International Journal of Agricultural Technology 2015 Vol. 11(5):1209-1218
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Screening of Antagonistic Bacteria for Controlling *Cercospora coffeicola* in Arabica Coffee

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Sirinunta, A. and Akarapisan, A. (2015). Screening of antagonistic bacteria for controlling *Cercospora coffeicola* in Arabica coffee. International Journal of Agricultural Technology 11(5):1209-1218.

Abstract Brown eye spot disease caused by *Cercospora coffeicola* were collected from Omkoi district, Chiang Mai, Thailand. Thirty isolates of antagonistic bacteria were obtained from rhizosphere of coffee plant. The isolates were purified and assay to inhibit growth of the *C. coffeicola*. The antagonistic bacteria isolates A74 and A75 showed the highest percentage of growth inhibit against *C. coffeicola* with 58.33% and 61.67% respectively. Based on the morphological and biochemical properties, isolate A74 and A75 were identified to be a *Bacillus megaterium* and *Bacillus badius* respectively. Isolates A74 and A75 were developed for the powder formulation, contained 20 ml of cell bacteria suspension were mixed with 43.5 g of rice flour, 1.5 ml of rice bran oil, 5 g of sucrose. After that, the mixture completely were oven dried at 45◦C for 12 hr and then bio-product were blender to form a powder. The antagonistic bacteria in bio-product can survive for more than 3 months under storage at room temperature. The efficacy for controlling leaf spot disease of bio-product in a greenhouse was tested. The result showed that the disease severity was reduced when spraying with A74 or A75 for 24 hr before or after the pathogen inoculation on coffee leaf. Therefore, bio-product A74 or A75 as a potential biological control agent against *C. coffeicola* in seedling.

Keywords: *Cercospora coffeicola*, Arabica coffee, Rhizosphere bacteria, Leaf Spot Disease, Highland

Introduction

Coffee (*Coffea arabica* L.) is the important cultivated in Thailand. It was grown in the northern of country. The coffee seedling was damaged by *Cercospora coffeicola*. Brown eye spot disease is found in coffee-growing areas wide and importance in nurseries and sometime also it attacks adult plant. The classic leaf symptom is circular spot with tan gray, or white centers; lesions may be irregular in shape and cause leaf blight. The margins of the lesions and

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dark brown to reddish brown or purplish to black in color. Lesions are sometimes surrounded or ringed by a bright yellowish "halo" which, is more visibly apparent on the upper leaf surface (Nelson, 2008). The fungal *Cercospora* sp. give rise to the leaf spot disease on numerous host plants in tropical regions and increase in the disease usually occurs in the rainy season (Agrios, 2004). The genus *Cercospora* shows wide variation in the infection process, and even the same species shows different pattern on different host such as, *Cercospora moricola* on mulberry and *Cercospora henningsii* on cassava form several germ tubes with or without appressoria formation (Gupta *et al.*, 1995; Babu *et al.*, 2007). Biological control can be effective when antagonists are applied as preharvest treatment to control leaf and fruit disease (Knudsen and Spurr, 1987). *Bacillus* species have been reported to be effective in the biocontrol of multiple plant disease owing to their production of several broad-spectrum antibiotics and their longer shelf lives as a result of their ability to form endospores (Emmert and Handelsman, 1999). The efficacy of the biological control agent would largely depend on the type of formulation and delivery technology (Lumsden *et al.*, 1995). Experimental formulations of *Bacillus* sp. that have effectively reduced plant disease (Osburn *et al.*, 1995); wettable powder, soluble concentrate and emulsifiable concentrate (Lee *et al.*, 2006); talc powder (EL-Hassan and Gowen, 2006); suspension concentrate formulation (Collins and Jacobsen, 2003; Kanjanamaneesathian *et al.*, 2013) and alginate microcapsules (Wiwattanapatapee *et al.*, 2013). The aim of this study was screened antagonistic bacteria strains to inhibit the growth of pathogen. The antagonistic bacteria were developed and powder bio-product for control brown eye spot disease.

Material and methods

Microorganisms and Screening of Antagonistic Bacteria

The causal organism *Cercospora coffeicola* was purified using single spore isolation (Choi *et al.*, 1999) from leaf showing typical spot symptom on water agar (WA) and were transferred to V8 juice agar (V8). Arabica coffee rhizosphere soil were collected from Omkoi district, Chiang Mai, Thailand and were isolated by serial dilution technique on nutrient agar (NA) and King's medium B (KB) incubated at room temperature for 24 – 48 ℃. The single colony of antagonistic bacteria were selected and re-isolation was performed on NA until pure culture. After that, all of antagonistic bacteria isolates were tested efficacy growth inhibition against *C. coffeicola* in potato dextrose agar (PDA)

by dual culture technique incubated at room temperature and assessed by measuring the size of the inhibition zone.

Identification of Antagonistic Bacteria

The most effective of antagonistic bacteria were tested by gram reaction, spore shape, catalase, urease, cellulose, citrate utilization, gelatin liquefaction, starch hydrolysis, Voges-Proskauer test (VP test), oxidase test and motility test. The morphological and biochemical properties were identified follow by *Bergy's Manual of Determinative Bacteriology*.

Activity of bacteria for plant growth promoting

Indole-3-acetic acid (IAA) production

The antagonistic bacteria was culture into 20 ml of Luria Bertani (LB) medium containing 0.5% (v/v) of L-tryptophan and incubated at 28 ± 2 ℃ for 15 days. Then, the bacteria culture were collected by centrifugation at 6,000 rpm for 30 min. Two ml of the supernatant was mixed with 2 drop of orthophosphoric acid and 2 ml of Salkowski's reagent (50 ml, 35% perchloric acid, 1 ml 0.5 M $FeCl_3$) modified method as described by Lwin *et al.* (2012) and kept in the dark for 30 min and observed for intensity of pink color. Optical density was check by UV-Vis spectrophotometer.

Ammonia (NH_3) production

Antagonistic bacteria isolates were tested for ammonia production was performed using the method of Cappuccino and Sherman (1992). The bacteria cells were cultured in 10 ml of peptone water incubated at 28 ± 2 ℃ for 48 – 72 hr. Bacteria suspension was added with 0.5 ml of Nessler's reagent in each tube. The development of yellow color as the positive resulted

Phosphate solubilization

Bacteria isolates were screened on Pikovskaya's agar plates (10 g glucose, 5 g tri-calcium phosphate, 0.2 g $NaCl_2$, 0.1 $MgSO_4$, 0.2 KCI, 0.002 $FeSO_4.7H_2O$, 0.5 yeast extract, 0.002 $MnSO_4$, 20 agar) containing 2.4 mg/ml bromophenol blue (Jasim *et al*., 2013). Firstly, 0.5 diameter of filter paper was touch on colony bacteria. Then, place on Pikovskaya's agar incubated at 28 ℃ for 7 days and observed for yellow zone around colony due to phosphate solubilization as the positive result.

Formulation of bio-product

The suspension of selected antagonistic bacteria 2 isolates, 3 ml of each isolate were cultured into 120 ml of nutrient glucose broth (NGB) and then shaken at 180 rpm for 96 hr. Bacterial cell was centrifuged at 5000 rpm for 10 min and washed with 0.85% (w/v) NaCl, centrifuged again at 3500 rpm for 5 min. The formulation contained 20 ml of cell bacteria suspension mixed with 43.5 g of rice flour, 1.5 ml of rice bran oil and 5 g of sucrose, the mixture completely dried in hot air oven at 45 ℃ for 12 hr and blender to form a powder. The survivability of antagonistic bacteria cells in bio-product were checked every month by drop plate method on NA.

Evaluation of bio-product in a green house

The seedling of Arabica coffee were grown in pots for 150 days. The plant were kept in the plastic box (Souza *et al.,* 2011) for 7 days before the pathogen inoculation. The spore suspension of *C. coffeicola* inoculum was spread on V8 agar plate incubated at 25 ℃ for 10 days. The spore suspension obtained was adjusted to 10^6 cfu/ml using a haemacytometer. The bio-product of antagonistic bacteria were prepared by 1 g mixed in distilled water 100 ml. The bio-product were applied by spraying 24 hours before or after the fungal pathogen inoculation treatment on coffee seedling. The disease severity sizes of the obvious lesions on the coffee leaf were measured until 30 days after pathogen inoculation. The 7 treatments were arranged in a randomized complete block design, with 6 replicates per treatment.

Results and Discussion

Microorganisms and Screening of Antagonistic Bacteria

The fungus *C. coffeicola* three isolates were found on coffee leaf showing typical symptoms (Fig 1). Thirty isolates of antagonistic bacteria were obtained from rhizosphere of Arabica coffee plant. The isolates A74 and A75 have the most effective growth inhibit mycelium *C. coffeicola* showed the highest percentage of growth inhibition with 58.33% and 61.67% respectively (Fig. 2). Srimai and Akarapisan (2014) report that previous studied of *Bacillus subtilis* LBF02 showed the highest percentage of growth inhibits against *Cercospora lactucae-sativae* leaf spot on lettuce with 80.82% inhibition. Muleta *et al.* (2007) report *in vitro* antagonistic effects of rhizobacteria associated with *Coffea arabica* L. against some fungal coffee pathogens were

studied. The most of antagonistic bacteria were found *Pseudomonas* spp. and *Bacillus subtillis*. Screen indigenous coffee-associated isolates for their inherent antagonistic potential against major coffee wilt diseases induced by *Fusarium oxysporum* and *Fusarium stilboides* were tested by dual culture method. The result showed that between rhizobacteria antagonists in inhibiting the mycelial expansion of *F. oxysporum* and *F. stilboides*, ranging from 40.1–71.8% and 37.3–73.6% radial growth inhibition, respectively.

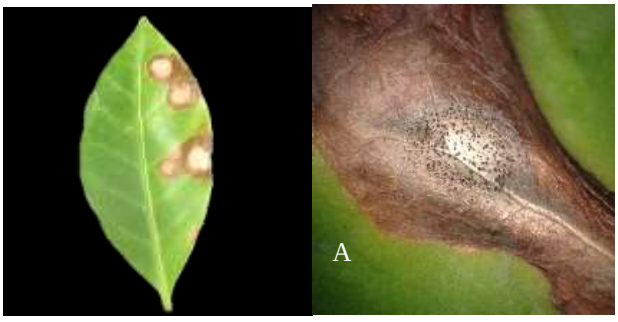

Figure 1. The symptoms of coffee leaf spot disease caused by *Cercospora coffeicola*: (A) brown spot had yellow halo around lesion; (B) closed-up detail of lesion showing.

Fig 2. The efficacy of antagonistic bacteria 2 isolates for growth inhibition of *Cercospora coffeicola*: (A) control; (B) A74; (C) A75.

Identification of Antagonistic Bacteria

Based on the morphological and biochemical tests according to *Bergy's Manual of Determinative Bacteriology*, the detailed characteristics of A74 and A75 as shown in Table 1. The antagonistic bacteria isolate A74 including gram-positive rod shaped, cellulose test was positive as well as citrate utilization, gelatin liquefaction, starch hydrolysis, oxidase test and motility test. The urease,

catalase and VP test were negative. For isolate A75 consisted gram-positive rod shaped, urease test was positive, as well as cellulose, gelatin liquefaction, starch hydrolysis, oxidase and motility test. The citrate untilization, catalase and Voges-Proskauer test were negative. These data indicated to a *Bacillus* group; A74 is *Bacillus megaterium* and A75 is *Bacillus badius*.

Table 1. The tested morphology and biochemical properties comparison for stain *B. subtillis* T01 with antagonistic A74 and A75

Properties	**Isolates**			**Properties**	**Isolates**		
	BS	**A74**	**A75**		**BS**	**A74**	**A75**
Gram staining	+	+	+	Citrate test	+	+	-
Cell shaped	rod	rod	rod	Gelatin test	+	+	+
Indole test	+	+	+	Motility test	+	+	+
NH_3 test	+	+	+	Starch hydrolysis	+	+	+
Phosphate test	+	+	+	Oxidase test	+	+	+
Catalase	+	-	-	Urease test	+	-	+
Cellulose	+	+	+	VP test	-	-	-

BS = *Bacillus subtillis* T01, "+" mean positive result and "-" mean negative result

Activity of bacteria for plant growth promoting

The antagonistic bacteria isolates *B. megaterium* and *B. badius* were developed to yellow color on peptone water was a positive test for NH_3 production. The presence of yellowish halo due to phosphate solubilization was taken as positive results. The indole-3-acetic acid (IAA) production by bacteria isolates have a quantitative of IAA was checked by UV-Vis spectrophotometer at 530 nm. The results showed that *B. badius* was the best IAA produced strain with 23.36 µg/ml. For the *B. megaterium* can be produced with 6.53 µg/ml. While, *B. subtillis* T01 (positive control) have the highest IAA produced concentration at 60.59 µg/ml. Lwin *et al.* (2012) report studied isolation of rhizobacteria 4 isolates of *Bacillus* (B1, B2, B3 and B4) were incubated on LB medium containing 0.5g/L of tryptophan for 2 weeks. At 10^{th} days found that IAA production by *Bacillus* spp. as 46.60, 52.10, 56.30 and 50.60 ppm respectively. As well as, report studied of Ahmad *et al.* (2008) assay for indole acetic acid (IAA) production *Bacillus* were cultured on NB containing different concentrations of tryptophan, 300, 400 and 500 µg/ml. The IAA was produced by *Bacillus* are 3.40, 5.03 and 7.03 µg/ml respectively.

Formulation of bio-product

The survivability of bacteria cells in each product A74 and A75 at 0, 1, 2, and 3 months after produced were checked by drop plate method for counting bacteria cells in bio-product. The results showed that the number of bacteria in the bio-product were seen to decline after produced, with A74 bacteria quantities at 1.8×10^{12}, 1.3×10^{8}, 2.8×10^{8} and 2.5×10^{8} cfu/g respectively and A75 at 1.4×10^{9}, 4.8×10^{8}, 3.5×10^{8} and 2.6×10^{8} cfu/g respectively. Therefore, the antagonistic bacteria cells in formulations were observed to survive more than 3 months under storage at room temperature.

Evaluation of bio-product in a green house

The efficacy for controlling leaf spot disease of bio-product in the greenhouse was tested. The evaluation of the damaged according to the wound size, when the data were analyzed statistically and compared, the mean of each treatment by RCBD (Randomized Complete Block Design). The pathogen *C. coffeicola* leaf spot disease severity was reduce when using A74 bio-product sprayed 24 hour before or after pathogen inoculation. The diameters of the wounds were measured to be about 2.08 and 2.27 mm respectively. The A75 bio-product sprayed 24 hour before or after pathogen inoculation, the diameters of the wounds were measured to be about 1.70 and 1.52 mm respectively, statistically significant not difference, when compared with the control pathogen inoculation (*C.coffeicola*) and sprayed with formulation without antagonistic bacteria the diameters of the wounds are 3.36 and 4.12 mm respectively had statistically significant difference (Table 2). Lee *et al.* (2006) evaluation of formulation of *Bacillus licheniformis* N1 for control gray mold on tomato plants caused by *Botrytis cinerea*. The result of pot experiments led to selection of the wettable powder formulation N1E (based on corn starch and olive oil and sucrose). The treatment resulted in the significant reduction of symptom development when N1E was applied before *B. cinerea* infection, but not after the infection. As well as Kuenpech and Akarapisan (2014) using liquid formulation of *Bacillus subtillis* B6 (including xanthan gum, phosphate buffer and glycerine) for reduced antracnose disease of Lady's Slipper leaves caused *Colletotrichum gloeosporioides*. The treatment of the Lady's Slipper leaves had significance, which clearly reduced on treatment with the B6 liquid formulation one day before the pathogen in comparison with the pathogen inoculated on its own and the pathogen inoculated one day before treatment with the B6 liquid formulation, which were about 3.00, 13.58, and 7.75 mm respectively. Moreover, reported the development of bioproduct from *Pichia* sp. Y2 (liquid

formulation; xanthan gum, phosphate buffer and glycerine) can reduced antracnose of Lady's Slipper as well (Kuenpech and Akarapisan, 2014).

Table 2. Efficiency of A74 and A75 bio-product in reducing leaf spot disease on Arabica coffee in a greenhouse for 30 days.

Treatment[1]	**Diameter of wound (mm)**
1. Control	0.00 c[2]
2. Pathogen inoculation (*Cercospora coffeicola*)	3.36 a
3. Spray with formulation without antagonistic bacteria and pathogen inoculation	4.12 a
4. Spray A74 formulation 24 hour before pathogen inoculation	2.08 b
5. Spray A74 formulation 24 hour after pathogen inoculation	2.27 b
6. Spray A75 formulation 24 hour before pathogen inoculation	1.70 b
7. Spray A75 formulation 24 hour after pathogen inoculation	1.52 b
LSD (P=0.05)	0.97
CV (%)	35.04

[1]The average was calculated using six replication data.
[2]The values within a column with different superscripts are significant (p=0.05)

Conclusion

The isolates of antagonistic bacteria A74 and A75 showed the highest percentage of growth inhibits against *C. coffeicola*. The characterized of antagonistic bacteria isolates A74 and A75 belonging to *Bacillus* group, isolate A74 is *Bacillus megaterium* and isolate A75 is *Bacillus badius*. Moreover, the isolates A74 and A75 were developed powder formulations. The bio-product showed a few decreasing of antagonistic bacteria when, compared with the first after produced and have ability to survive for more than 3 months under storage at room temperature. Finally, the efficacy for controlling leaf spot disease of bio-product in the green house. The result showed that the disease severity was reduced when, spraying with A74 or A75 for 24 before or after the pathogen inoculation on coffee leaf have statistically significant not difference.

Acknowledgements

We thank the Graduate School, Chiang Mai University, Chiang Mai, Thailand and Project of Potential and Capability in Marketing Competition of Thai Arabica Coffee in Upper Northern Thailand under Free Trade Area Agreement for their support.

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(Received: 24 April 2015, accepted: 28 June 2015)