Insecticidal Activity of *Bacillus thuringiensis* 656-3 Strain to Mushroom Flies in Oyster Mushroom House

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Bacillus thuringiensis **656-3 which was isolated from a soil sample of mushroom house and showed high toxicity to mushroom flies,** *Lycoriella mali* **and** *Coboldia fuscipes*, **was surveyed for insecticidal effect in the oyster mushroom house.** *B. thuringiensis* **656-3 was mass-cultured in the fermenter containing soybean cake (2%) and wheat bran (2%) as media source. Semi-formulation of** *B. thuringiensis* **656-3 was performed with metamorphic starch only. When the formulation suspension containing** 5×10^7 **cfu was sprayed on the mushroom in mushroom house, the insecticidal effect of** *B. thuringiensis* **656-3 to mushroom flies,** *L. mali* **and** *C. fuscipes*, **was maintained over 90% by the fifth day after starting spraying. The yield of oyster mushroom house with** *B. thuringiensis* **656-3 was significantly increased compared to control.** *B. thuringiensis* **656-3 represents a powerful biological insecticide for the control of mushroom flies.**

Key words: *Bacillus thuringiensis*, Biological control, *Coboldia fuscipes*, Insecticidal activity, *Lycoriella mali*, Oyster mushroom

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Introduction

Mushrooms have long been consumed as foods and herbs, and consumption has steadily increased. Among the marketed mushrooms in Korea, the oyster mushroom, *Pleurotus ostreatus*, is the most important in respect to the number of farmhouses and the market share.

Arthropod infestation is one of the major problems of mushroom culture. Mushroom flies, especially *Lycoriella mali* (Diptera: Sciaridae) and *Coboldia fuscipes* (Diptera: Scatopsidae), cause severe damage to mushroom: larvae feed on the mycelium and fruitbody of mushroom and adult flies transport germs such as nematodes, mites and mold spores (Clift, 1979; Clancy, 1981; Wetzel, 1981). *L. mali* is the most abundant mushroom pest, occurring all through the year in most mushroom houses in Korea (Bae *et al.*, 2001). This species is widely distributed in North America, Europe and Asia (Lee *et al.*, 1999). *C. fuscipes* is another major pest of oyster mushroom in Korea (Choi *et al.*, 2000; Bae *et al.*, 2001). However, the chemical insecticides for the control of mushroom flies are still limited. Therefore, biological control of mushroom flies gets an attention as a desirable control method. The increasing attention has been devoted to search for *Bacillus thuringiensis* strains which exert high toxicity to mushroom flies. *B. thuringiensis* is a gram-positive, rod-shaped, spore-forming soil bacterium widely used for the microbial control agent of insect pests. Many thousands of *B. thuringiensis* variants have been isolated from soil samples (Martin and Travers, 1989; Roh *et al.*, 1996; Park *et al.*, 1998; Chang *et al.*, 1998, 1999). Many *B. thuringiensis* isolates are toxic to lepidopteran larvae (Höfte and Whiteley, 1989; MacIntosh *et al.*, 1990) and, some are toxic to dipteran larvae (Goldberg and Margalit, 1977) or coleop-

teran larvae (Krieg *et al.*, 1983). In general, bipyramidal inclusions are toxic to Lepidoptera, ovoidal inclusions to Diptera and rhomboidal inclusions to Coleoptera (Yamamoto and Powell, 1993). We have previously isolated a *B. thuringiensis* 656-3 strain that shows high toxicity to mushroom flies, *L. mali* and *C. fuscipes* (Choi *et al.*, 2004). *B. thuringiensis* 656-3 which was isolated from the soil sample of mushroom houses produced bipyramidal inclusions and reacted with the H antiserum of *B. thuringiensis* subsp. *morrisoni* (H8a8b) (Choi *et al.*, 2004). In this study, *B. thuringiensis* 656-3 strain was mass-cultured and sprayed to oyster mushroom house for the control of mushroom flies. We report an insecticidal activity of *B. thuringiensis* 656-3 strain in oyster mushroom house.

Materials and Methods

Insect

The larvae of mushroom flies, *Lycoriella mali* (Diptera: Sciaridae) and *Coboldia fuscipes* (Diptera: Scatopsidae), were reared on a diet of oyster mushroom spawns (Choi *et al.*, 1997, 2000).

Bacterial strain and culture media

B. thuringiensis strain 656-3 was previously isolated from soil samples collected at mushroom houses in Korea (Choi *et al.*, 2004). For mass-culture of *B. thuringiensis* 656-3 strain, soybean cake and wheat bran were used as medium source (Kim, 1998).

Morphological observation

B. thuringiensis strain was grown in GYS medium at 30°C for 72 hrs and harvested in a 1.5 ml Eppendorf tube. The resulting parasporal inclusion pellet was dislodged from the tube bottom and fixed for 2 hrs in 3% glutaraldehyde [in 0.1 M cacodylate buffer (pH 7.4)]. After postfixation in 1% OsO_4 [in the same buffer], the pellets were dehydrated in an ethanol-propylene oxide series and embedded in an Epon-araldite mixture. Sections were cut with a Sorvall MT-5000 ultramicrotome and photographed in a transmission electron microscope (Hitachi H-600). For scanning electron microscopy, the fully lysed *B. thuringiensis* cells were air-dried, coated with carbon and stained with gold. The sample was observed by scanning electron microscope (Phillips SEM 515).

Mass-culture and semi-formulation

B. thuringiensis 656-3 strain was mass-cultured in the medium containing soybean cake (2%) and wheat bran (2%) mixed with 4% of final concentration by using the

fermenter with working volume of 5 l. For semi-formulation of *B. thuringiensis* 656-3 strain, the *B. thuringiensis* 656-3 cultures including toxin, spore, soybean cake and wheat bran particles were freeze-dried and mixed with metamorphic starch at a rate of 40 : 60 (dried *B. thuringiensis* 656-3 cultures : metamorphic starch). Two grams of *B. thuringiensis* 656-3 formulation, developed as powder formulation, was added to 750 ml of water. Seven hundred fifty milliliters of the formulation suspension containing 5×10^7 cfu was sprayed at 10 m^2 of mushroom bed.

Insect bioassays

The toxicity of *B. thuringiensis* 656-3 strain against mushroom flies, *L. mali* and *C. fuscipes,* was tested indoor oyster mushroom bed (1 m^2). The test was performed with spore-parasporal inclusion suspensions and independently repeated three times. For bioassay against *L. mali* and *C. fuscipes,* 300 third instar larvae were tested at various inclusion concentrations (1×10^6 to 1×10^8 cfu), respectively. In spray volume assay, *B. thuringiensis* 656-3 suspensions were performed at three volumes (25 ml, 50 ml and 75 ml per 1 m^2) including 1×10^7 cfu or 5×10^7 cfu, respectively. The spray period according to oyster mushroom growth was divided into two stages, spray at the germination or spray after germination. *B. thuringiensis* 656-3 suspension (5×10^7 cfu/50 ml/m^2) was sprayed at oyster mushroom bed and introduced 3rd instar larvae of mushroom flies on 1, 3, 5 and 7 after spraying. Dead larvae in all assays were scored daily and insecticidal activity was calculated for 3 days. LC_{50} values were calculated by Probit analysis (Russell *et al.*, 1977).

Mushroom house application

The insecticidal effect of *B. thuringiensis* 656-3 strain in oyster mushroom house was tested at oyster mushroom house. The test was performed with spore-parasporal inclusion suspensions (5×10^7 cfu) per oyster mushroom house (198 m^2). The insectcidal effect of *B. thuringiensis* 656-3 strain in oyster mushroom house was surveyed as the yield of mushroom. The yields of oyster mushroom in oyster mushroom house were scored for 4 harvest cycles and the mushroom yields were compared with controls.

Results and Discussion

In our previous report (Choi *et al.*, 2004), *B. thuringiensis* 656-3 which had toxicity against mushroom flies, *L. mali* and *C. fuscipes,* was isolated from the soil collected at mushroom houses and produced bipyramidal inclusions (Fig. 1). The serotype of *B. thuringiensis* 656-3 strain was
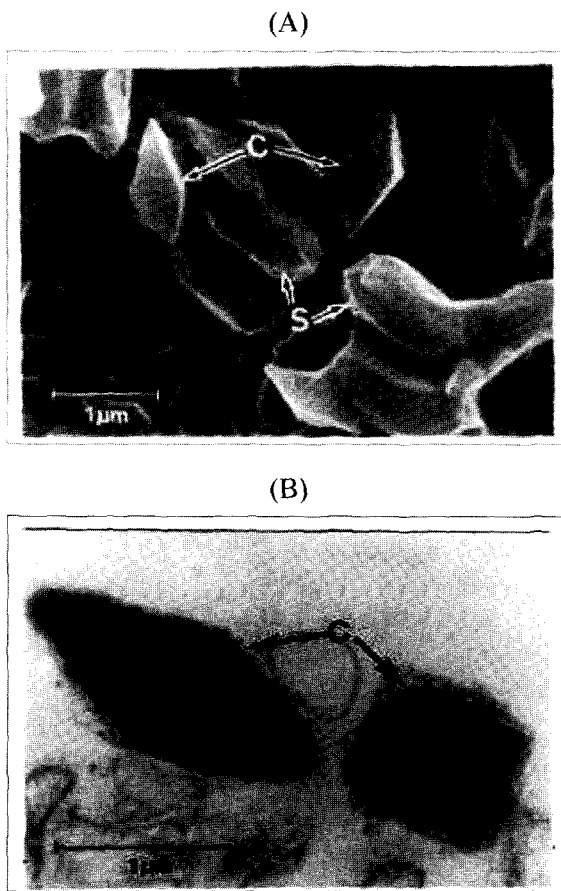
(A)

(B)

Fig. 1. Electron micrographs of spore and crystal of *B. thuringiensis* 656-3. (A) Scanning electron micrograph of the spore-crystal complex after autolysis and (B) Transmission electron micrograph of crystals. The spore and crystal are represented as S and C, respectively.

Table 1. Conditions for optimum fermentation of *B. thuringiensis* 656-3 with 7-liter fermenter

Condition	7 liter
Volume of medium[c]	5 liter
Aeration	1 vvm[a]
No. of impellers	2
Agitation	100 rpm
Incubation temperature	30°C
Starting pH	7.0
Final pH	7.75
End of log-phase of growth	48-60 hrs
End of sporulation phase	72-96 hrs
Completion of lysis	4 days
Age at harvest	4 days
Usual spore count[d]	$1 - 1.5 \times 10^{10}$ CFU[b]/m*l*

[a]pO2/volume of medium (5-liter air/5-liter medium).
[b]Colony forming unit.
[c]Soybean cake of 20 g + wheat bran of 20 g/liter.
[d]Number of viable spores (capable of germination and outgrowth) measured on nutriant agar plates.

Table 2. Insecticide activity of *B. thuringiensis* 656-3 from fermentation

Strain	Coboldia fuscipes Toxicity (LC_{50})[a]	Limits[b]	Lycoriella mali Toxicity (LC_{50})[a]	Limits[b]
B. thuringiensis 656-3	0.12	0.063~0.19	0.49	0.25~1.26

[a]50% lethal concentration ($\times 10^7$ /ml).
[b]Confidence limit.

identical to that of the reference strain, *B. thuringiensis* subsp. *morrisoni* PG-14 (H8a8b), which produced ovoidal inclusions (Krieg *et al.*, 1987; Padua *et al.*, 1984).

For mass-culture of *B. thuringiensis* 656-3 strain, soybean cake and wheat bran were used as medium source (Kim, 1998). *B. thuringiensis* 656-3 strain was cultured in the fermenter containing soybean cake (2%) and wheat bran (2%) mixed with 4% of final concentration. In this condition, the growth and spore forming of *B. thuringiensis* 656-3 strain were most effective: number of viable spores is approximately $1 - 1.5 \times 10^{10}$ cfu/ml at 4 days after culture (Table 1). This result shows that spore number of the mass-cultured *B. thuringiensis* 656-3 in the medium with soybean cake and wheat bran was similar to that of *B. thuringiensis* NT0423 (Kim, 1998), suggesting that soybean cake and wheat bran are available source for mass-culture of *B. thuringiensis* strains.

In the bioassay of *B. thuringiensis* 656-3 cultured in the fermenter, the LC_{50} values of *B. thuringiensis* 656-3 strain

are 0.12×10^7 for *C. fuscipes* and 0.49×10^7 for *L. mali*, respectively (Table 2). The toxicity of *B. thuringiensis* 656-3 strain was higher approximately 4 fold to *C. fuscipes* than that to *L. mali*, indicating that *C. fuscipes* was more susceptible to *B. thuringiensis* 656-3 strain than *L. mali*.

The semi-formulation of *B. thuringiensis* 656-3 strain was performed by mixing of *B. thuringiensis* 656-3 culture precipitates and metamorphic starch at a rate of 40 : 60. In this study, *B. thuringiensis* 656-3 formulation was developed as powder type.

In order to determine the optimum concentration of *B. thuringiensis* 656-3 formulation, the insecticidal activity of *B. thuringiensis* 656-3 formulation against mushroom flies, *C. fuscipes* and *L. mali*, was surveyed at three suspension concentrations (Fig. 2). When applied 1×10^7 for *C. fuscipes* and 5×10^7 for *L. mali*, the mortality of *B. thuringiensis* 656-3 formulation showed over 90% at the sec-
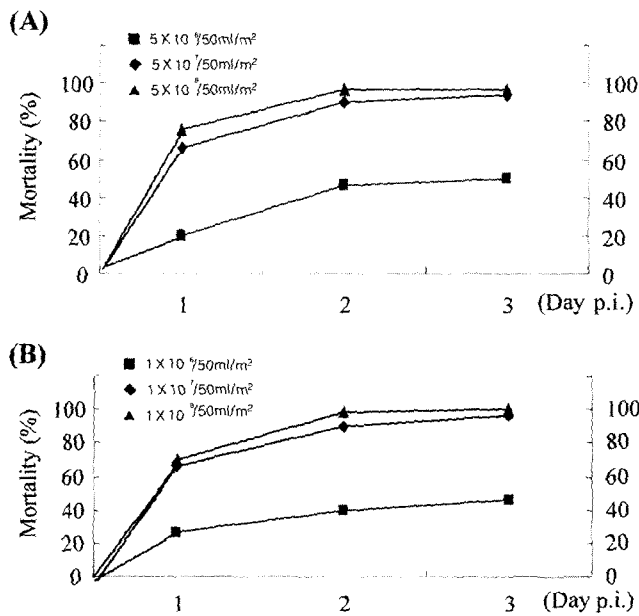
(A)

(B)

Fig. 2. Insecticidal effect of *B. thuringiensis* 656-3 formulation with various concentrations. Mortality of *B. thuringiensis* 656-3 formulation was assayed against 3rd instar larvae of *L. mali* (A) and *C. fuscipes* (B).
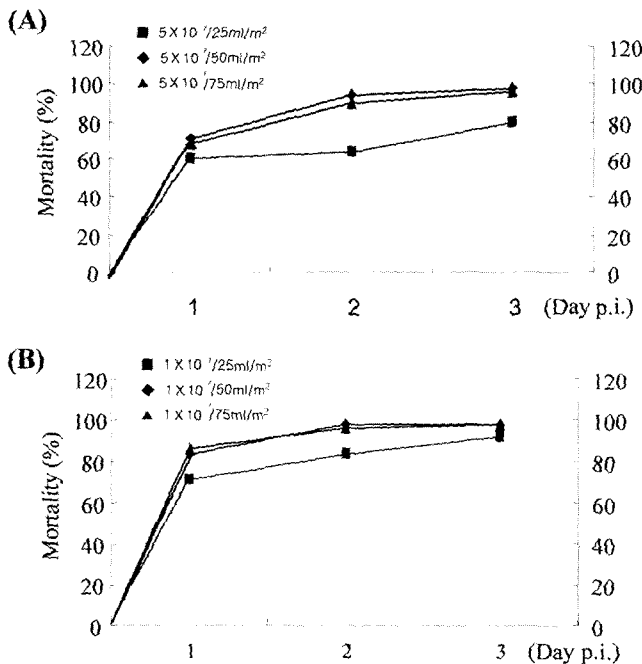
(A)

(B)

Fig. 3. Insecticidal effect of *B. thuringiensis* 656-3 formulation with various spraying volumes. Mortality of *B. thuringiensis* 656-3 formulation was assayed against 3rd instar larvae of *L. mali* (A) and *C. fuscipes* (B).

ond day after spraying.

The optimum spraying volume of *B. thuringiensis* 656-3 formulation was surveyed at three suspension volumes
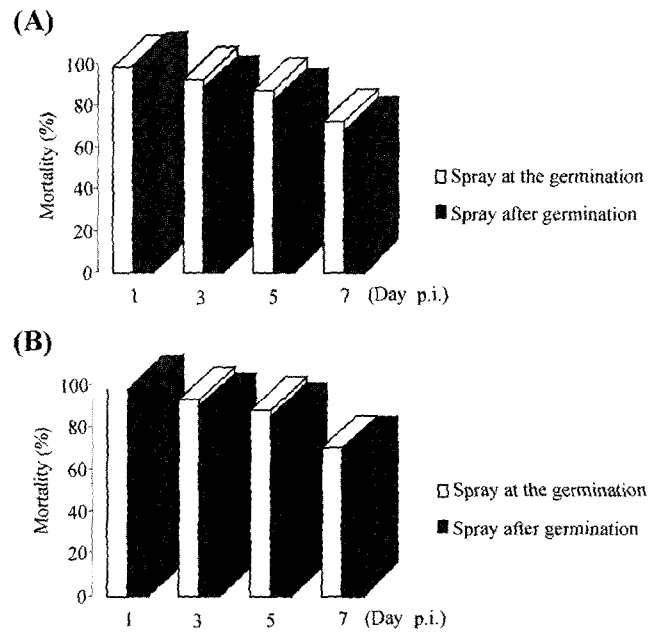
(A)

(B)

Fig. 4. Insecticidal effect of *B. thuringiensis* 656-3 formulation according to period after spraying and mushroom growth. Mortality of *B. thuringiensis* 656-3 formulation was assayed against 3rd instar larvae of *L. mali* (A) and *C. fuscipes* (B).

(Fig. 3). When applied 1×10^7 cfu/50 ml/m^2 for *C. fuscipes* and 5×10^7 cfu/50 ml/m^2 for *L. mali*, the mortality of *B. thuringiensis* 656-3 formulation showed over 90% at the second day after spraying. It is indicated that the optimum volume of *B. thuringiensis* 656-3 formulation was at least over 50 ml to 75 ml/m^2 for complete spraying on the mushroom bed.

To test insecticidal effect of *B. thuringiensis* 656-3 formulation according to mushroom growth and period after spraying, the formulation was sprayed at the germination or after germination of oyster mushroom and the mortality was respectively surveyed on 1, 3, 5 and 7 after spraying (Fig. 4). There was no significant difference between two growth stages, spraying at the germination or after germination. Although insecticidal activity of *B. thuringiensis* 656-3 formulation was continuously decreased with periods after spraying, the mortality of *B. thuringiensis* 656-3 formulation was maintained approximately 90% by the fifth day after spraying.

In Korea, oyster mushrooms are cultivated mostly for the spring and autumn seasons, and mushroom flies, *C. fuscipes* and *L. mali*, are serious pests in oyster mushroom houses. In general, these species simultaneously occur in oyster mushroom houses (Bae *et al.*, 2001). From these results, therefore, we have determined the final working suspension of *B. thuringiensis* 656-3 formulation with 5 $\times 10^7$ cfu/750 ml/10 m^2. In this condition, *B. thuringiensis* 656-3 formulation showed over 90% insecticidal effect
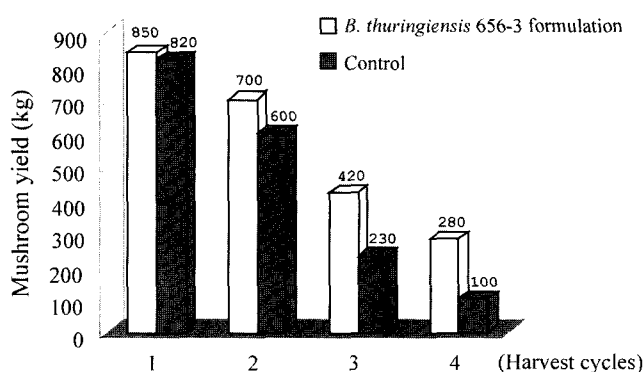
Fig. 5. Comparison of the mushroom yield in mushroom house with or without *B. thuringiensis* 656-3 formulation.

against mushroom flies, *C. fuscipes* and *L. mali*, by the fifth day after spraying.

Finally, insecticidal effect of *B. thuringiensis* 656-3 formulation to mushroom flies in mushroom house was surveyed as the yield of mushroom. The yields of oyster mushroom in oyster mushroom house were scored for 4 production cycles and the formulation was sprayed total 20 times with intervals of one time per 3 to 4 days. The yield of mushroom house with *B. thuringiensis* 656-3 formulation was increased approximately 7% for the first harvest, 17% for the second, 83% for the third, and 36% for the final harvest compared with control (Fig. 5). This result revealed that *B. thuringiensis* 656-3 formulation was effective to control of mushroom flies, especially from the second harvest cycle showing high occurrence of mushroom flies, in oyster mushroom house.

In conclusion, mushroom house application of *B. thuringiensis* 656-3 demonstrated that the mushroom yields in oyster mushroom house are significantly increased compared to control. Thus, *B. thuringiensis* 656-3 represents a powerful biological insecticide for control of mushroom flies.

Acknowledgments

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