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Original Research Article

Competencies of *Lecanicillium fungicola* in the face of *Agaricus bisporus* (white button mushroom)

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Abstract

The dry bubble disease is known as one of the most important and notorious diseases of the cultivated white button mushroom (*Agaricus bisporus* (Lange) Imbach). The causal agent *Lecanicilium fungicola* has a worldwide distribution. The control of the disease relies on strict hygiene and the use of fungicides. The epidemiology of the disease was investigated. The results approved statistically significant differences among transmission potential of alternative sources of casing materials. The relative inefficiency of the mechanical control as a method for the management of the disease and the internal effects of factors that involved in the epidemic of the dry bubble through mushroom farms were explained. It is also shown that there are direct and indirect relationships between the radial development of the *L. fungicola* and the disease incidence- related tothe inoculation depth of the pathogen, respectively, and that the pathogen colonizes the mushroom growth bed with the average rate of 2.3 mm per day.

Keywords: Casing soil, Epidemiology, *Lecanicillium fungicola,* Mechanical control

INTRODUCTION

Dry bubble disease caused *by Lecanicillium fungicola* (Preuss) Zare and Gamesis among the most threatening factorsin commercial cultivation of white button mushroom (*Agaricus bisporus* (Lange) Imbach)). Disease symptoms vary from small necrotic lesions on the cap or stopof the fruiting bodies to partially deformed fruiting bodies called stipeblowout or totally deformed and undifferentiated masses of mushroom tissue, known as scleroderma tissue or so-called dry bubble (Beyer et al., 2005). The primary source of the disease can be casing soil ingredients, especially peat carrying *L. fungicola* propagules (Wong and Preece, 1987; Fletcher and Gaze, 2008). The infection cannot occur before the casing time. If conidia land on the spawned compost, crop does not show disease during the crop cycle (Beyer et al., 2005; Fletcher and Gaze 2008). Once the disease established on mushroom farms, it can be spread by secondary vectors likeSciarid (Sciaridae); (Gandy1972; Fletcher and Gaze, 2008) and Phoride (Phoridae) (Kumar and Sharma, 1998) flies, dust particles, water droplets in the air coming from infected crops, (Gandy, 1972; Gaze, 2004; Beyer et al., 2005; Clift and Shamshad, 2009), pickers' hands, equipment and crates (Bech et al., 1982; Griensven 1988; Fletcher and Gaze, 2008).Currently Control management of the dry bubble disease relies mainly on chemical and mechanical means. Development of fungicide resistance in the pathogen, risks to human health and negative effects of many chemicals on A. *bisporus* as a fungus itself is among the limitations on the use of chemicals for the control of the dry bubble (Wuest et al., 1974; Bollen and Van Zaayen, 1975; Fletcher and Yarham 1976; Gea et al., 2005; Mehrparvar et al., 2013) Mechanical control of the disease manages by covering the mushroom infected tissues by Sodium salt, Paper towels soaked in formalin (1–1.5%) or upside down plastic cups. This is part of the operational measuresof hygiene, which is taken by the producers to prevent the spread of the disease on the rooms.

In Iran mushroom producers have their own casing soil mixing formulation which is usually inconsistent and

depends on the availability of ingredients in the region. Some amounts of black peat are also imported from Europe. This researched aimed to take a deeper lookinto the epidemiology and the complexity of thedisease to know why in spite of all the offered control measures, *L. fungicola* still maintained itself as one of the biggest concerns of commercial production of white button mushroom.

MATERIAL AND METHODS

Contribution estimate of each casing soil ingredients in transmission of the *L. fungicola* on mushroom farms

Distinct samples from either of the casing ingredients, i.e. peat moss from North Country woodlands, spent compost, sugarcane factory filtercake and Bagasse, Ardebil peat soil, BVB (imported black peat from the Netherlands) and Lithuania peat moss, were prepared from the stock depository of JDM Co. Iran. The PH of each soil sample was adjusted to 7–7.5 by adding the required amount of Calcium carbonate ($CaCo_3$). Commercial Compost blocks (60 × 40 cm, mean: 17.5 kg), inoculated with wheat spawn at 0.8 % of *A. bisporus* Syl-737 cultivar, were incubated at 25°C for a period of 14 d with 92 % relative humidity before casing at JDM CO. growing chamber. Casing was performed by each of distinct formerly prepared samples. Nine days after casing the room temperature was decreased to 16°C. The number of infected tissues was recorded with the beginning of the pining stage twice a day until the end of the first flush. The infected tissues were covered with plastic bags and gently removed. This experiment was conducted as a completely randomized design with 4 replications (each compost block) and 7 treatments (each distinct ingredient of casing soil). Statistical analysis to compare the effect of each ingredient of casing soil was performed by one-way ANOVA model with statistical software SAS Ver. 9.4 andthe comparison of means was performed by Duncan's multiple range test.

Efficacy of mechanical control in reduction of disease incidence

The occurrence of the disease was assessed on mushroom farms, based on the casing soil, natural noculums carrying potential, under two different treatments. Three rooms (at Jolge-Dez mushroom production Co. / 440m^2) were randomly selected for each treatment. On the first treatment, daily inspection of the mushroom growth bed was donefrom the beginning of the pinning stage. The infected tissues with dry bubble symptoms were immediately covered by placing upside down plastic cups plus 1.5% formaldehyde. When needed, the irrigation was not allowed until the completion of bed inspections. In the second treatment, the routine practice of mechanical control was performed by mushroom pickers that was not based on complete inspection of bed surface as precisely as the measures that were taken in the first treatment. The number of infected tissues and their location at shelves were recorded for both treatments. On the second treatment the number and location of infected mushrooms were recorded, regardless of had they been covered or not by the pickers.

Radial development of *L. fungicola* in mushroom growth bed

Plastic bags of 30cm wide, containing 4Kg of commercial compost inoculated with 0.8% wheat spawn of *A. bisporus*, Sylvan-737 cultivar, were incubated at 25°C for a period of 14 d with 92 % relative humidity before casing at JDM CO. Autoclaved soil, twice within 24 h, was used for the casing. The experiment consisted of three treatments, with placing inoculum plugs (5 mm diameter, removed fromthe edge of 14d-old cultures of *L. fungicola* var. *fungicola* on Potato Dextrose Agar (PDA) at the center of compost surface, the half soil layer and the soil surface. For each treatment, three replicates were considered. Control treatments were also considered with just inoculation of PDA plugs. Casing thickness was considered 5cm for all the treatments. To prevent the spread of spores via irrigation or during other mechanical handlings, inoculums plugs were placed upside down on the soil layers. Nine days after casing the room temperature was decreased to 16°C. With the beginning of the pining stage the disease incidence (number of the infected / total number of mushrooms) × 100) and distance of infected tissues from the center of the bags (radial development of the pathogen) were recorded daily until the end of the second flush. To prevent the spread of secondary inoculum, all the infected tissues were removed immediately after recording the data. Statistical analysis to compare the effect of inoculation depth on the disease incidence and the radial development of the pathogen on casing soil were performed by one-way ANOVA model with statistical software SAS.Ver.9.4 and the comparison of means was performed by Duncan's multiple range test. Multiple regression analysis was also used to predict the radial development of the pathogen and disease incidence-related to inoculation depth.

RESULTS

The results of this experiment demonstrated that the contribution of each casing soil ingredients in transmission of the disease could be varied greatly from zero to very high. Tables 1, 2 show analysis of variance

Table 1. Analysis of variance of the number of infected tissues/m^2 on mushroom growth beds using various sources of casing soil

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	2411.36	401.89	107.17**	<.0001
Error	21	78.75	3.75		
** : significant at 1% probability level					

Table 2. Comparison of the number of infected tissues/ m^2 mushroom growth bed on different sources of casing soil

Casing soil	N	Mean, infected tissues/m^2	Duncan's Grouping
North Peat moss	4	24	A
Lithuania peat moss	4	19	B
Spent compost	4	4	C
Ardabil peat moss	4	3.25	C
Sugarcane bagasse	4	0	D
Sugarcane filter cake	4	0	D
Black sphagnum peat	4	0	D
* Means with the same letters are not significantly different.			

of the number of infected tissues/m^2 on mushroom growth beds and mean comparison with Duncan's multiple range test of the various casing components, respectively.

Contribution estimate of each casing soil ingredients in transmission of the pathogen on mushroom farms

As the results demonstrated, the transmission potential of different sources of casing materials varies significantly. Application of these materials in soil recipe is not a set formula and varies with the accessibility and also knowledge and manner of the producers. Iranian peat moss had the most transmission potential with significant difference with other soil components. Since thesematerials come from the surface layers of forest lands, the active inoculum of *L. fungicola*, which is associated with a naturally growing wide range of cap fungi (Visscher, 1988), could easily find their way through mushroom farms. It seems that this is a good explanation for the high carrying capacity of Iranian peat moss. The same scenario could be considered for the Lithuania peat moss which placed in another group, but still with high potential of disease establishment. The spent compost which was used in this experiment was cooked out and piled for at least six months to decompose under natural condition, but still showed the potential of carrying the infection throughout the farms. Another component, Ardebil peat soil, which has recently composed more than 50% percent of the casing recipe of the country, also stayed in the same Duncan group with the spent compost. Sugarcane by-products along with Sphagnum peat stayed in another group without any carrying potential of the disease. Regardless of whether these materials come from, the most considerable matter is the role of casing materials as the main contributing factors in the establishment of the disease throughout the farms. These results are consistent with the Ware, 1933 who believed the casing soil as the main contributing factor in transmission of the disease to the mushroom farms. Sphagnum peat in terms of physical properties has many advantages in comparison with other materials which are used as casing. As there are no black peat resources in the country and the importing cost is too high, if used, it concludes less than 30% the casing recipe to reduce the production costs. Of course, the results of our experiments showed that the mushroom yield of some of these recipes in terms quality and quantity equals with the conditions that black peat is the sole casing material (unpublished data). It is noteworthy to remark thatapplication of agricultural waste products as casing material could be an environmentally friendly activity and a great help in achieving an economy based on creativity and cost efficiency with emphasize on the development of sustainable agriculture policies to save non-renewable natural resources.

Efficacy of mechanical control in reduction of disease incidence

Farmers apply different practices like covering the mushroom infected tissues with salt, formalin-soaked table napkin, upside down plastic cups and plastic sheets to prevent the spread of the disease throughout the farms. In general, it is believed that covering the newly formed infected tissues by continuous inspection of the mushroom growth bed could help preventing the spread of the secondary inoculum of the disease. Our

conclusions proved a great difference among the number of recorded infections/ m^2 mushroom growing bed inside each treatment (1.23, 2.4, and 10.8 under strict measures and 1.12, 7.12, 11.1 under routine practice farms). The results of the analysis of the data showed no difference between the two employed methods together with a high coefficient of variation which might be an indication of the influence of that the several other unmanageable factors, that only some of them were considered in this experiment, on the diseases development throughout the mushroom farms. We made a deeper consideration to understand and find out possible explanation(s) for the high amount of disease incidence fluctuations between and inside the treatments. Our recorded data from the understudied treatments and observations throughout the other farms lead to below conclusions:

1- Typically, the emergence of the primary infections in the first flush occurs randomly from one or more points at different shelves. These points can be called as the first foci of infections (FFI).

2- The frequency of the FFIvaries with the disease transmission potential of the casing materials.

3- There was not any correlation between the observed infection rates at two sides of every shelf.

4- Dissemination of the disease occurred transversely on the mushroom growth bed from the FFI points in consequence of the lateral application of the watering system.

5- The spread of secondary inoculums to the other shelves and thus the intensity of secondary infections in 2^{nd} flush were higher at farms where the FFI randomly located on upper shelves in compare with the situation that the FFI were at lower shelves. This again highlighted the role of watering in the washing and dissemination of the spores throughout the farms and the necessity of the replacement of the lateral irrigation system with the use of nozzle perpendicular to the growth bed to gently spray out the water to prevent the spread of the disease.

6- About 96% of the first flush recorded infections were of Sclerodermoid tissues. Since formation of the Sclerodermoid tissues is the consequence of the primordia infection, it could be attributed to the inoculum carrying potential of the casing recipe.

7- The diversity of the dry bubble disease symptoms increases during the 2^{nd} flush due to spread of the secondary inoculum of the pathogen and infection of mushrooms at different developmental stages.

8- High frequency and distribution FFIs could be a serious threat to the epidemic of the disease and complete yield loss. In spite of spending plenty of time for the precise and daily continuous inspection of the growth bed with skilled eyes from the initial stages of the primordia formation until the end of the 2^{nd} flush, management of the disease did not yield successful by the application of mechanical means. Depend on the frequency of the FFIs the trend of the disease spread and appearance of newly infected mushrooms around or beyond the primary infection points could reach to a frustrating and disappointing degreesin a way that desperate the farmers to continue the practice. It seems that the mechanical control can be counted, only when it is appliedas a second hand method for removing sporadic diseased mushrooms, if the farmers have taken the necessary measures to highly reduce the initial amount of the soil infectivity beforehand. Mechanical control also does not seem a logical approach because of the following reasons:

1- The possibility of human error and the need for expertise and experience in detecting diseased tissue.

2- The difficulty of detecting very small infected primordia and hidden diseased tissues under the mushroom clumps on the growth bed.

3- High saprophytic capability of *L. fungicola* to develop on the organic materials and the remnant of picking mushrooms on casing soil.

4- Risk of exposure to chemicals like formaldehyde.

5- Cost efficiency in terms of labor hour needed for complete inspection of the mushroom growth bed.

6- Increasing the possibility of the disease spread by manipulating the infected tissues.

Radial development of the pathogen in mushroom growth bed

Considering the time between casing and primordia appearance (about 20 d) and the optimal environmental conditions for the growth and development of the pathogen, there is plenty of time for the pathogen to build up plenty of secondary inoculum by saprophytic growth on casing organic materials. With the formation of primordia and being invaded by the pathogen the production of secondary inoculum increases dramatically where the pathogen has more than 10 days until the second flush have been picked completely to continue its development which can lead to an epidemic. This would be a good and convincing explanation for the dramatic development of the disease during the second and third flushes which can lead to complete destruction of the mushroom yield. We noticed that over the next few days, even after immediate covering of the infected primordia and before the pathogen have the chance of sporulation, some newly infected tissues appear a few centimeters far around the FFIs. These observations poked us into conduction of an experiment to understand whether/ how pathogen develops on the mushroom growth bed. Tables 3,4, 5 and 6 show the results of the ANOVA and mean comparisons of the data regarding the effects of inoculation depth on the radial development of *L. fungicola*/cm and the disease incidence under natural conditions on the mushroom growth bed, respectively.

The results of this experiment were primarily approved our hypothesis about the radial development of the *L.*

Table 3. Analysis of variance of the radial development of the *L. fungicola* /cm on the mushroom growth bed from the inoculation point in different casing depths

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	866.72	433.36	35.21**	<.0001
Error	175	2153.67	12.31		
Corrected Total	177	3020.39			
** : significant at 1% probability level					

Table 4. Duncan multiple range test results for the radial development of the *L. fungicola*/cm inoculated at three different casing depth of mushroom growth bed.

Inoculation depth	Tre	Mean	Duncan grouping
Compost surface	27	11.11	A
Soil Semi- depth	42	10.21	A
Soil surface	93	6.11	B
*Means with the same letters are not significantly different.			

Table 5. Analysis of variance of the disease incidence on mushroom growth bed from the inoculation points in different casing depths

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.11	0.052	16.84**	0.0035
Error	6	0.02	0.003		
Corrected Total	8	0.12			
** : significant at 1% probability level					

fungicola on mushroom growth bed. Interestingly, the average of pathogen radial development increased with the inoculation depths (Tables 3, 4). It seems that the lower average of radial development on the casing surface would be explainable by the natural formation of mushroom primordia on the casing surface and the ease of access to the mushroomhost tissues that triggers the establishment of the disease in the host- pathogen interaction.In contrast, with the increase of the inoculation depth the attempts of the pathogen at finding mushroom tissues increases by scavenging through casing soil.

The results also showed that the disease incidence significantly decreased with the accretion of inoculation depth. These findings were also consistent with the radial development -related to inoculation depths of the pathogen on the mushroom growth bed, suggesting that *L. fungicola* in presence of mushroom fruit bodies did not need to explore the growth bed and instead it serves more of its potential for invading the mushroom fruiting bodies around the point of inoculation site. Accordingly, corresponding to the increasing depth of inoculations on the mushroom growth bed, the average development speed of the *L. fungicola* in the time span from the inoculation to the end of 2nd flush were estimated at1.4, 2.63 and 3.16 mm per d (mean: 2.3mm/d), respectively (Table 7,8). It seems that *L. fungicola* use different strategies based on its location in casing profile and the accessibility to the host tissues by scavenging the growth bed or invading the host tissues relying on its saprophytic and/ or parasitic abilities. This fast colonizing ability of the *L. fungicola* might be among the reasons of its competence to be introduced as the most common and notorious diseases of the mushroom crop. Regression analysis of the relationships between theboth disease incidenceand radial development-relatedto the inoculation depths of the pathogen provides a better view of the strategies employed by the *L. fungicola* (Tables7,8).

The results documented adirect and significant relationship between the radial development and the inoculation depths of the pathogen on the mushroom growth bed (Table 7).

Equation 1 $y = 5.68 + 1.83x$

Equation 2 $y = 0.35 - 0.05x$

Equation 1 shows the estimation of the inoculation depth-related model for the radial development of *L. fungicola*, where x and y are the inoculation depth and the radial development of the pathogen on the mushroom growth bed. The results also documented the presence of an inverse relationship between the disease incidence and the inoculation depths, suggesting that the dry bubble disease incidence decreases with the increase of the inoculation depth. Equation 2 shows the estimation of the inoculation depth-related model for the dry bubble disease incidence, where x and y are the inoculation

Table 6. Duncan multiple range test results of the disease incidence on mushroom growth bed from the inoculation points in different casing depths

Inoculation depth	Tre	Mean	Duncan grouping
Compost surface	3	0.13	A
Soil Semi- depth	3	0.17	A
Soil surface	3	0.37	B
*Means with the same letters are not significantly different.			

Table 7. Regression analysis of the estimation of the inoculation depth- dependent radial development of *L. fungicola* on the mushroom growth bed.

Variable	DF	Parameter Estimate	Standard Error	t Value	Pr > |t|
Intercept	**1**	5.68	0.66	8.70**	<.0001
Treat	**1**	1.08	0.20	5.35**	0.0011
** : significant at 1% probability level					

Table 8. Regression analysis of the estimation of the inoculation depth- dependent incidence of the disease on mushroom growth bed.

Variable	DF	Parameter Estimate	Standard Error	t Value	Pr > |t|
Intercept	1	0.35	0.03	9.59**	< 0.0001
treat	1	-0.05	0.01	-4.40*	<0.0031
** : significant at 1% probability level					

depth and disease incidence of the pathogen on mushroom growth bed.

CONCLUSIONS

The economic importance of the dry bubble disease has ledto many studies over finding the approaches to at least manage the disease tocurb the epidemic over mushroom farms. Active control of the disease mainly relieson the hygiene measures and use of fungicides, but the chemicals used have become less effective as the pathogen has developed resistance and legislation is limiting their use. There are also some concerning reports of fungicide residuesabovethe MRLs (Maximum residue levels) from some regions of the country (Sobhanardakani et al., 2014; Abdi et al., 2015). In Iran failure in managing of the disease is the most important reason forthe tremendous yield loss or even the closure of both household and large-scale mushroom farms. Our findings approved that the main leading source of establishment of the disease through mushroom farms is the infected casing materials. Once the disease established in rooms, circulation of the conidia, due to poor hygiene, to the following crops would be added to the devastating potential of the disease. Besides, the difference between the intensity of the disease, which is sometimes seen in even neighboring rooms, could be attributed to the random locality of the FFIs as explained before and the lateral watering method. Therefore, it seems that any attempt to manage the disease could be started with using wholesome casing materials or finding ways for the decontamination of the casing soil at mixing area. Minimizing the arrival of the initial sources of the infection to the rooms would be the key prerequisite of the secondary measures to curb the circulation of the disease to the following crops. We believe that finding an economic, environmentally friendly and effective method for the large-scale decontamination of the casing materials, with greater efficacy and advantages than conventional methods, would be the next challenge that needed to be overcome in white button mushroom disease management strategies.

Disclosure

The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the Iran.

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