*Research article*

# Effects of Legume Residues with and without Allelochemicals on Biodiversity of Plant Growth Promoting Bacteria in Long-Term Organic Residues Amendment Systems

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**Abstract** Application of organic residues into infertile soil is one way to restore the fertility and productivity of arable soils. However, organic residues have different qualities (especially chemical compositions including allelochemicals) that may influence the activity and type of microorganisms present in the soil. Therefore, this study aims to investigate the effect of allelochemicals on plant growth promoting bacteria (PGPB) biodiversity in 17 years long-term field experiment continuous application of different quality residues, groundnut stover (*Arachis hypogaea*) (high quality organic residue; with allelochemicals) and tamarind (*Tamarindus indica* L.) (intermediate quality organic residue; without allelochemicals), compare to control (no organic residue applied). PGPB isolates were screened for their plant growth promoting factors such as production of indole-3-acetic acid (IAA), phosphate solubilizing activity and their ability to grow in nitrogen-free medium. In addition, the biocontrol activities which are protease production and antagonistic activity against *Fusarium* sp. were investigated. PGPB biodiversity was determined on a basis of amplified rDNA restriction analysis (ARDRA). The majority of bacteria were found to belong to the genera of *Bacillus*, *Staphylococcus*, and *Brevibacterium*. In addition, at 52 weeks after residue application, PGPB richness of tamarind treatment was higher than groundnut treatment. The findings of this study also indicated that long-term legume residues amendment with and without allelochemicals can affect PGPB richness and biodiversity.

**Keywords** plant growth promoting bacteria (PGPB), groundnut stover, tamarind residue, allelopathy

## INTRODUCTION

Application of organic residues into infertile soil is one way to restore the fertility and productivity of arable soils. In addition, incorporating organic matter into soil affects on the biological, chemical and physical properties of the soil and its overall health. However, organic residues have different qualities (especially chemical compositions including allelochemicals) that influence the number

and type of microorganisms present in the soil (Aciego Pietri and Brooks, 2009; Marschner et al., 2003). Among allelopathic plants, tamarind is well known for its allelopathic potential. There are several allelochemicals playing role as allelopathic substances were found in tamarind leaves such as tannins, phenolic compounds, flavoniod and alkaloids (Bhadoriya et al., 2011; Lewis and Neelakantan, 1964). Total phenols such as gallic acid, Caffeic acid were observed in tamarind leaves for 14 g/kg and tannic acid for 8 g/kg. These chemicals also showed antimicrobial activities (Lawongsa et al., 2016a) and leaf extract of tamarind which showed decreasing bacterial population in sandy soil (Somboon et al., 2017). Moreover, applications of different quality of organic residue also have a tendency to induce plant growth promoting bacteria (PGPB) in soil (Rungthong et al., 2013). The PGPB represent numerous species of soil bacteria which, when grown in association with a host plant, result in stimulation of growth of their host. PGPB are used as inoculants for biofertilization, phytostimulation and biocontrol (Bloemberg and Lugtenberg, 2001). PGPB can directly benefit plant growth by fixing nitrogen, which can subsequently be used by the plant, thereby improving plant growth when the amount of nitrogen in the soil is limiting (Vessey and Buss, 2002), produce phytohormone such as indole-3-acetic acid (IAA) (Ahemad and Khan, 2012; Sachdev et al., 2009) and phosphorus uptake (Rodriguez et al., 1999). Indirectly, by increase resistance to pathogen and stimulate activation of induced systemic resistance (ISR) and systemic acquired resistance (SAR) pathways (Duffy and Défago, 1999). The accumulation of legume residue with allelochemicals into soils beyond certain threshold levels to overcome soil degradation is may affect PGPB diversity.

## OBJECTIVE

The purpose of this study was to investigate the effect of allelochemicals on plant growth promoting bacteria (PGPB) biodiversity in 17 years long-term field experiment continuous application of different quality of legume residues, groundnut stover (*Arachis hypogaea*) (high quality organic residue; without allelochemicals) and tamarind (*Tamarindus indica* L.) (intermediate quality organic residue; with allelochemicals), compare to control (no organic residue applied).

## METHODOLOGY

### Study Sites and Soil Sampling

The long-term field experiments were established at the research station of the Agriculture and Co-operatives of Northeast at Tha Phra subdistrict, Khon Kaen province, Thailand (16°20′ N; 102°49′ E) since 1995. This experiment has been designed as the randomized complete block design (RCBD). Three treatments apart from control soil (C; soil with no organic residues applied), there were two legume residue treatments applied in early May every year including:, groundnut stover (*Arachis hypogaea*) as high quality organic residue (GN; C/N ratio: 17.1, Lignin: 67.6 g/kg; Polyphenols: 12.9 g/kg) and tamarind (*Tamarindus indica* L.) leaves + petiole litter as intermediate quality organic residue (TM; C/N ratio: 31.5, Lignin: 87.7 g/kg; Polyphenols: 31.5 g/kg) at the rate of 10 Mg/ha/year to bare soil plots. The organic materials were incorporated to a depth of 20 cm in a 4 x 4 $m^2$ plot. The average of soil moisture content in the control field, GN field and TM field all year were 6.24%, 6.26% and 6.6%, respectively. Weeds were controlled at approximately monthly intervals. For the present study, soil samples were obtained in April 2012, 17 years after the field experiment had started. Three random soil samples from each of three replicate plots of each treatment were collected at 0-15 cm depth for PGPB analysis at 0, 26, 52 weeks after residue application. The soil in this study was characterized as a Khorat sandy loam (fine loamy siliceous isohyperthermic Typic (Oxyaquic) Kandiustults (USDA, 2014). Soil texture of all plot sites were sand. The proportion of sand in the topsoil (0–15 cm depth) was between 93.86 - 94.93 %. The pH of all soil samples were found to vary from 5.1 to 6.62 which indicated the slight acidity of soils. The soil pH values of 5.10, 6.37 and 6.62 were obtained from C, GN and TM, respectively. The

electrical conductivity (EC) values of all soil samples were found in the range of 33.66 to 64.66 µS/cm indicated that all soil treatments were low in salinity. The soil samples were placed in plastic bags and stored at 4°C for further microbial analysis.

**Isolation of PGPB from Soil Samples**

Bacterial strains were isolated from soil samples by serial dilution plate technique on nutrient agar medium (NAM). The bacterial colonies were isolated and maintained on NAM slants at 4°C. One hundred isolates obtained from serial dilution plate technique of each treatment were screened for their plant growth promoting factors like production of indole-3-acetic acid (IAA), phosphate solubilizing activity and their ability to grow in N-free medium. In addition, their biocontrol activity like protease enzyme production as well as antagonistic activity against *Fusarium* sp. was investigated.

**Total Genomic DNA Isolation**

PGPB isolates were grown in a nutrient broth at 28°C overnight and then were harvested by centrifugation at 5,000xg for 5 min and washed twice in 500 µl of TEN buffer (50 mM Tris, 20 mM disodium EDTA, and 50 mM NaCl, pH 8.0). Cell lysates were prepared by mixing the cell pellet with 200 µl of 20% (w/v) sucrose in TEN buffer to this 20 µl of 2 mg/ml of lysozyme and 20 µl of 10 mg/ml of RNase was added. Cell mixtures were incubated at 37°C for 60 min. Then 75 µl of 5 M NaCl and 100 µl of 10% SDS were added before gentle mixing. The solution was purified twice by using phenol:chloroform:isoamyl-alcohol (25:24:1, by volume). The upper phase was collected and precipitated by using isopropanol and 3 M sodium acetate. The DNA pellet was resuspended in sterilized deionized-water and total genomic DNA was kept at -20°C before use (Sambrook and Russell, 2001).

**Amplified rDNA Restriction Analysis (ARDRA)**

The selected promising plant growth promoting traits were continued analyzed for biodiversity analysis using ARDRA. The 16S rDNA universal primers fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rP2 (5'-AAG GAG GTG ATC CAG CC-3') (Weisburg et al. 1991) were used to amplify a 1.5-kb internal region of the 16S rRNA gene. An initial denaturation at 95°C for 5 min was followed by 35 cycles with denaturation at of 95°C (30 s), annealing at 58°C (1 min) and extension at 72°C (2 min), and a final extension at 72°C for 7min. Restriction analysis was performed with 5 µl of amplified product and 10 µl of restriction buffer containing 2 U of either the restriction enzymes *Alu*I. After a 4 h digestion at the appropriate temperature, the enzyme was inactivated by heating the preparations at 65°C for 20 min. For each isolate, PCR amplification and restriction analysis were performed at least three times. Calculation of the pair-wise coefficients of similarity was based on the presence or absence of bands. A cluster analysis with the UPGMA algorithm was performed with the NTSYS-pc numerical taxonomy and multivariate analysis system. Then, Representatives of each group were selected for cloning and partial 16S rRNA gene sequencing to retrieve sequence similarity and bacterial identity from sequence databases.

**Diversity Index Analysis**

Based on these clusters, PGPB community richness and diversity index (Shannon) was calculated. The formula is as follows:

$$H' = -\sum_{i=1}^{s} p_i \ln p_i$$

where H' is the species diversity index, s is the number of species, and pi is the proportion of individuals of each species belonging to the species of the total number of individuals (Nolan and Callahan, 2006).

## RESULTS AND DISCUSSION

### PGPB Richness

The PGPB diversity was estimated by Shannon's index. Shannon value can reflect the species diversity of the community, affected by both species richness and species evenness, that is the two values also consider the abundance of each species. The greatest PGPB richness was observed in GN (0.14) and TM (0.14) at 26 weeks after residue application (Table 1). At 52 weeks after residue application, PGPB richness of tamarind treatment was higher than groundnut treatment. This study indicated that application of legume residue with allelochemicals like tamarind induced PGPB richness overtime. In a relatively study showed that a large number of bacteria and fungi have their ability to degrade allelochemicals (Zhang et al., 2010; Chen et al., 2011). These microorganisms could mineralize the allelochemicals as its sole source of carbon and energy. This could be certified to the fact that the PGPB richness can increase eventually.

**Table 1 PGPB richness from the three treatments**

| Treatments* | Shannon's index (weeks after residue application) | | |
|---|---|---|---|
| | 0 | 26 | 52 |
| C | 0.02 | 0.10 | 0.07 |
| GN | 0.02 | 0.14 | 0.10 |
| TM | 0.02 | 0.14 | 0.12 |

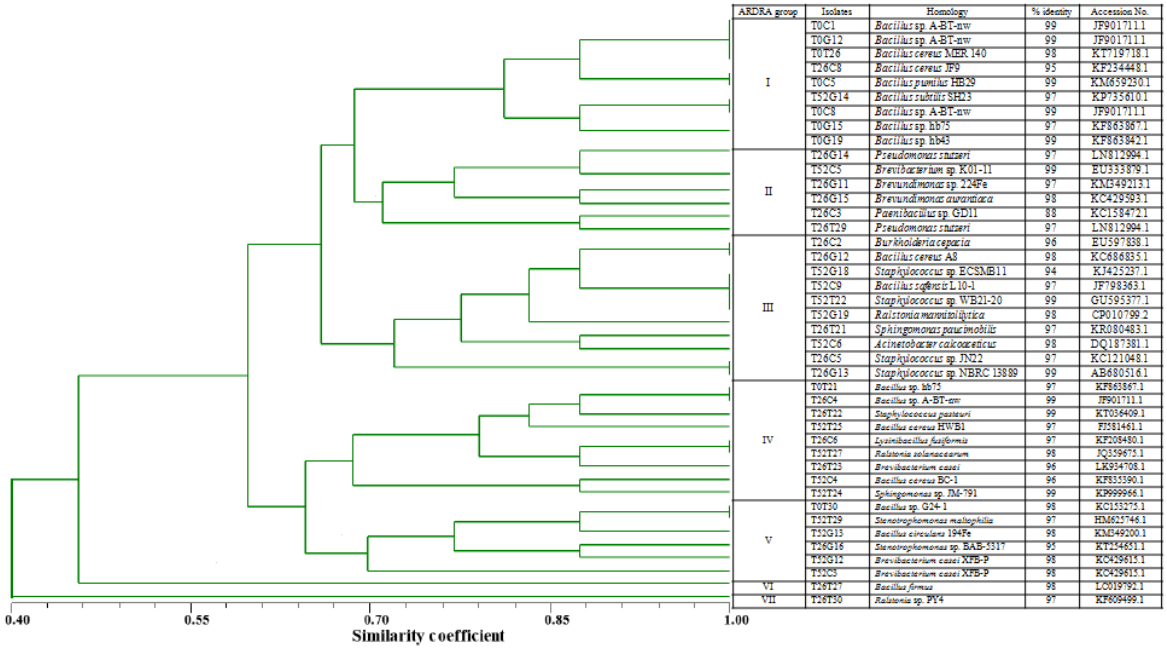
**Treatments (C; no long-term application of organic residue in tropical sandy soil, GN; long-term application of groundnut strover in tropical sandy soil, TM; long-term application of tamarind in tropical sandy soil)*

### ARDRA Analysis and 16S rRNA Gene Partial Sequencing on PGPB Isolates

The cluster dendrogram of ARDRA analysis of PGPB isolates obtained from soil samples of 17 years long-term field experiment is illustrated in Fig. 1. Digestion of amplified 16S rDNA with *Alu*I revealed seven main clusters of ARDRA dendrogram. Cluster I, II, III and V of ARDRA dendrogram showed representative of genera/species from all treatment. Cluster IV contained representative of PGPB genera/species only from groundnut treatment while, cluster VI and VII showed representative of PGPB genera/species only from tamarind treatment. Remarkably, Cluster I, IV and V of ARDRA dendrogram showed representative of genera/species from all weeks after residue application. No representative of PGPB genera/species isolated at 0 week after residue application was observed in cluster II, III, VI and VII of ARDRA dendrogram. In addition, no representative of PGPB genera/species isolated from 0 and 52 weeks after residue application was observed in cluster VI and VII of ARDRA dendrogram.

On the basis of the 16S rRNA gene sequence analysis, in excess of 1 kb fragments were sequenced for most of isolates, with similarities ranging between 88 and 99%. Thirteen isolates were identified as *Bacillus* spp., *Staphylococcus* spp., *Brevibacterium* spp., *Ralstonia* spp., *Pseudomonas* spp., *Brevundimonas* spp., *Sphingomonas* spp., *Stenotrophomonas* spp., *Paenibacillus* sp., *Burkholderia* sp., *Acinetobacter* sp. and *Lysinibacillus* sp.
Further, *Bacillus* spp., *Staphylococcus* spp. and *Brevibacterium* spp. were observed in all treatments. The PGPB in the genus of *Brevundimonas* was only found in groundnut treatment. In addition, the *Sphingomonas* spp. was only found in tamarind treatment.

The previous study reported that long-term application of organic residues strongly affected bacterial and fungal community structure and diversity (Lawongsa et al., 2016b; Kamolmanit et al., 2013). This study suggested that not only application of legume residue with or without allelochemicals can alter bacterial and fungal diversity, but also PGPB diversity.




| ARDRA group | Isolates | Homology | % identity | Accession No. |
|---|---|---|---|---|
| I | T0C1 | *Bacillus* sp. A-BT-nw | 99 | JF901711.1 |
| | T0G12 | *Bacillus* sp. A-BT-nw | 99 | JF901711.1 |
| | T0T26 | *Bacillus cereus* MER 140 | 98 | KT719718.1 |
| | T26C8 | *Bacillus cereus* JF9 | 95 | KP234448.1 |
| | T0C5 | *Bacillus pumilus* HB29 | 99 | KM659230.1 |
| | T52G14 | *Bacillus subtilis* SH23 | 97 | KP735610.1 |
| | T0C8 | *Bacillus* sp. A-BT-nw | 99 | JF901711.1 |
| | T0G15 | *Bacillus* sp. hb75 | 97 | KF863867.1 |
| | T0G19 | *Bacillus* sp. hb43 | 99 | KF863842.1 |
| II | T26G14 | *Pseudomonas stutzeri* | 97 | LN812994.1 |
| | T52C5 | *Brevibacterium* sp. K01-11 | 99 | EU333879.1 |
| | T26G11 | *Brevundimonas* sp. 224Fe | 97 | KM349213.1 |
| | T26G15 | *Brevundimonas aurantiaca* | 98 | KC429593.1 |
| | T26C3 | *Paenibacillus* sp. GD11 | 88 | KC158472.1 |
| | T26T29 | *Pseudomonas stutzeri* | 97 | LN812994.1 |
| III | T26C2 | *Burkholderia cepacia* | 96 | EU597838.1 |
| | T26G12 | *Bacillus cereus* A8 | 98 | KC686835.1 |
| | T52G18 | *Staphylococcus* sp. ECSMB11 | 94 | KJ425237.1 |
| | T52C9 | *Bacillus safensis* L10-1 | 97 | JF798363.1 |
| | T52T22 | *Staphylococcus* sp. WB21-20 | 99 | GU595377.1 |
| | T52G19 | *Ralstonia mannitolilytica* | 98 | CP010799.2 |
| | T26T21 | *Sphingomonas paucimobilis* | 97 | KR080483.1 |
| | T52C6 | *Acinetobacter calcoaceticus* | 98 | DQ187381.1 |
| | T26C5 | *Staphylococcus* sp. JN22 | 97 | KC121048.1 |
| | T26G13 | *Staphylococcus* sp. NBRC 13889 | 99 | AB680516.1 |
| IV | T0T21 | *Bacillus* sp. hb75 | 97 | KF863867.1 |
| | T26C4 | *Bacillus* sp. A-BT-nw | 99 | JF901711.1 |
| | T26T22 | *Staphylococcus pasteuri* | 99 | KT036409.1 |
| | T52T25 | *Bacillus cereus* HWB1 | 97 | FJ581461.1 |
| | T26C6 | *Lysinibacillus fusiformis* | 97 | KP208480.1 |
| | T52T27 | *Ralstonia solanacearum* | 98 | JQ359675.1 |
| | T26T23 | *Brevibacterium casei* | 96 | LK934708.1 |
| | T52C4 | *Bacillus cereus* BC-1 | 96 | KF855390.1 |
| | T52T24 | *Sphingomonas* sp. JM-791 | 99 | KP999966.1 |
| V | T0T30 | *Bacillus* sp. G24-1 | 98 | KC153275.1 |
| | T52T29 | *Stenotrophomonas maltophilia* | 97 | HM625746.1 |
| | T52G13 | *Bacillus circulans* 194Fe | 98 | KM349200.1 |
| | T26G16 | *Stenotrophomonas* sp. SAS-3317 | 95 | KT254851.1 |
| | T52G12 | *Brevibacterium casei* XPB-P | 98 | KC429615.1 |
| | T52C5 | *Brevibacterium casei* XPB-P | 98 | KC429615.1 |
| VI | T26T27 | *Bacillus firmus* | 98 | LC019792.1 |
| VII | T26T30 | *Ralstonia* sp. PY4 | 97 | KF609499.1 |

** The different isolates were designated T followed by the weeks after residue application (0, 26, or 52), treatment name (G;groundnut or T; tamarind) and by progressive numbers of PGPB isolation.*

**Fig. 1 Dendrogram of PGPB isolates representing each ARDRA group**

## CONCLUSION

In this study, thirteen bacterial strains isolated from 17 years long-term field experiment continuous application of different quality residues, groundnut stover (*Arachis hypogaea*) (high quality organic residue) and tamarind (*Tamarindus indica* L.) (intermediate quality organic residue), compare to control (no organic residue applied) were characterized. Most of the bacteria were member in the genera of *Bacillus* spp., *Staphylococcus* spp., *Brevibacterium* spp., *Ralstonia* spp., *Pseudomonas* spp., *Brevundimonas* spp., *Sphingomonas* spp., *Stenotrophomonas* spp., *Paenibacillus* sp., *Burkholderia* sp., *Acinetobacter* sp. and *Lysinibacillus* sp. The bacterial communities were dominated by *Bacillus* spp. (42.86%), *Staphylococcus* spp. (11.90%) and *Brevibacterium* spp. (9.52%). Interestingly, the richness of PGPB strains showed no different between groundnut treatment and tamarind treatment at 0 and 26 weeks after residue application. However, the richness of PGPB strains of tamarind residue had increased at 52 weeks after residue application. Notably, only the *Brevundimonas* spp. was only found in groundnut treatment. In addition, the *Sphingomonas* spp. was only found in tamarind treatment. These findings suggested that application of legume residue with allelochemicals like tamarind can alter PGPB richness and diversity. The direction of the future research should thus consider, apart from studying PGPB, also decomposing fungi and archaea in long term field experiment. This may include phylogenetic studies and functional genes to identify microbial community directly involved in the degradation of organic materials in soils.

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