and remove the extra gel which might have leaked on the side and below the support.
- Place the gel in the tank containing TBE 1X. Ensure that the gel is covered with buffer.

2) Loading

- Mix 3 µl of samples with 5 µl of loading dye.
- Load the total in the wells of the gel.
- Add a DNA ladder in at least one well so as to estimate the size of the fragment: dilute the stock solution of ladder 1/10 in loading dye and load 3 µl of this solution per well.

3) Running

- Put the cap on the tank and connect the cables to the power pack.
- Run at 150 V for 45-60 min for a gel with 1.5% agarose concentration. The higher the agarose concentration, the longer samples will have to run.
- Once the run is over, switch off the power pack.
- Disconnect the cables and remove the cap of the tank.
- Remove the support with the gel from the buffer and return the cap on the tank to avoid buffer evaporation.

4) Visualization

- Clean the glass tray of the Bio Rad Gel Doc XR+ system with distilled water and disinfectant (ethanol 70%) with a soft tissue paper and place the gel in the middle of it.
- Transfer the gel to the glass and gently press the gel to remove bubbles and excess liquid between the glass and gel, then close the drawer.
- Switch on the Gel Doc from the back, and also turn on the computer
- Open the Quantity One software on the computer and select Gel Doc XR+ from the File tab.
- Switch on the UV light and click then click auto-expose to view the gel.
- Click on the freeze button once the gel photo has been taken. Save the gel photo in the respective folders.

C. Safety

- Chemicals: Before using a new chemical, the information about its toxicity, conditions of use, risks and safety phrases... have to be understood and observed. Use the equipment for protection if needed (gloves, masks, hood...). The MSDS (Material Safety Data Sheet) of the different chemicals are also available in specifics files in the laboratory to get more details about the products.
- Special caution for:
    - Boric acid: May impair fertility. May cause harm to the unborn child.
- Electrophoresis tank: Before using, visually check the general aspect of the tank. The tank should be clean if calcareous deposits are visible. The maintenance has to be done regularly to avoid electrical problem and the results recorded.
- Power pack: Before using, visually check the general aspect of the power pack, connectors, cables... Strictly follow the instructions to start it. Always switch the power off before removing the cables or opening the tank.
- Solubilization of the agarose: As agarose is not soluble in TBE at cool or room temperature and must be warmed using a microwave. Check regularly how the solution behaves in the microwave since it can boil very fast and pour out of the flask. Use protection gloves (thick gloves) to remove the flask from the microwave and shake gently the solution when warm.
- UV lamp: UV poses a great danger to the skin and eyes. It can lead to burns and even cancer. The Bio Rad Gel Doc XR+ system has a security system which turns off the UV lamp when the door is open. However, never look directly at the lamp and if needed, wear individual protection items.

D. Quality control management

- The protocol, date of preparation, quantity prepared, calculations and any other relevant information are recorded in the lab book.
- The stock solutions are labelled with the date of reception, name or initials of the person who received it, the number of the container (x of n), date of opening, name or initial of the person who opened it.
- The reagents are labelled with the name of the contents, date of preparation, name or initials of the person who prepared them and any other relevant information.
- The photos of the gels are stored in a folder which is labelled with the name of the experiment. The photos are named so as to trace the samples, date of the analysis and any other relevant information.
- A copy of the photos is printed and added in the lab book.
- The buffer for the electrophoresis is changed regularly to ensure the quality of the results. The frequency depends on the frequency of the analysis: buffer should be replaced after 7 runs, if the machine has not been used for more than 2 weeks or if dust, deposits or other kinds of dirt are visible.

- Equipment maintenance: All the equipment should be regularly checked in regard to their specifications. The results are recorded and in case of repairs, the details about the intervention are recorded. Details about maintenance services and repairs

should be compiled in the Maintenance file, available in the office or in the laboratory.

- The general Good Laboratory Practices: They must be respected by everyone in the lab. The procedure "SOP-GA03/LH-V01: Hygiene and Safety rules in a laboratory" describes the general rules to be observed in the laboratory.
- In case of accident: Every accident must be reported to the lab manager and the staff if needed. Lab manager must put necessary measures in place to avoid the accident to occur again. For details on what to do in case of accident, read the procedure "SOP-GA03/LH-V01: Hygiene and Safety rules in a laboratory".
- Use of the equipment: For specific equipment, a form has to be filled when used. This may include the date and time of use (start and end), the name of the user, the notification of any deviations or problems and any relevant information about the equipment. These forms are also used to estimate the needs and the frequency of the maintenance.

E. Waste and decontamination

- Electrophoresis waste (solid waste, buffer as well as PCR products) can be considered as non-contaminated waste and is eliminated in the normal bin/sink.
- Non-contaminated glass waste (Pasteur pipette, slides, broken glassware...) are put in a separate container labelled with the mention: "Broken glass".

F. Cleaning

- The rooms are cleaned on a daily basis.
- Before starting and after manipulation, the hood and/or the bench is/are cleaned with a disinfectant.
- In case of contamination of the bench, floor, user..., it has to be cleaned and disinfected if needed before the work can be continued (see the procedure "SOP-GA03/LH-V01: Hygiene and Safety rules in a laboratory" for details).
- Non-contaminated or decontaminated items are cleaned with soap, rinsed with water and eventually rinsed with distilled water.

SOP-BM07 LH-V01: Restriction Fragment Length Polymorphism (RFLP)

I. Objective

Restriction Fragment Length Polymorphism (RFLP) is a technique which allows the detection of a difference in different DNA sequences, by the presence of fragments of different lengths, after digestion of the DNA samples with specific restriction endonucleases. During RFLP, the DNA sample (PCR product) is broken into pieces (digested) by restriction enzymes and the resulting restriction fragments are separated according to their lengths by gel electrophoresis.

This procedure describes how to digest the DNA samples and how to run the gel to get the pattern of bands.
Also read the procedure "SOP-BM01/V01: General information in a Molecular Biology laboratory" before starting to work in the Molecular Biology laboratory.

II. Definitions

- Good Laboratory Practices (GLP): The Principles of Good Laboratory Practice (GLP) have been developed to promote the quality and validity of results and of the analysis conducted in a laboratory. It is a managerial concept covering the organization and the conditions under which laboratory studies are planned, performed, monitored, recorded and reported. Its principles also include the protection of man and the environment.

- PCR product: the amplification of a template by PCR results in many copies of the same double stranded brand DNA as the template. After amplification in the PCR tube, the Taq polymerase is deactivated, the primers and the bases are in very low concentration, and the DNA quantity is high. This is called the PCR product and is used for the RFLP digestion. See « SOP-BM05/LH-V01: Polymerase Chain Reaction (PCR) » for details about how to run a PCR.

- RFLP mix: The RFLP mix contains the restriction buffer and the enzyme in the required concentration diluted in sterile water. This will be added to the template (PCR product) in the microtube for digestion. It avoids the problems of accuracy and pipetting very small volumes.

III. Abbreviations

- ^{o}C: Celsius degree
- g/l: gram per litre
- GLP: Good Laboratory Practices
- h: hour

- M: molar
- min: minute
- ml: millilitre
- MSDS: Material Safety Data Sheet
- PCR: Polymerase Chain Reaction
- RFLP: Restriction Fragment Length Polymorphism
- sec: second
- U/µl: Unit per microlitre
- UV: Ultra Violet
- V: Volt
- w/v: weight per volume
- µg/ml: microgram per millilitre
- µl: microlitre

IV. Procedures

A. Materials, furniture, reagents

1) Chemicals

- Agarose
- GelRed stain (10000X)/ RedSafe stain (20000X)
- Loading dye (Bromophenol blue, 0.05% (w/v); Sucrose, 40%; EDTA pH = 8, 0.1 M)
- Restriction buffer (specific of the enzyme, commercially sent with the enzyme)
- Restriction enzymes
- Sterile micro-pure water
- TBE 1 X (Tris Base, 10.8 g/l; Boric acid, 5.5 g/l; EDTA pH = 8, 0.002 M)

2) Instruments

- Balance
- Electrophoresis tank
- Gel documentation system
- Microwave
- PCR machine (thermocycler)
- Power pack
- Water bath

3) Materials

- 0.2 ml microtubes
- Conical flasks
- Gel support and comb
- Pipettes and tips

B. Description of analysis

1) PCR

Before restriction, the targeted fragment to be restricted must be amplified by PCR using specific primers.
For the analysis of the IGS fragment of the Rhizobia, the region between the 16S and 23S rDNA is amplified by PCR with primers FGPS 1490-72; 5'-TGCGGCTGGATCCCCTCCTT-3' (Normand *et al*., 1996), and FGPL 132-38; 5' CCGGGTTTCCCCATTCGG-3' (Ponsonnet and Nesme, 1994). PCR amplification is performed using the following programme:
Initial denaturation for 5 min at 94ºC, 35 cycles of denaturation for 30 secs at 94ºC, annealing for 30 secs at 58ºC and extension for 30 secs at 72ºC. Final extension for 7 min at 72ºC.

See the "SOP-BM05/LH-V01: Polymerase Chain Reaction (PCR)" for details.

2) Digestion

- The PCR machine can be used as an incubator for restriction. Select the incubation protocol and set the temperature at the optimized temperature for the reaction (depending on the restriction enzyme). For many enzymes such as MspI or HaeIII, the optimal temperature is 37ºC.
- Label 0.2 mL tubes.
- Prepare an enzyme solution with a final concentration of 5 U/µl.
- Combine the restriction buffer, water, and the enzyme in a separate tube: Mix RFLP. Prepare the RFLP mix including for one extra sample than what needs to be analysed.
- The composition of the mix <u>for one sample</u> is as following:
  - Enzyme: 5 U (1 µl of a 5 U/µl solution)
  - Restriction buffer: 2 µl
  - Sterile distilled (or micropure) water: 2 µl
- Aliquot 5 µl of the RFLP mix to each tube.
- Transfer 10 µl of each PCR product into labelled tube.
- Close the caps tightly to avoid evaporation during incubation.
- Briefly vortex the tubes and incubate at 37ºC for 2 h.
- Place at 4°C or on ice if the samples are not loaded immediately after digestion.

3) Staining, visualizing and photographing gel.

- Prepare a 3% agarose gel (w/v) and add the corresponding volume of GelRed/ RedSafe solution to obtain a final concentration of 2.5% (i.e. 3 µl for 120 ml of gel). Homogenise well. The Gel Red/ RedSafe can be added even if the agarose is still hot. See "SOP-BM06/LH-V01: Electrophoresis" for details about the gel preparation, loading of the samples, running and visualization.

- Load 7 µl of the restricted product with 7 µl of loading dye in the agarose gel and run at 100 V for 3 h.
- View under UV trans-illumination and photograph using gel documentation system.

C. Safety

- Chemicals: Before using a new chemical, the information about the toxicity, conditions of use, risks and safety phrases... must be understood and observed. Use the equipment for protection if needed (gloves, masks, hood...). The MSDS (Material Safety Data Sheet) of the different chemicals are also available to get more details about the products. MSDS files are available in the Preparation Room and in the office.
  Special caution for:
  - Boric acid: May impair fertility. May cause harm to the unborn child.
- PCR hood: If the hood is not working properly, it can lead to a fire risk. The maintenance must be done regularly, and the results recorded in a specific file (Maintenance file, available in the office).
- UV lamp: UV poses a great danger to the skin and eyes. It can lead to burns and even cancer. The Gel Documentation system has a security system which turns off the UV lamp when the door is open. However, never look directly at the lamp and if needed, wear individual protection items.

D. Quality control management

- The protocol, date of preparation, quantity prepared, calculations and any other relevant information are recorded in the lab book.
- The stock solutions of the enzymes are labelled with the date of reception, name or initials of the person who received it, the number of the container (x of n), date of opening, name or initial of the person who opened it.
- If the restriction products are stored at 4°C before electrophoresis, the rack must be labelled with the name of the samples, origin, date of storage, name or initial of the person who analysed and stored them, and any other relevant information.
- The gel photos are stored in a folder which is labelled with the name of the experiment. The photos are labelled to trace the samples, date of the analysis and any other relevant information.
- A copy of the gel photos is printed and added to the lab book.
- The buffer for the electrophoresis is changed regularly to ensure the quality of the results. The frequency depends on the frequency of the analysis and is noted in the machine notice.

- Equipment maintenance: All the equipment are regularly checked in regard to the specific specifications. The results are recorded and in case of repairs, the details about the intervention are recorded. Details about maintenance services and repairs are compiled in the Maintenance file, available in the office.

- The general Good Laboratory Practices: They must be respected by everyone in the lab. The "Hygiene and Safety rules in a laboratory" document describes the general rules to be observed in the laboratories.
- In case of accident: Every accident must be reported to the lab manager and the staff if needed. Lab manager must put necessary measures in place to avoid the accident to occur again. For details on what to do in case of accident, read the "SOP-GA03/LH-V01: Hygiene and Safety rules in a laboratory" document.
- Use of the equipment: For specific equipment, a form has to be filled when used. This may include the date and time of use (start and end), the name of the user, the notification of any deviations or problems and any relevant information about the equipment. These forms are also used to estimate the needs and the frequency of the maintenance.

E. Waste and decontamination

- Non-contaminated waste is eliminated in the normal bin.
- Non-contaminated glass waste (Pasteur pipette, slides, broken glassware...) are put in a separate container labelled with the mention: "Broken glass".
- Anything contaminated by microorganisms should be decontaminated before appropriate elimination/cleaning. Waste are put in a special autoclave bag and autoclaved for 20 min at 121°C. The autoclave bag can then be disposed off as non-contaminated waste. Re used material (glassware, small tools as sieves, pestles, ...) are autoclaved and then cleaned as non-contaminated items. Not reused glass instruments (pipettes, slides, cover glasses, broken glassware...) are put in a beaker containing Sodium hypochlorite solution for decontamination before being eliminated as non-contaminated glass waste.

F. Cleaning

A complete Cleaning Plan is available for details. Consult SOP-GA02/V01 "Cleaning plan".

- The rooms are cleaned on a daily basis.
- Before starting and after manipulation, the hood and/or the bench is/are cleaned with a disinfectant.
- In case of contamination of the bench, floor, user, ..., it has to be cleaned and disinfected if needed before the work can be continued (cf. "SOP-GA03/LH-V01: Hygiene and Safety rules in a laboratory" document).
- Non-contaminated or decontaminated items are cleaned with soap, rinsed with water and eventually rinsed with distilled water.
- Equipment: cf the SOP-GA02/V01 "Cleaning plan" for details.

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