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LEE, You Kyoung
Division of Crop Foundation, National Institute of Crop Science, Rural Development Administration

KIM, Hee Ji
Department of Applied Biology, College of Agriculture and Life Sciences, Chungnam National University

YU, Yong Man
Division of Crop Foundation, National Institute of Crop Science, Rural Development Administration

YASUNAGA-AOKI, Chisa
Laboratory of Insect Pathology and Microbial Control, Institute of Biological Control, Faculty of Agriculture, Kyushu University

他

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Control Effect and Applicability of *Bacillus Thuringiensis* for Control of *Lycoriella Ingenua* in *Agaricus Bisporus* Cultivation Plots

You Kyoung LEE[1, 2], Hee Ji KIM[2], Yong Man YU[1], Chisa YASUNAGA-AOKI* and Young Nam YOUN[2]*

Laboratory of Insect Pathology and Microbial Control, Institute of Biological Control, Faculty of Agriculture, Kyushu University, Fukuoka 819–0395, Japan
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A Bacillus thuringiensis strain, which is an entomopathogenic microorganism, was used for effective control of *Lycoriella ingenua* larvae, which are an intractable pest occurring during *Agaricus bisporus* cultivation. The effects of *B. thuringiensis* subsp. *kyushuensis* CAB464 strain that shows high insecticidal effects against *L. ingenua* larvae at low concentrations, which were sublethal concentrations, on the survival rate of *L. ingenua* larvae were reviewed and according to the results, the feeding and growth rates of the larvae were hindered, and it could be seen that while *B. thuringiensis* subsp. *kyushuensis* CAB464 strain directly acts on *A. bisporus* cultivation plots, it also has an insecticidal mechanism to inhibit feeding thereby starving the larvae to death. Pot tests were conducted to see the applicability and control effect of *B. thuringiensis* subsp. *kyushuensis* CAB464 strain on *A. bisporus* cultivation plots, and according to the results, *B. thuringiensis* subsp. *kyushuensis* CAB464 strain showed a control value of 80.1%, which was at least about 50% higher than that of the untreated control plot and showed a survival rate at least about 38% lower than that of the untreated control plot while the untreated control plot showed gradual increases in the survival rate. The control effects of *B. thuringiensis* subsp. *kyushuensis* CAB464 strain were tested in an *A. bisporus* cultivation plot in which *L. ingenua* in all age periods existed in mixture, and based on the results, the control value after two times of treatment was higher than that after one time of treatment as it was 75.6% after the first treatment and 81.7% after the second treatment, and it could be seen that the survival rate gradually decreased. The foregoing indicates that *B. thuringiensis* subsp. *kyushuensis* CAB464 strain is applicable as a novel microbial insecticide that can control *L. ingenua*.

Key words: *Bacillus thuringiensis, Lycoriella ingenua*, microbial insecticide, mushroom cultivation

INTRODUCTION

Globally, *Lycoriella ingenua* causes enormous damage and serious economic losses in the process of cultivation of not only *Agaricus bisporus* but also oyster mushrooms and shiitake mushrooms. In the case of mushroom cultivation in South Korea, the year round cultivation system was universalized so that the cultivation quantity is in an increasing trend. *L. ingenua*, which occurs at all stages ranging from inoculation to harvest to cause damage, not only reduces the yield and quality of mushrooms but also causes complex damage by mediating mushroom mites and nematodes by getting them on its body (Choi *et al.*, 2018). *L. ingenua* takes about 18 days from eggs to eclosion as it passes 3~5 days in eggs, 8~12 days in larvae, and 4~6 days in pupae. Since the larva period occupies about 70% of the total life cycle and the activity of larvae inside the mushroom medium is high, the damage caused by *L. ingenua* larvae is very serious (Kang *et al.*, 2016). The drugs registered for the control of *L. ingenua* show insecticidal effects by inhibiting chitin biosynthesis in the epidermal tissues of the insect thereby interfering with the normal metamorphosis or molting (Insect growth regulation, IGR), and are commonly applied by irrigation at intervals of two days after inoculation of the mushroom seeds and covering up the seeds with soil. However, the frequent use of organic synthetic pesticides, which have been used for long periods of time for the control of *L. ingenua* because there have been not many registered drugs, can increase the possibility of appearance of pests resistant to the drugs and can cause environmental pollution such as their residues in soil and mushrooms or toxicity to humans and livestock, and side effects such as ecosystem disturbance (Ahn *et al.*, 1989). In addition, because *L. ingenua* occurs continuously during the mushroom cultivation period, pesticides should be repeatedly used but there are many restrictions on the repeated use of pesticides, and since mushrooms are used as food, insecticide scattering is restricted a lot due to concerns about health and increased consumers' demand for the production of safe agricultural products (Kim *et al.*, 2004). As alternatives to chemical pesticides that cannot easily control the pests due to their limited time of application, control methods using biological control methods such as *Bacillus thuringiensis* products, entomopathogenic nematodes, and predaceous mites are being introduced. Since the insecticidal protein, the main mechanism of action of *B. thuringiensis*, which is the most widely used as a microbial insecticide, is safe against non–targeted organisms, it is used as a major agricultural material for crop protection in eco–friendly agriculture, and accounts for 80~90% of global biological control (Feitelson *et al.*, 1992). Studies on the

[1] Division of Crop Foundation, National Institute of Crop Science, Rural Development Administration, Wanju 55365, Korea

[2] Department of Applied Biology, College of Agriculture and Life Sciences, Chungnam National University, Daejon 34134, Korea

* Joint corresponding authors (E–mail: youngnam@cnu.ac.kr and yasunaga@grt.kyushu–u.ac.jp)

physiological and ecological characteristics of *L. ingenua* are insufficient, its generations occurred in the cultivations are mixed, and in particular, the control of old larvae is very difficult. Currently, most of the *B. thuringiensis* products, which are sold as microbial pesticides in South Korea, are used and studied for the control of Lepidoptera pests, and microbial pesticides registered for the control of Diptera pests are insufficient (Lee *et al.*, 2021). In addition, the problems of reduced insecticidal effects and resistance in the registered microbial insecticides came to the fore. Therefore, studies are necessary to select new *B. thuringiensis* products and maximize their effects with the method of use and treatment time. The main action mechanism of *B. thuringiensis* is the action of the toxin protein that forms small holes in the membranes of the epithelial cells of the midgut of the larvae thereby causing sepsis leading to the death of the insect (Bravo *et al.*, 2007). *B. thuringiensis* shows toxicity at the larva stage because it must be eaten through the mouthpart of the pest without fail to show activity (Jurat–Fuentes and Adang, 2006).

Therefore, in order to control *L. ingenua* larvae, which occur in *A. bisporus* cultivation plots and cause serious damage to mushrooms, this study was intended to investigate the applicability and control effects of *B. thuringiensis* subsp. *kyushuensis* CAB464 strain that shows high insecticidal activity on *L. ingenua* so that the study findings can be used as basic data for the development of microbial pesticides against *L. ingenua* larvae.

MATERIALS AND METHODS

Test insect

As for the *L. ingenua* used in the experiment, the adults were collected using an aspirator from an *A. bisporus* cultivation plot in Songguk–ri, Chochon–myeon, Buyeo–gun, Chungcheongnam–do and were momentarily paralyzed using carbon dioxide so that they could not move. Three males and three females among them were inoculated in a plastic Petri–dish (60×15 mm) containing a water agar medium to induce spawning. The spawning was confirmed under a microscope, and *A. bisporus* was used as food for rearing the hatched larvae. The *L. ingenua* was reared under the conditions of a temperature of 25±1°C, a light condition of 16L:8D, and relative humidity in a range of 50~60% (Lee *et al.*, 2018).

Feeding deterrence test

B. thuringiensis subsp. *kyushuensis* CAB464 strain was diluted into several concentrations, 200 μl each of the diluted solutions was sprayed on 0.5 g of *A. bisporus*, and each of the foregoing was inoculated with 10 *L. ingenua* larvae 5 days old after hatching. The foregoing process was carried out a total of three times repeatedly. The feeding rates for seven days were investigated and the degree of development of those larvae that fed on *B. thuringiensis* subsp. *kyushuensis* CAB464 strain was investigated through the pupation rate and eclosion rate of the larvae.

Pot control effect test

The *L. ingenua* culture medium was sprayed using a compressed automatic sprayer into small plastic boxes containing a straw fermentation medium inoculated with *A. bisporus* seeds through a conventional cultivation method. Yellow sticky traps were installed at the top and bottom of the pot to investigate the numbers of those adults that eclosed before treatment, 7 days after treatment, and 14 days after treatment. The foregoing process was undergone separately for the untreated control plot, the plot treated with *B. thuringiensis* subsp. *israelensis*, which is the reference strain, and the plot treated with *B. thuringiensis* subsp. *kyushuensis* CAB464 strain to compare and review the plots.

Test area in the mushroom cultivation house

The *A. bisporus*, which is the test crop, was the variety "Saehan" and the test was performed at a farm in Seokseong–myeon, Buyeo–gun, Chungcheongnam–do. Since various states of development of *L. ingenua* ranging from eggs to adults existed in mixture, yellow sticky traps (27.5×15 cm) were installed before strain treatment to investigate the number of *L. ingenua* adults (Fig. 1).

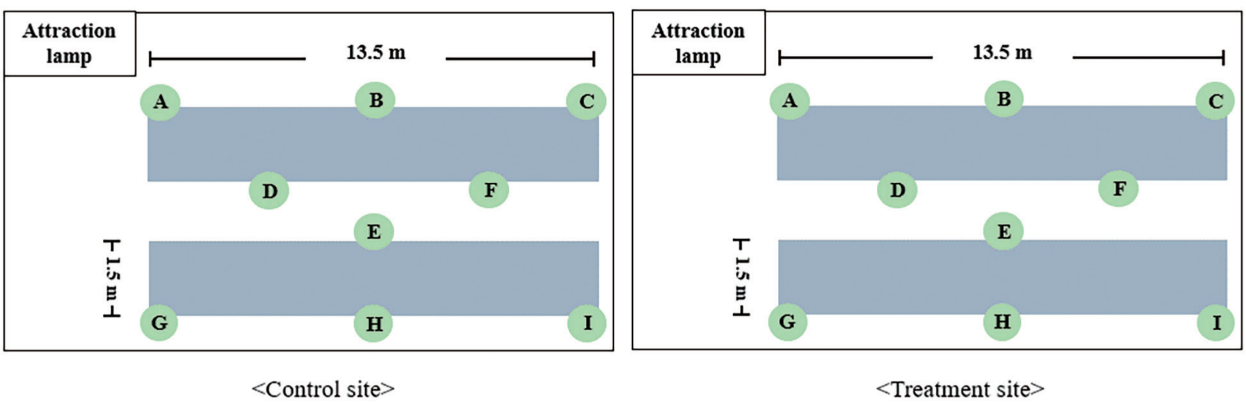


Fig. 1. Installment of yellow sticky traps (A–I) for monitoring of *L. ingenua* adults in mushroom cultivation.

Drug treatment method and investigation in the mushroom cultivation house

An appropriate amount of the *B. thuringiensis* subsp. *kyushuensis* CAB464 strain culture medium from mass cultivation by Kyungnong Co., Ltd. was diluted and sprayed with a sprayer for irrigation. As for the investigation method, the control effects were identified by examining the numbers of adults captured in the yellow sticky traps three times at 7 day intervals before and after treatment separately for the plot treated with *B. thuringiensis* subsp. *kyushuensis* CAB464 strain and the untreated control plot and the results were compared and reviewed.

RESULTS AND DISCUSSION

The effects of *B. thuringiensis* subsp. *kyushuensis* CAB464 strain treatment to inhibit the feeding and growth rate of *L. ingenua*

Although the *B. thuringiensis* strain directly acts on insects as an enterotoxin, it is reported to also have an insecticidal mechanism to deter feeding leading to death from starvation (Broderick *et al*., 2006). The deterrence of insects' feeding means that damage to crops can be reduced (Jin *et al*., 2009). Adding a certain amount of the *B. thuringiensis* strain not only directly affects the feeding behavior of larvae but also affects a series of physiological aspects such as sepsis in the body of the insects, eventually affecting the growth of larvae. It is reported that the toxin protein of the *B. thuringiensis* strain shows long–term effects of inhibiting and delaying the growth in addition to killing target pests (Erb *et al*., 2001).

In this experiment, *B. thuringiensis* subsp. *kyushuensis* CAB464 strain was used mainly at low concentrations around the sublethal level to review its effects on the survival rate of 4~5 days–old *L. ingenua* larvae. The treatment concentration of *B. thuringiensis* subsp. *kyushuensis* CAB464 strain had marked effects on the survival of the larvae (Fig. 2). When seven days passed after the drug treatment, the survival rates of the larvae at low concentrations of 10^2, 10^3 (cfu/ml) were markedly higher than the survival rate at 10^4(cfu/ml), and the survival rates showed a tendency to decreased relatively rapidly as the treatment concentration increased. In general, the lower the concentration, the higher the survival rate of larvae, indicating that the lower the treatment concentration, the smaller the amount of the toxin protein eaten by *L. ingenua*.

The larvae were fed with *B. thuringiensis* subsp. *kyushuensis* CAB464 strain and the effect of the feeding on the growth of the larvae was reviewed. At all the treatment concentrations of *B. thuringiensis* subsp. *kyushuensis* CAB464 strain, the feeding rates were remarkably lower than those of the untreated control plot, indicating that as the concentration increased, the feeding rate decreased rapidly (Fig. 3).

It could be seen that when the larvae were fed with the strain, the mortality of the larvae increased not only due to the effect of direct feeding but also due to the deterrence of feeding. Consequently, the treatment concentration of *B. thuringiensis* subsp. *kyushuensis* CAB464 strain was shown to have significant effects on

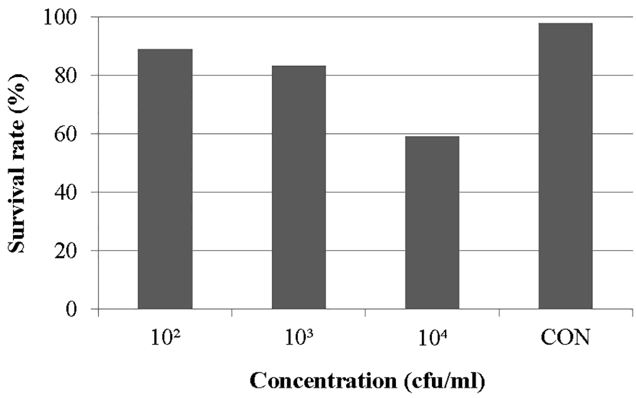


Fig. 2. Effects of the toxin of *B. thuringiensis* subsp. *kyushuensis* CAB464 on survival rate of larvae of *L. ingenua*.

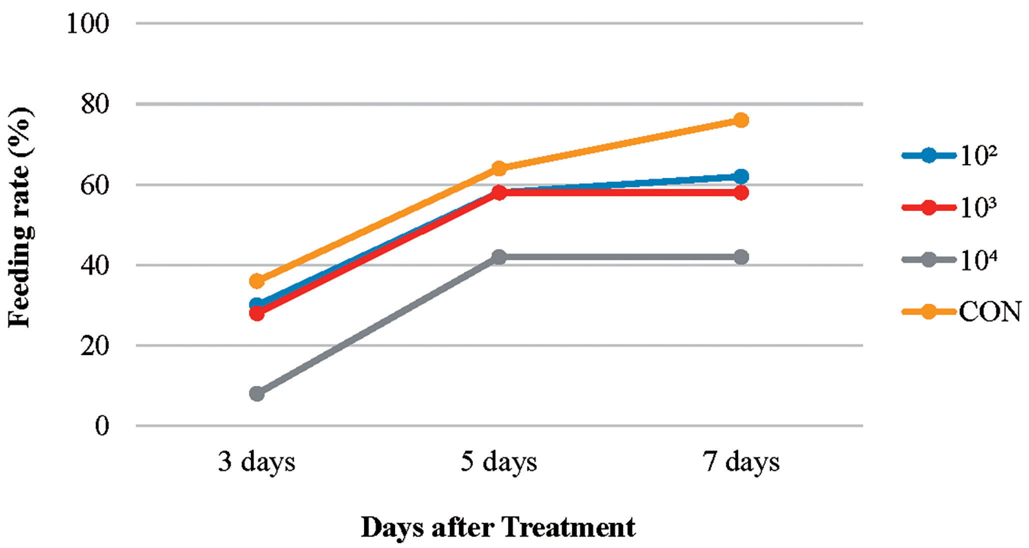


Fig. 3. Effects of the toxin of *B. thuringiensis* subsp. *kyushuensis* CAB464 on feeding rate of larvae of *L. ingenua*.

the growth of *L. ingenua* larvae (Table 1). The *L. ingenua* larvae fed with the toxin protein of *B. thuringiensis* subsp. *kyushuensis* CAB464 strain were not only affected greatly on their survival rate and feeding rate but also affected on their pupation and eclosion. Regardless of the treatment concentrations, the pupation rates were shown to be significantly lower than those in the control at all concentrations, and the higher the concentration was, the more rapidly the pupation rates tended to decrease. In addition, the toxin protein of *B. thuringiensis* subsp. *kyushuensis* CAB464 strain also affected the eclosion of *L. ingenua* adults, and it was identified that the eclosion rate decreased slowly when the concentration was low and as the concentration increased, the eclosion rate decreased rapidly.

From the results as such, it could be seen that, although the larvae die after feeding on *B. thuringiensis* due to the sepsis caused by the toxic protein, they also die from starvation due to the stop or avoidance of feeding and that the remaining larvae that survive take much longer time to grow or cannot grow normally. According to a study conducted by Zeng *et al.* (1999), the survival rate, weight, and growth period of pests that ingested the toxin protein of *B. thuringiensis* were not only directly related to the toxin concentration, but also different according to the degree of sensitivity of individuals. Sensitive individuals fed with the same concentration of the toxin protein showed lower survival rates and weight gains than resistant individuals but showed longer the development periods on the contrary. Therefore, it can be expected that by treating the target pest with the *B. thuringiensis* strain that has high activity on the target pest, the growth of the larvae can be deterred, and their development period can be delayed.

Table 1. Effects of the toxin of *B. thuringiensis* subsp. *kyushuensis* CAB464 on mortality, pupate and, emergence of *L. ingenua*

Treatment	7 days after treatment		
	Mortality (%)	Pupate rate (%)	Emergence rate (%)
Control	0.0± 0.0	87.8±14.8	97.8± 4.4
1.8×10^2	12.2±12.0	77.8±13.9	88.9±12.7
1.8×10^3	16.7±12.3	61.1± 7.8	83.3± 8.7
1.8×10^4	40.0±20.0	48.8±13.6	58.9±16.9

Pot test of *B. thuringiensis* subsp. *kyushuensis* CAB464 strain

A pot test was performed as a method to see the availability and control effect in the actual field where *L. ingenua* eggs, larvae, pupae, and adults exist simultaneously. Small boxes inoculated with *A. bisporus* seeds were treated with the culture media of the untreated control plot, the plot treated with *B. thuringiensis* subsp. *israelensis*, which is the reference strain, or the plot treated with *B. thuringiensis* subsp. *kyushuensis* CAB464 strain, and the numbers of adults were investigated. The survival rates of the untreated control plot, the plot treated with the reference strain, and the plot treated with *B. thuringiensis* subsp. *kyushuensis* CAB464 strain were shown to be 58.8, 51.5, and 33.3%, respectively, 7 days after the drug treatment and 72.1, 45.9, and 19.9%, respectively, 14 days after the drug treatment. Therefore, it was shown that whereas the survival rates of the plot treated with the reference strain and the plot treated with *B. thuringiensis* subsp. *kyushuensis* CAB464 strain decreased over time, the survival rate of the untreated control plot gradually increased (Fig. 4).

Control effects in mushroom cultivation houses

In order to measure the control effects in outdoor fields where all age periods exist in mixture, the experiment was conducted with treatment plots where *L.*

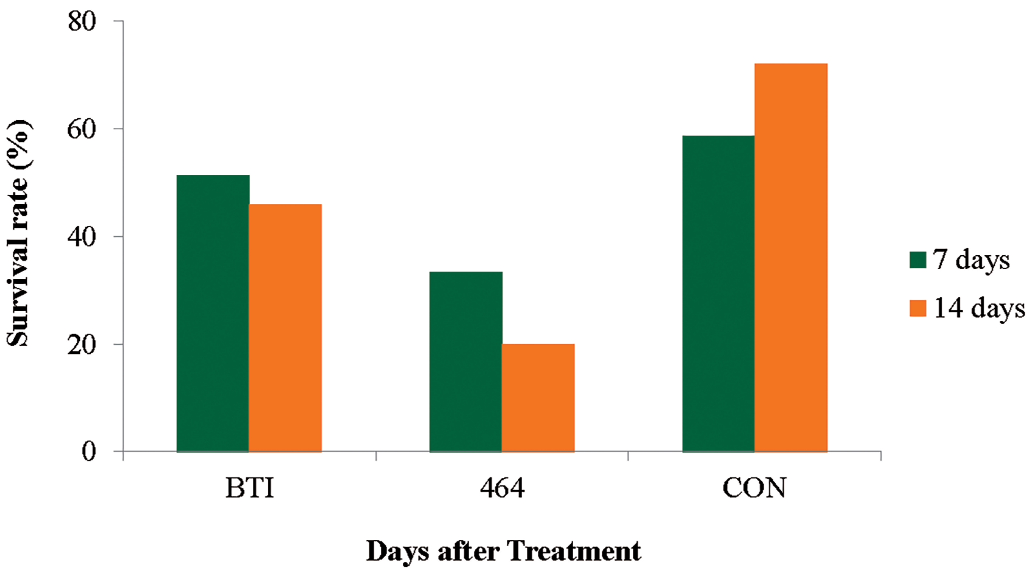
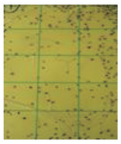
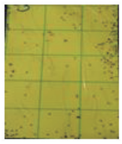
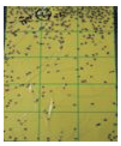
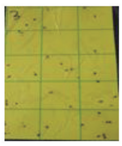
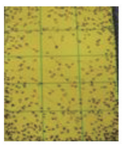
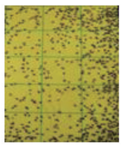


Fig. 4. Survival rate of *B. thuringiensis* subsp. *kyushuensis* CAB464 against *L. ingenua* on the *A. bisporus* pot bioassay.

ingenua adults, eggs, larvae, and pupae existed in mixture (Table 2). After one time of treatment, the control value was shown to be 44.1% when 7 days passed, increased to 63.9% when 14 days passed, and to 75.6% when 21 days passed so that it could be seen that the control value gradually increased over time (Fig. 5). It was identified that the survival rate decreased to 55.9, 36.1, and 24.4%, respectively, when 7, 14, and 21 days passed (Fig. 6). After two times of treatment, the control value was shown to be 54.8, 65.4, and 81.7%, respectively, when 7, 4, and 21 days passed (Fig. 7), and the survival rate was shown to be 48.1, 34.6, and 18.3%, respectively when 7, 4, and 21 days passed. Therefore, it could be seen that the control values were higher than those after one time of treatment and the survival rate gradually decreased (Fig. 8). On the other hand, in the case of the untreated control plot, the *L. ingenua* control value gradually decreased (Fig. 9) and the occurrence increased (Fig. 10). Through the foregoing, it could be seen that the *B. thuringiensis* product had great effects on the survival rate because it continuously led to the death of the larvae.

Choi *et al*. (1996) reported study findings indicating that even when excellent drugs are used, the selection pressure against natural pests will increase as the amount and times of use increase so that the occurrence of resistant pests is inevitable and said that the use of drugs for pest control should be based on accurate infor-

Table 2. Accumulated control value and survival rate of *L. ingenua* against *B. thuringiensis* subsp. *kyushuensis* CAB464 on the *A. bisporus* field

Treatments	Control value of weeks after treatment			Attraction lamp		Section	
	1	2	3	Before treatment	21 days after treatment	Before treatment	21 days after treatment
Once treatment	44.1±18.7	63.9±15.7	75.6±12.4				
Twice treatment	54.8±22.7	65.4±13.5	81.7±13.4				
Control	50.6±12.5	44.9±19.7	37.6±16.0				

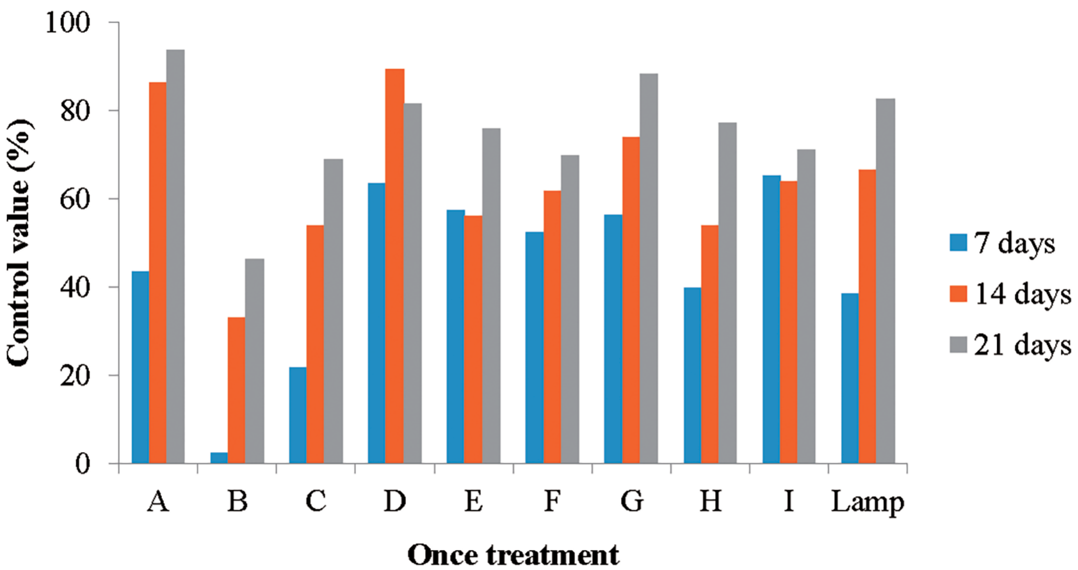


Fig. 5. After once treatment, control value of *B. thuringiensis* subsp. *kyushuensis* CAB464 against *L. ingenua* on the *A. bisporus* field.

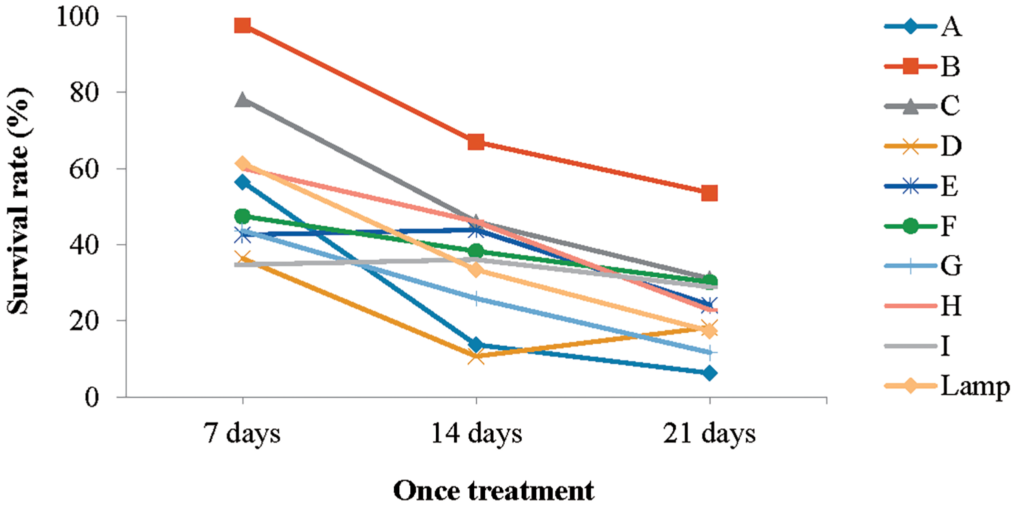


Fig. 6. After once treatment, survival rate of *B. thuringiensis* subsp. *kyushuensis* CAB464 against *L. ingenua* on the *A. bisporus* field.

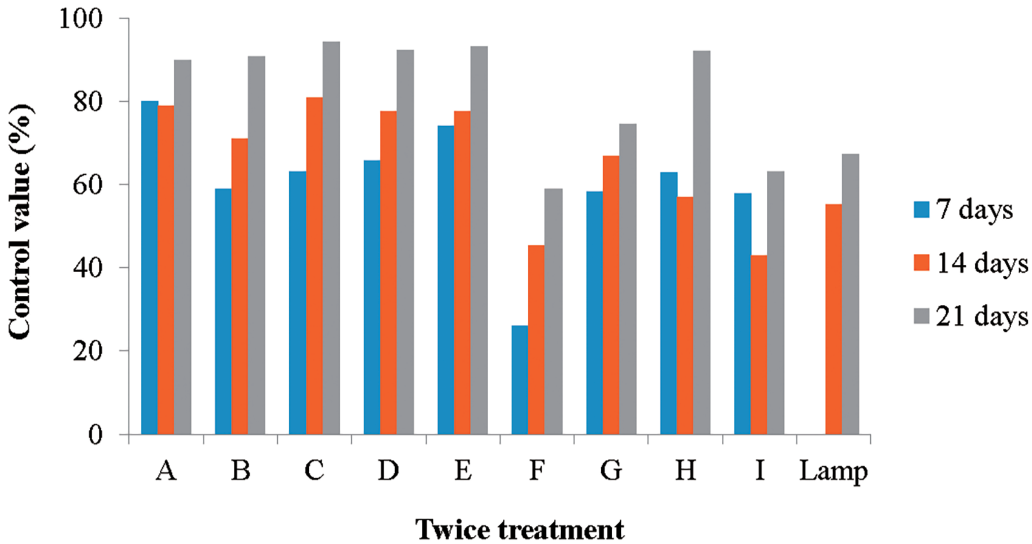


Fig. 7. After twice treatment, control value of *B. thuringiensis* subsp. *kyushuensis* CAB464 against *L. ingenua* on the *A. bisporus* field.

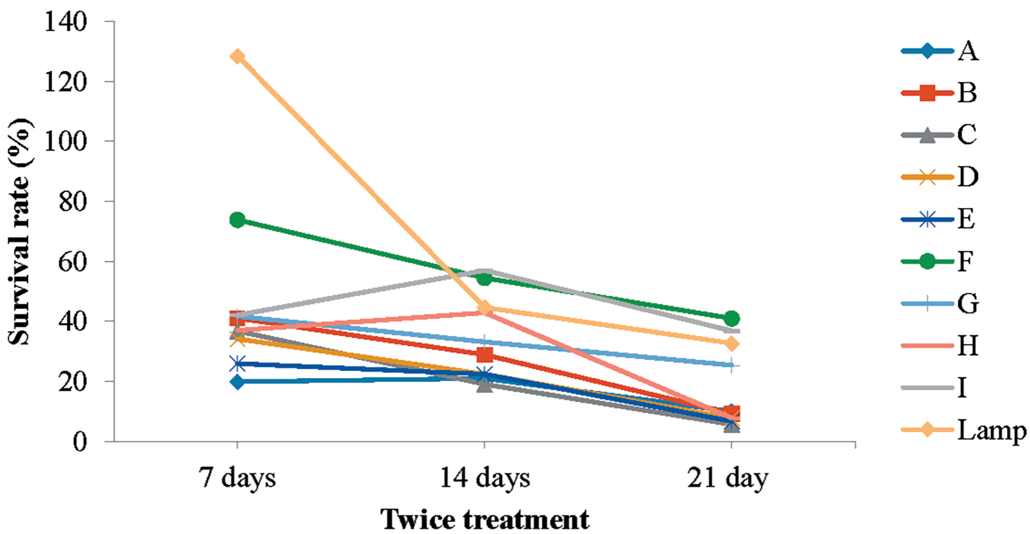


Fig. 8. After twice treatment, survival rate of *B. thuringiensis* subsp. *kyushuensis* CAB464 against *L. ingenua* on the *A. bisporus* field.

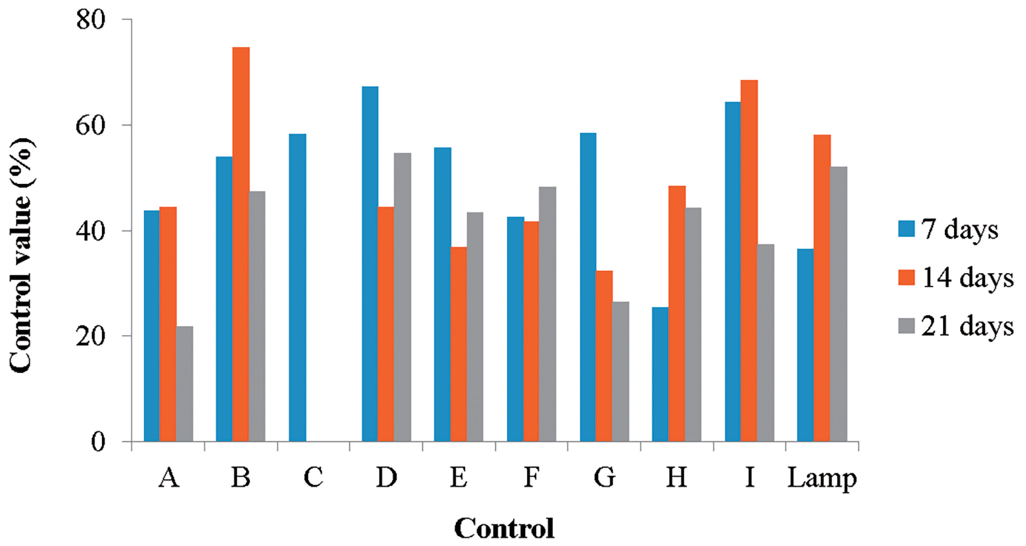


Fig. 9. After non treatment, control value of *B. thuringiensis* subsp. *kyushuensis* CAB464 against *L. ingenua* on the *A. bisporus* field.

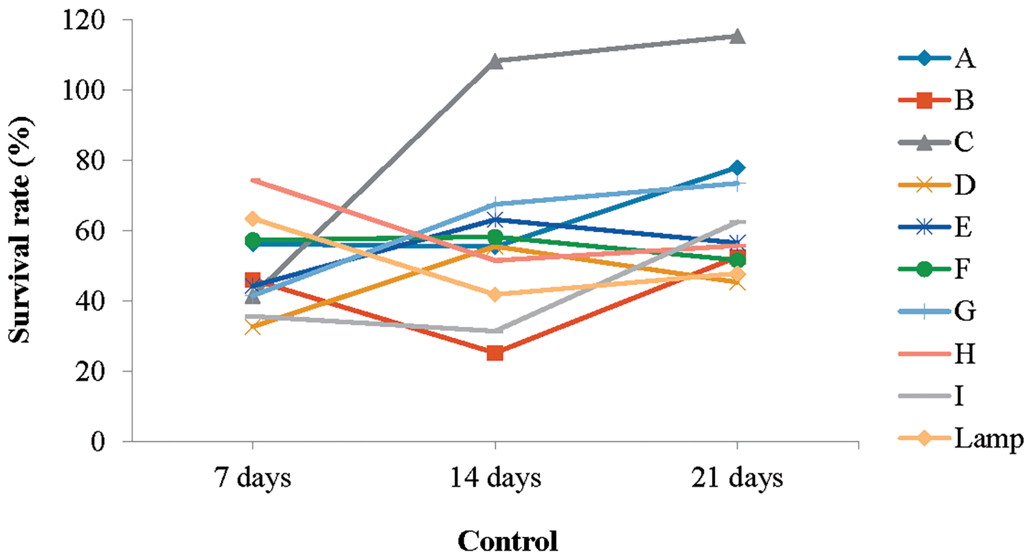


Fig. 10. After non treatment, survival rate of *B. thuringiensis* subsp. *kyushuensis* CAB464 against *L. ingenua* on the *A. bisporus* field.

mation on the insecticidal properties and acting characteristics of the drugs. Although both the primary and secondary treatments showed significant differences from before the treatment, since the developmental states of *L. ingenua* existed in mixture ranging from eggs to adults, the treatment with *B. thuringiensis* subsp. *kyushuensis* CAB464 strain alone is expected to have difficulties in control. Since the number of eco–friendly cultivation farms is increasing recently, the study findings described above are though to become baseline data for future studies to develop new microbial insecticides using *B. thuringiensis* strains that can be used to control *L. ingenua*, studies to monitor the occurrence pattern of *L. ingenua* and the growth conditions of mushrooms through outdoor field experiments to review actual applicability to farms, and other studies to measure the time of application and applicability of eco–friendly products in order to effectively control both the adults and larvae of *L. ingenua* simultaneously.

AUTHOR CONTRIBUTIONS

You Kyoung Lee designed the study, performed the comprehensive experiments, analyzed the data and wrote the paper. Hee Ji Kim performed the isolation of *B. thuringiensis* strains. Yong Man Yu edited the paper. Chisa Yasunaga–Aoki participated in the design of the study and discussed on the experiments and the results. Young Nam Youn supervised the work and wrote the paper. All authors assisted in editing of the manuscript and approved the final version.

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