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Morphological characteristics of wet bubble disease (*Mycogone perniciosa*) isolated from button mushroom (*Agaricus bisporus*) and assessment of factors affecting disease development and spread

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Wet bubble disease (*Mycogone perniciosa*) is a devastating disease in the crop production of mushrooms. In India, it has been reported to cause serious crop losses. It is also a common contaminant, occurring in mushroom houses in the Kashmir valley. The aim of the study was to investigate physiological characteristics of *M. perniciosa* isolated from button mushroom (*Agaricus bisporus*). The assessment of the factors affecting the development and spread of wet bubble disease was also done. The isolated fungus produced copious flocculent mycelia and the colour of colonies changed from white to pale brown and finally dark brown after 12-14 days of incubation at 24±1°C. The maximal radial growth (90 mm) was recorded on compost agar medium after 15 days, followed by potato dextrose agar (75.5 mm) and malt extract agar (72.0 mm). Microscopic examination of the pathogen reveals that the mycoparasite was both inter and intra-cellular. The conidiophores were erect, long and verticillately branched. Conidia were oval, single, 2-celled and thin walled. The pathogen was successfully re-isolated when admixed with the compost at 6th, 7th or 8th turnings, that is, on the 22nd, 25th and 29th day of composting with a pile temperature of only (0-30°C). The pathogen was also present in viable form in all the test samples of casing mixture in varied populations. The pathogen was continuously present in samples from spent compost of diseased trays. It was also observed that only the garden soil and the spent compost carried the wet bubble pathogen varying in populations each year. The peat soil, virgin soil, farm yard manure and sand did not yield any *M. perniciosa* propagules during either year.

Key words: Contaminant, conidia, compost, white, mycoparasite, mushroom.

INTRODUCTION

Cultivation of mushrooms is a very reliable and profitable option. Presently, mushroom culture represents the only major process in biotechnology which successfully converts various lignocellulosic, agricultural, industrial, forestry and horticultural wastes or their by-products into proteins (Stamets, 2000). Mushroom is an attractive crop to cultivate in developing countries for many reasons. Mushroom culture is a viable alternative venture for mini-

mising the ever increasing protein malnutrition gap and multitude of allied problems in developing countries. Mushrooms provide a rich addition to the diet in the form of proteins, carbohydrates and minerals. Mushrooms contain only 2 to 3% fat by dry weight and are a good source of essential amino acids and contain approximately 5-9% fiber (Yang et al., 2001). Mushrooms contain 19-35% protein on dry weight basis, having 70-90% digestibility. Mushrooms are also good source of minerals and B complex group vitamins like thiamine (B_1), riboflavin (B_2), niacin and biotin (Manzi et al., 2001; 2004). Apart from this, mushrooms are also known to have various medicinal properties. Mushrooms possess various anticancer, antitumour, anticholestrol, immune-modulating and antioxidative properties and are currently used as dietary supplements (Selegean et al., 2009; Lavi et al., 2010).

India has a great potential for mushroom production as it is blessed with varied agro-climate, abundance of agricultural wastes and manpower, making it most suitable for cultivation of temperate, sub-tropical and tropical mushrooms. In India, only three edible mushrooms; *Agaricus bisporus* (Button mushroom), *Volvariella* spp. (Paddy straw mushroom) and *Pleurotus* spp. (Oyster mushroom) are in commercial or semi-commercial cultivation. From a production stand point, the white button mushroom has the highest growth rate and potential for production. In India, Button mushroom (*A. bisporus* L.) is the most popular mushroom grown especially in Kashmir valley. It is an important cash crop which utilizes large quantities of farm residues, additives and supplements for its cultivation and harbours a variety of microbial pathogens and competitor moulds. In most farms, seasonal cultivation of this mushroom is being practiced, where unpasteurized composted substrate is used and poor hygiene and insanitary practices are prevalent at farmer's level. Such conditions invite a number of parasitic diseases which take a considerable toll on the produce each year (Fletcher et al., 1986; Bahl, 2000). Of these, the incidence of wet bubble disease (*Mycogone perniciosa*) of white button mushroom causes extensive damage by bringing soft rot or decay of whole fruiting bodies. *M. perniciosa* Magn. (Perfect state; *Hypomyces perniciosa*) belongs to the division Eumycota, sub-division Deuteromycotina, class Hypomycetes, order Moniliales and family Moniliaceae. Its telomorph, that is, *Hypomyces* belongs to division Eumycota, sub-division Ascomycotina, class Pyrenomycetes, order Sphaeriales and family Hypomycetaceae. The mycelium of *M. perniciosa* is white compact, felt-like. The hyphae are branched, interwoven, septate, hyaline and 3-5 µm broad. It produces conidia and chlamydospores. Conidiophores are short, slender, branched, hyaline measuring 200 x 3-5 µm and have subverticillate to verticillate branches, which bear thin celled conidia measuring 5-10 x 4-5 µm. Large two-celled chlamydospores are also produced with upper cell warty, thick walled, globose, bright coloured measuring 15-30 x 10-20 µm and the lower cell hyaline, smooth and measuring 5-10 x 4-5 µm (Sharma and Singh, 2003).

Although *M. perniciosa* is not host-specific, the cultivated mushroom *A. bisporus* seems to be its main host worldwide. This mycopathogen has been reported to attack a large number of wild fleshy fungi (Sharma, 1995). Figueiredo and Mucci (1985) revealed that *M. perniciosa* is capable of infecting other mushroom species like *Agaricus compestris.* The disease is most prevalent in India in temperate areas although reports of its occurrence exist in subtropics also. The primary source of infection on most farms is casing material containing dormant thick-walled chlamydospores. Besides this, infection can also occur through air infected trash and spent compost.

Once the pathogen is established in the crop, the secondary spread is by means of conidia which are carried by water splashes and run-off water. Pickers also spread the disease/pathogen through their hands. The pathogen remained viable up to 3 years in the post mushroom substrate under natural day conditions in the form of chlamydospores (Tu and Liao, 1989; Singh and Sharma, 2002).

The problem of this pathogenic fungus is a serious impediment in the planned development of mushroom cultivation and expansion programme in India too. The study was undertaken to characterize *M. perniciosa* associated with Button mushroom cultivation, based on morphological characteristics of this fungus. The study also included the assessment of factors affecting development and spread of this disease.

MATERIALS AND METHODS

Morphological characters of the isolated pathogen

The morphological characteristics of the causal organism on host and in artificial culture were studied in the laboratory. The important characters studied were the cultural characters such as mycelial colour and growth and the morphological characters such as shape, size, colour and septation of hyphae, conidiophores, conidia and chlamydospores.

Assessment of factors responsible for disease development and spread

Contaminated compost

The pathogen was mixed with compost at different stages *viz*., on

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stacking (0 day), and 1st, 2nd, 3rd. 4th, 5th, 6th, 7th and 8th turnings (Turning : in turning, the compost is turned up down so that the whole materials will be decomposed uniformly) on 6th, 10th, 13th, 16th, 19th, 22nd, 26th and 29th day of composting. At each stage 10 ml of *M. perniciosa* spore suspension (1x107 spores/ml) prepared in sterilized distilled water from 7-day old culture, was admixed with 100 g of composting mixture. The inoculated compost was filled in sterilized glassbottles, tight lidded, labeled and then kept in the centre of the pile. Five such bottles were maintained for each composting stage. The bottles were taken out from the compost heap on the filling day. For examining the presence of live pathogen in the compost, one gram of the compost was taken out from each bottle, serially diluted in sterilized distilled water and 1 ml aseptically poured on PDA in Petri-plates. The plates were incubated at 23±2°C for 6 h and the germinating conidia/chlamydospores counted.

Contaminated casing soil

Presence of pathogen in casing materials: Composite samples of different casing materials such as peat, garden soil, virgin soil, spent compost (Spent mushroom compost is the residual compost waste generated by the mushroom production industry), farm yard manure (FYM) and sand were collected, sieved through a 200 mesh sieve and put in pre-sterilized plastic vials. One gram of the material was separately used for assessing the *M. perniciosa* propagules using serial dilution method.

Spent compost as inoculum source

500 g of composite samples of spent compost were collected in triplicate from the production trays, showing the signs of wet bubble disease soon after termination of spring and autumn cropping season of 2008 and placed separately in plastic jars. The jars were kept outdoors under natural conditions and 5 g of compost samples drawn at monthly intervals for assessing the presence of pathogen *M. perniciosa* using serial dilution method.

Pathogen survival in casing material

Sterilized casing material comprising of soil and peat in the ratio of 2:1 was admixed with a spore suspension of test pathogen having a spore load of 6 x 10^8 spore ml^{-1} and prepared from seven days old culture in distilled sterilized water. The inoculated casing mixture was filled in perforated plastic jars and kept outdoors under natural conditions. From each jar samples were drawn at monthly intervals and examined for presence of the pathogen by dilution plate technique.

Air currents

The infected fruit bodies/sclerodermoid masses having abundant conidia were placed 15 cm away from a small electric fan in the inoculation room. Petri-dishes containing PDA, amended with Rose Bengal were exposed for 5 s at right angles to air flow at various distances from 20 to 100 cm away from infected sporophores, the plates were incubated at 24±1°C and the data on number of the pathogen colonies developed recorded after 48 h of incubation.

Spread through water splash

Water was sprayed on to the mushroom trays containing *M. perniciosa* infected sporophores/sclerodermoid masses and the water splashes collected in pre-sterilized Petri-plates at different distances such as 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 cm. The water collected was later examined for spore density by the method adopted by Hsu and Han (1981) and Singh and Sharma (2002) and expressed as number of spores per microscopic field.

Flies

To assess the influence of sciarid flies infestation on dispersal of wet bubble disease, different population (10, 20, 50, 75 and 100) of flies collected from infested *Pleurotus* bags were allowed to acquire inoculum from wet bubble infected sporophores on diseased trays (kept in insect cages) for varied periods (30, 60, 90 and 120 min) and subsequently released on healthy trays also kept in insect cages. The percentage of infestation in each tray was calculated by the following formula:

$$\text{Percentage of infested sporophores} = \frac{\text{No. of infested sporophores}}{\text{Total number of sporophores per tray}} \times 100$$

RESULTS

Morphology of the fungus

The cultural and morphological characters of the pathogen were studied both in culture and on host.

Morphology of fungus in culture

The isolated fungus cultured on potato dextrose agar medium (Plate 1) produced copious flocculent mycelium with even or uneven edges. The amount of aerial mycelium varied from dense to sparse, while the colour of colonies turned from white to pale brown and finally dark brown after 12-14 days of incubation at 24±1°C (Plate 2). The maximal radial growth (90 mm) was recorded on compost agar medium after 15 days, followed by potato dextrose agar (75.5 mm) and malt extract agar (72.0 mm).

The pathogen in culture produced septate and hyaline mycelium. The hyphae were branched and interwoven, 3-5 µm broad. Conidiophores were short, slender, branched, hyaline sub-verticillate to verticillate with apical conidium measuring 150 to 180 x 3 to 5 µm. Conidia were oval, single, 2-celled, thin walled, 12-14 x 4-5 µm and relatively short lived. Chlamydospores or aleurospores were bi-celled with upper cell thick walled, warty, globose, bright coloured, measuring 12-15 x 24-26 µm and lower cell hyaline, smooth and measuring 12 x 14 µm. The chlamydospores are produced terminally (Plate 2).

Morphology of fungus on host

The mycoparasite was both inter and intra-cellular, the conidiophores were erect, long and verticillately branched. Under humid conditions, aerial growth of mycelium con-

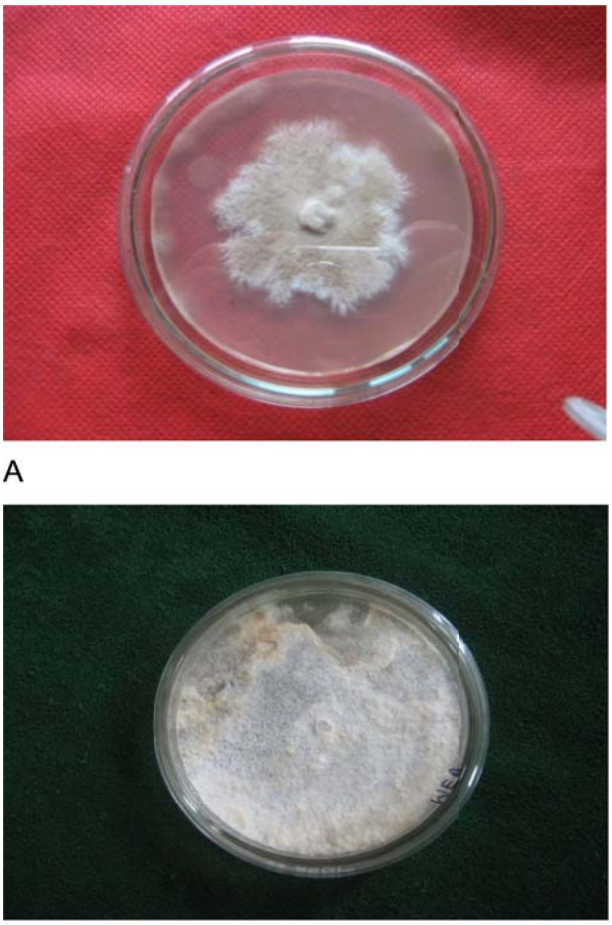

Plate 1. *In vitro* growth of *Mycogone perniciosa.* A. Mycelial growth on PDA; B. mycelial growth on MEA

tinued over the surface of infected fruit bodies. However, there was no variation in the colour, size, shape and septation of conidiophores, conidia and mycelium either on the host or in culture medium.

Factors affecting disease development and spread

Contaminated compost

The recovery and re-isolation of the pathogenic fungus from the infested compost samples kept in the central core of compost heap at stacking and during various turnings were made through dilution plate technique. The results (Table 1) reveal that the pathogen admixed with compost either at stacking (0 day of composting) or at 1st (6th day of composting), 2nd (10th day of composting), 3rd (13th day of composting), 4th (16th day of composting) or 5th turnings (19th day of composting) could not be re-isolated. However, the pathogen was successfully re-isolated when admixed with the compost at 6th, 7th or 8th turnings, that is, on 22nd, 25th and 29th day of composting. The pile temperature recorded during composting indicated that the temperature was minimum at stacking (0-30°C), which increased to 65-70°C from 1st to 3rd turnings and gradually decreased thereafter reaching minimum (25-30°C) at 8th turning, when the pile was ready for filling.

Contaminated casing soil

Pathogen presence in casing mixture (soil and peat): Samples were drawn from the pathogen-infested casing soil kept outdoors under shade at monthly intervals starting from May 2008 to March 2009, and examined for the presence of viable pathogen propagules through dilution plate technique. It is evident from the Table 2, that the pathogen was present in viable form in all the test samples in varied populations. The population was maximum (6×10^4 cfu g^{-1} soil) in the first month of sampling, that is, in May 2008 which decreased gradually as the season advanced reaching minimum (3×10^1 cfu g^{-1} soil) in February 2009. Thereafter, the population again increased slightly (1.0×10^2 cfu g^{-1} soil) in March and April, 2009.

Spent-compost as a source of inoculum

The spent compost collected from healthy and diseased mushroom production trays at the end of spring crop of 2008 was kept outdoors and examined for the presence and population dynamics of *M. perniciosa* at the end of each month by dilution plate technique. It is clear from Table 3, that the pathogen was continuously present in samples from spent compost of diseased trays compared to samples from spent compost of healthy trays where no pathogen was detected. However, the pathogen population was maximum (3×10^7 cfu g^{-1} compost) immediately after placing outdoors in May 2008, the population gradually decreased every month reaching appreciably low (6×10^3 g^{-1} compost) in October 2008 and the lowest (3×10^2 g^{-1}) in March-April 2009.

Population of *M. perniciosa* in different casing materials

Composite samples of different casing materials utilized for preparation of casing mixture, collected from growers houses, were examined for the presence and population density of *M. Perniciosa*. The result (Table 4) reveals that only the garden soil and the spent compost carried the wet bubble pathogen in populations varying each year. The garden soil harboured maximum pathogen population of 2×10^4 and 5.5×10^2 cfu g^{-1} soil, compared to 4.5×10^2 and 3.5×10^2 cfu g^{-1} during 2008 and 2009, respectively. The peat soil, virgin soil, farm yard manure

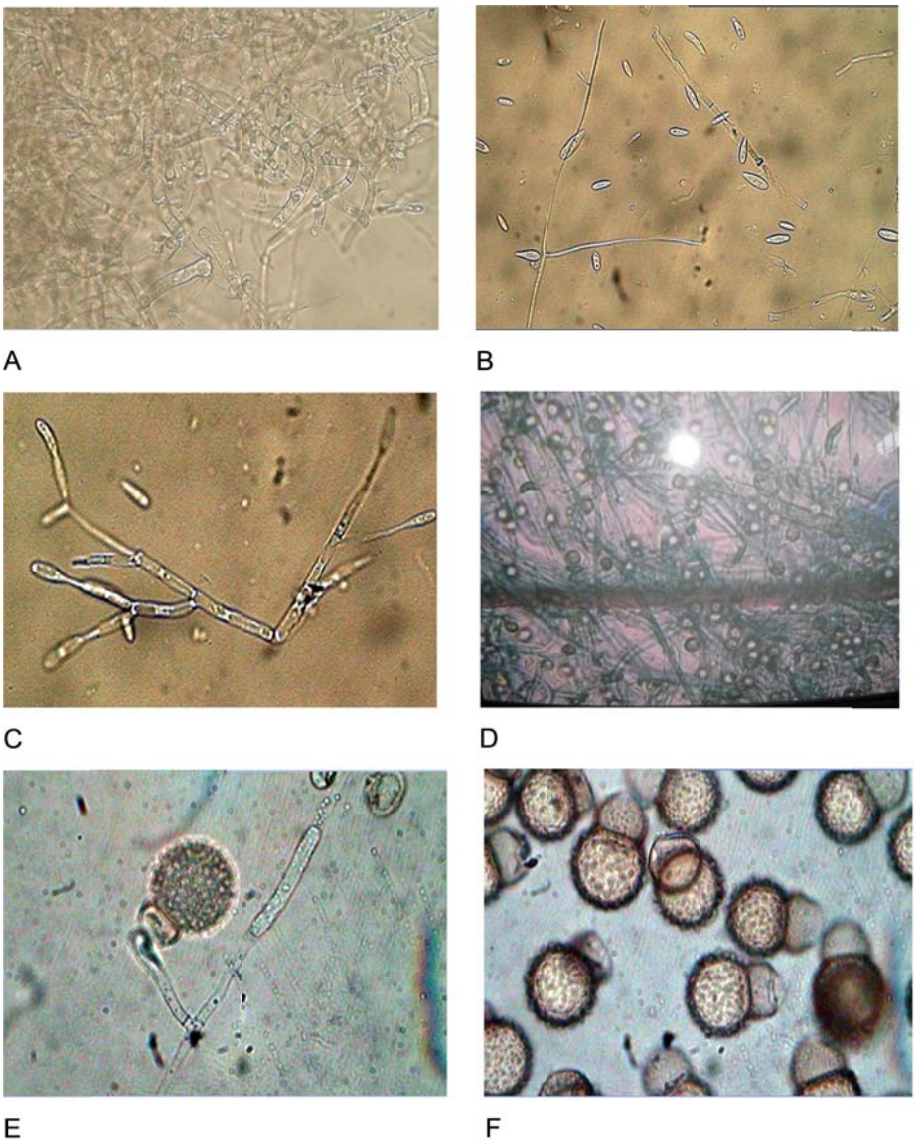

Plate 2. Morphological characters of *Mycogone perniciosa. A.* Mycelium; B. single celled conidia; C. sub-verticillate to verticillate conidiophores; D. chlamydospores on host; E. terminal chlamydospore; F. bi-cellular chlamydospore.

Table 1. Isolation of *M. perniciosa* incorporated in compost at different stages of composting.

Pathogen incorporation stage	Temperature of pile (°C)	Isolation status
Stacking (0 day)	0-30	-
1st turning (6th day)	65-70	-
2nd turning (10th day)	65-70	-
3rd turning (13th day)	65-70	-
4th turning (16th day)	60-65	-
5th turning (19th day)	50-55	-
6th turning (22nd day)	40-45	+
7th turning (25th day)	30-35	+
8th turning (29th day)	25-30	+

+ = Successful isolation; - = pathogen could not be isolated.

Table 2. Population of *Mycogone perniciosa* propagules in samples drawn at monthly intervals from casing mixture (soil and peat) placed outdoors under natural conditions during May 2008 to April 2009.

Month in which sample drawn	Pathogen population ($cfug^{-1}$ casing mixture)
May 2008	6×10^4
June 2008	4×10^3
July 2008	8×10^2
August 2008	3×10^2
September 2008	6×10^2
October 2008	2.5×10^2
November 2008	2×10^2
December 2008	2×10^2
January 2009	1×10^2
February 2009	3×10^1
March 2009	1×10^2
April 2009	1×10^2

Table 3. Population of *M. perniciosa* propagules in samples drawn at monthly intervals from mushroom spent-compost collected from apparently diseased and healthy production trays of spring crop 2008 and placed outdoors.

Sampling month	Pathogen population ($cfug^{-1}$ compost)
May 2008	3×10^7
June 2008	3×10^7
July 2008	6×10^5
August 2008	4×10^5
September 2008	3.5×10^5
October 2008	6×10^3
November 2008	5×10^3
December 2008	3×10^3
January 2009	8×10^2
February 2009	4×10^2
March 2009	3×10^2
April 2009	3×10^2

No pathogen propagule was detected from spent compost of healthy production trays placed outdoors.

Table 4. Population of *Mycogone perniciosa* in different casing materials during 2008 and 2009.

Casing material	*M. perniciosa* population ($cfug^{-1}$ casing mixture)	
	Spring 2008	Autumn 2009
Peat soil	-	-
Garden soil	2×10^4	5.5×10^2
Virgin soil	-	-
Spent compost	4.5×10^4	3.5×10^2
Farmyard manure	-	-
Sand	-	-

- = Not isolated.

Table 5. Number of *Mycogone perniciosa* colonies developed on PDA plates exposed to air currents of different velocities at different distances

Air current velocity	Distance from inoculum source					Mean
	20 cm	40 cm	60 cm	80 cm	100 cm	
High (7 meters/sec.)	15.67	11.33	7.00	1.00	0.00	7.00[a]
Medium (5 meters/sec.)	9.33	3.67	2.00	0.00	0.00	3.00[b]
Low (2 meters/sec.)	2.67	1.33	0.33	0.00	0.00	0.87[c]
Mean	9.22[A]	5.44[B]	3.11[C]	0.33[D]	0.00[E]	
		SE±			CD(p=0.05)	
Distance		0.53			1.08	
Speed		0.41			0.83	
Distance x Speed		0.91			1.87	

*Figures are the number of *M. perniciosa* colonies.

Table 6. Per cent *Agaricus bisporus* sporocarps infected with *Mycogone perniciosa* after release of different populations of sciarid flies fed on diseases sporocarps

Acquisition time (min)	Sciarid fly population					Mean
	10	20	50	75	100	
30 min	5.33*	8.33	11.00	18.00	19.00	12.33
60 min	12.00	15.67	16.67	23.00	29.67	19.40
90 min	16.67	25.00	35.00	37.33	42.67	31.33
120 min	27.00	31.33	42.00	46.67	52.00	39.80
	15.25	20.08	26.16	31.25	35.83	
		S.E±			CD (p=0.05)	
Fly population (FP)		1.09			2.20	
Acquisition time (AT)		0.97			1.97	
Pop. x Time (FP x AT)		2.18			4.40	

*Figures are the per cent infected sporocarps.

and sand did not yield any *M. perniciosa* propagules during both the years.

Air currents

The dispersal of *M. perniciosa* spores from the site of production with the help of varied air currents was assessed by counting the number of pathogen colonies developed on PDA plates exposed to the air currents at different distances. The results (Table 5) revealed an average maximum dispersal (7 colonies/plate) occurring at high air current velocity (7 m/s) followed by that (3 colonies/plate) at medium air current velocity (5 m/s), with minimum dispersal (0.87 colonies/plate) occurring at low velocity (2 m/s). The high air velocity (7 m/s) could drift *M. perniciosa* spores to a distance of up to 80 cm. However, the spores were dispersed up to a maximum distance of 40-60 cm only with air currents of low to medium velocity.

Sciarid fly infestation

Different populations of sciarid flies were allowed to acquire inoculum from wet bubble infected sporophores for varied periods and subsequently released on healthy mushroom trays to assess their influence on the spread of the disease. The results (Table 6) reveal an average maximum sporocarp infection (39.80%) occurring with the maximum acquisition period of 120 min followed by that (31.33%) with 90 min acquisition period. Least average sporocarp infection (12.33%) was recorded with acquisition period of 30 min. The population of sciarid flies also influenced the disease spread symptoms significantly. On an average, the maximum test population of 100 flies/tray yielded maximum sporocarp infection (35.83%) followed by a population of 75

Table 7. Effect of water sprays on dispersal of *M. perniciosa* spores from infected sporophores of white button mushroom.

Distance from infected mushroom sporophores (cm)	No. of aleurospores/hlam chlamydospores/ml water splash
0	176.00
10	149.33
20	94.67
30	74.33
40	42.67
50	29.00
60	12.00
70	8.00
80	2.00
90	0.0
100	0.0
Distance	
S.E±	**CD(p=0.05)**
2.16	4.47

flies/tray (31.25%); the minimum fly population of 10/tray even resulted in 15.25% sporocarp infection. A significant interaction also existed between fly population and acquisition period in spreading the wet bubble disease. The maximum fly population of 100/tray with the maximum acquisition time of 120 min resulted in the maximum sporocarp infection of 52% followed by a fly population of 75/tray with the same acquisition period (46.67%). The least fly population of 10-20/tray with minimum acquisition period of 30 min resulted in the least sporocarp infection (5.33-8.33%).

Water splashes

The influence of water spray on the dispersal of *M. perniciosa* spores was observed by collecting water splashes at different distances from the infected mushroom sporophore and counting the spores per ml. The result (Table 7) reveal that the population was maximum (176 cfu/ml of splash water) nearest the source (0 cm) and minimum (2 cfu/ml) at a distance of 80 cm from the infected mushroom sporophore. None of the pathogen spores was observed in water splashes collected beyond 80 cm distance from the infected mushroom sporophore.

DISCUSSION

Wet bubble disease is a nuisance in the profitable cultivation of white button mushroom (*Agaricus bisporus* L.). It is caused by a cosmopolitan fungal pathogen, *M. perniciosa* which is responsible for a huge crop failures in Kashmir valley. This mycoparasite parasitizes the fruit bodies/sporophores and/or growing mycelium and causes variable yield losses depending upon the stage of infection, amount of inoculum and the prevailing ecological factors inside the mushroom production rooms.

The pathogen was isolated from infected sporophores of button mushrooms. The morphological characters of the associated pathogen were studied and compared with the standard authentic description from the literature (Hsu and Han, 1981; Kaul et al., 1978), establishing its identity as *M. ssperniciosa* authority etc. and re-confirming through from ITCC, New Delhi, India under Accession No 7493.09.

Mycelium of the pathogen was initially compact, felt like, septate, hyaline but later turning amber brown with age. The pathogen produced conidia and chlamydospores, conidiophores sub-verticillate to verticillately branched, well septated bearing thin walled 1-2 celled conidia which were relatively short lived. The chlamydospores are terminal, two celled, with a very thick walled terminal cell. The average time between inoculation and symptom expression varied between 2-12 days depending upon the time of infestation.

The pathogenic fungus of wet bubble disease was not re-isolated from the pathogen inoculated compost samples at the time of filling when kept within central region of the heap at the time of stacking; 1st, 2nd, 3rd, 4th and 5th turning however it got easily isolated when the samples were kept during 6th, 7th and 8th turning. The peak heating temperature eliminated the pathogen from the sick compost samples.

Pathogen infested casing soil serves the main source of infection of wet bubble disease. The pathogen survived and remained viable for all months worked out in infested casing soil when kept outdoors. The population of pathogen remained maximum in the first month, the experiment was laid out and later declined gradually.

Diseased spent compost also serves as a source of infection of wet bubble disease. Samples drawn each month from healthy and diseased spent compost recorded presence of pathogen during the month observed. No pathogen was isolated from healthy spent compost. The pathogen population in diseased spent compost was maximum (3×10^7 g^{-1} compost) immediately after placing outdoor then declined gradually.

Only the garden soil and spent compost was found to harbour the wet bubble pathogen. The peat soil, virgin soil, farm yard manure and sand did not yield any propagule of *M. perniciosa.*

Dispersal of *M. perniciosa* spores was found to be carried out by air currents, sciarid flies and water splash. The dispersal by air current is highly influenced by air velocity (7 colonies/plate) was carried out by higher air current velocity (7 m/s) and minimum (0.87 colonies/plate) at low velocity (2 m/s).

Sciarid fly also carried the spores to different distances, dispersal by sciarid flies are influenced by inoculum acquiring period and number of flies visiting diseased sporophores. Maximum sporocarps got infected with maximum acquisition period of 120 min and higher population of flies visiting diseased sporocarps.

The dispersal was also found be carried by water splashes during water spray on trays each day. The maximum colony forming units were observed during spray nearest the infected sporocarps and it declined with the increase in distance of splash.

Conflict of interests

The author(s) did not declare any conflict of interest.

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