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Bacillus subtilis LBF02 as Biocontrol Agent Against Leaf Spot Diseases Caused by *Cercospora lactucae-sativae* in Lettuce

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Abstract

More than 51 isolates of bacteria were obtained from leaves, crushed leaves and rhizosphere of lettuce plants. The bacteria isolates were purified and assayed against *Cercospora lactucae-sativae* on PDA plate by dual culture technique. Four isolates showed zone of inhibition against the pathogens. The antagonistic bacteria isolate LBF02 showed the highest percentage of growth inhibits against *C. lactucae-sativae* leaf spots with 80.82% inhibition, compared with the control. Based on morphological and biochemical tests, isolate LBF02 was identified as belonging to the *Bacillus subtilis* group. The LBF02 isolate was chosen for the formulation development. The formulation contained 40 ml of cell suspension, 89 g of rice flour, 1 ml of vegetable oil and 10 g of sucrose. The biocontrol of leaf spot diseases was tested by using a formulation applied in greenhouse experiments. The result showed that spraying 1 hour before or after the pathogen inoculation on lettuce plants was more effective in suppressing leaf spot disease than simply pathogen inoculation alone. Moreover, the antagonistic bacteria in formulation have the ability to survive for more than 6 months under storage at room temperature and survive for up to 15 days on lettuce leaves.

Keywords: biocontrol, *Cercospora lactucae-sativae*, *Bacillus subtilis*, lettuce

1. Introduction

Lettuce (*Lactuca sativa* L.) is the most popular leafy salad vegetable in Thailand. But, the vegetable is susceptible to leaf spot disease caused by *Cercospora lactucae-sativae*; this is disease that has important economic implications around the world and normally, this disease has been a major damage-causing one in Thailand (To-Anun et al., 2011). The effects of the pathogen are reduced yield and low qualities produce of the lettuce in greenhouses, hydroponics and fields (Hsieh & Goh, 1990). The fungal *Cercospora* sp. give rise to the leaf spot disease on numerous host plants in tropical regions and an increase in the disease usually occurs in the rainy season (Agrios, 2004). Crowded planting, high humidity and bad ventilation are conducive to the disease outbreak (Chupp, 1954; Koohakan et al., 2008). In the year 2004, due to an attack of just the *Cercospora longissima* comm losses of up to 68% were reported in the specific conditions (Gomes et al., 2004). All these antagonistic bacteria that have been isolated from the soil surrounding the plants and from the plant surfaces (Weller, 1988; Kim et al., 1997; Sindhu et al., 2002; Todorova & Kozhuharova, 2010) are active under the general mechanism of competitive exclusion or reduction of growth by other microorganisms, which is the interference process of the pathogens. Moreover, the antagonistic bacteria manufacture a diverse range of secondary metabolites due to enzymatic activity and therapeutics due to various mechanisms of secretion and are capable of catalyzing various biochemical reactions with novel enzymes of lytic enzymes, siderophores and antibiotics (Das et al., 2006). One of the bacteria biocontrol agents that have received much attention is the genus *Bacillus*. The *Bacillus* sp., because they produce active antagonistic metabolites, is abundant in soils and readily form endospores that survive under adverse environmental conditions (Silo-Suh et al., 1994). *Bacillus subtilis* showed inhibition against *Cercospora beticola* (Lindow & Brandl, 2003), *Cercospora beticola, Colletotrichum gloeosporioides* (Collins et al., 2003), *Pseudocercospora purpurea* (Eeden & Korsten, 2006) and *Rhizoctonia solani* Kühn (Kai et al., 2007). The antagonistic bacteria were chosen for formulation development such as for the formulation development of powder formulation and granule formulations of bacteria and used for controlling fungi growth (Chumthong et al., 2008; Kim et al., 2007; Lee et al., 2006). The formulations were applied by spraying on the plant leaves in the greenhouse experiments and fields.

2. Materials and Methods

2.1 Microorganisms and Screening of Antagonistic Bacteria

The fungus *C. lactucae-sativae* was isolated from lettuce by single spore isolation method (Choi et al., 1999) and maintained on V-8 juice agar (V8) medium. The antagonistic bacteria were isolated from lettuce leaf samples that were healthy. There were isolated by leaf wash technique using 10 g of leaf with 100 ml sterile distilled water. The bacteria isolated from crushed leaves, 3 g of plant leaf mixture was treated with 30 ml sterile distilled water. For the bacteria isolation from rhizosphere by soil dilution plate method, 1 g of soil rhizosphere was suspended in 10 ml of sterile distilled water and shaken at 180 rpm for 2 hours. Subsequently, they were serial diluted to 10^{-3} fractions and spread on plate under nutritive agar (NA) incubation at room temperature for 48 hours. The single colony of bacteria was selected and re-isolation was performed on NA until pure culture was obtained.

2.2 Dual Culture Inhibition Assays

Tests were performed on antagonistic bacteria isolates for inhibition effect against *C. lactucae-sativae* in potato dextrose agar (PDA). The antagonistic bacteria were touch single colony with paper dish at the defined medium. *C. lactucae-sativae* NH04 isolates were then inoculated on either side of the bacterial growth in four replicate plates incubated at room temperature for 30 days, assessed by measuring the size of the inhibition zone.

2.3 Inhibition Against Germination of Spores of Fungus

The antagonistic bacteria were cultured in 50 ml NGA broth and shaken at 160 rpm for 2, 4 and 6 days. The culture was separated by centrifugation at 5,000 rpm for 5 min at 4^{o}C. The cultures were then filtered using Whatman No. 1 filter paper. The supernatants of filtrated culture medium (FM), non-filtrated culture medium (NFM), NA and sterile distilled water were mixed with the spores of the fungus in the ratio 1:1 and spread on plate on water agar (WA). The spore was examined for germination at 3, 6, 9, 12 and 24 hrs, and the percentage of germination of the spores was counted.

2.4 Identification-Biochemical Studies

The test of the ability of the study bacteria LBF02 to isolate for physiological and biochemical characterization was carried out as described in *Bergey's Manual of Systematic Bacteriology* (Holt et al., 1984; Todorova & Kozhuharova, 2010; Zheng et al., 2007). The following sources of carbon were used: glucose, lactose, maltose, fructose, sucrose, mannitol, sodium citrate and urease. The remaining physical-biochemical conditions were as follows: growth at different temperatures, growth at different concentrations of NaCl, reduction of nitrates, Voges-Proskauer test, methyl red test, formation of indole, disintegration of casein, disintegration of gelatin, hydrolysis of starch and catalatic activity.

2.5 Bio-Product of Formulation

To generate formulations of the bacteria LBF02 isolate, 3 ml culture of bacterial cells were inoculated into 150 ml of nutrient glucose broth (NGB) and shaken at 150 rpm at 28°C for 5 days. The cells were harvested by centrifugation under 4°C at 5,000 rpm for 10 min and washed with 0.85% (w/v) NaCl and then centrifuged at 3,500 rpm for 5 min. The formulation contained 40 ml of cell suspension LBF02, 89 g of rice flour, 1 ml of vegetable oil and 10 g of sucrose, with the mixture completely dried at 45°C in a drying oven for 12 hours and subsequently ground in a blender to form a powder. The formulation without the bacteria was prepared in an identical way and referred to as "control" and maintained at room temperature. The viability tests consisted of spray formulation on plant leaves for 15 days, carried out after formulation and at 2-month intervals during storage at room temperature (28 ± 2^{o}C). Two plant leaves were suspended in 10 ml of sterile distilled water and shaken at 160 rpm for 30 minutes, after which the cfu was counted. The value (cfu/ml) of viable bacteria was taken as the average of three replications (three drops) per dilution.

2.6 Greenhouse Testing of Formulation for Control of C. lactucae-sativae

The formulation was performed under greenhouse condition. The lettuce plants were grown in pots to become 30-days-old. The *C. lactucae-sativae* inoculum was found to grow on the V8 medium for 7 days at room temperature. The spore suspension obtained was adjusted to 10^4 conidia using a haemacytometer. The formulations were prepared as 1 g mixed with 100 ml sterile distilled water before being sprayed on the lettuce plants. The products were applied by spraying 1 hour before or after the fungal pathogen treatment and sprayed 3 days before or after the fungal pathogen treatment on the lettuce plants. The plants were maintained under controlled growth for 5 days. The treatment had four replications, with the plants in each pot arranged in a Completely Randomized Design (CRD). The disease severity index of the leaf spot symptoms was recorded and graded on a 0 - 10 scale of Poonponkun et al. (2007), with the modification rating scale as follows: 0: plants did not

show any symptoms, 1: 1-10%, 2: 11-20%, 3: 21-30%, 4: 31-40%, 5: 41-50%, 6: 51-60%, 7: 61-70%, 8: 71-80%, 9: 81-90% plants showed evidence of spot symptoms and 10: 91-100% plants exhibited spot symptoms.

3. Results and Discussion

3.1 Microorganisms and Screening of Bacillus sp. Strains With an Antifungal

The fungus *C. lactucae-sativae* nine isolates were found on the leaf spot diseased lettuce. A total of 51 isolates were bacteria isolated from leaves, crushed leaves and rhizosphere of healthy lettuce. Four isolates of the antagonistic bacteria showed the highest percentage of inhibition of the pathogens. All of them were obtained from the crushed leaves as LBF02 and LBF03 isolates and from the rhizosphere as SRR02 and SRF08 isolates.

3.2 Dual Culture Inhibition Assays

The results of screening for antagonistic bacteria against *C. lactucae-sativae* produced 51 antagonistic bacteria isolates on PDA using the dual culture technique. Inhibition assays were conducted on the four isolates by forming zones of inhibition. These antagonistic bacteria isolates which were LBF02, LBF03, SRR02 and SRF08, showed the highest percentage of growth inhibition of *C. lactucae-sativae* with 80.82%, 79.30%, 75.12% and 73.67%, respectively. The LBF02 isolated exhibited the most pronounced antagonism against *C. lactucae-sativae* when compared with the control. Todorova & Kozhuharova (2010) report that previous studies of the antagonistic *Bacillus subtilis* strains TS 01 and ZR 02, which were isolated from soil, reveal that they showed the highest antifungal activity against *Alternaria solani*, *Botrytis cinerea*, *Monilia linhartiana*, *Phytophthora cryptogea* and *Rhizoctonia* sp. Favorable results were also found by Korsten & Jager (1995) who demonstrated the efficiency of *Bacillus subtilis* (isolate B246), *Bacillus cereus* (isolates B247 and B249) and *Bacillus licheniformis* (isolate B248) in hibiting effectively avocado post-harvest pathogens *Colletotrichum gloeosporioides, Phomopsis perseae, Drechslera setariae, Pestalotiopsis versicolor* and *Fusarium solani.*

3.3 Inhibition Against Germination of Spores of Fungus

The 100 conidia of *C. lactucae-sativae* were made to undergo germination in 3 hours in the sterilized distilled and NB culture methods. The results of the treatment of the spores which were treated using non-filtrated culture medium (NFM) and filtrated culture medium (FM) methods at 2, 4 and 6 days revealed that the NFM of 2, 4 and 6 days old culture demonstrated inhibition of spore germination of *C. lactucae-sativae* with 20, 23 and 28 spores, respectively. At 6 hours of cell treatment, the germ tubes were swelling and at 9 hours the spores of *C. lactucae-sativae* stopped germinating. The treatments of FM at 2, 4 and 6 days old culture demonstrated inhibition of spore germination, with 7, 8 and 10 spores, respectively (Figure 1). The spores of *C. lactucae-sativae* mixed FM experienced a reduction in the spore germination after 9 hours compared with the sterilized distilled water and the NB culture methods. Alderman & Beute (1986) reported about the conidia of *Cercospora arachidicola* atomized onto peanut leaves which began germinating after 2 hours; in that experiment, the maximum germination that is 82-85% occurred within 24-48 hours. Additionally, Folman (2004) reported using *Lysobacter enzymogenes* strain 3.1T8 as a potential biocontrol agent of *Pythium aphanidermatum* in cucumber. The antagonistic bacteria showed the activity, hemolytic activity and the production of a surface active compound, which decreased in the media of increasing strength, one or more low molecular compounds, caused rapid immobilization of zoospores of *P. aphanidermatum* and inhibited cyst germination. Moreover, Bacon, and Hinton (2006) reported the antagonistic bacteria as having the ability produce diverse range of enzymatic activities which can be sources of secondary metabolites and therapeutics by various secretion mechanisms and capable of catalyzing various biochemical reactions with novel enzymes of lytic enzymes, siderophores and antibiotics.

3.4 Identification-Biochemical Studies

Based on the biochemical and morphological tests according to *Bergey's Manual of Systematic Bacteriology* (Holt et al., 1984; Todorova & Kozhuharova, 2010; Zheng et al., 2007), the detailed physiological characteristics of LBF02 were investigated and compared with *Bacillus subtilis*, as shown in Table 1. The strain LBF02 consisted of spore-forming, Gram-positive rod shaped, bacteria colonies which were convex colonies with wrinkled surface, circular, white-cream, entire and opaque on nutrient agar. Catalatic reaction was positive, as well as liquefaction of gelatin, starch hydrolysis, nitrate reduction, Voges-Proskauer test, urease, methyl red test, hydrolysis of casein, as well as characters like oxidase, anaerobic growth, acid and no gas formation from different sugars. However, oxidase reaction was negative; so were the results of the tests for indole and H_2S production. These data indicated that strain LBF02 resembled a member of the Bacillus genus. Fritze (2004) reported about the conspicuous morphological feature of endospore formation that has lent itself from early on as an easily recognized property for taxonomic differentiation. The genus *Bacillus* groups were surely the largest and the most prominent. Based on

morphology, physiological tests (Todorova & Kozhuharova, 2010; Mishra et al., 2009), biolog and the 16S rDNA sequence, bacteria strain ZJB-063 was identified as *Bacillus subtilis* (Zheng et al., 2007).

3.5 Bio-Product of Formulation

The antagonistic bacteria of formulation survival tests were counted using the drop plate method on day 0, 1, 3, 5, 7, 10 and day 15 after spraying the formulation on the lettuce leaves. The results was that the numbers of bacteria on the lettuce leaves were seen to decline over time, with the bacteria quantities at 2.67×10^4, 1.33×10^4, 1.0×10^4, 1.0×10^4, 8.35×10^3, 3.35×10^3 and 2.83×10^3 cfu/ml respectively. The antagonistic bacteria in formulation was observed to survive more than 6 months under storage at room temperature and in the sprayed formulation on the leaves the antagonistic bacteria were seen to survives for up to 15 days on the lettuce leaves. Chumthong et al. (2008) reports that the result of the granule formulation of *B. megaterium* because of spraying with the formulation on leaf sheaths and leaf blades at the 7 days was that the number of bacteria on the surfaces of both the rice tissues was observed to be approximately 10^6 cfu/g of the plant.

3.6 Greenhouse Testing of Formulation for Control of C. lactucae-sativae

The tests conducted in the greenhouse experiments analyzed the effectiveness of formulation from isolate LBF02 on lettuce diseases, *C. lactucae-sativae* on lettuce that had grown up to 30-days. Upon evaluation of the damage according to the disease severity, when the data were analyzed statistically and compared, the mean of each treatment by CRD (completely randomized design) the confidence level was found to be 95 percent. The evaluations effective of nine treatments were tested the formulation on lettuce leaves. The formulation LBF02 was used by spraying it 1 hour before or after and by spraying 3 days before, the pathogen infection was found to have significantly reduced the infection indexes to 1.62, 1.87 and 2.12 respectively, whereas the control was found to be infested with 4.62. The inhibition is significantly at the 95 percent confidence level when compared with the control (Table 2 and Figure 2). Dhitikiattipong et al. (2011) reports that the result efficiency of powder formulation of antagonistic bacteria to control rice bakanae disease bacterized with BAK-131 and BAK-088 demonstrated bakanae incidences of 8.9% and 9.7%, respectively, which is comparable to the 8.2% of the mancozeb+carbendazim treatment and the 10.9% of the control treatment. In addition, Arunyanart et al. (2008) applied the powder formulation of the antagonistic bacteria *Bacillus subtilis* No. 33 and tested their effectiveness against the fungi *Curvularia lunata* (Wakk.) Boed, *Cercospora oryzae* Miyake, *Helminthosporium oryzae* Breda de Haan, *Fusarium semitectium* Bark & Rav, *Sarocladium oryzae* Sawada and *Trichoconis padwickii* Ganguly. Moreover, *Pseudomonas fluorescens* strain Pf1 was developed as powder formulation and applied as a seed treatment and foliar spray for the bacteria on the leaves. This has effectively controlled the *Pyricularia oryzae* disease and increased the grain yield (Vidhyasekaran et al., 1997).

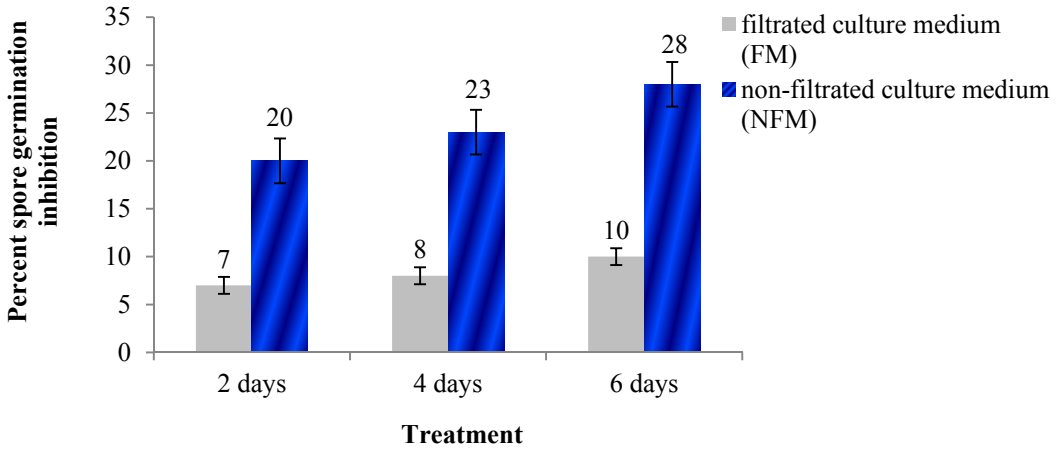

Figure1. The effect of 2 days, 4 days and 6 days old filtrated culture medium (FM), non-filtrated culture medium (NFM) treatment on the inhibition spores germination of *C. lactucae-sativae* on WA plates. The 100 percent of conidia *C. lactucae-sativae* germination took place under a condition of sterilized distilled water and NB culture treatment

Error bars represent the standard deviation of four replications.

Table1. Comparison of phenotypic characteristics for strain *B. subtilis* and LBF02 using conventional chemical tests

Characteristics	**Result**		**Characteristics**	**Result**	
	B. subtilis	**LBF02**		***B. subtilis***	**LBF02**
Gram staining	+	+	Methyl Red test	+	+
Cell shape	Rod	Rod	Indole production	-	-
Spores	+	+	Urease	+	+
Spore shape	Ellipsoid	Ellipsoid	Formation of H_2S	-	-
Spore position	Central	Central	Hydrolysis of casein	+	+
Mobility test	+	+	Utilization of citrate	+	+
Catalase	+	+	D-Glucose	+	+
Oxidase	+	+	Lactose	+	+
Liquefaction of gelatin	+	+	Maltose	+	+
Starch hydrolysis	+	+	Fructose	+	+
Nitrate reduction	+	+	Sucrose	+	+
VogesProskauer test	+	+	D-mannitol	+	+

"+" means positive and "-" means negative.

Table 2. Control of leaf spot disease on lettuce at five days after spray formulation treatments and pathogen inoculation in plants grown in pots under greenhouse conditions

Treatments*	**Severity of leaf spot****
1. Sprayed with sterile distilled water	0.00 d***
2. Sprayed with formulation without antagonistic bacteria	0.00 d
3. Sprayed with formulation of antagonistic bacteria	0.00 d
4. Sprayed with pathogen inoculation (*C. lactucae-sativae)*	4.62 ab
5. Sprayed with formulation without antagonistic bacteria and spray pathogen inoculation	4.12 b
6. Sprayed with formulation of antagonistic 1 hour before pathogen inoculation	1.62 c
7. Sprayed with formulation of antagonistic 1 hour after pathogen inoculation	1.87 c
8. Sprayed with formulation of antagonistic 3 days before pathogen inoculation	2.12 c
9. Sprayed with formulation of antagonistic 3 days after pathogen inoculation	5.37 a
CV (%)	32.20

* In treatments 1, 2 and 3, the lettuce plants were not inoculated with *C. lactucae-sativae*, while in treatments 4, 5, 6, 7, 8 and 9 the lettuce plants were inoculated with *C. lactucae-sativae*

** Severity index of leaf spot disease on the lettuce plants was defined as the percentage of diseased leaf area, where 0: plants did not show any symptoms, 1: 1-10%, 2: 11-20%, 3: 21-30%, 4: 31-40%, 5: 41-50%, 6: 51-60%, 7: 61-70%, 8: 71-80%, 9: 81-90% and 10: 91-100%.

*** Values within a column with different superscript are significant ($P < 0.05$).

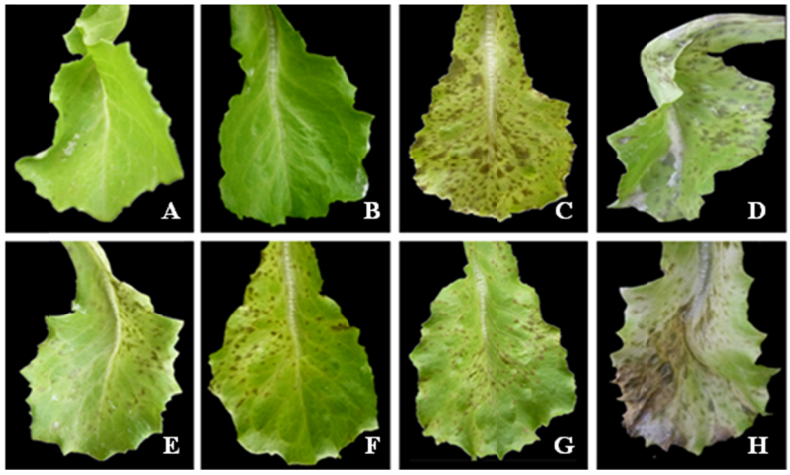

Figure 2. The comparison of efficiency upon the spraying of formulations after five days post inoculation for control of *C. lactucae-sativae* under greenhouse conditions. The following are the explanations: sprayed with formulation without the antagonistic bacteria (A), sprayed with the formulation of antagonistic bacteria (B), sprayed with the pathogen inoculation (*C. lactucae-sativae*) (C), sprayed with the formulation without antagonistic bacteria and spray pathogen inoculation (D), sprayed formulations were applied 1 hour prior to inoculation with *C. lactucae-sativae* (E), sprayed formulations were applied 1 hour after inoculation with *C. lactucae-sativae* (F), sprayed formulations were applied 3 days prior to inoculation with *C. lactucae-sativae* (G), sprayed formulations were applied 3 days after inoculation with *C. lactucae-sativae* (H)

4. Conclusion

The antagonistic bacteria LBF02 isolated showed the highest percentage of inhibition against *C. lactucae-sativae*. Non-filtrated culture medium (NFM) and filtrated culture medium (FM) treatment of LBF02 showed inhibition of the spore germination compared with the control. These data clearly indicate that the isolate LBF02 can be identified resembling a member of the *Bacillus subtilis* group. As for the greenhouse experiment, the results showed that using the formulation spray one hour before or after the pathogen inoculation on the lettuce plants was more effective in suppressing and managing leaf spot disease.

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