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INFLUENCE OF *TRICHODERMA HARZIANUM* METABOLITES ON THE DEVELOPMENT OF GREEN MOULD DISEASE IN THE OYSTER MUSHROOM

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ABSTRACT

Green mould disease in Oyster mushroom (*Pleurotus ostreatus*) caused by *Trichoderma harzianum* results in considerable inhibition of growth of mycelium and fruit bodies of Oyster mushroom lowering the yield substantially. The study examined how mushroom growth is inhibited while *T. harzianum* growth is accelerated. Diseased mushroom bags were collected from mushroom houses within the Kandy district and *T. harzianum* was isolated. The most dominant strain was identified as *T. harzianum* biotype II (Th_2) from the colony and growth characteristics. When the two fungi were grown on dual culture, *T. harzianum* overgrew the colonies of *P. ostreatus* rapidly. Diffusible metabolites produced by *T. harzianum* in culture significantly reduced the growth of *P. ostreatus*. Volatile metabolites of *T. harzianum* slightly stimulated the mycelial growth of *P. ostreatus* initially but *P. ostreatus* soon reverted to its normal growth. Growth of *T. harzianum* was not stimulated by *P. ostreatus* metabolites. Understanding of the metabolic interactions between the two organisms may be useful for developing measures that counter the inhibitory effect of *T. harzianum* metabolites on mushroom growth and overcome green mould growth.

Key words: Oyster mushroom, green mould disease, *Pleurotus ostreatus, Trichoderma harzianum*

INTRODUCTION

Green mould disease in mushrooms incited by *Trichoderma spp*., first reported by Beach (1937), was long considered to be of minor importance to the mushroom industry (Sharma *et al.,* 1999). However, different levels of losses in *Agaricus bisporus,* up to 80% in some occasions, have been reported from various parts of the world, Ireland (Seaby, 1996*b*), England, Scotland (Staunton, 1987), North America, Germany, Belgium, Japan, India (Seaby, 1987), South America, Canada and Australia (Goltapeh and Danesh, 2000). In late 1985, green mould disease caused by *T. harzianum* reached epidemic proportions in the Northern Ireland mushroom industry (Seaby, 1996*b*). The green mould disease in Sri Lankan Oyster mushroom (*Pleurotus ostreatus*) is caused by *T. harzianum* (Wickramasinghe *et al.* 1998).

The main symptom of green mould disease is the appearance of greenish mycelium in the compost, bagging layer or fruiting bodies of *P. ostreatus*, 2–5 weeks after the beginning of production cycle. The pathogen inhibits the growth of mushroom and in severe outbreaks the fruit bodies are not produced. Even if mushrooms do appear in diseased bags, they are not desirable for marketing.

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Comparison of *T. harzianum* isolates collected from diseased mushrooms in Northern Ireland revealed the occurrence of three biotypes of *T. harzianum*, biotype I (Th_1), biotype II (Th_2) and biotype III (Th_3) which varied in growth rate and the form and timing of sporulation (Seaby, 1996*a*). Th_1 and Th_2 are commonly found in compost raw materials but rarely cause problems during mushroom production.

The most common mushrooms cultivated in Sri Lanka are *Pleurotus* species (Oyster mushroom), *Volvariella volvacea* (Paddy straw mushroom), *Agaricus bisporus* (Button mushroom). Among these, cultivation of Oyster mushroom has become a popular cottage industry. Pests and diseases are a major constraint to the Oyster mushroom cultivation in Sri Lanka. The green mould accounts for over 20% yield loss in Oyster mushroom cultivation in Sri Lanka (Wickramasinghe *et al.,* 1998). In addition, deformations of mushroom heads have frequently been reported (Illankoon and Wijesekara, 2002). Yield losses due to green mould in Oyster mushroom industry continue to occur in Sri Lanka as there are no measures taken to control the disease.

The objective of this study was to establish the dominant strain/s of *T. harzianum* causing green mould disease in Sri Lanka and examine whether the growth of the mushroom is affected by the metabolites produced by *T. harzianum*. Understanding of metabolic interactions between the two organisms was thought to be useful in developing measures to overcome *T. harzianum* colonization of mushroom perhaps through nullifying the effects of such metabolites.

MATERIALS AND METHODS

The dominant biotype of *T. harzianum* affecting Oyster mushroom in Sri Lanka

Samples of Oyster mushroom bags with green mould growth were collected from different mushroom houses in and around Kandy (Central Province) during the period from November 2001 to August 2002. The sampling included compost bags with and without visual growth of *P. ostreatus* and also compost bags containing or without fruit bodies. In the laboratory, pieces of mycelium taken from green mould affected areas of each sample were placed on 2% Malt Extract Agar (MEA) using a sterilized inoculating needle. The plates were incubated at room temperature (27^{o} C) until fungal growth was visible. The fungi were then sub-cultured on fresh MEA medium for identification and biotype characterization. More than 20 isolates of *Trichoderma* were obtained during the period. Two culture media, Potato Dextrose Agar and 2% Malt Extract Agar (Atlas, 1995) with chloramphenicol and streptomycin were also used for culturing the mushroom and the green mould.

Radial growth of *T. harzianum* isolates

Radial growth rate of *Trichoderma* isolates was determined at two different temperatures, 17 ^{o}C and 27 ^{o}C. Discs of mycelium cut from the margin of an actively growing 2 day old colony of each *Trichoderma* isolate were placed at the edge of MEA plates. Four replicate plates were used per isolate. One set of plates was incubated at room temperature (27 ^{o}C) and the other set at 17 ^{o}C. The margin of each colony was marked with ink 48 hours after incubation and thereafter daily for four consecutive days. At the end of the 4th day the plates were removed and the colony diameter was measured at two locations right angle to each other and the average diameter was calculated for each day.

Effect of diffusible metabolites produced by *T. harzianum* and *P. ostreatus* on each other's growth

To assess the effect of diffusible secondary metabolites released by *T. harzianum* on *P. ostreatus*, first *T. harzianum* was cultured on 2% MEA medium overlaid with a sterilized cellophane membrane (Dennis and Webster,1971). A mycelial disc (4 mm diameter) cut from the margin of an actively growing *T. harzianum* colony was placed on the centre of cellophane laid on MEA medium of one set of three plates. The other set of three plates had cellophane membrane overlaid but was without *T. harzianum*. After incubating the plates at 25 ^{o}C in dark for 2 days, the cellophane membranes in all plates were removed and a disc (4 mm diameter) of mycelia cut from the margin of a 7 day old *P. ostreatus* culture was placed at the center of each plate. The plates were incubated at 25 ^{o}C for 7 days and the diameter of the *P. ostreatus* colony in each plate was measured. The experiment was repeated twice.

To observe the effect of diffusible metabolites of *P. ostreatus* on *T. harzianum*, the same experiment was carried out using MEA medium by placing discs of mycelia of *P. ostreatus* on the cellophane membrane laid over MEA. Control plates contained no *P. ostreatus* on cellophane. Following two days of incubation, the cellophanes were removed and the medium was inoculated with *T. harzianum*.

The effect of volatile metabolites released by *T. harzianum* on the growth of *P. ostreatus*

A set of three MEA (2%) plates was inoculated with discs (4 mm diameter) of mycelia cut from the margin of actively growing 2 day old *T. harzianum* culture. After 2 days of incubation, when the mycelium has attained some growth, the lids of each plate were removed and replaced with the bottom half of plates containing 7 day old *P. ostreatus* colony grown on 2% MEA (Dennis and Webster, 1971). In the controls, the lids of 2% MEA plates were removed and replaced with the bottom half of the plates containing 7 day old colony of *P. ostreatus* grown on 2% MEA. The two Petri plate halves were held together by an adhesive tape. All the plates were incubated at 27 ^{o}C for 7 days and the diameter of *P. ostreatus* colony in each plate was measured daily for 10 days. Percent inhibition/acceleration of *P. ostreatus* growth by volatiles of *T. harzianum* was calculated by first taking the difference in average diameter of *P. ostreatus* colony between the treated and control plates and multiplying by 100.

To examine the effect of *P. ostreatus* volatiles on *T. harzianum,* first *P. ostreatus* cultures were grown on 2% MEA and after 7 days of incubation at 27 ^{o}C, the lid of each pate was removed and replaced with a bottom half of another plate containing *T. harzianum* colony grown on 2% MEA. The two halves were held together by adhesive tape. The lids of 2% fresh MEA plates replaced with a bottom half of plates containing *T. harzianum* colony on 2% MEA were taken as controls. All the plates were incubated at 27 ^{o}C for 7 days and the diameter of *T. harzianum* colony in each plate was measured daily for 10 days. Percent inhibition/acceleration of *T. harzianum* growth by volatiles of *P. ostreatus* was calculated by first taking the difference in average diameter of *T. harzianum* colony between the treated and control plates and multiplying by 100.

Possibility of direct inhibition of *P. ostreatus* by *T. harzianum*

P. ostreatus was grown on 2% MEA at 25 ^{o}C. After 3-4 days, a disc (4 mm diameter) of mycelium cut from the growing edge of 3 days old *T. harzianum* culture was placed on each agar plate opposite to the *P. ostreatus* mycelial disc. Discs of mycelia of *T. harzianum* and *P. ostreatus* placed alone at the centre of a set of 2% MEA plates were used as controls. Four replicate plates were prepared and all the plates were incubated at 25 ^{o}C. The diameter of each colony was measured daily for 8 days. Percent inhibition of the two fungi was calculated separately using the equation,

$$\% \text{ Inhibition} = \frac{D_1 - D_2}{D_2} \times 100$$

D_1 = Colony diameter in the control
D_2 = Colony diameter in treated

In another study 4 mm diameter mycelial discs cut from a 3 days old *T. harzianum* culture were placed over a 7 days old *P. ostreatus* culture on MEA medium without disturbing the *P. ostreatus* colony. The changes in the appearance of *P. ostreatus* colony were recorded for a period of 10 days.

Data analysis

Data were statistically analyzed using *t*-test at 95% confidence limit using MINITAB Release 14 statistical software.

RESULTS

Seventeen fungal isolates were obtained from diseased mushroom bags collected within the Kandy district and all 17 were identified as *T. harzianum*. The fungus was found growing mainly in the compost and occasionally colonizing the fruit bodies.

All *T. harzianum* isolates grew rapidly on MEA, at an average rate of 0.90 – 1.06 mm/h at 27 ^{o}C. The growth of *T. harzianum* was slower at 17 ^{o}C averaging 0.511$\pm$ 0.057 mm/h. Average growth rate ratio between the two temperatures (27/17 ^{o}C) was 2.069 $\pm$ 0.116. The colonies had a smooth edge and aerial mycelium was initially thick and whitish. From the fourth day onwards when sporulation began, the colony turned green colour and the periphery of the colonies remained white. The conidia were small (3.0 – 3.5 µ), round, smooth and thin-walled.

T. harzianum Th_2 appears to produce extra cellular, diffusible metabolites, which could suppress the growth of *P. ostreatus* (Figure 2 and 4). The presence of these metabolites resulted in a significantly (P = 0.05) reduced the growth of *P. ostreatus*, notably during the first 72 h after introduction of *P. ostreatus* when the highest inhibition (over 90%) was recorded. The extent to which *P. ostreatus* was inhibited was around 90% during the period of incubation. Results also indicated that the growth rate of *T. harzianum* Th_2 was not adversely affected by the diffusible metabolites produced by *P. ostreatus* but slightly stimulated at times, 9.0 % and 4.0 % on the second and third day respectively (Figure 3). However, the stimulation of *T. harzianum* growth was not significant (p = 0.05) compared to the control.

Volatile metabolites

The effect of volatile metabolites produced by *T. harzianum* Th_2 and *P. ostreatus* was studied and the results are summarized in the Figure 5 and 6. The volatile metabolites of *T. harzianum* Th_2 did not affect the growth of *P. ostreatus* adversely (Figure 5). Instead they were slightly stimulatory. The stimulatory effect on *P. ostreatus* was, however, not significant (P = 0.05).

The effect of *P. ostreatus* volatile metabolites on the growth of *T. harzianum* Th_2 was similar to that of *T. harzianum* on *P. ostreatus.*

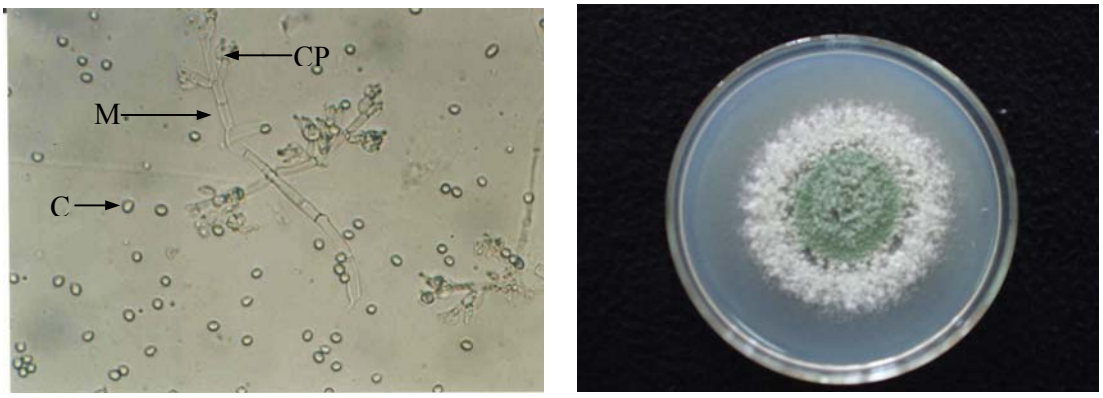

Figure 1. Mycelium (M), conidia(C), conidiophore(CP) (10 × 45) (Left) and a (3 days old) colony of *T. harzianum* on MEA (Right).

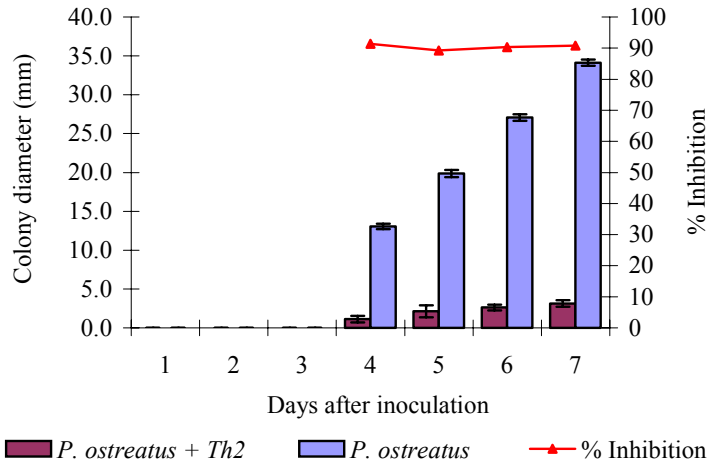

Figure 2. The effect of diffusible metabolites released by *T. harzianum* Th_2 on the growth of *P. ostreatus* (Bars indicate the Standard Error of the mean of three replicates).

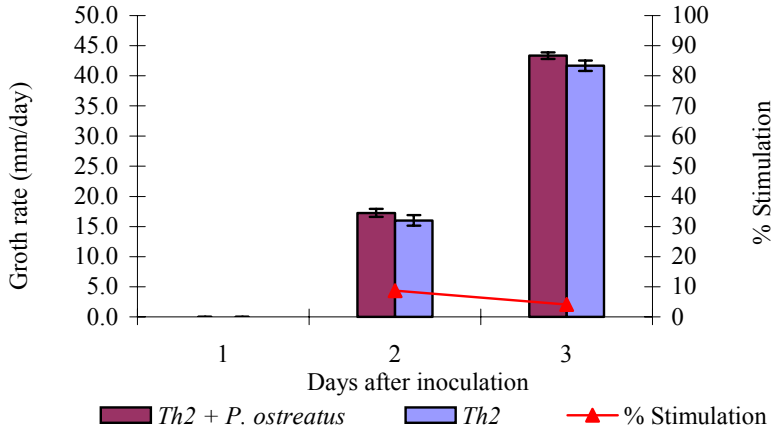

Figure 3. The effect of diffusible metabolites released by *P. ostreatus* on the growth rate of *T. harzianum* (Bars indicate the Standard Error of the mean of three replicates).

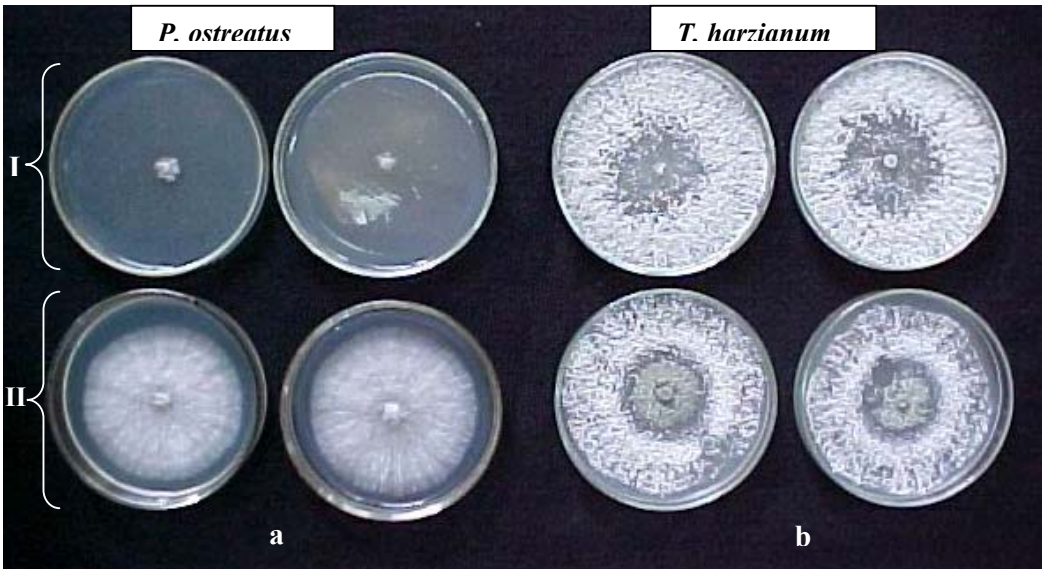

Figure 4. (a) *P. ostreatus* growth on diffusible metabolites of *T. harzianum* (b) and the *T. harzianum* growth on diffusible metabolites of *P. ostreatus*, five days after incubation (I –Treated, II– Control).

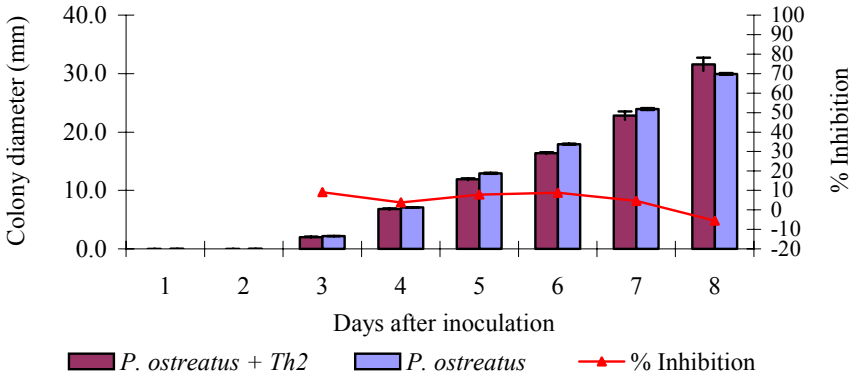

Figure 5. The effect of volatile metabolites produced by Th_2 on the growth of *P. ostreatus.* Bars indicate the Standard Error of the mean of three replicates.

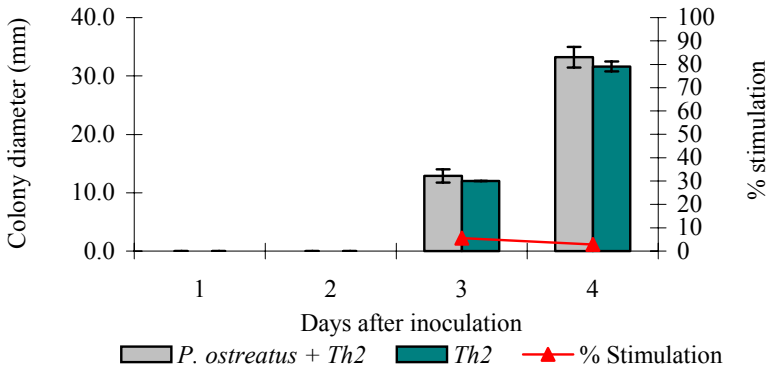

Figure 6. The effect of volatile metabolites of *P. ostreatus* on the growth of *T. harzianum* Th_2. Bars indicate the Standard Error of the mean of three replicates.

Inhibitory effect of *T. harzianum* against *P. ostreatus* on dual culture

P. ostreatus and *T. harzianum*, when cultured singly on MEA grew rapidly covering the entire agar surface. However, on a dual culture, *P. ostreatus* grew slower than *T. harzianum* and the area occupied by the colony was less than 50%. There was more *T. harzianum* growth covering a larger area on the plate and the colony extended its growth over the *P. ostreatus* colony within a period of four days. Green colour mycelial and conidia masses were observed on the *P. ostreatus* colony (Figure 7). There was no zone of inhibition produced in between the colonies of *T. harzianum* and *P. ostreatus*. When the disc of mycelium of *T. harzianum* was placed touching on a fully-grown *P. ostreatus* culture, a burned patch was observed on the surface of the *P. ostreatus* colony after two days (Figure 8).

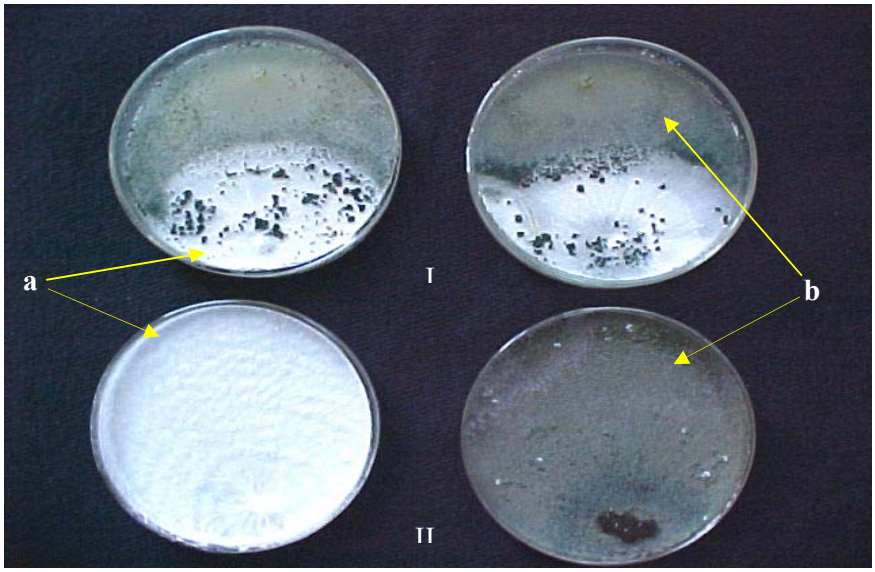

Figure 7. Colonies of *P. ostreatus* (a) and *T. harzianum* (b) grown alone (II) and together (I) in dual culture on MEA. Bars indicate the Standard Error of the mean of three replicates.

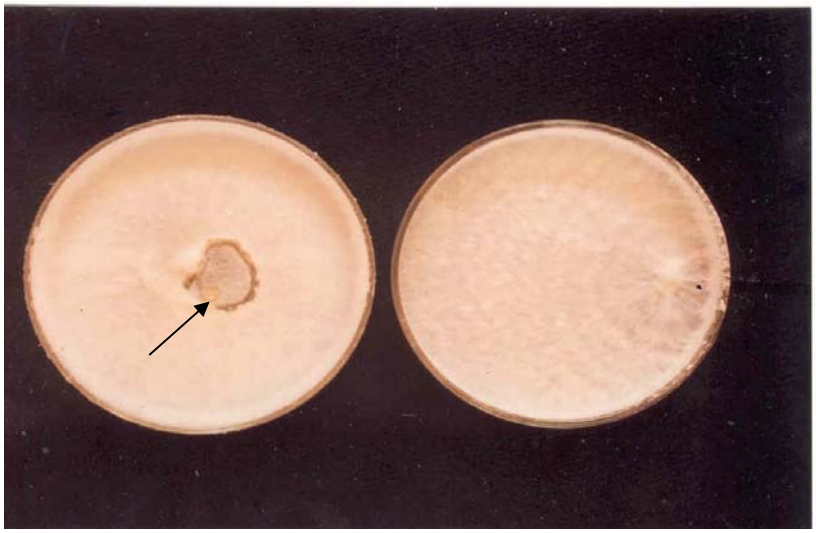

Figure 8. *P. ostreatus* culture showing a `burnt' patch (arrow) after placing a mycelial disc of *T. harzianum*.

DISCUSSION

For identification of *Trichoderma*, the morphological characters such as size and shape of spores, phialides and phialide grouping have a moderate use. Spore characteristics, when used as a main diagnostic feature for *T. harzianum,* are not sufficiently consistent, because *T. viride* as well as *T. citrinoviride* display both obovoid as well as round spores. However, different spore forms are not reflected in different DNA patterns (Seaby, 1996*a*). Among the colony characters, time taken for the appearance of spores is the main character for identification of biotypes of *T. harzianum* (Seaby, 1989). The *T. harzianum* isolates obtained in the present study sporulated within four days. Their growth rate on MEA was comparable with the growth rate of *T. harzianum* Th_2 recorded by Seaby (1996*a*). The growth rate ratio taken at two temperatures, 27 ^{o}C and 17 ^{o}C for the 17 *T. harzianum* isolates is also closer to growth rate ratio reported by Seaby (1996*a*) for *T. harzianum* Th_2. These suggest that the biotype found in this study is *T. harzianum* biotype II (Th_2). The fungus was predominantly found in compost bags during this study except in one occasion where the fungus was found on the fruiting body of *P. ostreatus*.

T. harzianum Th_2 successfully grew on a cellophane membrane-lined MEA medium and *P. ostreatus*, when introduced on to the same agar medium after removing the cellophane membrane with the colony, grew 90% slower than the controls. Certain diffusible metabolites released by *T. harzianum* Th_2 may have caused this inhibition while the growth of *T. harzianum* may also have resulted in depletion of to acertain degree nutrients and this may have reduced the growth of *P. ostreatus*. *Trichoderma* species produce both volatile and non-volatile metabolites that adversely affect growth of different fungi (Mumpuni *et al.*, 1998). Moreover *T. harzianum* Th_2 is capable of producing antibiotics and compete for substratum (Goltapeh and Danesh, 2000). When the two fungi were grown opposite to each other on the same agar plate there was no clear zone observed in between the two colony fronts showing that none of them produced antibiotic-like substances that directly inhibit the other one. Different *Trichoderma* species are known to secrete enzymes such as chitinases, β–glucanase and cellulases, which hydrolyze fungal cell walls and play a role in the mycoparasitism of the fungus (Goltapeh and Danesh, 2000). Two *T. harzianum* biotypes Th_1 and Th_2 that have been found associated with the green mould disease in button mushroom (*Agaricus bisporus*) have been observed to cease *A. bisporus* growth in mushroom bags. Of the two isolates, Th_2 significantly reduced the growth of *A. bisporus* (Mumpuni *et al.*, 1998). The diffusible metabolites of *P. ostreatus* on the other hand were stimulatory to *T. harzianum* Th_2, however, this effect was not significant.

The present study also revealed that the volatile metabolites produced by *T. harzianum* had no significant adverse effect on *P. ostreatus*. The growth of *P. ostreatus* was slightly reduced initially by the volatile metabolites of *T. harzianum*. However, after the initial slow down *P. ostreatus* recovered quickly and grew covering the entire plate within 8 days. These results are also in agreement with the earlier findings on *T. harzianum* and *A. bisporus* interactions (Goltapeh and Danesh, 2000) where the volatile metabolites did not cause a permanent inhibition. It has also been reported that antagonism between mushroom and *Trichoderma spp*. varied with nutritional conditions (Thomas *et al.*, 1984).

The growth of *P. ostreatus* was much slower on MEA compared to *T. harzianum*. Often *T. harzianum* overgrew *P. ostreatus* colonies. Similar observations were made by Goltapeh and Danesh (2000) when *Agaricus bisporus* and *T. harzianum* were cultured together. Similarly on compost bags *T. harzianum* colonized fruiting bodies of *P. ostreatus*. It appears that the diffusible metabolites produced by *P. ostreatus* promoted the growth of *T. harzianum* Th_2 while the inhibitory effect of *T. harzianum* Th_2 metabolites simultaneously checked the growth of *P. ostreatus* allowing rapid colonization of the green mould. This relationship holds until *T. harzianum* Th_2 colonization of compost reaches a certain level and nutrients become limited and at that time a change in the competitive balance occurs in favor of Th_2. The growth of myceliim, production of fruiting structures by *P. ostreatus* and their development are suppressed as a consequence. The relative ability of three biotypes of *T. harzianum* Th_1, Th_2 and Th_3 to colonize compost and *A. bisporus* and to compete with *A. bisporus* and influence *A. bisporus* growth was also associated with the nature and production of secondary metabolites (Mumpuni *et al*., 1998). The understanding of secondary metabolites involved in interaction between the two organisms could be utilized in developing counter measures for the inhibitory effect of *T. harzianum* metabolites on mushroom growth and thereby overcome the green mould disease.

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