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Isolation and identification of *Mycogone perniciosa*, causing wet bubble disease in *Agaricus bisporus* cultivation in Kashmir

Shaheen Kouser and Shaiesta Shah*

Division of Plant Pathology, Sheri-Kashmir Agricultural University of Science and Technology (SKAUST-Kashmir) Shalimar campus, Srinagar-191121, Kashmir, India.

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Button mushroom is an important edible fungus cultivated in Kashmir, but it is often attacked by disease causing organisms, including fungi, bacteria and viruses. Some of them cause huge losses by reducing the yield significantly or result in serious crop failures, depending upon the severity and stage of appearance. One of the mycoparasite, *Mycogone perniciosa*, the cause of wet bubble disease, was observed in samples collected from mushroom farms of three districts: Srinagar, Budgam and Pulwama of Kashmir Valley. This fungal pathogen was constantly associated with the disease and produced typical and characteristic symptoms of wet bubble disease. Isolation of the pathogen was made from diseased fruiting bodies. The pathogenicity proved in accordance with the Koch's postulates both *in vivo* as well as *in vitro*. The pathogen was found to attack button mushroom at all the growth stages but the immature mushroom were found more susceptible than the mature ones, and characteristic symptoms developed on all the inoculated sporophores. The mushrooms were malformed with swollen stipes and with deformed caps. The *in vitro* interaction between *Agaricus bisporus* and *M. perniciosa* mycelia indicated the hyphal collapse of the former at the point of contact between the two fungi. There was no zone of inhibition, found in this interaction.

Key words: Yield, symptoms, susceptible, sporophores, interaction, hyphal collapse.

INTRODUCTION

Mushroom production represents one of the commercially important microbial technologies for large-scale recycling of agro wastes and relieves pressure on arable land owing to its cultivation under controlled conditions. Mushroom culture represents the only major process in biotechnology which successfully converts cellulosic into useful foods and by-products.

Edible mushrooms include 2000 species from more than 30 genera, but only 80 species are grown experimentally, 40 are cultivated economically, 20 are cultivated commercially and only 4 to 5 are produced on an industrial scale (Chang, 1990). Only three species of edible mushrooms, *Agaricus bisporus* L. (white button mushroom) *Volvariella* spp. (paddy straw mushroom) and *Pleurotus* spp. (oyster mushroom) are in commercial or semi-commercial cultivation in India. From a production stand point, the white button mushroom has the highest growth rate and potential for production. It contributes about 90% of total country's production.

Button mushrooms comprise a good nutritious diet for all ages and under all conditions of health. They are rich in proteins with lysine and tryptophan that are normally

*Corresponding author. E-mail: shaiestashah@gmail.com.

deficient in cereals. The carbohydrate content ranges from 4.5 to 5.0% but are in the form of glycogen, chitin and hemicellulose instead of starch. The fat is as low as 0.3% but is rich in linoleic acid, which is an essential fatty acid (Yang et al., 2001). Cholesterol is absent and is replaced by ergo-sterol, which gets converted to vitamin D in the human body. Button mushrooms are a good source of vitamin C and vitamin B complex, particularly thiamine, riboflavin, niacin, biotin and pantothenic acid (Mattila et al., 2000; Manzi et al., 2004). Folic acid and vitamin B12 which are generally absent in most vegetables are present in these mushrooms. Such vitamins also supply a range of valuable minerals especially potassium and iron.

The cultivation of button mushroom has emerged as an important agro-based industry in Kashmir division. New farms with environmental control system and compost pasteurization facilities are coming up in the state, but the number of such farms is very few (Munshi and Ghani, 2003). By the end of 2010, the mushroom production of Jammu and Kashmir State has reached 950 metric tons per annum of which valley contributed 250 metric tons. The mushroom industry in the valley is in the revival phase and more and more people and entrepreneurs are taking up this venture to earn their livelihood by adopting the year round cultivation. Its cultivation in the Kashmir valley has increased manifold, but the major constraint in the popularization of this crop are diseases and pests, which happen to be devastating and perpetuate easily from one season to another. The cultivation of the mushroom is susceptible to many competitive organisms and weed fungi (Brown, 1937; Davis, 1938), causing substantial economic loss to the growers due to decrease yields. *Mycogone perniciosa* Magn. is the most common fungi causing severe losses in yield of *A. bisporus* throughout the country. Wet bubble disease of white button mushroom also called as La mole, white mould, bubble, *Mycogone* disease has been reported as one of the serious diseases from almost all major mushroom growing countries of the world. In India, this disease was reported for the first time in 1978 from some mushroom farms in Jammu and Kashmir (Kaul et al., 1978), later this disease was reported from the states of Himachal Pradesh, Haryana and Maharashtra (Sharma and Kumar, 2000; Bhatt and Singh, 2000; Sharma and Singh, 2003).

Wet bubble disease is reported to be a devastating disease in the crop production of button mushrooms. It is a common contaminant, occurring in mushroom houses in the Kashmir Valley. It has been reported to cause complete crop failures in cases of severe infections. The pathogen, *M. perniciosa*, causes pathological changes in fruit bodies of *A. bisporus*, to form undifferentiated primordia. The primordia are of vastly hyperplastic, tumorous character. They produce large, irregular, confluent lumps of *A. bisporus* with no sign of differentiation or organogenesis (Umar et al., 2000).

These timorous bodies often showed dripping amber liquid as teardrops.

Keeping in view the devastating nature of this disease, the present study has been undertaken to isolate, characterize and identify the pathogen responsible for the disease.

MATERIALS AND METHODS

In the present study, diseased material (sporophores along-with infected casing soil) was collected in paper bags/containers from three districts (Srinagar, Budgam and Pulwama) of Kashmir Valley. The fungi were cultured on fresh Potato Dextrose Agar (PDA) medium for isolation, identification and characterization. The pathogen was purified and maintained by repeated sub-culturing after every month and kept in a refrigerator at 2 to 5°C for the following studies:

Isolation, identification and establishment of pure culture

Isolation of the pathogen

Isolation of the associated micro-organism was made from the mushroom sporophores, showing typical symptoms of wet bubble disease following routine pathological techniques (Holliday, 1980). The diseased sporophores/sclerodermoid masses were first examined for the associated fungus by teasing the diseased portion with the help of a teasing needle and observed under microscope. For isolation of the causal fungus, 5 mm small flesh disc segments were taken from the infected sporophore, with the help of sterilized cork borer, surface sterilized with 0.1% mercuric chloride for 30 s followed by rinsing thrice with sterilized distilled water, blotter dried and inoculated under aseptic conditions on Rose Bengal-amended PDA medium in sterilized Petri-dishes, incubating the plates at 23 ± 2°C.

Purification and maintenance of pathogen

The culture was purified by hyphal tip (Pathak, 1972) and single spore isolation (George, 1947) methods. The pure culture, thus obtained, was maintained by repeated sub-culturing at monthly intervals. The stock culture in PDA slants was stored at 4°C in a refrigerator. The isolations were made afresh in each cropping season to avoid possible loss of pathogenic behaviour of the isolated fungus by repeated sub-culturing.

Identification of pathogen

The isolated micro-organism was identified on the basis of its morphological and cultural characteristics comparing it with the standard descriptions of *M. perniciosa* given by Hsu and Han (1981) and Singh and Sharma (2002). The identity of the pathogen was also confirmed from National Bureau of Agriculturally Important Micro-organisms (NBAIMS) New Delhi, and the culture deposited under accession No. 7493.09.

Pathogenicity test

In vitro test

The isolated micro-organism and the *A. bisporus* was grown in dual culture in Petri-plates at 23±2°C and observed for the type of

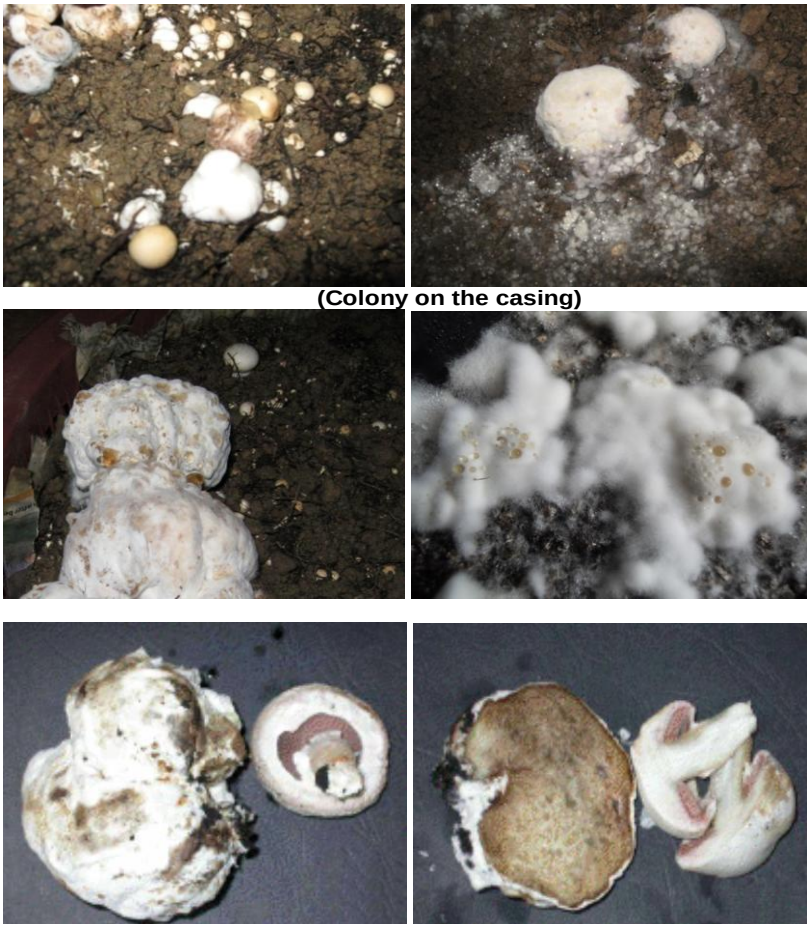

Plate 1. Natural symptoms of wet bubble disease of white button mushroom.

interaction. The slower growing *A. bisporus* was inoculated first and the colony was allowed to reach a diameter of 15 to 20 mm before the fast growing pathogen was inoculated on opposite sides of 90 mm Petri-plates. The radial growth of the pathogen and host was measured at the time of colony contact. Upon contact between the host and the pathogen, the interaction between the two mycelial colonies was noted.

***In vivo* test**

In order to prove the pathogenicity of the isolated micro-organism and also to record the symptoms and development of infection on the sporophores, two sets of experiments were carried out in plastic baskets of 5 kg compost capacity. The baskets were disinfected with 2% formalin and rinsed thrice with sterilized distilled water. The baskets were filled with pasteurized compost, spawned and kept in spawn run room at 24 ± 2°C. In the first set of experiment, aleuriospore suspension (2×10^6 ml^{-1}) was inoculated in sterilized casing mixture at the time of casing.

In the second set of experiment, the isolated micro-organism was inoculated on healthy pinheads and fruit-bodies in the form of aleuriospore suspension (2×10^6 ml^{-1}) and the mycelial discs from the colony, to observe the wet bubble symptom development. After inoculation, the baskets were kept in an isolated room at a temperature 21 ± 1°C with relative humidity of more than 85%. An un-inoculated basket was also maintained under similar conditions to serve as check and kept apart to avoid contamination. Both the sets of pathogenicity tests were closely monitored for symptom development.

Re-isolations of the micro-organism from the artificially inoculated sporophores/sclerodermoid masses and diseased pinheads were made and the resultant cultures compared with the original inoculant to satisfy Koch's postulates.

RESULTS

The results obtained during the course of this study are divided into the following sub-headings as follows.

Symptomology

The natural symptoms of wet bubble disease included development of whitish mouldy growth of the mycelium on the casing surface and on portions of fruit bodies, which eventually spreads covering the entire cap and causing distortion of the affected mushrooms (Plate 1). The infected pin heads have amorphic shapes, not resembling the typical mushrooms. Similar types of

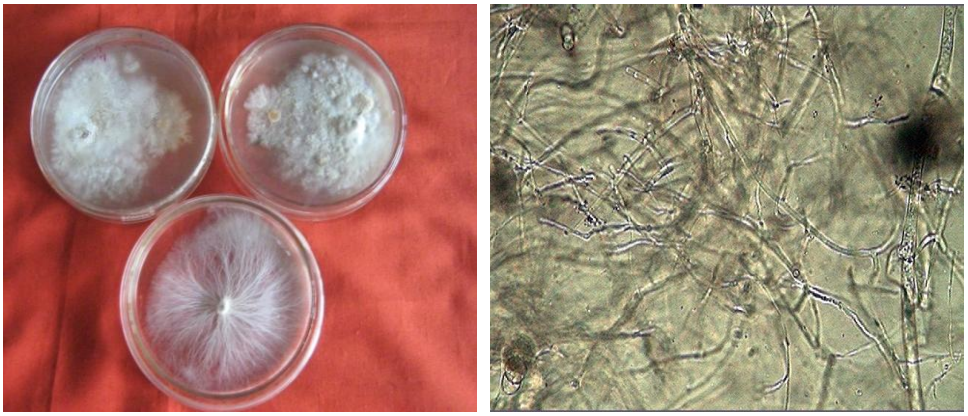

Plate 2. *In vitro* pathogenicity test.

symptoms were produced on inoculation of pinheads and mature fruit bodies with mycelial bits from spore suspension sprays or inoculation with agar culture discs of the pathogen. However, the appearance of symptoms and the subsequent disease development was much quicker when fruit bodies of the mushroom (*A. bisporus*) were inoculated with mycelia. Characteristic symptoms in this case appeared within 1 to 2 days of inoculation. The reactions of undifferentiated primordia were vastly hyperplastic and of tumorous nature. Finally, large irregular lumps of mushroom tissues were formed with no sign of differentiation or organogenesis. Dripping out of foul smelling amber liquid as teardrops on the timorous bodies was consistently a typical sign of the wet bubble disease.

Pathogenicity

Observations recorded on the response of white button mushroom to the isolated fungus (Plate 2) inoculated in sterilized casing mixture at the time of casing indicated its pathogenic behaviour, which ultimately resulted in the development of typical symptoms (Plate 3). The symptoms started appearing as white fluffy patches of mycelium on the surface of casing from the 7^{th} day after inoculation, whereas when inoculation was done on young pinheads, symptoms started appearing within 1 to 2 days after inoculation. Mushrooms attacked before differentiation looked as a blob. Inoculation of mature stages of mushroom showed an initial browning. A large portion of the gill tissue got colonized when the mature mushrooms got infected, well after the differentiation of stalk, cap and gills. When the infected mushrooms were incubated at 18 to 20°C at a high relative humidity of 95% maintained, enormous number of spores appeared on the mycelial mat of *A. bisporus*. The large mushrooms when inoculated also showed swollen stipes or deformed caps with tissue discolouration and also fuzzy growth on mushroom cap. The blobby surface of mushroom became wet, and slightly tinted extracellular fluid was discharged. Re-isolations from infected sporophore masses yielded typical culture of the fungus and satisfied the Koch's postulates.

DISCUSSION

Wet bubble disease is severe impairment in the profitable cultivation of white button mushroom (*A. bisporus* L.). It is incited by an important and cosmopolitan fungal pathogen, *M. perniciosa* Magn. which is responsible for frequent crop failures in Kashmir Valley. It is believed that the frequent incidence of mould disease including that of wet bubble disease in Kashmir Valley and the resultant crop failure of maximum frequency, owing to lack of knowledge to effectively and efficiently manage the disease led to abandoning of its cultivation in early eighties. The 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 production rooms.

Like other diseases, the wet bubble of button mushrooms is also identified by the manifestations of certain characteristic symptoms. Two main types of symptoms were observed during the present studies-infected sporophores and brown tumorous undifferentiated sclerodermoid masses, which resulted due to infection at different stages in the development of sporophores. Sporophores inoculated with pathogen spore suspension developed characteristic symptoms within 24 h, whereas the infestation of the casing soil with spore suspension symptoms developed 9 to12 days after inoculation depending upon the time of infestation of casing soil (in Materials and Methods it was mentioned that inoculation was done at the time of casing and on pinheads in the second experiment as well). Shortest incubation period of 4 to 5 days was recorded when the inoculum was sprayed at pinhead formation stage and the longest of

Plate 3. *In vivo* Pathogenicity test.

12 days when sprayed 1 day after casing. The findings indicate maximum susceptibility and vulnerability of pinheads and buttons. The symptoms produced in inoculated material were similar to those recorded under natural conditions during survey. The undifferentiated primordia which were less than 5 to 6 mm in diameter, responded to the inoculation with hyperplasia and tumor induction. Large, irregular confluent *A. bisporus* lumps developed with no signs of differentiation or organogenesis. These tumorous bodies were often studded with coloured tear drops especially in conditions of very high humidity. Sometimes partial differentiation of the primordia resulted in partly formed caps showing protuberances on their surfaces. At later stages (10 to 12th day of infections, these tumorous masses were found infected the various types of bacteria. Similar description of the disease was also given by several other researchers (Smith, 1924; Bech et al., 1982; Geijn, 1977; Fletcher and Ganny, 1968; Sharma and Kumar, 2000; Umar et al., 2000).

Isolation of the pathogen was made from infected sporophores and pathogenicity proved in accordance with the Koch's postulates both *in vivo* and *in vitro*. The pathogen was found to attack all the growth stages of this mushroom but the immature stages were found more susceptible than the mature ones, and characteristic symptoms developed on all the inoculated sporophores. The morphological characters of the associated pathogen were studied and compared with the standard authentic description from the literature (Smith, 1924; Atkins and La Touche, 1948; Hsu and Han, 1981; Kaul et al., 1978), establishing its identity as *M. perniciosa* authority etc. and re-confirming through from ITCC, New Delhi, India under Accession No 7493.09

The *in vitro* interaction between *A. bisporus* and *M. perniciosa* mycelia indicated the collapse of *A. bisporus* hyphae at the point of its contact with *M. perniciosa* hyphae without production of any zone of inhibition, indicating thereby operation of mycoparasitism between the two fungi. Hyphal collapse at the point of contact between the two fungi was also reported earlier (Gray and Morgan, 1981; Sharma and Vijay, 1996). Khanna et al. (2003) also reported that no clearing zone of inhibition occurred between *A. bisporus* and *Verticillium fungicola* mycelium.

Conclusion

The pathogen was found to attack all the developmental stages of the button mushroom *A. bisporus*. The disease is mainly characterized by the presence of white mouldy growth on the sporophores, leading to their putrefaction with the production of golden brown exudates. The

mushrooms became malformed with swollen stipes and with reduced or deformed caps. When these distorted masses were cut across, the affected tissue was generally dark in colour and wet in appearance. When differentiated, sporophores were attacked; the stalk was colonized, with a brown streak, reaching the cap and the gills.

Isolation of the pathogen was made from diseased sporophores and the pathogenicity proved in accordance with the Koch's postulates both *in vivo* as well as *in vitro.* The pathogen was found to attack all the growth stages of this mushroom but immature stages were found to be more suitable than the mature ones and characteristic symptoms developed on all the inoculated sporophores. Mycelium of the pathogen was initially compact, felt like, septate, hyaline but later turning amber brown with age, produced conidia and aleuriospores, conidiophores sub-verticillate to verticillately branched, well septated bearing thin walled 1 to 2 celled conidia which were relatively short lived. The aleuriospores were terminal, two celled, with a very thick walled terminal cell. Further, the interaction between *A. bisporus* and *M. perniciosa* mycelia studied in dual cultures revealed the hyphal collapse of *A. bisporus* at the point of contact with *M. perniciosa* without any zone of inhibition, indicating thereby mycoparasitism between the two fungi.

On the basis of cultural and morphological characters of the isolates fungus as compared with the authentic description together with its pathogenicity on *A. bisporus,* the fungus was identified as *M. perniciosa* Magn. and its identity confirmed through Indian type collection centre, New Delhi under Accession No. 7493.09.

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