

SCREENING OF *Bacillus* spp. SUPPRESSING THE INFECTION OF *Trichoderma* sp. IN MUSHROOM CULTIVATION

Titiya Chittihunsa¹, Eakaphun Bangeekhan², Nisa Wongsamitkul¹,
and Tipaporn Subsomboon³

¹Department of Biology, Faculty of Science, ²Department of Microbiology, Faculty of Science, and ³Department of Biotechnology, Faculty of Engineering and Industrial Technology, Silpakorn University, Nakorn Pathom 73000, Thailand

ABSTRACT

The aim of this study is to search for potential *Bacillus* spp. that can suppress the infection of *Trichoderma* sp. in Shiitaki mushroom (*Lentinus edodes*) and oyster mushroom (*Pleurotus sajor-caju*). Preliminary screening of 30 isolates of *Bacillus* spp. was performed against the green mold, *Trichoderma* sp., one of the pathogens of several mushroom cultivation. All bacilli except the commercial product, PlaikaowTM (*B. subtilis* PKTM) were isolated from infected and non infected mushrooms with *Trichoderma* sp. Antifungal efficiency was examined by agar diffusion and dual culture assay. Microscopic study revealed that the bacilli caused abnormalities in mycelium, such as cytolysis, vacuole formation, cell-shrink and cell-size reduction. API 50 CHB was used to identify the species of 5 isolates and 4 isolates were found to be *B. subtilis* (designated as Bs 02-SU₂, Bs 05-SU₃, Bs 07-SU₄ and Bs 09-SU₅) while another species was *B. licheniformis* (Bl 01-SU₁). For *in vivo* test in mushroom house, bacterial cell suspensions from 24 hr. incubation time, pH 7 at 37 °C, containing 10⁶ cell/ml of the 5 selected isolates were injected into the spawn cultivated mushrooms and followed by 10⁶ spore/ml of *Trichoderma* sp. 2 days later. *Trichoderma* sp. infected-mushrooms were counted twice a week, for a period of 6 weeks. It was found that the percentage of green mold infection by Bl 01-SU₁ on *L. edodes* and *P. sajor-caju* was 58.23 and 80, respectively. Its efficacy was equivalent to Bs PKTM ($P > 0.05$) but was significantly different ($P \leq 0.05$) from the rest of other bacterial isolates. In addition, all tested bacteria exhibited significantly higher suppression for *Trichoderma* sp. infected *P. sajor-caju* than those infected *L. edodes*.

KEYWORDS: microbial control, microbial antagonism, *Bacillus*, green mold, *Trichoderma*, mushroom, *Lentinus edodes*, *Pleurotus sajor-caju*

*Corresponding author: Tel: 089-496-7408 Fax: 034-217-046
E-mail: titiya @ su.ac.th

1. INTRODUCTION

Apart from good taste and flavor, there is several evidence showing that most mushrooms contain high healthy nutrients. Some mushrooms are good sources of protein, vitamins and minerals, for instance, Shiitake contains proteins, lipid, carbohydrates, vitamins A, B₁, B₂, C, D₂, niacin, phosphorus, iron, calcium, and other minerals. In addition, some bioactive compounds in mushrooms can lower serum cholesterol and function as anti-tumor and anti-viral or anti-microbial pathogens [1]. Cultivation of a number of high value mushrooms has grown rapidly due to the high incomes and accessible technology. The ability to grow Shiitake in sawdust plastic bags instead of on wood logs at the Northern and North Eastern parts of Thailand makes it more practical, economical and convenient to mushroom growers. The shorter distance of transportation, freshness, good taste, good flavor and high healthy nutrients have created an increasing demand for mushrooms as well as the rapid growth of mushroom supplies.

Unfortunately, cultivating mushrooms in some regions are seriously facing the infection of *Trichoderma* sp., a causative agent of green mold disease in mushroom. One of the common species is *T. harzianum* which has been widely used to control some plant pathogens in some area of Thailand for almost 2 decades [2]. Therefore, mushroom cultivated in or near the area that farmers use *T. harzianum* is at high risk to get infection.

Currently, there are strict regulations on the use of fungicidal chemicals owing to their toxic residues contaminating food and deteriorating the environment. In addition, there is a political pressure to remove hazardous chemicals from the market as soon as possible. Therefore, numerous microbes and microbial products have become interesting alternatives to fungicides [3]. Abundant species or strains of *Bacillus* have been developed as commercial products, since they are not normally regarded as human pathogen. In addition, they possess a number of good characteristics, for instance, rapid growth, inexpensive culture media, production of several antifungal substances [4] and some plant nutrients [5-6]. Besides, their endospore which can resist adverse environments and can be stored for a long time are properties of good biopesticides [7].

In some areas of mushroom cultivation in Thailand, a local commercial product PlaikaowTM belonging to *B. subtilis* (produced by the Agricultural Technology Transfer Forum, Kasetsart University, Bangkok and some local well-trained members) is widely used among the well trained-mushroom growers for suppressing the green mold infection [8]. In accordance to the use of several microbes for control of plant diseases, resistance has been reported for those have been used for a long time [9]. Therefore, after the utilization over a long period, the green mold disease may possible develop resistance to PlaikaowTM. Thus, we aimed to search for some new strains of *Bacillus* spp., not only as an alternative to PlaikaowTM but also to provide more options for mushroom growers to choose the appropriate inhibitory microbes to suppress *Trichoderma* sp. infection under different environmental conditions and circumstances.

2. MATERIALS AND METHODS

2.1 Mushroom

Pure cultures of *Lentinus edodes* and *Pleurotus sajor-caju* were obtained from a mushroom farmhouse at Pakchong, Nakorn Ratchasima province and were maintained on potato dextrose agar (PDA) at 4 °C.

2.2 Preparation of Sawdust Spawn

The bag culture of mushroom was performed. The fruiting substrate is composed of 100 kg of Para rubber sawdust, 5 kg of rice bran, 2 kg of Ca (OH)₂ (slaked lime), 1 kg of sucrose, 0.5 kg of CaSO₄ (gypsum), 0.2 kg of Na₂SO₄ with a 60-65% moisture content (all components were purchased from local market). Three hundred grams of the mixed substrates were packed in 6 x 9 inch plastic bags and sterilized by autoclave. The grain spawn (from the Agricultural Technology Transfer Forum, Pak-Chong School, Nakorn Ratchasima Province) was inoculated to the bag and incubated at 25 °C for 7 days.

2.3 Microorganisms

Mushroom green mold disease; Pure cultures of *Trichoderma* sp. was isolated from green mold infected mushrooms by piercing a sterile needle through green mold-infected and non infected mushrooms and isolated as a single colony by streaking on PDA plate. Culturing of *Trichoderma* sp. was performed on PDA plate (pH 7) at 37 °C and was maintained on slant at 4 °C.

Bacteria; All *Bacillus* spp. were isolated from green mold-infected and non infected mushrooms by homogenizing 1 g of surface sterile mushroom in 9 ml of sterile distilled water then boiled in 100 °C 10 mins. A single colony of endospore forming and Gram positive *Bacillus* was obtained by streaking the heated culture on the nutrient agar (NA). The commercial product Plaikaow™ was kindly provided by the Agricultural Technology Transfer Forum, Pak-Chong school, Nakorn Ratchasima province, and was used as a reference isolate for comparing the inhibitory efficiency with the screened *Bacillus* spp. Pure cultures of bacterial isolates were maintained on NA slant at 4 °C.

2.4 Antifungal Assay

2.4.1 Agar diffusion assay; All bacterial isolates were cultured in nutrient broth (NB) at 37 °C (pH 7) with constant shaking at 150 rpm for 36 hrs (late log phase) and bacterial concentrations were adjusted to 10⁶ cell/ml. One centimeter diameter punched-wells were made by cutting the PDA (1.5% agar) plate with a sterile 1 cm diameter cork-borer and a drop of agar was added to the bottom of the well in order to avoid leakage. One hundred microliter of culture broth from each bacterial isolate was filled into each punched-well. All these wells were 1 cm distance apart from the tip of a 3 day-old mycelium which was seeded on the center of a 9 cm diameter Petri dish. Equal volume of NB (without bacteria) served as control. Inhibition zones were measured when mycelium of *Trichoderma* sp. reached the edge of the control well. Five replications were performed for each isolate. Only promising bacteria were selected for further dual culture assay.

2.4.2 Dual culture assay; The active area of *Trichoderma* sp. mycelium (cultured similarly to 2.3) was punched with a 5 mm diameter sterile cork borer and was placed in each well containing 1 ml of PDB of a 24 well-plate. One milliliter of promising bacteria (prepared similarly to 2.4.1) was placed into the same well. The cultures were incubated for 48 hrs at 30 °C (pH 7) on an orbital shaker at 120 rpm. The negative control group was prepared similarly but without the bacterial culture. The mycelial dry weight was measured. The percentage of inhibition for the growth of *Trichoderma* sp. was calculated as described by Nawa [10] (% inhibition = {[A– B]/ A} X 100 When A is dry weight of mycelium in control group; B is dry weight of mycelium in treated group).

2.5 Inhibitory Effect on Hyphal Morphology

The abnormality of *Trichoderma* sp. mycelium treated with *Bacillus* spp. and stained with lactophenol cotton blue was examined under a light microscope.

2.6 Bacterial Species Identification

Bacteria were identified by API 50 CHB test kit, in which each capsule was preloaded with dehydrated substrates. All procedures were performed accordingly to the instruction of Biomerieux Company.

2.7 In vivo Test

This experiment was performed in the mushroom farmhouse in Pak-Chong School. The 24 hr suspension culture of each selected bacterial isolate prepared similarly to 2.4.1 and 1 ml was injected by syringe at the bottom, middle and top of spawn of mushroom cultured in a plastic bag. Two days later, 10^6 spores/ml of green mold was applied. The green mold infected-mushroom was counted twice a week for a period of 6 weeks. Mushrooms treated without microbe was used as a control group. Twelve replications were performed per each experimental group. The percentage of green mold inhibition was calculated as described in 2.4.2; ($\% \text{ inhibition} = \{[A - B] / A\} \times 100$ when $A = \% \text{ of green mold infection in control group}$; $B = \% \text{ of green mold infection in treatment group}$).

2.8 Statistical Analysis

Results from experiments 2.4.1, 2.4.2 and 2.7 were analyzed statistically by the analyses of variance (ANOVA) and Tukey's Test at the 5% level.

3. RESULTS AND DISCUSSION

Thirty isolates of *Bacillus* spp. were isolated from green-mold infected and non infected mushrooms. Ten isolates were selected as promising isolates by agar diffusion (Figure 1 and Table 1) and only 5 isolates were considered to be the potential bacteria owing to the great inhibitory effect in the co-inoculation test (Figure 2 and Table 1). They were designated as *B. 01-SU₁*, *B. 02-SU₂*, *B. 05-SU₃*, *B. 07-SU₄* and *B. 09-SU₅*.

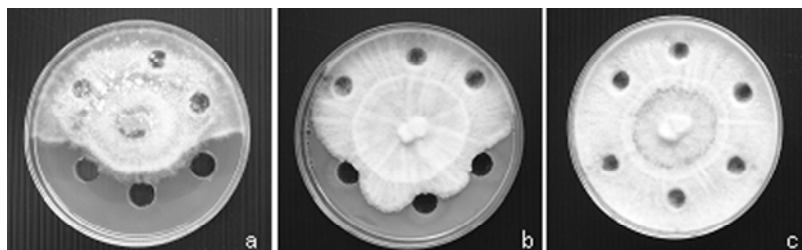


Figure 1 Inhibitory tests using agar diffusion method were demonstrated. The best efficacy, *B. 01-SU₁* (a); moderate, *B. 02-SU₂* (b) and no efficacy *B. 12-SU₁₂* (c)

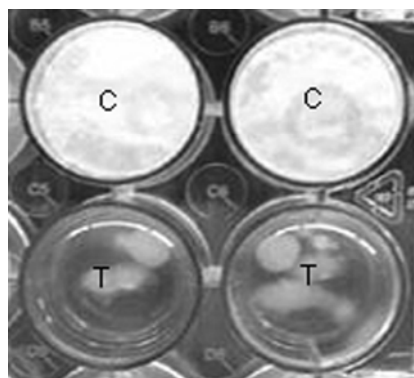


Figure 2 Dual culture between each selected bacterial isolate and the green mold mycelium in 24 well plate. The great inhibitory effect of *B* 01-SU₁ was demonstrated in well “T” comparing with the negative control well in “C”.

Table 1 The inhibitory effect of *Bacillus* spp. against the growth of *Trichoderma* sp. mycelium

Bacteria isolates	Inhibition zone* (agar diffusion test)	% Inhibition** (co-inoculation test)
control	0	0
Plaikaow	+++	78 ± 1.3^c
B01	+++	74 ± 2.4^c
B02	+++	68.6 ± 2.9^{bc}
B03	++	40 ± 3.8^b
B04	+	40 ± 2.6^b
B05	++	58 ± 4.8^b
B06	+	53 ± 2.6^b
B07	+++	65.4 ± 4.9^{bc}
B08	+	37.5 ± 0.5^a
B09	+	65.6 ± 2.4^{bc}
B10	+	40 ± 3.7^a

* + = clear zone less than 1 mm.

++ = clear zone between 1-5 mm.

+++ = clear zone more than 5 mm.

** Means of percent inhibition significantly different ($P \leq 0.05$) were indicated by different lower cases.

Table 1 revealed that the inhibitory effects tested by 2 different methods were almost similar except the isolates B05 and B09. Isolate B05 showed higher efficiency than B09 by agar well diffusion assay but less efficiency by dual culture. This may imply that the inhibitory effect of B05 is based on its excretion while B09 is based on nutrient competition and on excretion. Bacterial isolates from PKTM and B01 showed the highest percentage inhibition ($78 \pm 1.3\%$ and $74 \pm 2.4\%$, respectively) against the mycelium of *Trichoderma* sp. They caused cytolysis to the treated mycelium (Figure 3c). B02-SU₂ and B05-SU₃ caused vacuole formation (Figures 3d and 3e) while B07 SU₄ caused shrinkage in mycelium cell (Figure 3f) and the others such as B09-SU₅ caused a slightly reduction in the size of mycelium (Figure 3g). Results from Table 1 and Figure 3 might be possible to predict that the main mechanism of suppression of PKTM and B01-SU₁

caused by their excretion which might cause pore formation on cell membrane and forced cytoplasm to leak out from the cell. This result agreed with several reports that several species of *Bacillus* can secrete a number of antimicrobial lipopeptide substances, such as iturin, surfactin which can cause pore formation at the cell membrane [11]. For the rest of bacterial isolates, we preliminary predicted that the main mechanism of the control was the competition for environmental nutrients. Normally, those producing antifungal substances should be superior to those acting as parasites or nutrient competitors. However the synergism of multiple mode of actions will provide more capability to suppress the growth of green mold.

Since screening is a critical step for further studies, the drawback of dual culture test is a tedious task, and both tests normally possess high variable effects. To improve such step by labeling the interesting bacteria with some chemicals such as fluorescent may speed up the screening process and also provide more reliable results but high cost is needed [12].

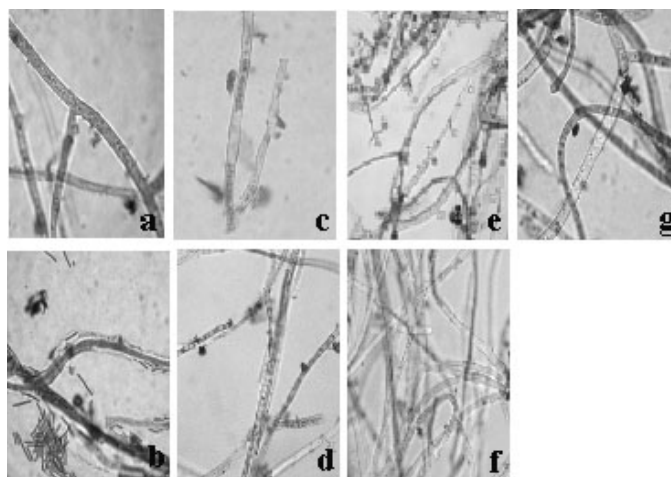


Figure 3 The abnormalities of *Trichoderma* sp. mycelium caused by inhibitory effect; normal mycelium in control group (a) mycelium adhering by bacteria at the beginning of co-inoculation (b), cytolysis, B01-SU₁ (c) vacuole formation, B 02-SU₂ and B05-SU₃ (d, e), slightly shrinkage in cell, B07-SU₄ (f), reduced in mycelium size, B0₃-SU₃, B04-SU₄ B06-SU₆, B08SU₈ and B09-SU₅ (g)

From bacterial species identification, 4 isolates were *B. subtilis* and 1 isolate was *B. licheniformis* (Figure 4). This finding agreed with Wang and Ng [13], who reported that these 2 species were the ubiquitous soil bacteria and they may produce high amount and effective antimicrobial substances.

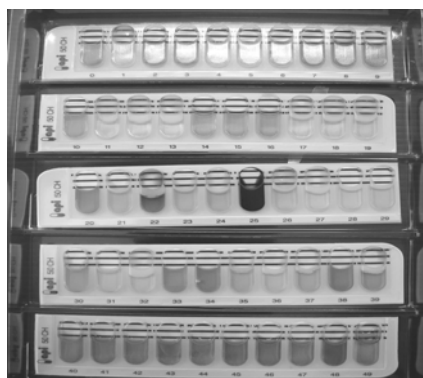


Figure 4 API 50 CHB revealed that four selected bacteria were *B. subtilis* (designated as Bs 02-SU₂, Bs 05-SU₃, Bs 07-SU₄ and Bs 09-SU₅), one was *B. licheniformis* (Bl 01-SU₁)

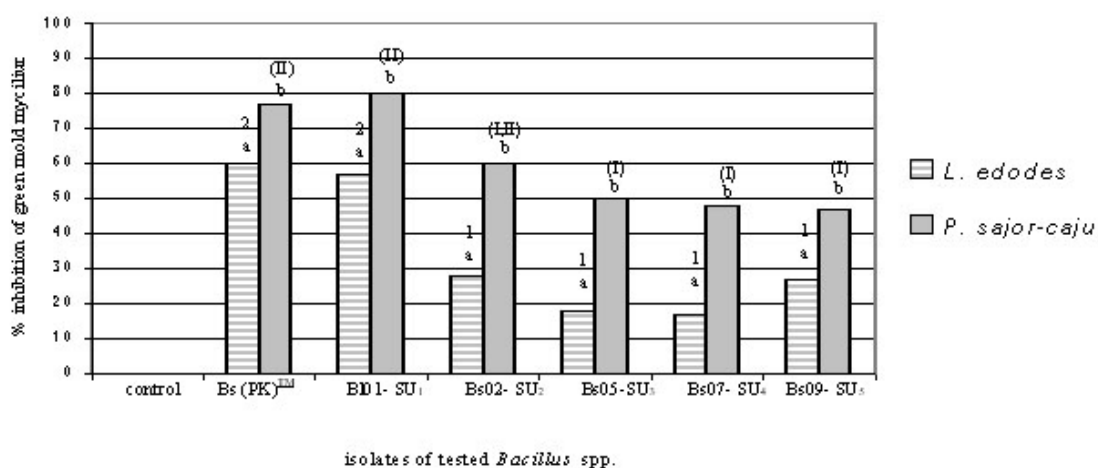


Figure 5 Percentage of inhibition by 5 tested bacterial isolates on *Trichoderma* sp. mycelium growth on *L. edodes* and *P. sajor-caju*

Note: 1) Means of % inhibition by the different bacterial isolates that inhibited the growth of *Trichoderma* sp. mycelium on *L. edodes* were indicated by Arabic numbers

2) Means of % inhibition by the different bacterial isolates that inhibited the growth of *Trichoderma* sp. mycelium on *P. sajor-caju* were indicated by Roman numbers.

3) Means of % inhibition by the different bacterial isolates on the growth of *Trichoderma* sp. mycelium in comparison of *L. edodes* and *P. sajor-caju* was indicated by lower cases.

Results from *in vivo* test in the mushroom farmhouse indicate that Bl 01-SU₁ was the most effective isolate against *Trichoderma* sp. infection on *L. edodes* and *P. sajor-caju* (percentage of inhibition was 58.23 and 80, respectively). Its effectiveness was almost equivalent to *B. subtilis* PKTM (percentage of inhibition on *Le* 60 and *P. sajor-caju* was 78.24, respectively). Percentage of inhibition from Bs02-SU₂ was 28.05 on *Lentinus edodes* and 60 on *P. sajor-caju*. Its effectiveness was higher but not significantly different ($P > 0.05$) from those of

Bs05-SU₃, Bs07-SU₄ and Bs09-SU₅. All tested bacterial isolates could suppress *Trichoderma* sp. mycelium infected on *Le* significantly ($P \leq 0.05$) more than on *P. sajor-caju* as shown in Figure 5. It may be due to the more rapid growth of the mycelium of *P. sajor-caju* than those of *L. edodes*. so *P. sajor-caju* is able to compete with *Trichoderma* sp. mycelium much better than *L. edodes*. Wang and Ng and Chu *et al.* [13-14] reported that *P. sajor-caju* produced antifungal substances like pleurostrin and eryngin which may be involved in suppressing the growth of green mold. However, there are still several further studies to be carried out to confirm the ability of B1 01-SU₁. These include the test for its safety to non target organisms, the test for its consistency effectiveness in various environments, etc. Development of products with appropriate formulations and long self -life is the crucial factors that should be considered.

4. CONCLUSIONS

Currently, the use of inhibitory microbial agent is one of the most possible methods for controlling some plant diseases. In mushroom cultivation, only PlaikaowTM is the only commercial product available for Thai mushroom grower. There are considerable reports of host resistance to pathogens used as biological agents [10] and based on several good characteristics of *Bacillus* spp. as stated previously. Therefore, it is reasonable to collect and screen for more microbes with high activity to suppress the green mold and be affordable for the use as commercial product. We found only 1 promising isolate, B1 01-SU₁, which is different from *B. subtilis* PKTM and showed almost equivalent effectiveness to *B. subtilis* PKTM both *in vitro* and *in vivo*. However, there are still several further studies to be carried out as stated previously. Moreover, breeding for mushroom that can resist *Trichoderma* sp. is one of the interesting aspects [15- 16] that will make mushroom cultivation a success.

5. ACKNOWLEDGEMENTS

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