Research article

Effects of Pesticide on Phenotypic Characteristics of Plant Growth - Promoting Rhizobacteria (PGPR) in Cassava Production Systems

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Received 12 October 2013 Accepted 1 March 2014 (*Corresponding Author)

Abstract Cassava (*Manihot esculenta* Crantz) is an important economic crop in Thailand. Nowadays, insect pest is the major problem that causes severe damage to cassava leading to considerable yield losses. Since, using chemical pesticide is always presence of chemical residues on soil and may affect the population and diversity of soil microorganism. Therefore, the aim of the study was to determine the effect of thiamethoxam on plant growth promoting rhizobacteria (PGPR) population in cassava production system. A total of 400 bacteria were isolated from 4 sites, including 1 (cassava production system without thiamethoxam and fertilizer application 2 (cassava production system with thiamethoxam application 3 (cassava production system with thiamethoxam and organic fertilizer application 4 (cassava production system with thiamethoxam and chemical fertilizer application. These isolates 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 and chitinase enzyme production and siderophore production as well as antagonistic activity against *Fusarium* sp. were investigated. The findings of this study indicated that the application of thiamethoxam in cassava production system can affect PGPR population. In this study, numbers of bacterial isolates demonstrate that they are a potential source to be used as microbial inoculant for crop production system.

Keywords Plant growth promoting rhizobacteria (PGPR), thiamethoxam, cassava

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is one of the most important economic crops in Thailand. Cassava can be used for various food products, animal feed, raw material to produce ethanol etc. In addition, an increasing demand for alternative energy products will increase the market value of cassava in the near future.

Nowadays, insect pest is the major problem that caused damage of cassava production. In particular, the most of farmers prefer to use of chemical pesticide such as thiamethoxam, insecticide in the group of neonicotinoids which is one of the most widely used, is resulting in chemical residue in soil. This may disturb the soil environment by affecting microorganisms in soil, and also the physical -chemical properties of soil leading to infertility of soil. Biological control is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture.

The using of introduced microorganisms with induced systemic resistance against plant disease and stimulate plant growth has been extensively studied (Compant et al., 2005; Welbaum et al., 2004). The introduction of plant growth promoting rhizobacteria (PGPR) offers a promising alternative to manage plant pathogen and enhance plant growth. PGPR can directly benefit plant growth by increasing nitrogen and phosphorus uptake (Rodriguez et al., 1999) and indirectly by increasing resistance to pathogen. PGPR may suppress plant pathogens by producing antimicrobial metabolism (Duffy, 2004) and also by producing enzymes and/or fungicidal compounds (Bloemberg and Lugtenberg, 2001; Haas and Défago, 2005). This study was designed to investigate how chemical pesticide shaped the bacterial populations as well as plant growth promoting activities for successful application of PGPR in cassava production systems to reduce the use of chemical pesticide and soil fertility could be maintained.

OBJECTIVE

The purpose of this study was to investigate the influence of chemical pesticide on the population and phenotypic characteristics of PGPR in cassava production system.

METHODOLOGY

Study Sites and Soil Sampling

Study sites used in this study were selected from Khon Kean province and Kalasin province (N 16° 28' 43", E 102' 49 and 37" N 16° 38' 3", E 103° 15' 15", respectively). Soil samples were collected from rhizosphere of cassava cultivar Rayong 11 with three replications of each treatment was collected at 0-15 cm depth. Pesticide used in this study for cassava stake priming was thiamethoxam 25 WG. Four treatments used in this study, including 1) cassava production system without thiamethoxam and fertilizer application 2) cassava production system with Thiamethoxam application 3) cassava production system with thiamethoxam and organic fertilizer application, 1000 kg/1,600 Square meter 4) cassava production system with thiamethoxam and chemical fertilizer application 15-7-18, 100 kg/1,600 Square meter. The rhizosphere samples were placed in plastic bags and stored at 4 °C for further microbial analysis. In addition, soil samples were collected from 0-15 cm depth for the examination of physical and chemical properties of soil.

Isolation of PGPR from Cassava Rhizosphere

Bacterial strains were isolated from cassava rhizosphere by serial dilution plate technique on nutrient agar medium (NAM) (Dubey and Maheshwari, 2002). 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 and chitinase enzyme production and siderophore production as well as antagonistic activity against *Fusarium* sp. were investigated.

Phenotypic Characterization of PGPR

Indole-3-acetic acid (IAA) production: IAA production was determined using the method described by Lawongsa (2008) with slight modification. Bacteria 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% (w/v) agar (Nautiyal, 1999). Five microliters of each bacterial culture was dropped on NBRIP agar plates. The development of halo zone around the bacterial colony indicated 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 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.

Chitinase assay: Chitinase activity was determined using colloidal chitin (Cattelan et al, 1999). Five microliters of each bacteria culture were dropped on colloidal chitin agar (Colloidal Chitin 10 g, K_2HPO_4 0.5 g, $MgSO_4$ $7H_2O$ 0.5 g, Na_2HPO_4 0.5 g, $NaNO_3$ 3.0 g, Yeast extract 1 g, Agar 20 g, pH 7) and incubator at 28 °C for 7 days. The development of halo zone around the bacterial colony indicated chitinase 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.

Antagonistic activity: An inhibition of phytopathogen by bacterial strains on potato dextrose agar (PDA) plates was performed as detailed in previously study (Keel et al., 1996) with slight modification. Briefly, bacteria was grown overnight in NB (Nutrient broth) medium, and 5 µl of each culture was spotted 2 cm from the edge of the plate (four spots per plate) and 0.3 cm square plug from a culture of *Fusarium* sp. was placed at the center of the plate. The results were assessed after 5 days by measuring the distance between the edges of the bacterial colony and the fungal mycelium.

Statistical analysis: The data collected was analyzed statistically using Analysis of variance (ANOVA) along with least significant differences (LSD) by analytical software STATISTIC 8.

RESULT AND DISCUSSION

Soil Analysis

The data of physical and chemical properties of soil are shown in Table 1. The results showed that soil samples of all treatment were sandy soil and contained low fertility.

Effect on Bacterial Population of Rhizospheric Soil of Cassava

Total count of bacteria was found to decrease with the application of thiamethoxam (Table 2). Interestingly, when the experimental plot received organic fertilizer as well as chemical fertilizer,

there was slight increase in the bacterial count as compared to cassava production system without any fertilizer.

Table 1 Physical and chemical properties of soil

Treatment	1	2	3	4
Organic matter; OM (%)	0.227 c	0.360 b	0.432 a	0.368 b
Total N (%)	0.014 a	0.009 b	0.013 a	0.009 b
Available P (mg/kg)	7.230 c	16.793 bc	51.890 a	26.330 b
Exchangeable K (mg/kg)	31.470 ab	24.037 b	33.057 ab	43.870 a
Organic carbon (%)	0.132 c	0.209 b	0.250 a	0.213 b
Soil pH	4.411 a	3.553 b	4.556 a	3.450 b
Electrical conductivity (dS/m)	0.045 a	0.026 b	0.029 b	0.032 b
Cation exchange capacity (cmol/kg)	1.725 b	5.433 a	3.333 ab	6.000 a
Bulk density (g/cm^3)	1.504 a	1.411 a	1.353 a	1.442 a
Soil moisture (%)	3.372 a	2.935 a	3.396 a	3.009 a
Soil texture	sand	sand	sand	sand

Treatment: 1) cassava production system without thiamethoxam and fertilizer application. 2) cassava production system with Thiamethoxam application. 3) cassava production system with thiamethoxam and organic fertilizer application, 1000 kg/1,600 Square meter. 4) cassava production system with thiamethoxam and chemical fertilizer application 15-7-18, 100 kg/1,600 Square meter. Means values followed by different letters are significantly different (ANOVA; LSD test, P<0.05)

Table 2 Effect of pesticide application on bacterial population

Treatment	Bacterial count (cfu/g soil)
1	5.94 x 10^{11} a
2	4.51 x 10^{10} b
3	5.42 x 10^{10} b
4	4.78 x 10^{10} b

Treatment: 1) cassava production system without thiamethoxam and fertilizer application. 2) cassava production system with Thiamethoxam application. 3) cassava production system with thiamethoxam and organic fertilizer application, 1000 kg/1,600 Square meter. 4) cassava production system with thiamethoxam and chemical fertilizer application 15-7-18, 100 kg/1,600 Square meter. Means values followed by different letters are significantly different (ANOVA; LSD test, P<0.05)

Phenotypic Characterization of Bacterial Isolates for Plant Promotion and Biocontrol Traits

A total of 100 cultivable bacterial isolates of each treatment obtained after serial dilutions and plating onto NAM were screened for plant growth promoting traits through growth promoter assays and biological control assays.

The bacterial ability to produce IAA in presence of L-tryptophan as precursor was tested. A total of 305 isolates showed the ability to produce IAA, 60%, 64%, 89% and 92% of isolates that have ability to produce IAA were obtained from treatment 1, 2, 3 and 4, respectively (Fig. 1).

Phosphate solubilizer produce clear zone around the bacterial colonies on media containing insoluble mineral phosphate. A total of 245 isolates showed the ability to solubilize tricalcium phosphate, 60%, 64%, 77% and 44% of isolates that have ability to solubilize phosphate were obtained from treatment 1, 2, 3 and 4, respectively (Fig. 1).

A total of 310 isolates showed the ability to grow in nitrogen-free medium, 55%, 75%, 98% and 82% of isolates that have ability to grow in nitrogen-free medium were obtained from treatment 1, 2, 3 and 4, respectively (Fig. 1).

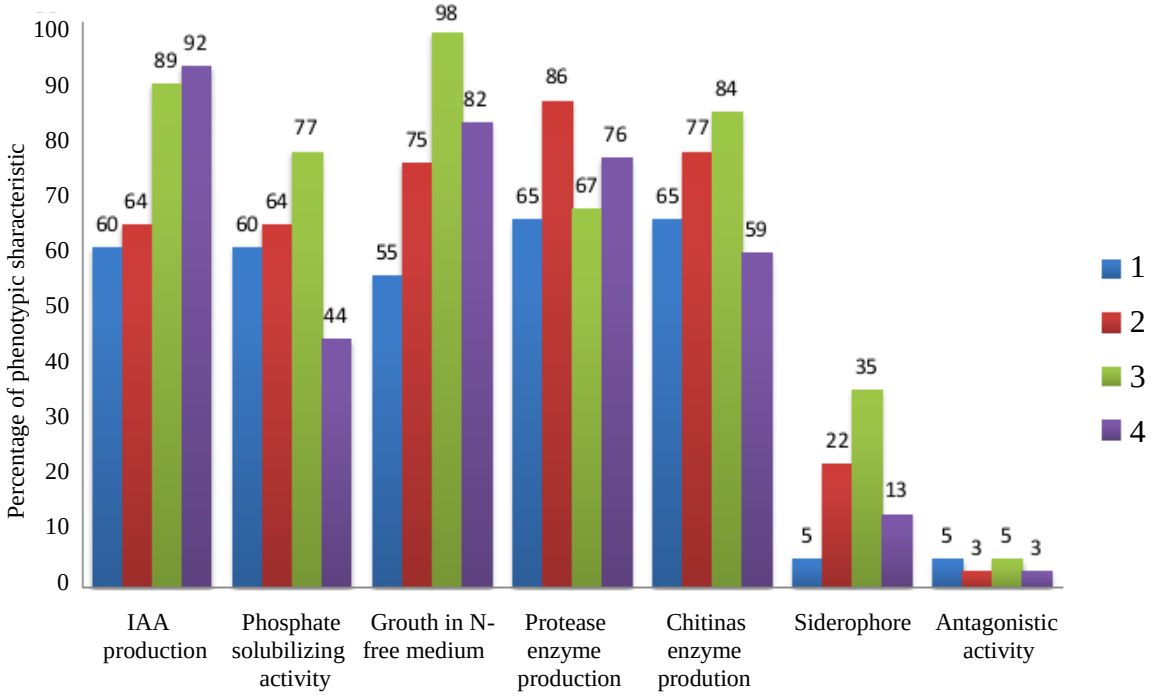
Production of fungal cell wall degrading enzymes (protease and chitinase enzymes), an important mechanism of fungal inhibition, was analyzed. A total of 294 isolates could produce halo zone on skim milk agar that showed protease activity, 65%, 86%, 67% and 76% of isolates that have ability to produce protease were obtained from treatment 1, 2, 3 and 4, respectively. A total of 285 isolates could produce halo zones on colloidal chitin agar that showed chitinase activity, 65%, 77%, 84% and 59% were obtained from treatment 1, 2, 3 and 4, respectively (Fig. 1).

Bacterial isolates which have the ability to produce siderophore and showed clear zone on CAS agar were considered as positive isolate. A total of 75 isolates could produce clear zone that

showed siderophore production, 5%, 22%, 35% and 13% were obtained from treatment 1, 2, 3 and 4, respectively (Fig. 1).

In vitro antagonistic activity against *Fusarium* sp. was investigated. The result revealed that only 16 isolates showed the ability to control *Fusarium* sp. growth among 400 isolates, 5%, 3%, 5% and 3% of isolates were obtained from treatment 1, 2, 3 and 4, respectively (Fig. 1).

From this present study, the results showed that bacteria showed variation in their growth promoter and biocontrol characteristics. Numbers of bacteria still showed the ability to promote plant growth directly and indirectly way even in the presence of pesticide. This could be certified to the fact that certain soil bacteria can degrade pesticides (Sethunathan, 1973). In addition, certain soil bacteria might have utilized pesticide as energy sources (Ahemad and Khan, 2011). Furthermore, the results showed that the application of organic fertilizer can promote plant growth promoting activities of bacteria.



Treatment: 1) cassava production system without thiamethoxam and fertilizer application 2) cassava production system with Thiamethoxam application 3) cassava production system with thiamethoxam and organic fertilizer application, 1000 kg/1,600 Square meter 4) cassava production system with thiamethoxam and chemical fertilizer application 15-7-18, 100 kg/1,600 Square meter.

Fig. 1 Number of bacterial isolates showing phenotypic characteristic at each sampling site displayed as a percentage of the total site number

CONCLUSION

This study has shown that thiamethoxam not only affect the bacterial population but also have an impact on their plant growth promoting activities. In addition, the use of organic fertilizer can slight increase the number of bacteria that exert the positive effects on plant growth promoting via direct and indirect mechanisms. The higher numbers of bacterial isolates which have the ability to produce IAA, solubilize phosphate, grow in nitrogen-free medium, produce chitinase enzyme and produce siderophore were found in cassava production system with thiamethoxam and organic fertilizer addition when compared to control with thiamethoxam. Therefore, it can be concluded that the use of pesticides should be applied with organic fertilizer, to maintain the biodiversity of soil microorganisms. Further study should be carried out with such efficient PGPR isolates to achieve the successful cassava management.

ACKNOWLEDGEMENTS

This work was supported by a grant from National Science and Technology Development Agency and Agricultural Biotechnology Research Center for Sustainable Economy, Khon Kaen University.

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