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Evaluation of epidemiological factors and mushroom substrate characteristics influencing the occurrence and development of *Trichoderma* green mold

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ABSTRACT: One of the major diseases currently impacting cultivated *Agaricus bisporus* mushroom production worldwide is *Trichoderma* green mold, caused by the fungus, *Trichoderma harzianum.* The epidemiology of this fungal disease is poorly understood and controversial. Based on the results of an on-farm survey the spawn grain, flies and compost conditions were identified as important components of the *Trichoderma* disease complex. This research studied several different parameters that influenced *Trichoderma* competitiveness with *Agaricus.* Results suggested that *T. harzianum* growth and disease development is influenced by spore concentration and compost moisture. Different fungicide-treated spawn and spawn-run compost as spawn was determined to be effective in delaying disease development. Different spore infested materials were removed after Phase II, *T. harzianum* failed to survive the Phase II temperatures and *T. harzianum* was killed soon after the start of Phase II composting.

1 INTRODUCTION

One of the worst hazards of mushroom growing are the diseases which growers think can strike without warning and must come from some mysterious and undetectable source. Their sudden and devastating emergence provides weight to the mystery surrounding them. Such is not usually the situation, though now and then a disease appears to be unique in a certain farm or a particular location. The recent outbreak in Pennsylvania of *Trichoderma harzianum,* biotype Th4, green mold has caused some growers to feel they have been cursed with a new plague and in fact this disease has impacted worldwide production of the cultivated mushroom, *Agaricus bisporus* (Lange) Imbach.

T. harzianum green mold colonizes mushroom compost, competes with *A. bisporus* mycelium for space and nutrients, and results in large areas of the growing beds that do not produce mushroom fruiting bodies. Yield losses from this disease can be catastrophic and therefore, it is essential to develop an understanding of the epidemiology of *T. harzianum* green mold disease. Unfortunately, the epidemiology of this fungal disease is poorly understood and controversial (Grogan, et al., 1995, Seaby 1996, Rinker, 1997). Worldwide, growers and researchers had a wide range of opinions on how to eliminate the green mold or prevent it from moving around the farm. Growers changed farm sanitation, crop management procedures, Phase II programs, spawning rates, and supplement types in attempt to reduce the incidence and severity of the disease. Farmers in Pennsylvania found that all spawning supplements were equally susceptible to disease development.

Based on the results of the on-farm survey, the spawn grain, flies and compost conditions were identified as important components of the *T. harzianum* disease complex (Beyer et al. 1998). Observations made at farms in Pennsylvania suggest that composting (Phase I and/or Phase II), procedures were often associated with disease development (Beyer, 1996). The occurrence of *T. harzianum* green mold at some farms, even with the increased level of sanitation, appeared related to compost not being nutritionally selective for the mushroom mycelium. Wet

compost, which is poorly aerated during Phase I and Phase II, was often related to increased severity or incidence of disease on the farms. Grogan (1995) reported that some compost factor was involved in disease development. Fletcher, 1997 was the first to report the importance of the spawn grain in disease development.

This project was undertaken to study other compost characteristics that may favor *T. harzianum* in competition with *A. bisporus.* Other objectives were: 1) to determine how different spawning materials influence disease severity and occurrence; 2) to determine if *T. harzianum* survived Phase II composting temperatures.

2 MATERIAL AND METHODS

Throughout the study, isolates of *T. harzianum,* biotype Th4 were stored in liquid nitrogen, subcultured as needed and maintained on sterilized spawn grain one week prior to testing. Subcultures from this isolate were used in all experiments. The standard procedures for the Phase I and II composting at the Mushroom Test Demonstration Facility (MTDF) were used in all experiments (Beyer and Beelman, 1995). Standard spawn growing and cropping procedures at the Mushroom Research Center (MRC) were used in those experiments where spawn run and cropping were needed (Schroeder and Schisler, 1981). The crop numbers referred to in this paper correspond to the numbers assigned each crop at the MTDF or MRC. Unless otherwise noted spawn growing temperatures were maintained between 24-25°C. Air temperature was held at 18°C during cropping.

2.1 *Different concentrations of T. harzianum spore suspensions*

Several techniques were tested to determine an inoculation method necessary to produce individual colonies on the surface of the compost, casing or media. Dry spores blown from a petri dish culture of *T. harzianum* onto media did not produce discrete individual colonies. A petri dish with a 10 d old colony of *T. harzianum* was inverted on top of an uninoculated petri dish and gently tapped to dislodge spores. This technique failed to produce individual colonies of *T. harzianum.* A liquid spore suspension was used to determine the *T. harzianum* spore concentration necessary to achieve single colony growth on agar. Serial dilutions were made to the following concentrations: 1 x 10 , 1 x 10^4, 1 x 10^3, 1 x 10^2, and 1 x 10 . These concentrations, with the addition of a sterile distilled water (SOW) control, were then used for inoculation. Three percent malt agar (DIFCO, Detroit, MI) and Potato Dextrose with Yeast extract Agar (PDYA) plates were inoculated by sterile aspiration with each of the different spore suspensions and the control. The agar surface was held 10 inches from the tip of the aspirator as 1 single spray was placed on each of the plates. The plates were incubated at 24°C and observed for single spore growth for 5 days.

To determine spore concentration for compost trials, standard MTDF compost was spawned with off-white hybrid commercial spawn. Different concentrations of *T. harzianum* spore suspensions were prepared and used as inoculum at the rate of 1 ml per 2.2 kg compost. Concentrations of 0; 1 x 10^7; 5 x 10^7; 1 x 10^8 were used. After inoculation, the tubs were covered with plastic wrap and placed in a 25°C incubator. Observations were made for the incidence of *T. harzianum* green mold every four d for 44 d. Results are reported in Table 1.

2.2 *Different spawn and spawn carrying materials*

To determine the effect of the mycelial carrier (spawn) on the development of disease different materials were used to spawn compost and then inoculated with *T. harzianum.* Treatments consisted of fresh spawn (purchased and used within 7 d); old spawn (stored for 90 d); non-grain spawn (Speed Spawn™ Full House Spawn and Supplement Co., Blandon, PA); Benomyl-gypsum treated fresh spawn (1.34 g/kg of Benomyl and 66.8 g/kg of gypsum); compost spawn (CS), (21 d spawn run compost) were used to spawn standard MTDF compost. The CS rate was 11 kg per 110 kg of compost per tray. A 1 x 10 spore suspension was used in each tray was infested with *T. harzianum* at the rate of 1 ml of inoculum per inoculation site. Each tray was inoculated at 4 different sites. Inoculum was placed directly onto spawn carrying material at two

sites and placed onto the compost 2.5 cm away from spawn carrying material at two other sites. Observations for the incidence and percent infested area continued over the course of the crop and are reported in Table 2.

2.3 *Compost moistures*

MTDF compost after Phase I, was prepared at standard oven-dry moisture content of 72.4% and wet compost was prepared at 75.6%. The composts were weighed in 1-pound allotments and spawned with off-white hybrid at a rate of 3 grams per 2.2 kg wet wt. compost. Compost completely colonized with *A. bisporus* mycelium (with no grain) was used to spawn compost at the rate of 0.1 kg per 1.0 kg wet wt. compost. Sixteen plastic tubs were filled with 2.2 kg wet wt. spawned compost. Five treatments with 4 repetitions each were prepared. Compost was inoculated at the rate of 1 ml per container, using 1x10 spore suspension of *T. harzianum* prepared in a sterile distilled water (SDW)-Tween 80 solution. After inoculation, the tubs were covered with plastic wrap and placed in a 27°C incubator. They were observed for the quality of spawn growth and the incidence of *T. harzianum* growth for 34 d. Results are reported in Table 3.

2.4 *Compost moisture and fungicide-treated spawn*

In the second compost moisture test, MTDF compost after Phase I, at filling, had a oven-dry moisture content of 72.3% and wet compost was prepared at 74.5%. All compost was conditioned during Phase II, however the wet compost was more difficult to clear ammonia and Phase II was extended for an additional 2 days. Before spawning the wet compost had an "off almost sewer-like odor to it. Composts were spawned with 3g of commercial off-white hybrid, either untreated, or treated with Benomyl-gypsum or Mertect fungicides into 2.2 kg wet weight portions. Spawned compost was packed into plastic tubs and infested with 1 ml of an aqueous suspension of *T. harzianum* spores standardized to a concentration of 1×10^7 spores per ml. After infestation, the tubs were covered with plastic and incubated at 27°C. Tubs were observed for the rate of spawn growth and the presence of *T. harzianum* green mold for 33 d. Results are reported in Table 4.

2.5 *Pasteurization survival*

Phase I substrate preparation was followed by an indoor Phase II compost process of seven days. For Phase II the substrate is filled into wooden 1.5 m trays, 25 cm deep and placed into rooms with a computer-controlled environment. Several tests were run to determine the survival rate of *T. harzianum* spores during Phase II. Standard pasteurization at the MRC occurs 48 h after filling, when the compost and air temperatures are raised to 60°C for 2 h. The highest compost temperature reached during pasteurization was 62°C. Before and after pasteurization the substrate temperatures were maintained between 46-57°C.

The fungus *T. harzianum* was inoculated onto sterilized corncobs and rye grains, then incubated at about 27°C for 28 days. In addition, compost infested with *T. harzianum* was removed from a previous crop after harvesting was completed and before post crop steaming. The infested compost, corncobs, and grains were placed separately into compost, after filling. *A. bisporus* spawn was also placed into the compost at this time. In another test, *T. harzianum* inoculated rye grains, corn cobs and *A. bisporus* spawn were placed at different depths into the compost after filling. The three materials were placed on the compost surface; 7.5 cm into the compost and in the center of the substrate, 15 cm into the substrate. At the end of Phase II, in 8 d, the infested materials were removed and plated onto Potato Dextrose with Yeast extract Agar (PDYA) amended with 0.1 g/1 streptomycin sulfate (ICN Biomedicals Inc., Aurora, OH) and incubated for 12 d to ascertain the presence of *Trichoderma, Agaricus* or other microbes.

In a subsequent test to determine if *T. harzianum* spores would survive before and after pasteurization, infested materials were placed into compost at filling. At 24 h, 40 h and 64 h after filling samples of the infested material were removed and plated onto PDYA amended with 0.1 g/1 streptomycin sulfate and incubated for 12 d to ascertain the presence of *Trichoderma, Agaricus* or other microbes. Results are reported in Table 5.

Table 1. *T. harzianum* growth in compost inoculated with different spore concentrations.

Spore concentration	7 d	15 d	17 d	22 d	32 d	44 d
0						
1×10^7		+	+	++	++	+++
5×10^7	+*	+	++	++	++	++
1×10^8	+	+++	+++	+++	+++	+++

The number of "+" indicates the number of repetitions with visible signs of *T. harzianum.*

Table 2. The incidence and isolation of *T. harzianum* Green Mold from compost spawned with different spawning materials.

Treatment Description	Disease incidence of *T. harzianum* (%)[a]				Total surface
	At Case	Break 1	Break 2	Break 3	area infested (%)
Fresh Spawn uninoculated		-	-	-	0%
Fresh Spawn + *T. harzianum*	20%**	20%	100%	100%	68%
Benomyl Spawn + *T. harzianum*		-	50%	50%	9%
Aged Spawn + *T. harzianum*		20%	100%	100%	39%
Non-Grain Spawn + *T. harzianum*		40%	40%	80%	31%
CS + 7*. *harzianum*		-	40%	40%	14%

Tray positive 5 days prior to casing.

[a] Disease incidence is defined as the percentage of the replicates that developed green mold.

Table 3. The incidence of *T. harzianum* from different moisture composts spawned with different spawn materials.

Treatment #	Disease incidence [a]
Control compost: Fresh Spawn + *T. harzianum*	75.0%
Control compost: Benomyl-treated spawn + *T. harzianum*	0.0 %
Wet compost: Fresh spawn + *T. harzianum*	25.0 %
Control compost: CS + *T. harzianum*	0.0 %
Wet compost: CS + *T. harzianum*	10.0 %

[a] Disease incidence is defined as the percentage of the replicates that developed green mold.

3 RESULTS AND DISCUSSION

3.1 *Different concentrations of T. harzianum spore suspensions*

The earliest observation of *T. harzianum* green mold on the compost was at the concentrations 5×10^7 and 1×10^8 after 7 days of incubation (Table 1). By fifteen days of incubation 1×10^7 and 1×10^8 had 2 more replicates with visible signs of the *T. harzianum.* At 17 and 22 days, an additional replicate at 5×10^7 and one more at 1×10^7, respectively, displayed green mold symptoms. The only other visible *T. harzianum* developed after 44 days incubation and in one more replicate at the 1×10^7 spore concentration. Compost samples from randomly selected inoculated tubs that displayed no visible symptoms of *T. harzianum* were plated after 44 days incubation. No additional *T. harzianum* was isolated from any of those samples. The results of this test indicated that the earliest green mold symptoms develop in the compost with the higher spore concentrations. By 21 days incubation, compost with the heaviest and moderate rates of inoculum had substantial *T. harzianum* growth, whereas compost with the lowest rate of inoculum was just beginning to grow. However, disease incidence continued to rise at the lowest inoculum level throughout the test's end.

In another experiment it was demonstrated that spores did not germinate at concentrations less than 1×10^2 (data not shown) Concentrations of 1×10^2 and 1×10^3 had single spore colonies develops days after inoculation. 1×10^4 and 1×10^5 developed single colonies in 2 days after inoculation and by day 3 the plates were fully colonized with *T. harzianum.* The result of this experiment is important to mushroom growers and researchers because if only a few spores are

present < 1 x 10^1 they may not readily germinate. Therefore, it may be suggested that a low spore load in compost may result in slow or no disease development.

3.2 *Different spawn and spawn carrying materials*

Disease developed faster and more severely in those treatments where the spawn carrying material was untreated fresh grain spawn, Table 2. The incidence and severity of the *T. harzianum* was also higher on aged grain spawn and non-grain spawn and in all treatments when inoculum was placed directly on the grain or spawn carrying material. Disease developed slower in the compost with Benomyl-treated spawn and compost spawn (CS) when compared to other spawn materials.

The use of Benomyl as a pretreatment of the spawn, and CS appeared to have the most control on *T. harzianum* green mold growth and development. With these treatments not only is the appearance of the green mold later in the crop, but the severity of the disease is greatly reduced. Benomyl-treated spawn is now registered for the mushroom industry and is widely used to control *Trichoderma* green mold on farms. However, the commercial application of CS by growers is not recommended. The use of aged and non-grain spawn seemed to reduce the amount of infestation when compared to fresh spawn. Although the occurrence of *T .harzianum* was earlier in this treatment, the severity of the disease was less.

These results suggested that non-grain spawn materials, non-grain spawn and CS, are not as susceptible to *T. harzianum* infestation as grain spawn. The reason for this may be that these materials have less readily available carbohydrates that *T. harzianum* seems to prefer. Because the compost is a selective substrate, the spawn grain may provide a readily available source of carbohydrate for the *T. harzianum* to become established in the substrate. Once the *T. harzianum* is established in the compost, it causes problems later in production. Further testing of alternatives to grain spawn is needed to provide a solution should *T. harzianum* develop resistance to Benomyl in the future. The results showed that *A. bisporus* and *T. harzianum* are indeed in competition for food or each other in mushroom compost. The faster growth rate of *T. harzianum* provides it with an advantage in this competition for nutrients. However, when *A. bisporus* successfully colonized compost before *T. harzianum* the incidence and severity of the disease is reduced.

3.3 *Compost moistures*

Spawn growth was heaviest and most uniform in those tubs spawned with the spawn run compost. Spawn growth was slower to develop in the other composts. The surface growth on the wet compost was much weaker and slower. Growth of *T. harzianum* was present in control compost with fresh spawn 75% of the time, Table 3. Wet compost with fresh spawn developed *T. harzianum* after 18 days of incubation in 25% of the replicates and with the Spawned Compost as spawn *T. harzianum* developed only in one small area in one replicate near the end of the experiment. No *T. harzianum* appeared in normal composts containing Benomyl-treated spawn or Spawned Compost used for spawn. In all appearances of green mold, growth was seen first at the edges of the container, then with continued incubation, would spread over the surface area.

3.4 *Compost moisture and fungicide-treated spawn*

Spawn grew more rapidly, more uniformly, and denser in the compost with normal moisture content. In the wetter compost, spawn growth was delayed by approximately 5 days. The two fungicide treatments had no influence on the quality of the spawn growth in the normal compost. However, in the wet compost, Benomyl-treated spawn appeared to cover the surface of the compost in a thicker, more even growth pattern than the untreated or Mertect-treated spawn.

No visible *T. harzianum* developed in either of the two uninoculated controls. In the infested treatments, *T. harzianum* developed faster in all treatments with the control compost than it did in the same treatment in the wet compost. Additionally, the *T. harzianum* covered the compost surface more completely in the control compost. At the end of the experiment 75% of the untreated or Benomyl-treated spawn in wet compost displayed visible *T. harzianum* compared to 50% of the repetitions in their normal compost, Table 4. The Mertect-treated spawn treatments,

both in normal and wet compost, had only 25% of the repetitions per treatment with visible *T. harzianum.*

Development of visible *T. harzianum* in treatments with Benomyl-treated spawn was slower than the untreated control. In the normal compost, 50% of the repetitions of untreated spawn had visible green mold within 11 days of infestation. Fifty percent of the replicates of Benomyl-treated spawn developed *T. harzianum* within 13 d of spawning. In the infested treatments with wet compost, one repetition of Mertect-treated spawn had *T. harzianum* growth developed in 13 d, an additional one at 22 d, and a third at 28 d following infestation. With the Benomyl-gypsum treated spawn, one replicate had visible *T. harzianum* after 15 d, and two more developed it by 20 d after infestation. The severity of the disease, as defined by the extent of surface coverage, was greatest in wet compost with Benomyl-treated spawn. These results suggest less effectiveness of the Benomyl-treated spawn in wet compost.

These results support the theory that the development of *T. harzianum* green mold in commercial mushroom crops is dependent upon the presence of *A. bisporus* spawn. The results also support the theory that when *T. harzianum* is present the healthy development of the spawn may accelerate the development of the disease in normal compost, suggesting that *A. bisporus* is either eliminating an antagonist(s) to *T. harzianum* in compost or *A. bisporus* produces a metabolite(s) that favors *T. harzianum* growth. Although *T. harzianum* was slower to develop in wet

Table 4. Severity and incidence of *T. harzianum* green mold on different compost moisture and fungicide treatments.

Treatment description	Quality of spawn growth [a]	Disease incidence [b]	Severity [c]
Control compost + untreated spawn + uninoculated	3.0	0.0%	0.0%
Control compost + untreated spawn + *T. harzianum*	2.5	50.0%	50.0%
Control compost + Benomyl-spawn + *T. harzianum*	3.0	50.0%	46.3%
Control compost + Mertect spawn + *T. harzianum*	3.0	25.0%	7.5%
Wet compost + untreated spawn + uninoculated	2.0	0.0%	0.0%
Wet compost + untreated spawn + *T. harzianum*	2.0	100.0%	11.3%
Wet compost + Benomyl-spawn + *T. harzianum*	2.5	75.0%	66.3%
Wet compost + Mertect spawn + *T. harzianum*	2.0	25.0%	1.3%

"Quality of Spawn Growth Scale:

0 = No growth; 1 = Growth only at the spawn grains, not spreading over the compost surface; 2 = Thin, stringy mycelial growth, patchy surface coverage; 3 = Moderate mycelial growth with even surface coverage; 4 = Heavy mycelial growth with even surface coverage.

[b] Disease incidence is defined as the percentage of the replicates that developed green mold.

' Severity is defined as surface area of the compost that developed *T. harzianum* growth

Table 5. Summary of all experiments investigating survival of *T. harzianum* during Phase II pasteurization and composting processes.

Treatment	*Agaricus* or *T. harzianum* growth after pasteurization[a]
A. bisporus spawn	0.0
T. harzianum infested grain	0.0
T. harzianum infested compost/casing	0.0
T. harzianum infested compost pre-post crop	0.0
T. harzianum infested corn cobs	0.0
T. harzianum infested grain removed 24h after filling	0.0
T. harzianum infested grain removed 40 h after filling	0.0
T. harzianum infested grain removed 64 h after filling	0.0
T. harzianum infested grain on compost surface	0.0
T. harzianum infested grain 7.5 cm into compost	0.0
T. harzianum infested grain 15.0 cm into compost	0.0

[a]% fungal growth detected in 12 d

[b]% spore germination in 12 d

compost, the disease incidence was higher in wet compost when compared to the control compost.

The consistency of disease incidence and severity on Benomyl-treated spawn varied between these two reported experiments. This difference demonstrates some of the inconsistencies these researchers have encountered working with a complex system such as compost and with the pathogen, *T. harzianum.*

3.5 *Pasteurization survival*

The fungus *T. harzianum* failed to survive the Phase II temperatures in all tests. The results of the first experiment showed that neither *T. harzianum* nor *A. bisporus* survived the Phase II composting process. *T. harzianum* and *A. bisporus* could not be isolated from the infested grains, corncobs, substrate and casing, Table 5.

In the next experiment, infested grain was removed from the Phase II compost at 24 h, 40, and 64 h after filling. The first two extracts were before pasteurization and the last extraction after pasteurization. The results show that *T. harzianum* could not be isolated from infested grain at any of the treatment times. These results suggest that *T. harzianum* spores are killed soon after filling. It was not determined whether the temperatures or ammonia levels before pasteurization were effective in killing the spores.

In the next experiment, infested materials were placed at different depths within the substrate filled trays. After a standard Phase II and pasteurization, *T. harzianum* could not be isolated from any of the materials at the different depths. The results of all these tests demonstrate that the spores of *T. harzianum* do not survive pasteurization temperatures above 60°C or the Phase II process and that the compost must be infested with the fungus sometime during cooldown, spawning or later.

4 SUMMARY AND CONCLUSIONS

Tests were conducted to determine the effect of different spawn and spawn carrying materials on the growth and incidence of *Trichoderma* green mold. Results suggest that fully colonized spawn run compost, fungicide-treated spawn and non-grain spawn, used as the source of spawn, resulted in less incidence and severity of disease, when compared to fresh grain spawn. These results confirm that *A. bisporus* and *T. harzianum, biotype Th4,* are in competition for food in the mushroom compost, yet *T. harzianum* growth is dependent of a healthy ,4. *bisporus* growth. The difference in growth rate of the two fungi provides *T. harzianum* with the advantage in the competition. However, if given sufficient time to colonize the compost prior to the introduction of *T. harzianum, A. bisporus* is capable of successful colonization and production. This study also demonstrated the difficulty is working with *T. harzianum* in compost systems, since the repeated experiment showed some variation in the results.

Tests were developed to begin to assess the effect of compost moisture on the growth and development of *T. harzianum* in compost. Excess compost moisture has been suggested as a predisposing factor to an increase in severity and incidence of *T. harzianum* growth and disease development. These results also suggested that slow colonization of *A. bisporus,* hindered by excess moisture, increased the likelihood of *T. harzianum* dominance over time.

Results of this study have shown conclusively that the spores of *T. harzianum* do not survive pasteurization temperatures above 60°C or the Phase II process and that the compost must be infested with the fungus sometime during cooldown, spawning or later.

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