BIOCONTROL OF GREEN MOULD DISEASE OF OYSTER MUSHROOM (*Pleurotus ostreatus*) USING *Bacillus amyloliquefaciens*

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ABSTRACT
The occurrence of *Trichoderma harzianum* and *T. asperellum* in cultivation of oyster mushroom (*Pleurotus ostreatus*) frequently results in serious crop losses and considerable inhibition of growth of mycelium and fruiting bodies of oyster mushroom thus lowering the yield substantially. *Bacillus amyloliquefaciens* strain isolated from groundnuts proved very effective in antagonizing the oyster mushroom pathogenic *T. harzianum* and *T. asperellum* without having a negative effect on *P. ostreatus* mycelia. *Bacillus amyloliquefaciens* produced diffusible and volatile organic compounds and this strain is a potential biocontrol agent for *Trichoderma* green mould due to the lack of antagonistic activity towards *P. ostreatus* mycelia. However, field studies on this isolate as substrate inoculant in oyster mushroom are required in order to establish its actual performance.
Keywords: *Bacillus amyloliquefaciens*, Green mould, Mushroom, Biocontrol

INTRODUCTION

Trichoderma green mould infection in edible mushrooms has a long history. This green mould disease of cultivated oyster mushroom (*Pleurotus ostreatus*) has been reported in several countries where large scale production of oyster mushroom is practised (Hatvani *et al*., 2008). Oyster mushroom is the third most important commercially grown basidiomycete worldwide and its cultivation has significantly increased in the world during the last few years. Among many pests and diseases in oyster mushroom cultivation and the most serious crop losses are due to *Trichoderma* green mould infections. Previously, the fungus responsible for the green mould disease of *P. ostreatus* was reported to be *T. aggressivum* (Hatvani *et al*., 2007; Komon-Zelazowska *et al*., 2007). However, new species including *T. harzianum* and *T. asperellum* have emerged and reported (Park *et al*., 2006).

Parameters of mushroom cultivation e.g. high carbon and nitrogen, high relative humidity, warm temperatures, and the absence of light during spawn production are also ideal environmental conditions for moulds and these can easily lead to a contamination (Chen and Moy, 2004). In these conditions moulds exhibit fast growth, thus competing for space and nutrients more successfully than the mushrooms (Hatvani *et al*., 2008). Additionally, they are able to produce extracellular enzymes, toxic secondary compounds as well as volatile organic compounds, which can result in a substantial decrease in production or wiping out entire crop. Pathogenic green moulds may colonize the substrate or grow on the surface of the emerging mushrooms, which become severely spotted and often distorted. In serious outbreaks, no fruiting bodies are produced. *Trichoderma* spp. produce whitish mycelia which are not easily distinguished from those of the mushrooms during spawn run, making it difficult to recognize the infection at an early stage (Largeteau-Mamoun *et al*., 2002).

The main symptom of green mould disease is the appearance of greenish mycelium in the compost and the bagging layer or fruiting bodies of *P. ostreatus* at 2–5 weeks after the beginning of production cycle. The pathogen inhibits the growth of mushroom and in severe outbreaks the fruiting bodies are not produced. This severely affects the markets of the mushrooms as most of the oyster mushroom farms are affected by the *Trichoderma* green mould problem. Although the first flush of the production can be saved with very strict hygiene, *Trichoderma* green mould often reduces the yield of the second flush by 20-30% (Nagy, 2012). Prevention has therefore, to play a central role in green mould management. However, if the infection has already occurred at a farm, it has to be controlled.

Biological control offers vital alternatives to synthetic chemicals. It has proved to be economical, environmental-friendly and an alternative to chemical fungicides for managing oyster mushroom diseases and contamination. *Bacillus* is a genus of gram positive and rod shaped bacteria and are capable of forming stable dormant structures called endospores in nutrient void and stressful environmental conditions. Their spores are generally viable for a long period even under harsh conditions. The sporulation ability and easy cultivation of *Bacillus* species are attractive for their practical use as inoculants (Ross *et al*., 2001; Tiago *et al*., 2004).

There have been no reports on the use of microorganisms for the biological control of mushroom green mold. It is considered that effective biological control of green mold relies both on the selection of antagonists that act specifically against the pathogens which cause the green mold and that these same antagonists have no inhibition on the mycelial growth and fruiting bodies formation of mushrooms. The use of bacteria like *Bacillus* spp., have been investigated because of their ability to produce antifungal metabolites that protect plants from fungal infection (Moita *et al*., 2005; Siddiqui *et al*., 2005); Nourozian *et al*., 2006).

Microorganisms produce compounds that have high specificity against target plant pathogens, easy degradability and low cost for mass production. *Bacillus* spp. are widely distributed in soils and substrates, have high thermal tolerance, rapid growth in media culture and readily form resistant spores. They are considered safe biological agents and their potency is high (Kim *et al*., 2003; Saharan and Nehra, 2011). This study was therefore conducted to select prospective antagonists for the biological control of green mold of mushrooms caused by *Trichoderma* spp. It explored the potential of *B. amyloliquefaciens* as a biological control agent of green mold of oyster mushrooms.

MATERIALS AND METHODS

Bacterial and Fungal Isolate

The *B. amyloliquefaciens* was isolated from healthy groundnuts and identified according to the morphological, biochemical and physiological tests as described by Collee *et al*. (1996). *Trichoderma* fungal isolates were isolated from the spent mushroom substrate and from infected wheat grain spawn. These included *T. harzianum,* and *T. asperellum*. The isolates were characterized and identified based on their colonial morphology and microscopic characteristics using different identification keys and methods (Eastburn and Butler, 1988). The bacterial isolate was maintained on nutrient agar (NA) slants while fungal pathogens were maintained on potato dextrose agar (PDA) slants. Slant cultures were stored at 4°C in the refrigerator until use.

Antagonistic Effect of *B. amyloliquefaciens* Isolate (*In Vitro*)

Bacillus amyloliquefaciens isolate was used in *in vitro* sensitivity tests against the fungal isolates. Potato dextrose agar plates were inoculated with antagonistic isolate of *B. amyloliquefaciens* as two streak lines with a loop-full of 2 days-old culture on the periphery of the petri plate. The plates were doubly inoculated with 5-mm diameter mycelial disc of an actively growing culture of the pathogen placed 5 cm opposite to the other edge of the petri plate and incubated at 25 °C for 7 days (Plate 1).

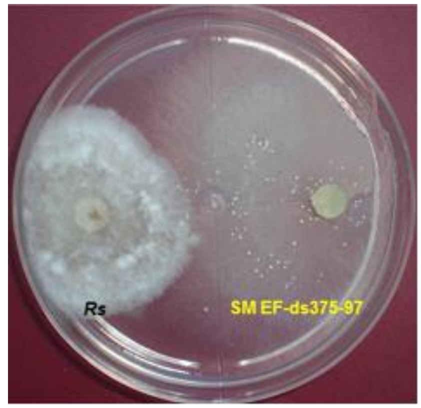

Plate 1: Dual culture interaction between *B. amyloliquefaciens* and *T. harzianum*

Plates with *B. amyloliquefaciens* and the respective pathogens alone were used as checks. Inhibition zones (the distance between the edge of antagonistic bacterial growth and the edge of tested fungal isolates) were measured. The percentage inhibition of the growth of the pathogen was calculated using the formula:

L = (C-T)/CX100, Where:
L = inhibition of radial mycelial growth,
C = radial growth of pathogen in control,
T = radial growth measurement of pathogen in the presence of antagonist.

Radial growth of pathogen was recorded and percentage inhibition calculated in relation with control according to Hajieghrari *et al*. (2008). The experiment was done twice and each test was done in five replicates. The percentage inhibition, L, was categorized on a growth inhibition category, GIC scale from 0-4, Where:

0 = no growth inhibition,
1 = 1 - 25% growth inhibition,
2 = 26 - 50% growth inhibition,
3 = 51 - 75% growth inhibition
4 = 76 - 100% growth inhibition

Mycelial growth of the pathogen was measured and observations were recorded on formation of inhibition zone, over growth and lysis of pathogen mycelium. The data obtained were statistically analyzed using the Statistical Analysis System (SAS).

Production of Volatile Antifungal Metabolites

Production of volatile metabolites by *B. amyloliquefaciens*, having antagonistic activity against *Trichoderma* pathogens was tested by paired plate technique as described by Fiddaman and

Rossall (1993) with some slight modifications. A petri dish containing PDA medium was streak inoculated with a loopful of 48 hours old *B. amyloliquefaciens* isolate. The top of the petri dish containing PDA was inoculated with a 5 mm plug of the actively growing *T. harzianum* and another set with *T. asperellum* separately, at the centre. Both half plates were sealed together and the paired plates were incubated at 25º C for seven days. This incubation ensured that both organisms were growing in the same culture conditions although they were physically separated. Control set of paired plates was designed with only the test pathogens on PDA half plate inverted over unstreaked PDA half plate. The experiment was repeated twice and conducted in five replicates. After incubation period, the paired plates were observed for inhibition of fungal growth as compared to the control. The radial growth diameter of the pathogens was measured and compared with the control set. Percentage inhibition of radial growth of the pathogens was calculated as mentioned before.

Slide Cultures

For slide cultures, a clean slide was placed on an L-shaped glass rod in a 9 cm diameter petri dish and autoclaved. Then a small amount of molten PDA was poured and evenly spread over the slide to make a thin agar film. One end of the slide was kept free of the medium to facilitate handling. Inocula from each *B. amyloliquefaciens* or *Trichoderma* isolates were placed separately on the slide 1 cm apart from each other. Two ml of sterile water was added to the petri dish to prevent drying, and the slide incubated at 25ºC for 3-5 days. *Trichoderma* species alone were used as controls. At the end of incubation period, regions where the *B. amyloliquefaciens* met the hyphae of the pathogen were observed under a light microscope for the presence of coil formation and penetration structures, or wall disintegration.

RESULTS

Effect of *B. amyloliquefaciens* on the Growth pf *Trichoderma* Pathogens *in Vitro*

Bacillus amyloliquefaciens grew faster than *T. harzianum* and *T. asperellum* on PDA media under the same culture conditions. *B. amyloliquefaciens* grew in all possible directions and came into contact with the pathogenic fungi on the fifth day after inoculation and started to suppress further growth of the pathogens. No inhibition zone was formed around the contact area between these species (Plate 2). Thus initially, *B. amyloliquefaciens* inhibited *T. harzianum* and *T. asperellum* by competing for space and nutrients. Later the mycelia of *T. harzianum* collapsed and died completely indicating that *B. amyloliquefaciens* produced antibiotics.

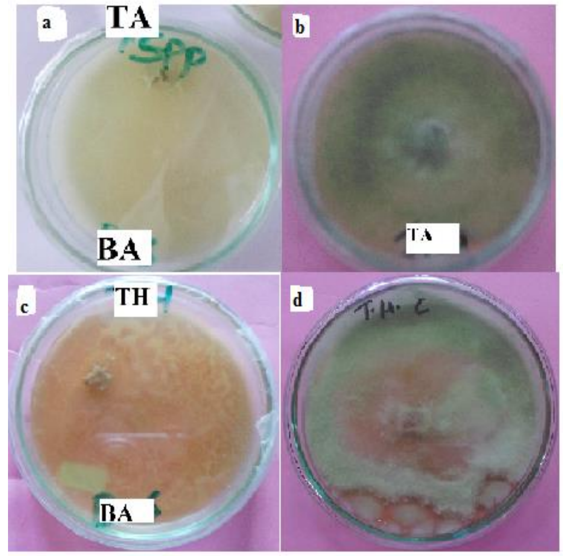

Plate 2: Antagonism of *B. amyloliquefaciens* (BA) against *T. harzianum* (TH) and *T. asperellum* (TA); a and c are treated; b and d are controls

Production of Volatile Compound by *B. amyloliquefaciens* Against *Trichoderma* species

The antagonistic potential was noted to vary through volatile metabolites produced by *B. amyloliquefaciens*, and direct parasitism on the two pathogens. In addition, a change in mycelia colour which was different from the mycelium colour of the control, close to the colony was observed. A stronger antibiosis mechanism of antagonistic *B. amyloliquefaciens* and a higher pathogen inhibition through volatile metabolites were noted. Volatile toxic substances produced by antagonists spread easily and inhibit pathogens growth *in vitro* (Plate 3).

The volatile organic compounds (VOCs) produced by *B. amyloliquefaciens* reduced the mycelial growth of *T. harzianum* and *T. asperellum* (Pate 2, 1 and 2) in comparison with the control (Plate 2, 3 and 4). The VOCs decreased the length of fungal mycelia, and colonies seemed to be significantly reduced (P = 0.05). The inhibition of *T. harzianum* and *T. asperellum* by VOCs was about 71 and 72.8% (Figure 1) respectively compared with the control after seven days, suggesting that the bacterial VOCs were unable to completely kill these two pathogens but had a significantly inhibitory effect on fungal mycelia. The colour of the mycelia also changed from green to white indicating that there was no sporulation in the treated plates (Plate 3, 1 and 2).

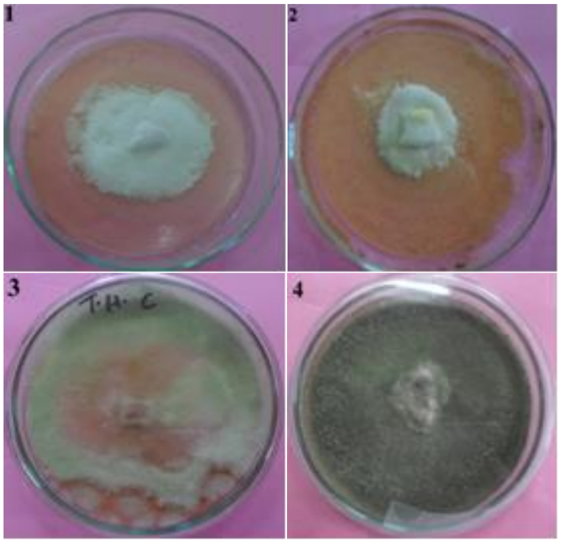

Plate 3: Effects of volatile compounds *of B. amyloliquefaciens*

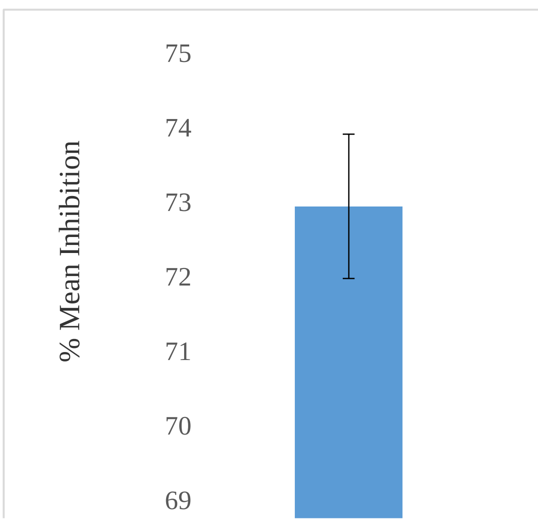

Figure 1: Percentage mean inhibition of *T. harzianum* and *T. asperellum* by volatile compound of *B. amyloliquefaciens*

DISCUSSION

The production of antifungal compounds and siderophores is a primary mechanism in suppressing disease by *Bacillus* spp (Smitha *et al*., 2015). Peptide antibiotics and several other compounds which are toxic to plant pathogens have been recovered from several *Bacillus* strains (Yu *et al*., 2002). Antagonism was evident in petri dishes through the different magnitudes of the *Trichoderma* suppression by the *B. amyloliquefaciens.* The control plates without *B. amyloliquefaciens*, were completely covered by pathogen mycelia showing no fungus growth inhibition. Sporulation was also inhibited completely compared to the control. The mean mycelium growth inhibition on the tested pathogens by this bacterial isolate revealed that the antagonist is highly effective.

A compound microscope was used to make observations and it was clear that the appearance of the mycelium collected from the interface region of *T. harzianum* and *T. asperellum* was modified by *B. amyloliquefaciens*. Some of the modifications included: mycelia colour changing from dark green to white, a coagulation of the fungal cytoplasm which could be observed up to the hypha, presence of small vesicles and the appearance of big vacuoles. In this case, the destructive effect of the *Trichoderma* spp by *B. amyloliquefaciens* was high, resulting in serious damage of the hyphae, associated with a series of degradation events. The mycoparasitic potential of *Bacillus* spp. is well documented (Johri *et al*., 2003; Saharan and Nehra, 2011). Thus, this phenomenon has often been used as a means for *in vitro* screening of biocontrol agents (Jat and Agalave, 2013). Similar conclusions have been reported by El Hassni *et al*. (2007). They reported a modification of the fungal mycelium appearance, due to antifungal secondary metabolite production. Generally, biocontrol capacity through antagonistic bacteria involves either competition or bacterial metabolite production. Bacterial metabolites produced for antagonism towards plant pathogens include siderophores, hydrogen cyanide, antibiotics or extracellular enzymes (Kamilova *et al*., 2005; Sang *et al*., 2006). It has been reported that *Bacillus* spp. contains various biocontrol characteristics including secondary metabolites, the colonizing potential, and the production of competitors (Yoshida *et al*., 2001; Schmidt *et al*., 2004).

According to the observations made in this study, production of diffusible and volatile organic compounds seems to be a primary source of inhibition of the tested fungal pathogens. This agrees with the work done by Prashar *et al.* (2013), who reported that isolate TNAM5 belonging to *Bacillus* spp. was found to be a strong producer of volatile and diffusible antifungal compounds, a character that has been previously established for various strains of *Bacillus* (Wang *et al*., 2007; Dunlap *et al*., 2011).
Nonvolatile antibiotics, including lipopeptides, have strong antifungal activities. However, these nonvolatile antibiotics cannot spread over long distances and only when these antagonists directly colonize the mushroom mycelia they can prevent pathogenic fungi from infecting the mushroom crop. Volatile organic compounds spread over long distances, producing fungistatic microenvironments around the pathogenic communities. In addition, the antifungal VOCs produced by bacteria can kill

surviving spores in the mushroom substrate and limit both the production and the establishment of the green mould disease in spawn preparation and in mushroom houses where mushrooms are growing.

CONCLUSION

The *B. amyloliquefaciens* used in this study is effective in suppressing mushroom pathogenic fungi, including *T. harzianum* and *T. asperellum,* the causative agents of green mould disease of oyster mushrooms. It exhibited broad-spectrum antifungal properties by producing both volatile and nonvolatile organic compounds hence a good potential for biological control of green mould disease. However, a detailed investigation is recommended to evaluate this isolate for its field performance as a biocontrol.

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