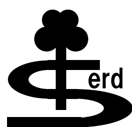

Research article

Diversity of Plant Growth Promoting Rhizobacteria Communities Associated with Thiamethoxam in Cassava Production Systems

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Abstract The use of chemical pesticide always leaves chemical residues on soil and may affect the population, function and diversity of soil microorganisms. Therefore, the aim of the study was to determine the effect of thiamethoxam, pesticide in the group of neonicotinoid to control insect pest, on diversity of plant growth promoting rhizobacteria (PGPR) communities in cassava production systems. Bacteria were isolated from rhizosphere of cassava grown in the field of 5 treatments, including cassava production system without thiamethoxam and fertilizer application, cassava production system with thiamethoxam application, cassava production system with thiamethoxam and organic fertilizer application, cassava production system with thiamethoxam and chemical fertilizer application, and cassava production system with thiamethoxam, organic and chemical fertilizer application, and then screened for plant growth promoting traits. The genotypic diversity of isolates was determined on a basis of amplified rDNA restriction analysis (ARDRA). The findings of this study indicated that the majority of bacteria were found to belong to the genera of *Bacillus*, *Ochrobactrum*, and *Brevibacillus*. Interestingly, the application of thiamethoxam in cassava production system has no effect on PGPR diversity.

Keywords Plant Growth Promoting Rhizobacteria (PGPR), Thiamethoxam, Cassava

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) represent numerous species of soil bacteria which, when grown in association with a host plant, result in stimulation of growth of their host. PGPR are used as inoculants for biofertilization, phytostimulation, biocontrol (Bloemberg and Lugtenberg, 2001) and bioformulation (Prathap and Ranjitha Kumari, 2015). PGPR 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 limited (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 and Fraga, 1999). Indirectly, by increase resistance to pathogen, PGPR may suppress plant pathogens by producing antimicrobial metabolites (Duffy et al., 2004) as well as by producing enzymes and/or fungicidal compounds (Bloemberg and Lugtenberg, 2001; Haas and Défago, 2005). In addition, PGPR can produce siderophore induced disease resistance, which can be enhanced by the simultaneous activation of induced systemic resistance (ISR) and systemic acquired resistance (SAR)

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pathways (Duffy and Défago, 1999). However, accumulation of chemical pesticides into the soils beyond certain threshold levels due to its frequent application in the fields to overcome plant pests damages the rhizosphere microorganisms and their activities (Wani et al., 2005) and may alter diversity of the microorganism. Nowadays, thiamethoxam, pesticide in the group of neonicotinoid, was normally used for cassava stake soaking to control aphids. However, the data of whether thiamethoxam affects soil microorganism diversity remains scarce.

OBJECTIVE

The purpose of this study was to investigate the influence of chemical pesticide (thiamethoxam) on the diversity of PGPR communities in cassava production systems.

METHODOLOGY

Study Sites and Soil Sampling

Study sites used in this study were selected from Kalasin province (N16°38'3", E 103° 15 '15"), Thailand. This experiment has been designed as the randomized complete block design (RCBD). A sampled area was set up at 5m x10 m for each block. Soil samples were collected from rhizosphere of cassava cultivar Kasetsart 50 (KU50) at a spacing of 1m x 1m with three replications at 0, 15, 30, 45 and 60 days after planting (DAP). Thiamethoxam 25 WG was used in this study for cassava stake soaking at the rate 4g/20 liters of water. Five treatments used in this study, including cassava production system without thiamethoxam and fertilizer application (T1), cassava production system with thiamethoxam application (T2), cassava production system with thiamethoxam and organic fertilizer application (T3), cassava production system with thiamethoxam and chemical fertilizer application (T4), and cassava production system with thiamethoxam, organic and chemical fertilizer application (T5). Organic fertilizer used in this study is chicken manure mixed with rice husk, 1000 kg/1,600 m^2 and chemical fertilizer used in this study is formula 15-7-18, 50 kg/1,600 m^2. The rhizosphere samples were placed in plastic bags and stored at 4°C for further microbial analysis.

Isolation of PGPR from Cassava Rhizosphere

Bacterial strains were isolated from cassava rhizosphere 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 included production of indole-3-acetic acid (IAA), phosphate solubilizing activity and their ability to grow in N-free medium. In addition, their biocontrol activity included protease enzyme production as well as siderophore production was investigated.

Assays for Growth Promoting Abilities of Isolates

Indole-3-acetic acid (IAA) production: IAA production was determined using the method described by Lawongsa et al. (2008) with slight modification. Bacterial isolates were cultured in Tris-TMRT (D-mannitol 10 g, yeast extract 0.2 g, $CaCl_2.2H_2O$ 0.2 g, $MgSO_4.7H_2O$ 0.25 g, tris-base 1.21 g, pH 6.8) supplemented with tryptophan 0.5 mM for 48 h. The measurement of IAA was done by adding 2 ml of 0.01 M $FeCl_3$ in 35 % $HClO_4$ into 1 ml of Tris-TMRT culture broth. The mixture was incubated in the dark at 30 °C for 30 min. The detection of IAA was determined by the development of pink color.
Phosphate solubilizing assay: Solubilization of tricalcium phosphate was detected in national botanical research institute's phosphate growth medium (NBRIP) agar plate supplemented with 1.5 %

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(w/v) agar (Nautiyal, 1999). Five microliters of each bacterial culture was dropped on NBRIP agar plates. Plates were incubated for 7 days at 28 °C. The development of halo zone around the bacterial colony was considered as positive for phosphate solubilizing activity.

Nitrogenase activity: For rapid determination, nitrogenase activity was assayed after bacterial strains were streaked onto N-free minimal medium supplemented with 1.5 % (w/v) agar and incubated at 28 ± 2 °C for 3 days (Desnoues et al., 2003). Bacterial growth indicated nitrogenase activity.

Protease assay: Bacteria were isolated for protease enzyme (casein degradation) using a method described by Sjödahl et al. (2002). Samples were inoculated on Luria-Bertani (LB) agar plates containing skim milk (20 %), then incubated at 28 ± 2 °C for two days (Uyar et al., 2011). The development of clear zone around the bacterial colony indicated protease enzyme activity.

Siderophore assay: Siderophore was determined by chromazurol sulphonate agar (CAS) using the method described by Clark and Bavoil (1994). Bacterial inoculum was spotted into the center of a CAS agar plate. After incubation at 28°C for 5 days, siderophore production was assayed by clear zone formation around the cell.

Total Genomic DNA Isolation

PGPR 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 (0.1 M Tris-Cl, 0.01 M ethylenediaminetetraacetic acid (EDTA), and 1 M 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% Sodium dodecyl sulfate (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 16S rDNA universal primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-AAGGAGGTGATCCAGCC-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 nucleotide sequence databases.

RESULTS AND DISCUSSION

Plant Growth Promoting Properties of Bacterial Strains

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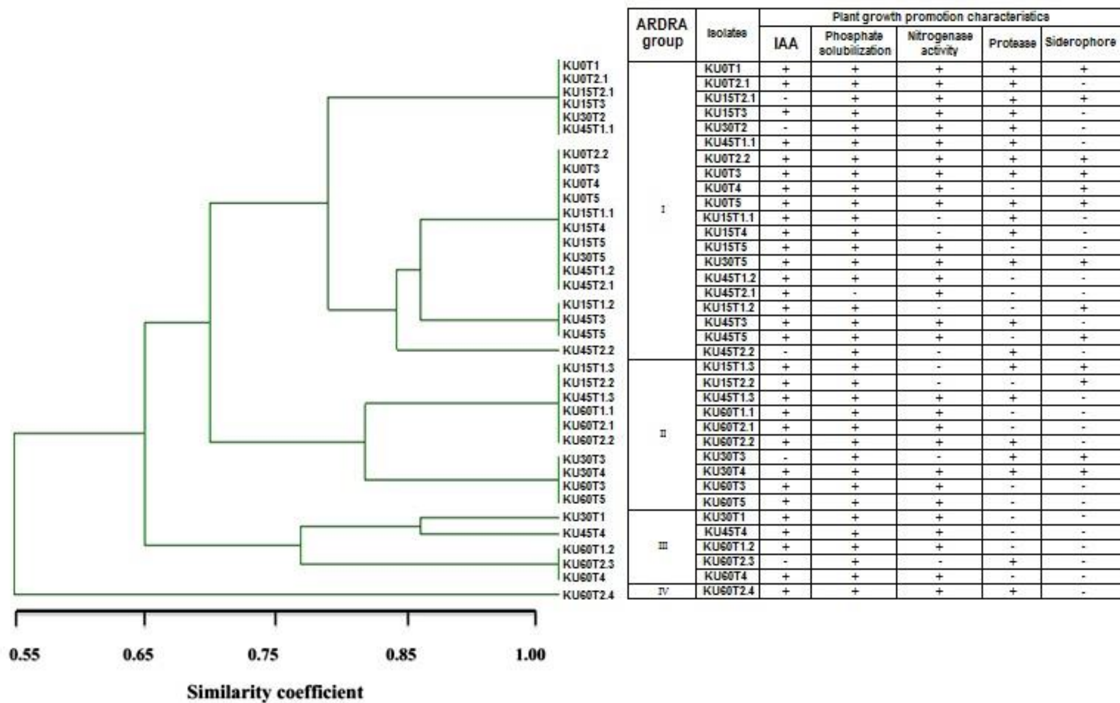
A total of 100 cultivable bacterial isolates of each treatment obtained after serial dilutions were screened for plant growth promoting traits. Subsequently, community of representatives of PGPR genera/species of each treatment was distinguished by ARDRA analysis. Detailed data of the phenotypic characterization of these isolates are shown in Fig. 1. Ninety five percentage of PGPR isolates obtained from ARDRA cluster I showed the ability to solubilize phosphate and 85% of PGPR isolates showed the ability to produce IAA. In ARDRA cluster II, 100% of PGPR isolates obtained from ARDRA cluster I showed the ability to solubilize phosphate and 90% of PGPR isolates showed the ability to produce IAA. In ARDRA cluster III, 100% of PGPR isolates obtained from ARDRA cluster I showed the ability to solubilize phosphate, 80% of PGPR isolates showed the ability to produce IAA and 80% of PGPR isolates showed the ability to grow in N-free medium. Meanwhile, in ARDRA cluster IV, PGPR isolate showed the ability to produce IAA, protease can solubilize phosphate and be able to grow in N-free medium but did not show the ability to produce siderophore.

ARDRA Analysis and 16S rRNA Gene Partial Sequencing on PGPR Isolates

The cluster dendogram of ARDRA analysis of PGPR isolates obtained from cassava rhizosphere is illustrated in Fig. 1. Digestion of amplified 16S rDNA with *Alu*I revealed four main clusters of ARDRA dendogram. Cluster I and II of ARDRA dendogram showed representative of genera/species from all treatment. Cluster III contained representative of PGPR genera/species from T1 (cassava production system without thiamethoxam and fertilizer application), T2 (cassava production system with thiamethoxam application) and T4 (cassava production system with thiamethoxam and chemical fertilizer application) while, cluster IV showed representative of PGPR genera/species only from T2.



ARDRA group	Isolates	Plant growth promotion characteristics				
		IAA	Phosphate solubilization	Nitrogenase activity	Protease	Siderophore
I	KU0T1	+	+	+	+	+
	KU0T2.1	+	+	+	+	-
	KU15T2.1	-	+	+	+	+
	KU15T3	+	+	+	+	-
	KU30T2	-	+	+	+	-
	KU45T1.1	+	+	+	+	-
	KU0T2.2	+	+	+	+	+
	KU0T3	+	+	+	+	+
	KU0T4	+	+	+	-	+
	KU0T5	+	+	+	+	+
	KU15T1.1	+	+	-	+	-
	KU15T4	+	+	-	+	-
	KU15T5	+	+	+	-	-
	KU30T5	+	+	+	+	+
	KU45T1.2	+	+	+	-	-
	KU45T2.1	+	-	+	-	-
	KU15T1.2	+	+	-	-	+
	KU45T3	+	+	+	+	-
	KU45T5	+	+	+	-	+
	KU45T2.2	-	+	-	+	-
II	KU15T1.3	+	+	-	+	+
	KU15T2.2	+	+	-	-	+
	KU45T1.3	+	+	+	+	-
	KU60T1.1	+	+	+	-	-
	KU60T2.1	+	+	+	-	-
	KU60T2.2	+	+	+	+	-
	KU30T3	-	+	-	+	+
	KU30T4	+	+	+	+	+
	KU60T3	+	+	+	-	-
	KU60T5	+	+	+	-	-
III	KU30T1	+	+	+	-	-
	KU45T4	+	+	+	-	-
	KU60T1.2	+	+	+	-	-
	KU60T2.3	-	+	-	+	-
	KU60T4	+	+	+	-	-
IV	KU60T2.4	+	+	+	+	-

**The different isolates were designated KU followed by the time after planting (0, 15, 30, 45 or 60), treatment number and by progressive numbers of PGPR isolation.*

Fig. 1 Dendogram of PGPR isolates representing each ARDRA group and plant growth promotion characteristics presented by bacterial isolates and their ARDRA groups

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Interestingly, no representative of PGPR genera/species isolated at 60 DAP was observed in cluster I of ARDRA dendogram. In addition, no representative of PGPR genera/species isolated from 0 DAP was observed in cluster II of ARDRA dendogram. In ARDRA cluster III, no PGPR genera/species isolates at 0 and 15 DAP was obtained. Moreover, In ARDRA cluster IV, only PGPR genera/species isolates at 60 DAP from T2 was found. Additionally, PGPR isolates from treatment applied with organic fertilizer were not found in ARDRA cluster III and IV.

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 95 and 99%. Six isolates were identified as *Ochrobactrum anthropi*, three as *Ochrobactrum ciceri*, One as *Ochrobactrum intermedium*, fifteen as *Bacillus* spp., one as *Bacillus silvertri*, four as *Bacillus thuringiensis*, two as *Bacillus megeterium* and four as *Brevibacillus reuszeri* (Table 1). Interestingly, *Brevibacillus* spp. was not found at 0, 15, 30 and 45 DAP, but was only obtained at 60 DAP.

Table 1 Similarity analysis based on 16S rRNA gene partial sequences of PGPR isolates by comparing to the GenBank and their ARDRA groups

ARDRA group	Isolates	Homology	% identity	Accession No.
I	KU0T1, KU0T2.1, KU15T2.1, KU15T3, KU30T2, KU45T1.1	*Ochrobactrum anthropi* DJ3	97	KC992296.1
	KU0T2.2, KU0T3, KU0T4, KU0T5, KU15T1.1, KU15T4, KU15T5, KU30T5, KU45T1.2, KU45T2.1	*Bacillus* sp. SGE119	99	HM566648.1
	KU15T1.2, KU45T3, KU45T5	*Ochrobactrum ciceri* L22	96	JX646649.1
	KU45T2.2	*Ochrobactrum intermedium* SYF-18	98	JN048652.1
II	KU15T1.3	*Bacillus* sp. DoB56	98	JQ359104.1
	KU15T2.2	*Bacillus* sp. SGE39	99	HM566736.1
	KU45T1.3	*Bacillus silvestris* SAFN-010	95	AY167818.1
	KU60T1.1, KU60T2.1, KU60T2.2	*Bacillus* sp.YY-13	99	JX575604.1
	KU30T3, KU30T4, KU60T3, KU60T5	*Bacillus thuringiensis* Ou2	98	KP128698.1
III	KU30T1	*Bacillus megaterium* H2	99	JQ579631.1
	KU45T4	*Bacillus megaterium* ML257	98	KC692200.1
	KU60T1.2, KU60T2.3, KU60T4	*Brevibacillus reuszeri* NBRC15719	99	AB680946.1
IV	KU60T2.4	*Brevibacillus reuszeri* BCX-22	98	KM378576.1

**The different isolates were designated KU followed by the time after planting (0, 15, 30, 45 or 60), treatment number and by progressive numbers of PGPR isolation.*

These findings clearly showed that thiamethoxam had no effect on PGPR diversity as ARDRA cluster 1, 2 and 3 shared the same soil bacterial ribotypes including PGPR isolated from T1 and PGPR isolated from T2. This could be certified to the fact that certain soil bacteria can degrade pesticides. *Bacillus* spp., *Ochrobactrum* spp. and *Brevibacillus* spp. are common soil bacteria easily cultured from most agricultural soils. The previous study showed that *Bacillus megaterium* has potential application in bioremediation of contaminated soil and water system (Sogani et al., 2014). *Ochrobactrum* spp. is also found to be able to degrade pesticide in neonicotinoid group such as imidacloprid (Hu et al., 2013). In addition to species of the *Ochrobactrum* genus, many individual isolates have been found to be able to degrade pesticide in neonicotinoid group, such as *Bacillus* spp. and *Brevibacterium* spp. (Sabourmoghaddam et al., 2015). Moreover, certain soil bacteria might have utilized pesticide as energy sources as well (Ahemad and Khan, 2011).

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CONCLUSION

In this study, thirteen bacterial strains isolated from cassava KU50 were characterized. Most of the bacteria were member of the Bacillaceae, Brucellaceae and Paenibacillaceae families. The bacterial communities were dominated by *Bacillus* spp. (61.11%), *Ochrobactrum* spp. (27.78%) and *Brevibacillus* spp. (11.11%). Interestingly, the findings of diversity analysis of thirty six bacterial isolates selected at random but representing each field site by ARDRA suggested that thiamethoxam had no effect on PGPR diversity as application of thiamethoxam was not be able to distinguish soil bacterial ribotypes.

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