

**Biological Control of Anthracnose Disease in Lady's Slipper Using *Bacillus subtilis* Isolate B6**

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Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, ThailandAccepted 24<sup>th</sup> January, 2014**ABSTRACT**

The objective of this study was to examine the antifungal activity demonstrated by the *Bacillus subtilis* isolate B6 against the plant fungal pathogen; *Colletotrichum gloeosporioides* were isolated from the anthracnose disease in the Lady's Slipper orchid. Filtrated culture medium and non-filtrated culture medium of the B6 inhibited spore germination, and the hyphal growth of *C. gloeosporioides*. The effect of the B6 non-filtrated culture medium treatment was significantly higher than that of the filtrated culture medium treatment also; it was found that treatment with the 4-day culture medium was significantly more effective than treatment with the 2-day and 6-day culture medium. Additionally, the morphology of the germ tube had changed and had turned into abnormal shapes. The B6 was developed to two bioproduct formulations namely powder formulation and liquid formulation. The antagonistic bacteria in formulations can survive for more than six months under storage at room temperature. The treatment of the Lady's Slipper leaves by co-inoculation with the formulation 24 hours before the application of the pathogen would clearly serve to reduce anthracnose disease severity as compared to just pathogen inoculation. Therefore, evidently the *B. subtilis* isolate B6 can be developed for the biocontrol of the anthracnose disease in Lady's Slipper.

**KEYWORDS:** anthracnose, *Bacillus subtilis*, biological control, formulation, Lady's Slipper

**INTRODUCTION**

*Paphiopedilum concolor* (Bateman) Pfitzer. (Lady's Slipper) belongs to the family Orchidaceae. (Nattha, 2005). The Lady's Slipper is protected under conservation of Plant Varieties Act B.E. 2518 and forest prohibition of Forestry Act B.E. 2484. Moreover, the export of the Lady's Slipper is forbidden as per CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) pact. *Paphiopedilum* is susceptible to anthracnose disease caused by *Colletotrichum gloeosporioides* (Penz.) Sacc. and in Brazil the anthracnose in *Paphiopedilum insigne* is caused by *Colletotrichum gloeosporioides* (Raginaldo *et al.*, 2005). The damage caused is severe and stunning with substantial decrease production and economic value.

Biological control agents are living organisms including, bacteria. The antagonistic bacteria *Burkholderia capacia* and *Pseudomonas aeruginosa* were screened from the surface of fruits. These bacteria demonstrated the ability to control *Colletotrichum gloeosporioides* in papaya by the bacterial volatile compound producing (Rahman *et al.*, 2007). The crude extracts of the antifungal compound were produced from *Bacillus subtilis* SSE4 for use against *Colletotrichum gloeosporioides*. These were observed to have the highest efficacy in the prevention of anthracnose in orchid leaves (Benjaporn *et al.*, 2012).

Our objective was to isolate the anthracnose pathogen in *Paphiopedilum concolor*. There after the efficacy of the antagonistic bacteria against *Colletotrichum* sp. which causes the anthracnose disease was evaluated which was finally used to determine the best microbial against the control of anthracnose in *Paphiopedilum concolor*.

**MATERIALS AND METHODS****Isolation of plant pathogenic fungi**

The *Colletotrichum* sp. were isolated using the single spore isolation method (Yuen-Wah *et al.*, 1999) and incubated at room temperature (28–30°C), and then observed periodically. The anthracnose pathogens were cultured on V8 agar for inoculation. All isolates were made to undergo the pathogenicity test on the Lady's Slipper leaves by following Koch's postulate. The inoculated leaves were kept in humid conditions at room temperature (28–32°C). The data were collected as lesion diameter (cm) on leaves, and analyses of variance using completely randomized design (CRD) with four replications.

**Evaluation of antagonistic activity**

The *Bacillus subtilis* isolate B6 were grown in nutrient broth supplemented with glucose 2% (NGB); 150 ml of NB in 250 ml Erlenmeyer flasks were inoculated and then incubated on a shaker (150 rpm) at 28°C. After 2, 4, and 6 days of incubation, filtrated culture medium of bacteria were obtained by passing the supernatant through a sterile syringe filter (pore size 0.45 µm). The bacterial evaluation was conducted in two ways:

In the first test, the B6 filtrated culture medium (FM) and the B6 non-filtrated culture medium (NFM) was analyzed for their effects on the spore germination of *C. gloeosporioides* using the slide culture technique.

The spore suspension of *C. gloeosporioides* was prepared by keep it for 10 days on V8 agar culture. The concentration of the spores was determined using a hemacytometer and adjusted with sterile distilled water containing 100–150 spores/ml, which was pipetted into each slide culture. The FM and NFM were mixed with spore suspension of *C. gloeosporioides* in a 1:1 ratio and then 40 µl of the same were made to drop on water agar (WA) slides. The slides were incubated in a humid chamber at room temperature for 12 h. The data on the spore germination and the germ tube shape were recorded for 3, 6, and 12 h. under a compound microscope.

In the second, tested the NFM and the FM were tested for mycelium growth inhibition of *C. gloeosporioides* using the well test method on Potato Dextrose agar (PDA). The pathogen was cultured for 5 days on PDA. Hyphal plugs of *C. gloeosporioides* were placed on one side of the PDA plate (Petridishes 9 cm in diameter) and 60 µl of the NFM and the FM were dropped into the well on the other side of plate, and then incubated at room temperature. The data were collected by measuring the colony diameter for 10 days of growth of *C. gloeosporioides* as percent growth inhibition. The percentage of the radial growth inhibition (PIRG) of the pathogen was computed by way of analyses of variance using completely randomized design (CRD) with four replications.

**Formulation**

The antagonistic bacterial isolate B6 was grown in NGB; 150 ml of the isolation in 250 ml Erlenmeyer flasks were inoculated and then incubated on a shaker (150 rpm) at 28°C during 96 h. Fresh cells from the NGB were harvested by centrifugation at 5000 rpm. When the growth was considered enough for the preparation of the different cell suspensions, the cells were recovered in phosphate buffer solution (10<sup>10</sup> CFU/ml). To prepare the bacterial suspensions it was necessary to dissolve the glycerol in two thirds of the final volume of the phosphate buffer solution. The bioproduct were found to have two formulations, including powder formulation and liquid formulation of B6. The powder formulation including talcum powder, wheat bran, sodium alginate, glycerine, sucrose, and yeast extract was obtained (modified from Pervin and Mehmet, 2008, and Mokhtarnejad *et al.*, 2009). The liquid formulation including xanthan gum, phosphate buffer and glycerine was then added (when it was the case) and mixed together for 12 h with a magnetic stirrer and sterilized at 121°C for 15 min in an autoclave and then allowed to cool to room temperature. Thereafter, the bacterial suspensions were added to the sterilized solution (Patin<sup>o</sup>-Vera *et al.*, 2005).

**Application of formulation on *Paphiopedilum concolor***

In vivo assay for the prevention of the anthracnose caused by *C. gloeosporioides* was performed using the leaves of *Paphiopedilum concolor*. The experiment used a completely randomized design which was divided into nine treatments (T1–T9). The formulations of the antagonistic bacteria were applied 10 µl of 1 mg/ml ratio for the powder formulation and applied 10 µl of 1 ml/ml ratio for the liquid formulation. Spore suspensions of *C. gloeosporioides* were concentrated to 10<sup>4</sup> spores/ml and 10 µl of the same was applied. The sizes of the visible lesions on the orchid leaves were measured for 7 days (Prapagdee *et al.*, 2008).

**RESULTS AND DISCUSSION****Isolation of plant pathogenic fungi**

The result showed that ten of the *Colletotrichum* sp. were isolated from ORCHIDACEAE including three isolates from Lady's Slipper leaves, one isolates from Lady's Slipper flower, two isolates from Dai orchid (*Dendrobium chrysotoxum* Lindl.), Friederick's Dendrobium (*Dendrobium friedericksianum* Rchb.f), Dendrobium orchid hybrids, *Cymbidium orchid hybrids* and Cattleya orchid (*Cattleya labiata* Lindl.). In the same manner as given in the report of Raginaldo *et al.* (2005), the fungi were identified following sporulation and pure cultures were stored at 4°C on PDA slants. The *Colletotrichum gloeosporioides* isolate PAL2 was found to be the highest in disease severity on the Lady's Slipper leaves (Table 1).

**Table 1** Isolation of *Colletotrichum* sp. from Orchid's Anthracnose Using Single Spore Isolation Method and Pathogenicity Test on Lady's Slipper Leaves on at Seventh Day.

Location	Orchids host	Isolate	Diameter of wound* (mm)
Fang district, Chiang Mai	Dai orchid ( <i>Dendrobium chrysotoxum</i> Lindl.)	DEC1	5.62 <sup>d**</sup>
		DEC2	5.00 <sup>de</sup>
Division of Horticulture, Faculty of Agriculture, Chiang Mai University	Lady's Slipper leaves ( <i>Paphiopedilum concolor</i> [Bateman] Pfitzer.)	PAL1	8.62 <sup>ab</sup>
		PAL2	9.25 <sup>a</sup>
		PAL3	8.12 <sup>bc</sup>
	Lady's Slipper flower	PAF	7.62 <sup>c</sup>
	Friederick's Dendrobium ( <i>Dendrobium friedericksianum</i> Rchb.f)	DEF	5.62 <sup>d</sup>
	Dendrobium orchid hybrids	DEH	4.75 <sup>e</sup>
	<i>Cymbidium</i> orchid hybrids	CYH	5.50 <sup>de</sup>
Cattleya orchid ( <i>Cattleya labiata</i> Lindl.)	CAL	4.88 <sup>de</sup>	
LSD (p=0.05)			0.77
CV (%)			8.25

**Note:**\* The average was calculated using six replication data.

\*\*The values within a column with different superscripts are significant (p=0.05)

**Evaluation of antagonistic activity**

The result of the B6 filtrated culture medium (FM) and the B6 non-filtrated culture medium (NFM) showed 100% spore germination inhibition at the 3rd hour. In 6th hours, all of the spores could have germinated; however, the inhibitory effect of the B6 culture medium had effectively slowed down the germination time. Additionally, as regards the morphology of the germ tube, there shapes had become abnormal in comparison with the spore germination tube, which was treatment with sterile NGB (control).

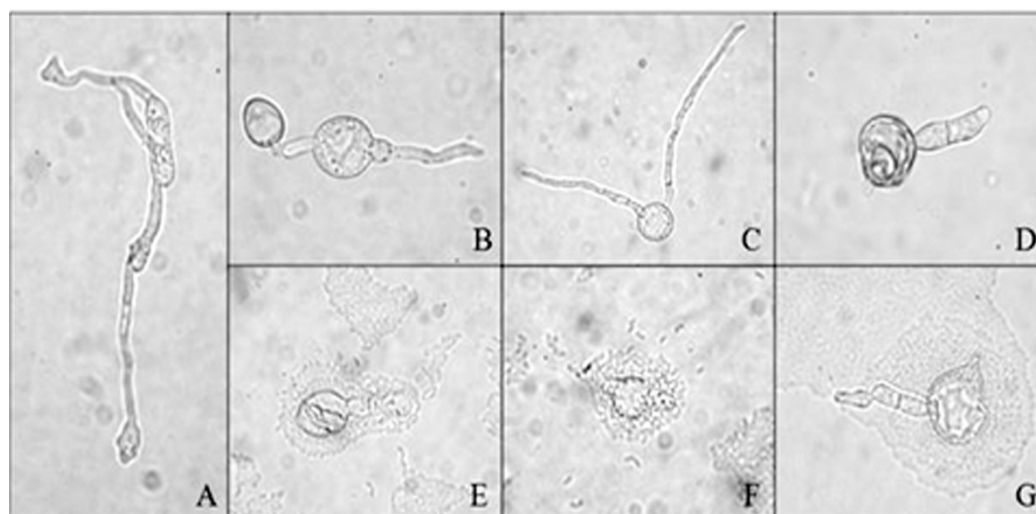
The spores on the control treatment were observed to have germinated before 3 hours, and the normal shape of the germ tube, which was found to have become hypha elongated and a colony was observe to have grown.

The spore on treatment with the B6 culture medium showed abnormality in the shape of the germ tube in the form of swelling and shorter tube within 12 hours when compared with the control (Figure 1). However, the antagonistic activity of the NFM was observed to have better effects against spore

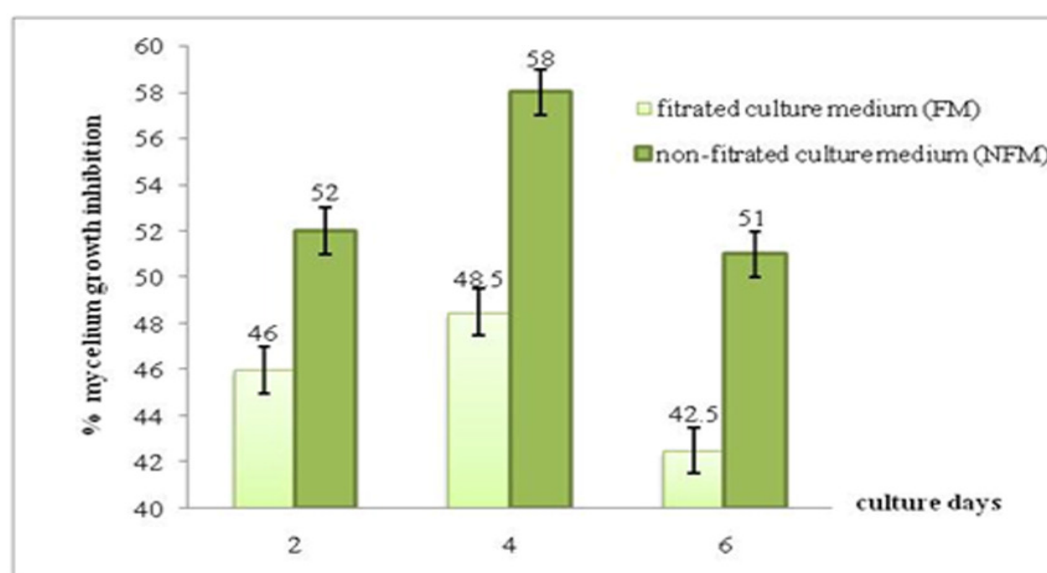
germination than the FM. Also, highly effective activity against *C. gloeosporioides* spores was demonstrated by the 6-day NFM treatment (Figure 1-D), same as by the 6-day FM treatment (Figure 1-G). The 4-day NFM showed 58% of mycelium growth inhibition of *C. gloeosporioides* this effect was the highest percentage of significance (p=0.05) compared with the control (Figure 2).

The effect of the NFM demonstrated that it had a higher percentage of growth inhibition than the effect of the FM. Additionally, the 2-day FM showed 46% higher effect than 6 days of FM (42.5%) but lower than 4 days of FM (48.5%), which was significant (p≤0.05).

Furthermore, 2 days of NFM and 6 days of NFM showed 52% effect and 51% effect, respectively, which were not significant. The antagonistic activity of a crude extract of extracellular antifungal compounds produced from *Bacillus subtilis* SSE4 inhibited the mycelium growth, in additional to inhibiting the spores germination of *Colletotrichum gloeosporioides* and controlling the anthracnose disease in the *Dendrobium* orchid (Choochuen et. al, 2010).



**Figure 1.** The antagonistic activity of *Bacillus subtilis* isolate B6, whereby it inhibited the spore germination of *Colletotrichum gloeosporioides* on WA slide culture at 12 hours; spores germinated after the treatment with sterile NGB (A); spores germinated after the treatment with 2-days of the B6 filtrated culture medium (FM) (B); spores germinated after the treatment with 4-days of FM (C); spores germinated after the treatment with 6-days of FM (D); spores germinated after the treatment with 2-days of the B6 non-filtrated culture medium (NFM) (E); spores germinated after the treatment with 4-days of NFM (F); spores germinated after the treatment with 6-days of NFM (G) were observed under light microscopes (40×).



**Figure 2.** The effect of *Bacillus subtilis* isolate B6 inhibiting *Colletotrichum gloeosporioides* mycelium growth in the well test method at room temperature incubated for 10 days on PDA; the first bar show the percentage of inhibition effect by 2, 4, and 6 days of the B6 filtrated culture medium (FM) about 46%, 48.5% and 42.5%, respectively and the second bar show the percentage of inhibition effect by 2, 4, and 6 days of the B6 non-filtrated culture medium (NFM) about 52%, 58% and 51%, respectively.



## Formulation

The bioproduct were found to have two formulations, including powder formulation and liquid formulation of B6. The antagonistic bacteria in formulations are known to be able to survive for more than six months under storage at room temperature, but the initial viable count of  $3.3 \times 10^9$  cfu/ml was found to have decreased to a final viable count of about  $10^6$  cfu/g or ml.

Additionally, it was detected that they survived on the phyllosphere for more than 15 days after the application. In the same way, it was observed that the shelf life of the *Bacillus subtilis* isolate ENB-24 liquid formulations at the ambient temperature of 21–32 C was seven months. (Ramanujam *et.al*, 2012).

## Application of formulation on *paphiopedilum concolor* leaves

The application of the B6 liquid formulation 1 hour before the advent of the pathogen (T6) helped to reduce the severity of the anthracnose disease in comparison with the pathogen inoculated 1 hour before the treatment with B6 liquid formulation (T7), and the diameters of the wounds were measured to be about 4.08 mm and 9.17 mm, respectively (Table 2).

The result obtained upon the treatment of the Lady's Slipper leaves had significance ( $p=0.05$ ), which clearly reduced on treatment with the B6 liquid formulation one day before the pathogen (T8) in comparison with the pathogen inoculated on its own (T2) and the pathogen inoculated one day before treatment with the B6 liquid formulation (T9), which were about 3.00, 13.58, and 7.75 mm, respectively (Figure 3). In addition, the application of sterilized formulation only (T3) and B6 formulation only (T5) did not affect the Lady's Slipper leaves when compared with the negative control (T1), as there were found to be wound measuring 3.00 mm in diameter.

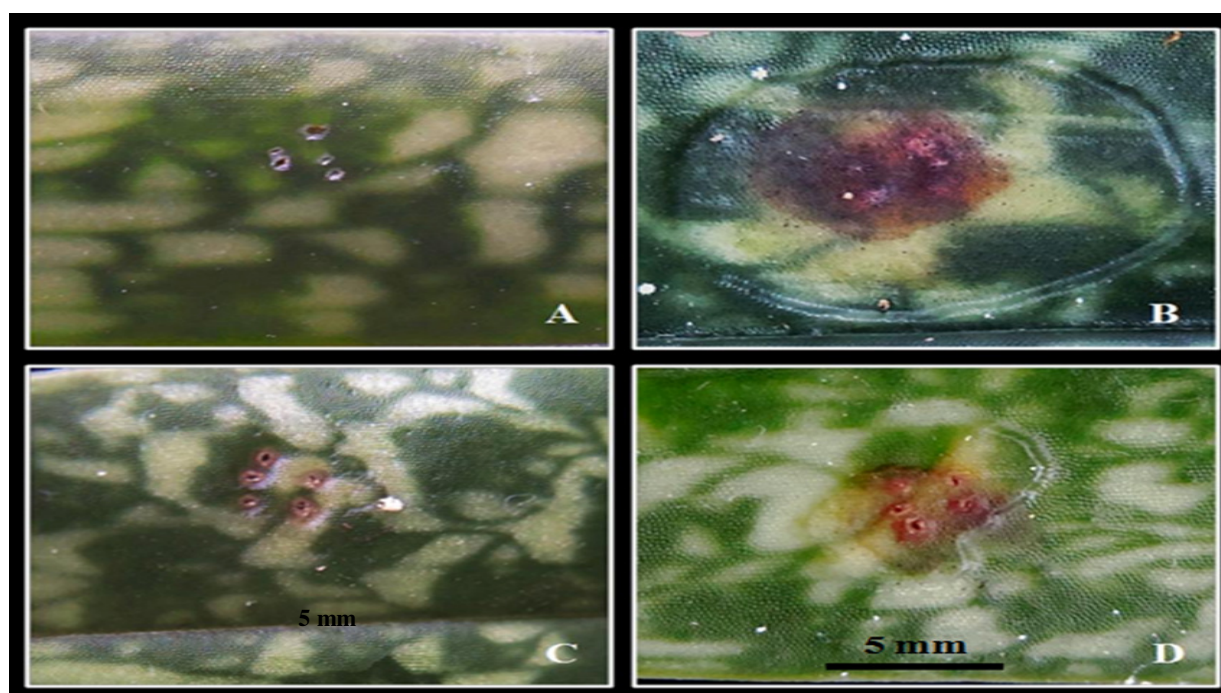
The effect of the liquid formulation in reducing the anthracnose disease in Lady's Slipper leaves was not found to be markedly different from that of the powder formulation. Ramanujam *et.al* (2012) reported about the liquid formulations of *Bacillus subtilis* isolate ENB-24, as, based on fruit bioassay studies they were identified as potential antagonists to the chilli anthracnose pathogen, *Colletotrichum capsici*. Moreover, the powder formulations of *Bacillus licheniformis* have been observed to reduce post-harvest mango fruit diseases (anthracnose and stem-end rot [SR]) when used for its application in the dip treatment of mangoes in a pack house (Veloshinie and Lise, 2006).

**Table 2.** Efficiency of *Bacillus subtilis* Isolate B6 Formulation in Reducing Anthracnose Disease on Lady's Slipper Leaves Room Temperature in Seven day.

Treatments	Diameter of wound (mm)*	
	Powder formulation	Liquid formulation
T1 Negative control (applied with sterile distilled water only)	3.00 g**	3.00 g
T2 Positive control (pathogen inoculation only)	13.58 a	13.58 a
T3 Applied with sterile formulation only	3.00 g	3.00 g
T4 Applied with sterile formulation and pathogen inoculation	8.25 cd	8.25 cd
T5 Applied with B6 formulation only	3.00 g	3.00 g
T6 Applied with B6 formulation 1 hour before pathogen inoculation	4.17 f	4.08 f
T7 pathogen inoculation 1 hour before application with B6 formulation	9.00 bc	9.17 b
T8 Applied with B6 formulation 1 day before pathogen inoculation	3.50 fg	3.17 g
T9 pathogen inoculation 1 day before application with B6 formulation	7.08 e	7.75 de
	LSD ( $p=0.05$ )	0.85
	CV (%)	12.28

Note:\* The average was calculated using six replication data.

\*\*The values within the table with different superscripts are significant ( $p=0.05$ ).



**Figure 3.** The efficiency of the *Bacillus subtilis* isolate B6 liquid formulation reduced the anthracnose disease on Lady's Slipper leaves at room temperature in 7 days; the negative control was applied with sterile distilled water only (A); the positive control was applied with pathogen inoculation only (B); applied with B6 formulation one day before pathogen inoculation (C); pathogen inoculation one day before application with B6 formulation (D).

## CONCLUSION

The fungal pathogen *Colletotrichum gloeosporioides* isolate PAL2 from the anthracnose disease in *Paphiopedilum concolor* showed high disease severity. Subsequently, the *Bacillus subtilis* isolates B6 filtrated culture medium (FM) and non-filtrated culture medium (NFM) inhibited the pathogen mycelium growth and the pathogen spore germination. Moreover, the *B. subtilis* isolate B6 was developed to two biproduct formulations, including powder formulation and liquid formulation. The antagonistic bacteria in both the formulations are able to survive for more than six months under storage at room temperature. Additionally, the B6 formulation demonstrated the ability to reduce the anthracnose disease on Lady's Slipper leaves upon application

with the B6 formulation one day before the pathogen inoculation. It was also observed that the effect of the liquid formulation in reducing the anthracnose disease in the Lady's Slipper leaves be not different when compared with the powder formulation.

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