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Full Length Research Paper

Diversity of legume nodulating bacteria as key variable of coffee agro-ecosystem productivity

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Abstract

The high diversity of Legume Nodulating Bacteria (LNB) was found in soil of agro-ecosystem of legume trees that has been established for long time. Incorporating legume trees as shading in coffee agro-ecosystems would conserve LNB diversity and provide ecosystem services such as N fixation, dinamic shading, and nutrient cycling. The aim of the experiment was to determine of wether legume shaded coffee agro-ecosystems have higher LNB diversity and higher coffee productivity than those of non-legume or sun (no shade) coffee agro-ecosystem. The experiment was conducted in West Lampung District, Indonesia, throughout survey and experimental plot of coffee agro-ecosystems consisted of 4-5 years old coffee plot and a quassy experiment plot of 15-16 years old coffee. Types of coffee agroecosystems to be examined were *Coffea canephora* with shading of legume trees of *Gliricidia sepium* and *Erythrina sububrams*, non-legume shade trees of *Michelia champaca*, and sun coffee (without shade tree). The results showed that coffee agro-ecosystems shaded by legume trees had higher LNB diversity than those of coffee agro-ecosystems shaded by nonlegume trees (*Michelia champaca*) and coffee agro-ecosystem without shade trees. Diversity of legume nodulating bacteria in the soil may indicate productivity of coffee agro-ecosystems.

Keywords: LNB diversity, Legume tree, Coffee agro-ecosystem, Productivity.

INTRODUCTION

Nitrogen fertilization is a key factor for coffee production but creates environmental problems such as water contamination through nitrat leaching (Harmand et al., 2007). Nitrogen is the most limiting nutrient for the productivity of coffee agro-ecosystem. Relying on nitrogen fertilization may costly due to high price of nitrogen fertilizer and low efficiency of fertilization which only 30-40% is absorbed by the coffee plants. Shade tree is a key factor for improving coffee productivity (Vaast et al., 2005). Incorporating shade trees in coffee agro-ecosystem reduces nitrate leaching (Babbar and Zak 1995), reduces weed growth (Senarathne and Perera 2011), increases organic matter input through litter fall, absorbs nutrients by deep roots, generates N fixation by root nodule of legume trees (Tscharntke et al., 2011; Young 1990) and leads to reduction on N fertilization in coffee agro-ecosystem (Wintgens 2004) and improves coffee production sustainability (Vaast et al., 2005).

Biological nitrogen fixation is mainly carried out by in legume nodulating bacteria (Herridge et al., 2008). The majority of studies on N fixing bacteria focused on growth characteristics, nodulation capacity, and N fixing efficiency but legume nodulating bacteria (LNB) diversity is poorly performed (Lavay and Burdon 2007). Mwangi et al. (2011) found less diversity of LNB in agroforestry with no legume trees. Rodriguez-Echeverria et al. (2007) reported that high diversity of LNB was found in soil of agro-ecosystem of legume trees that has been established for long time. Bala et al. (2003) found high diversity of LNB in soil of agroforestry legumes from the tropical continents. The success of the tree legumes growth in the tropical continents is the result of their relative promiscuity allowing nodulation with diverse indigenous rhizobial types. Lafay and Burdon (2007) concluded that tropical areas are centres of LNB

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biodiversity. Hsu and Buckley (2009) found positive relation between diversity of diazotroph bacteria and rate of N fixation.

Method of ribosomal intergenic spacer analysis (RISA) has been successful to descriminate close relatives of bacteria. Intergenic spacer to be amplified has different size of base pairs as seen by silver staining (Ranjard et al., 2001). Molecular analysis of bacterial diversity could be done by DNA extracting from samples such as soil, water, and media culture (Gabor et al., 2002). In this study, nodules of Siratro planted in soils of coffee agro-ecosystems were used to extract DNA of nodulating bacteria. The objective of the research was to determine of wether legume shaded coffee agro-ecosystems have higher LNB diversity and higher coffee productivity than those of non-legume or sun (no shade) coffee agro-ecosystem.

MATERIALS AND METHODS

Plot Experiment

The research was based on survey and experimental plot of coffee agro-ecosystems, conducted at benchmark of Conservation and Sustainable Management of Below-Ground Biodiversity (CSM-BGBD), in Sumberjaya Subdistrict, West Lampung, Indonesia, situated at $4^{o}64'$ – $5^{o}10'$S and $104^{o}15'$ – $104^{o}20'$E. Types of coffee agro-ecosystems to be examined were *Coffea canephora* with shade trees of *Gliricidia sepium*, *Erythrina sububrams*, *Michelia champaca*, and sun coffee (without shade tree). Two plots consisted of 4-5 years coffee and 15-16 years coffee were established.

In experimental plot 1, shrub was cleared and Robusta coffee seedlings were planted with four shade treatments as mentioned above. The experiment used randomized complete block design (RCBD) with three replications. Coffee trees spaced at 2 x 2 m while shade trees spaced at 4 x 4 m. Fertilizer dose of 75-25-50 kg ha^{-1} of NPK was applied. Plot 2 was a quassy experiment of mature Robusta coffee fields of 15-16 years old with the same types of shade trees. Fertilizer of NPK (150-50-100 kg ha^{-1}) was applied (Evizal et al., 2012).

Productivity and Soil Analysis

Harvesting was conducted at age of 4 and 5 years for young coffee and at age of 15 and 16 years for mature coffee trees. Coffee productivity was calculated based on yield of fresh bean per plot (Haggar et al., 2011). Biomass production was determined based on the production of litter fall, pruning of coffee and shade trees, and weed harvesting. Litter fall was sampled using 3 litter traps of 1x2 m wide per plot. Standing litter and weed biomass was sampled base on quadrant of 1x1 wide (Evizal et al., 2009). Weed biomass was harvested every 3 months before weeding. At each plot replications, soils were sampled at 0-20 cm depth to analyze diversity of legume nodulating bacteria (LNB) and content of organic C (Walkley and Black) and total soil N (Kjeldahl).

Nodulation and N fixation Activity

Siratro (*Macroptilium atropurpureum*) was used to trap nodulating bacteria from soil of different type of coffee agro-ecosystems. Seedlings were grown in plastic pouch filled with free N medium of Fahreus. After 2 months, nodules were harvested. N fixation activity was measured by method of *Acetylene Reducing Assay* (Hsu and Buckley, 2009). Sampled nodules were put into venoject tub, added with 1 ml of acetylene and incubated for 3 hours. When incubation has completed, ethylene gas inside the venoject was analyzed using gas chromatography.

DNA Extraction

Nodule samples of siratro (0.3 g) were washed, soaked in 95% ethanol for 3 min, and soaked in 0.1% sublimate for 3 min. Using sterilized pin-set, nodules were broken and dissolved in 0.5 ml of sterile distilled water, centrifuged and settles for 5 minutes. Supernatant (0,4 ml) was harvested and filled in micro tubes of Fast DNA Spin Kit (Biomedical, USA) to extract DNA of bacteria following the protocol. Homogenization was done using Fast Prep 24 MP (Biomedical, USA) at the speed of 6,0 m sec^{-1} for 40 sec.

Ribosomal Intergenic Spacer Analysis

To analyze LNB diversity, method of Ribosomal Intergenic Spacer Analysis (RISA) was used. The intergenic spacer region on bacteria DNA was amplified in 20 µl PCR mixtures of 10 µl Mega-Mix-Blue (Microzone, UK), 6 µl sterile distilled water, 1 µl each of 1406F and 23sR primer (25 pmol/µl), 2 µl DNA (25 ng/µl). DNA was amplified in a GeneAmp PCR System 9700 (Biosystems, USA) as follows: denaturation of DNA at $95^{o}C$ for 5 min; 30 cycles of denaturation ($95^{o}C$ for 1 min), annealing ($53^{o}C$ for 45 sec), and elongation ($72^{o}C$ for 1 min 30 sec) with final elongation time of 7 min at $72^{o}C$. The product of RISA were analyzed in 8% Polyacrilamide Gel Electrophoresis (PAGE).

Data Analysis

Intensity of PAGE bands as representation of Operational Taxonomic Unit (OTU) (Cetecioglu et al., 2009) were

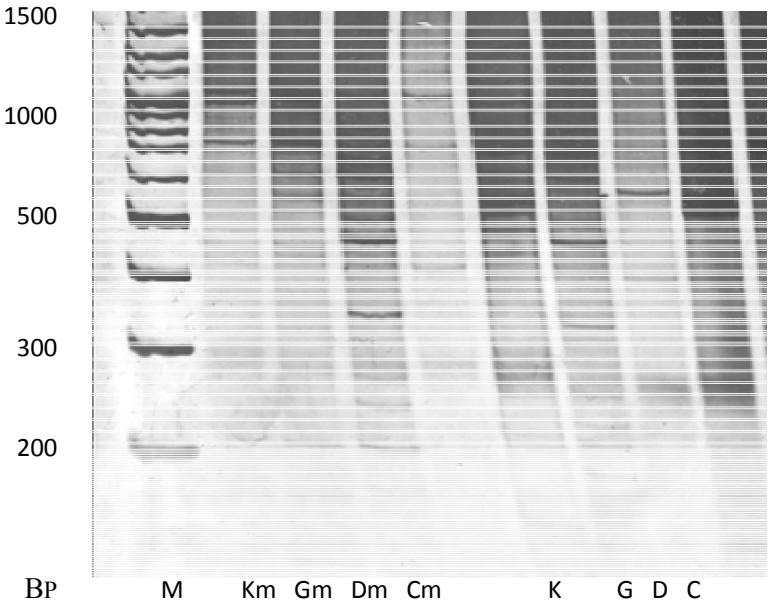

Figure 1. The profile of LNB diversity in the soil of 5 years coffee agro-ecosystems (Km, Gm, Dm, Cm = no shade, *G. sepium* shade, *E. sububrams* shade, M. champaca shade) and 15 years coffee agro-ecosystems (K, G, D, C = no shade, *G. sepium* shade, *E. sububrams* shade, *M. champaca* shade), M = marker

Table 1. Shannon-Weaver diversity indices of LNB from coffee agro-ecosystems based on RISA

Coffee agro-ecosystems	Shannon diversity indices	
	Coffee 5 years	Coffee 15 years
No shade	2.7883 a	2.5770 B
G. sepium shade	2.8125 a	2.9112 A
E. sububrams shade	2.8834 a	3.0056 A
M. champaca shade	2.8194 a	2.7098 B

Note: Within column, means followed by the same letter are not significantly different by Duncan's test at α 0.05

quantified by Adobe Photoshop software (Evizal et al., 2012). Using software of Biodiversity Analysis Package, Shannon-Weaver diversity indices (H') were calculated based on formula (Ge et al., 2008):

$H' = - \Sigma pi(\ln pi)$ where

$pi = n/N$

n = intensity of *operational taxonomic unit* (OTU)

N = total intensity of OTU.

Analysis of variance and Duncan multiple range test was done using SAS software. Cluster analysis was done using NTSys software. Regression analysis and t-test was done using SPSS software.

RESULTS AND DISCUSSION

The result showed that types of coffee agro-ecosystems affected diversity of legume nodulating bacteria (LNB) that extracted from sample nodules (Figure 1). Based on Shannon diversity indeces, diversity of LNB in the soil of 15-years-old coffee agro-ecosystem shaded with legume trees was higher than those of coffee agro-ecosystem shaded with non-legume trees or without shade trees. In the soil of 5-years-old coffee agro-ecosystems, diversity of LNB was not significantly different (Table 1). The roots of legume trees as shade of new planting coffee agro-ecosystems (5 years old) has not grown vigorously yet, and nodulation was found only 1 m around the trunk. Mwangi et al., (2011) reported that agroforestry systems of legume trees that have been established for long time, had higher diversity of LNB than those of new establishment of legume trees or no legume trees.

Shaded coffee agro-ecosystems had higher LNB diversity than no shade coffee agro-ecosystems. Coffee agro-ecosystems shaded by legume trees had higher LNB diversity than those of coffee agro-ecosystems

Table 2. Contrast test of LNB diversity from 15-year-old coffee agro-ecosystems

Contrast among agro-ecosystems	F calculated	Significant
No shade vs with shade	86.032	**
Legum shade vs nonlegum shade	56.159	**
G. sepium shade vs *E. sububrams* shade	4.249	ns

Note: ** Significant at α=0.01, ns = no significant at α 0.05

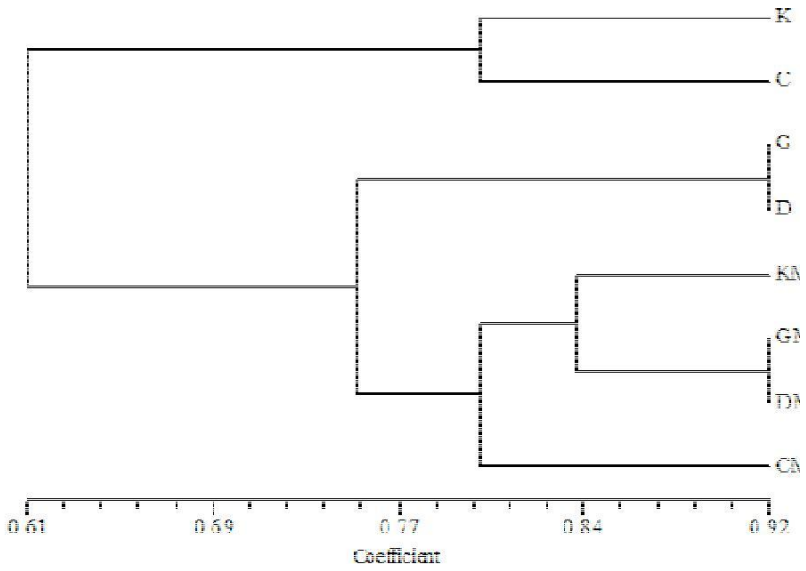

Figure 2. Dendrogram of LNB diversity in soil of 5 years coffee agro-ecosystems (Km, Gm, Dm, Cm = no shade, *G. sepium* shade, *E. sububrams* shade, *M. champaca* shade) and 15 years coffee agro-ecosystems (K, G, D, C = no shade, *G. sepium* shade, *E. sububrams* shade, *M. champaca* shade).

Table 3. N fixation activity of siratro nodules based on ARA

Coffee agro-ecosystems	N fixation (mmol h^{-1} g^{-1})**
Coffee 5 year no shade	0.4398 cd
Coffee 5 year *G. sepium* shade	1.0193 b
Coffee 5 year, *E. sububrams* shade	0.8086 bc
Coffee 5 year, *M. champaca* shade	0.4838 cd
Coffee 15 year, no shade	0.3318 d
Coffee 15 year, *G. sepium* shade	1.5853 a
Coffee 15 year, *E. sububrams* shade	0.9253 b
Coffee 15 year, *M. champaca* shade	0.4132 cd

Note: Means followed by the same letter are not significantly different by Duncan's test at α 0.05

shaded by nonlegume trees (*Michelia champaca*). Table 2 showed the contrast test of LNB diversity among types of coffee agro-ecosystems. Between legume tree coffee agro-ecosystems, there was no significant different between LNB diversity of *G. sepium* and *E. Sububrams* – coffee agro-ecosystem.

Based on LNB diversity, the dendrogram of cluster analysis (Figure 2) supported the same finding that: (1) Agro-ecosystems of 5 years old coffee gathered in a cluster, (2) agro-ecosystems of 15 years coffee separated in cluster of legume shade trees and cluster of non-legume shade tree and no shade tree, (3) coffee agro-ecosystem of *G. sepium* and *E. sububram* had high similarity level of LNB diversity.

N fixation activity of siratro nodules showed in Table 3. LNB from *G. sepium* shade coffee agro-ecosystem had high activity, followed by LNB from *E. sububram* shade coffee agro-ecosystem, non-legume shade (*M.*

Table 4. Coffee productivity in average of year 15-16 harvesting

Coffee agro-ecosystems	Coffee productivity (kg ha^{-1})
No shade	641.01 C
G. sepium shade	822.36 B
E. sububrams shade	961.52 A
M. champaca shade	512.13 C

Note: Means followed by the same letter are not significantly different by Duncan's test at α 0.05

Table 5. Linear regression of some variables on coffee production

Variables	Coefficient	t calculated	Significant
Constant	-5.708	-1.466	0.161
Shannon Weaver diversity indeces of LNB (Ln)	3.325***	5.431	0.000
Weed productivity (Ln kg/ha)	0.888***	5.353	0.000
Soil organic C (Ln %)	0.74**	2.527	0.022
Soil total N (Ln %)	-1.099**	-2.730	0.041
Total biomass (Ln kg/ha/thn)	-0.141^{ns}	-0.334	0.742
Standing litter (Ln ton/ha)	0.308*	1.854	0.081
F calc (signification)		19.943 (0.000)	
R^2		0.876	

Note: ns = non significant

champaca) and no shade or coffee agro-ecosystem. This suggested that bacteria formed nodule of siratro was able to form nodule with legume shade trees. Evizal et al., (2010) reported that nodules of *G. sepium* had higher N fixation activity than nodules of *E. sububrams*, but *E. sububrams* trees form bigger nodules and produced more nodules (total fresh or dry weight). Futhermore, *E. sububrams* trees produced more abundance of litter fall than *G. sepium* trees that affected the coffee yield.

Types of agro-ecosystems had not significant effect on bean productivity of 5-6 years old coffee agro-ecosystems. Table 4 showed the productivity of bean coffee from different types of 15-16 years coffee agro-ecosystems. Types of agro-ecosystems influenced the productivity of 15-16 year old coffee. Both coffee agro-ecosystems with legume shade trees had higher productivity than those of non legume or no shade trees. Beside its capacity of N fixing, legume trees also produced abundance N from litter fall and provided suitable shading for coffee (Evizal et al., 2009). Productivity of coffee agro-ecosystem shaded by *E. sububrams* was higher than those of coffee agro-ecosystem shaded by *G. sepium*.

Table 5 showed linear multiple regression of some variables on coffee productivity. Diversity of LNB together with weed productivity, soil organic C, and standing litter had positive regression on coffee productivity. In contrast, ralated to LNB diversity, soil total N had negative regression on coffee productivity. Palmer and Young (2000) reported that soil N content had negative effect on diversity of LNB in soil. This result suggested that maintaining of weed biomass (by proper weeding), soil organic C, and standing litter (as representative of litter fall) was significant for high productivity of coffee agro-ecosystems. Diversity of legume nodulating bacteria (LNB) in the soil may indicate productivity of coffee agro-ecosystems.

CONCLUSION

Coffee agro-ecosystems shaded by legume trees had higher LNB diversity than those of coffee agro-ecosystems shaded by nonlegume trees (*Michelia champaca*) and coffee agro-ecosystem without shade trees. Diversity of LNB together with weed productivity, soil organic C, and standing litter had positive regression on coffee productivity. Diversity of LNB in the soil may indicate productivity of coffee agro-ecosystems.

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