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Morphological variability in *Verticillium fungicola* isolates collected from different mushroom farm of Haryana state

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ABSTRACT

In present investigation, the morphological variability were studied by collecting seventeen isolates of *V. fungicola* from different mushroom farms of Haryana state and out of these eight were isolated, purified on PDA medium and which coded as MHS (Hisar), BFT (Fatehabad), NJN (Jind), RHT (Rohtak), TPN (Panipat), BSN (Sonipat), FDB (Fridabad) and SKK (Kurukshetra) and pathogenicity was proved on host *A. bisporus*. Regarding morphological variability, the radial growth was fastest in isolate BSN (44.66 mm), followed by TPN (43.86 mm), FDB (43.33 mm), SKK (42.16 mm) and RHT (41.50 mm), while isolates MHS, BFT and NJN had slow growth *i.e.* 35.83, 34.50 and 38.00 mm, respectively after 12 days of incubation. Isolates MHS, BFT and NJN showed uneven and less feathery colony growth, while rest of isolates had even, profuse feathery and raised colony. Colony pigmentation of *V. fungicola* isolates was white and underside light yellow in MHS, BFT, NJN and RHT, whereas, isolates TPN, BSN, FDB and SKK having dark yellow colour. The size of conidia also varied among the isolates and it ranges from 2.4-5.1×1.2-2.1µm (BFT) to 3.0-7.9×1.1-2.5µm (BSN) whereas, isolates MHS (2.8-4.1×1.0-2.1 µm), BFT (2.4-5.1×1.2-2.1µm) and NJN (2.6-3.1×1.0-2.4 µm) having small size conidia and rest one *i.e.* RHT (2.9-5.5×1.0-2.1µm), TPN (2.9-6.5×1.1-2.3 µm), BSN (3.0-7.9×1.1-2.5 µm), FDB (3.0-6.5×1.0-2.2 µm) and SKK (2.8-4.1×1.0-2.1 µm) had large sized conidia.

Keywords: *Agaricus bisporus*, isolate, pathogenicity, variability, *Verticillium fungicola*

The commercial production of edible mushroom converts different types of agricultural and house-hold wastes into nutrition rich food which helps in addressing the problems of quality food, health and environmental sustainability. In view of increasing demand of high quality food with an increasing world population, mushrooms will be an important source of proteins that can replace meat and vegetables and milk products for a major part (Wani *et al*., 2010). About 1.5 million species of fungus are known (Hawksworth, 1991) and out of these it has been estimated that 14,000 species produce fruiting bodies that are desirable to be considered as mushrooms (Hawksworth, 2001). About 7,000 species of edible mushrooms are known out of which 200 are experimentally grown and 10 have been produced at the industrial scale (Chang and Miles, 2004). Presently, three geographical regions *viz.,* Europe, America and East Asia contribute 96 per cent of world mushroom production. India contributes 0.13 million metric tons and in Haryana; the annual mushroom production is about 10,207 metric tons (Sharma *et al.,* 2017). Button mushroom are a popular and valuable food, low in calories, fat and high in minerals, essential amino

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acids, vitamins and fibers and supply a range of valuable minerals especially potassium and iron (Mattila *et al*., 2002).

In India, mostly four species of edible mushrooms *viz*., *Agaricus bisporus* (white button mushroom), *Volvariella* spp. (paddy straw mushroom), *Pleurotus* spp. (oyster mushroom) and *Calocybe indica* (milky mushroom) are commercially cultivated. Mushroom cultivation is affected by a large number of biotic and abiotic factors. Fungi, bacteria, viruses, nematodes, insects and mites are different biotic factors that damage the mushroom crop directly or indirectly (Sharma *et al*., 2011). Among the various factors responsible for low production and productivity of mushroom in our country, fungal diseases play a major role. The fungal pathogens, *Verticillium fungicola*, *Mycogone perniciosa*, *Trichoderma* spp. and *Papulaspora byssina* are the predominant mycopathogens. Amongst these, *Verticillium fungicola* var. *fungicola* (Preuss) is the important pathogen of the *Agaricus bisporus* (Lange) Imbach and annual losses to the growers are estimated to be 2–4% of total revenue (Berendsen *et al*., 2010). The pathogen induces various symptoms like bubbles (undifferentiated spherical masses), bent and/or split stipes (blowout) and spotty caps. Inoculation of *A. bisporus* crop with isolates of *V. fungicola* var. *fungicola* of various degrees of aggressiveness showed that the more aggressive isolates induced higher numbers of bubbles (Largeteau and Savoie, 2008). The *Verticillium* dry bubble is the most prevalent disease and if left uncontrolled in the mushroom growing environment; the disease can wipe out an entire crop in 2–3 weeks (Sharma *et al*., 2002).

Dry bubble was first detected in a commercial planting in North America in 1981. However, literature on dry bubble has been published in India as early as 1960, proving that it has been an economic problem for mushroom growers during 20th century. It mainly affects three different species of mushrooms *viz. Agaricus bisporus*, *A. bitorquis* and *Pleurotus ostreatus*. Though, the infection by *V. fungicola* does not decrease the weight of the mushrooms, but has the potential to decrease the total number of mushrooms produced (Berendsen *et al*., 2010). *V.fungicola* when grown on potato-dextrose agar at room temperature, the colonies are white and the underside of the plates is colourless to yellow. Some cultures may have a low profile with rounded edges and under a microscope the measure of spores would be about 8-10 mm long by 1-3 mm wide and tapering to 0.5-1.0 mm at the tip (Calonje *et al.,* 1997).

Meagre information is available on *V. Fungicola* in India, especially regarding cultural and morphological variability for effectively management of disease. Therefore, kkeeping this in view; the present studies were undertaken with the objectives of variability studies in terms of cultural and morphological characteristics of *Verticillium fungicola* isolates.

MATERIALS AND METHODS

The present investigation entitled "morphological variability in *Verticillium fungicola* isolates collected from different mushroom farm of Haryana state" was carried out at Mushroom Technology Laboratory, Department of Plant Pathology, CCS Haryana Agricultural University, Hisar, during 2015-2017 seasons. Hisar is situated at a latitude of 29^{o}10'N, longitude 75^{o}46'E and an altitude 215.2 m above mean sea level and fall in semi-tropical regions of Western Zone of India. Details of the materials used and methodology adopted during the course of this investigation are given below.

Samples collection, isolation, purification and pathogenicity test

In the present study, 17diseased samples in triplicate were collected from different parts of the Haryana, out of these *V. fungicola* was isolated from eight samples. Samples were washed thoroughly with tap water, dried and then kept in paper bags for further isolation of pathogen. The fungi was cultured on fresh potato dextrose agar medium for isolation,

purification and pathogenicity test. The pathogen was purified and maintained by repeated sub-culturing of each isolates after every month and kept in a refrigerator at 4°C for the further studies. Isolation of the *V. fungicola* was made from the infected fruiting bodies, which showing typical symptoms of dry bubble disease. The diseased fruiting bodies were first examined for the associated pathogen by teasing the diseased portion with the help of a needle and observed under microscope. For isolation of the causal fungus, five mm small disc segments were cut from the infected sporophore with the help of sterilized cork borer, surface sterilized with 0.1% mercuric chloride for 30 seconds followed by rinsing thrice with sterilized distilled water, blotter dried and inoculated under aseptic conditions on PDA medium in sterilized Petri dishes and incubated at 25±1°C (Sabharwal and Kapoor, 2014). The pathogen culture was purified by hyphal tip culture method (Pathak, 1972). The pure culture was obtained and maintained by repeated sub-culturing at monthly intervals. The stock culture in PDA slants was stored at 4±1°C in a refrigerator. The repeated sub-culturing was done for further studies to avoid the possible loss of pathogenic behavior of the test pathogen. In order to prove the pathogenicity of *V. Fungicola* and development of infection on the sporophores, two sets of experiments were carried out. In the first set plastic baskets of five kg compost capacity were used and disinfected with two per cent formalin and rinsed three times by sterilized distilled water. The baskets were filled with pasteurized compost, spawned and kept in spawn run room at 24±1°C. In the first set of experiment, spore suspension (2×10^6 ml^{-1}) was inoculated in sterilized casing mixture at the time of casing. However, in the second set of experiment, the isolated pathogen was inoculated on healthy pinheads and the fruit bodies with spore suspension (2×10^6 ml^{-1}) and also with the mycelial discs from culture plate of the pathogen to observe the development of dry bubble symptom. After inoculation, the baskets were incubated in an isolated room at a temperature 25±1°C with high relative humidity (>85%). An un-inoculated basket was also maintained under similar conditions as check in isolation apart to avoid any contamination of mushroom fruiting bodies. Both the sets of pathogenicity tests were closely monitored for the development of disease symptoms. Re-isolation of the micro-organism was done from the diseased fruiting bodies to prove the Koch's postulates (Sabharwal and Kapoor, 2014). In the present investigation the morphological studies were performed on the potato dextrose agar (PDA) medium. The sterilized 25 ml PDA was poured aseptically in each Petri plates and inoculated after 24 hrs with five mm mycelia disc from the periphery of actively growing colony of 12 days old cultures of all different isolates and then incubated at 25±1°C. The morphological features such as radial growth (mm), complete colonization (days), colony pattern, colony pigmentation and size (µm) of conidia were recorded after incubation. For each isolate three replications were maintained in completely randomized design (CRD). The conidial size of different isolates was measured by using ocular and stage micrometer (Gams and Van Zaayen, 1982). The observations were measured by the number of divisions of stage micrometer coinciding with division of the ocular micrometer, the calibration factor or ocular index (µm) was calculated by the formula as follows:

$$\text{Ocular index } (\mu) = \frac{\text{Number of division of stage micrometer coinciding withdivision in ocular}}{\text{Number of divisions of ocular micrometer coinciding withdivision in stage}} \times 10$$

RESULTS AND DISCUSSION

Samples collection, isolation, purification and pathogenicity test

During variability studies in *V. fungicola*, the 17 diseased samples of white button mushroom were collected from different parts of the Haryana and from these *V. fungicola* was isolated from eight samples. Isolation of *V. fungicola* was done from the diseased samples and pathogenicity was proved on white button mushroom. The cultures were purified and maintained

Fig. 1. *Verticillium fungicola* isolates collected from different locations

on potato dextrose agar medium (Plate-1). The purified *V. fungicola* isolates were coded (Table-1) as MHS (Hisar), BFT (Fatehabad), NJN (Jind), RHT (Rohtak), TPN (Panipat), BSN (Sonipat), FDB (Fridabad) and SKK (Kurukshetra).

Morphological variability of *V. fungicola* isolates

To study the variability in *V. fungicola* isolates were cultured on PDA medium and incubated at 25°C to study the various morphological characteristics. The isolates exhibited variations among themselves regarding radial growth, colony pattern, pigmentation and size of conidia. It was depicted from the Table-2 and Plate-1, radial growth varied between 34.50 to 44.66 mm amongst isolates after 12 days of incubation on potato dextrose agar media at 25±1°C. The mycelial growth was fastest in isolate BSN (44.66 mm), followed by TPN (43.86 mm), FDB (43.33 mm), SKK (42.16 mm) and RHT (41.50 mm), on the other

Table 1. Collection of dry bubble disease samples from different parts of the Haryana

Isolates of *Verticillium fungicola*	White button mushroom diseased samples collection		
	Isolates Code	Village/ City	District
VF-1	MHS	Matarshyam	Hisar
VF-2	BFT	Bhattu	Fatehabad
VF-3	NJN	Narwana	Jind
VF-4	RHT	Rohtak	Rohtak
VF-5	TPN	Taharpur	Panipat
VF-6	BSN	Bainyapur	Sonipat
VF-7	FDB	Fridabad	Fridabad
VF-8	SKK	Sudha mushroom lab, Kurukshetra	Kurukshetra

Table 2. Morphological variability amongst *V. fungicola* isolates isolated from different location

Isolates Code	Mycelial characteristics* of *Verticillium fungicola* isolates on PDA#					
	Location	**Radial growth **(12DAI)**	**Radial growth (45mm)**	**Colony pattern**	**Colony pigmentation**	**Size of conidia (um)**
MHS	Hisar	35.83	17	Uneven-less feathery	Whiteunderside –light Yellow	2.8-4.1×1.0-2.1
BFT	Fatehabad	34.50	17	Uneven-less feathery	Whiteunderside –light Yellow	2.4-5.1×1.2-2.1
JNJ	Jind	38.00	15	Uneven-less feathery	Whiteunderside –light Yellow	2.6-3.1×1.0-2.4
RHT	Rohtak	41.50	15	Even-feathery, Raised	Whiteunderside –light Yellow	2.9-5.5×1.0-2.1
TPN	Panipat	43.86	12	Even- very feathery, Raised	Whiteunderside – dark Yellow	2.9-6.5×1.1-2.3
BSN	Sonipat	44.66	12	Even-very feathery, Raised	Whiteunderside – dark Yellow	3.0-7.9×1.1-2.5
FDB	Fridabad	43.33	12	Even-very feathery, Raised	Whiteunderside – dark Yellow	3.0-6.5×1.0-2.2
SKK	Kurukshetra	42.16	15	Even-feathery, Raised	Whiteunderside – dark Yellow	2.8-4.1×1.0-2.1
DMR-Reference	DMR- Solan	45.00	12	Even-very feathery, Raised	Whiteunderside – dark Yellow	3.0-8.5×1.0-2.5

*Average of three replications, **Growth (mm) 12 DAI, #Potato dextrose agar medium

hand the isolates MHS, BFT and NJN having mycelial growth very slow *i.e.* 35.83 mm, 34.50 mm and 38.00 mm, respectively. Similarly the isolates BSN, TPN and FDB are fast growing which completed 45 mm radial growth after 12 days of incubation while the isolates SKK, RHT and NJN completed 45 mm radial growth after 15 days incubation so these are medium growing. The isolates MHS and BFT are slow growing which took 17 days for completion of 45 mm radial growth.

Three isolates MHS, BFT and NJN showed uneven and less feathery colony growth, while, the rest of isolates had even, very feathery and raised colony growth. The colony pigmentation of all *V. fungicola* isolates is white and underside light yellow in MHS, BFT, NJN and RHT, whereas, isolates TPN, BSN, FDB and SKK showed dark yellow. The size of conidia also varied with different isolates and it ranges from 2.4-5.1×1.2-2.1µm (BFT) to 3.0-7.9×1.1-2.5µm (BSN) whereas, isolates MHS (2.8-4.1×1.0-2.1 µm), BFT (2.4-5.1×1.2-2.1µm) and NJN (2.6-3.1×1.0-2.4 µm) having small size conidia and rest one RHT (2.9-5.5×1.0-2.1µm), TPN (2.9-6.5×1.1-2.3 µm), BSN (3.0-7.9×1.1-2.5 µm), FDB (3.0-6.5×1.0-2.2 µm) and SKK (2.8-4.1×1.0-2.1 µm) had large sized conidia. These finding are in complete agreement with those of Calonje *et al.* (1997) who also reported that *V.fungicola* when grown on potato dextrose agar medium at 25±1°C, had white colonies and the underside as light yellow. Collopy *et al*. (2001) reported that colonies growing moderately fast on PDA, reaching diameter 31 mm in 10 days at 20!. The colonies are white, flocculose and underside of the plates is whitish to cream. Conidiophores erect, with several whorls of 3-5 phialides and phialides subulate usually 12.0-26.0 × 0.7-1.9 µm. Conidia are ellopsoidal to short-cylindrical, one-celled, 2.8-4.1×1.0-2.1 µm. Sharma *et al*. (2007) reported that *V. fungicola* produces single celled, cylindrical conidia having the diameter 3.5-15.9 x 1.5-5.0 µm.

On the other hand, Gams and Van Zaayen (1982) also observed that colonies of *V. fungicola* look white, reverse pale yellowish and conidia of unequal size (3.8-7.2 × 1.2-2.4 µm). Similarly, Sabharwal and Kapoor (2014) also reported different morphological

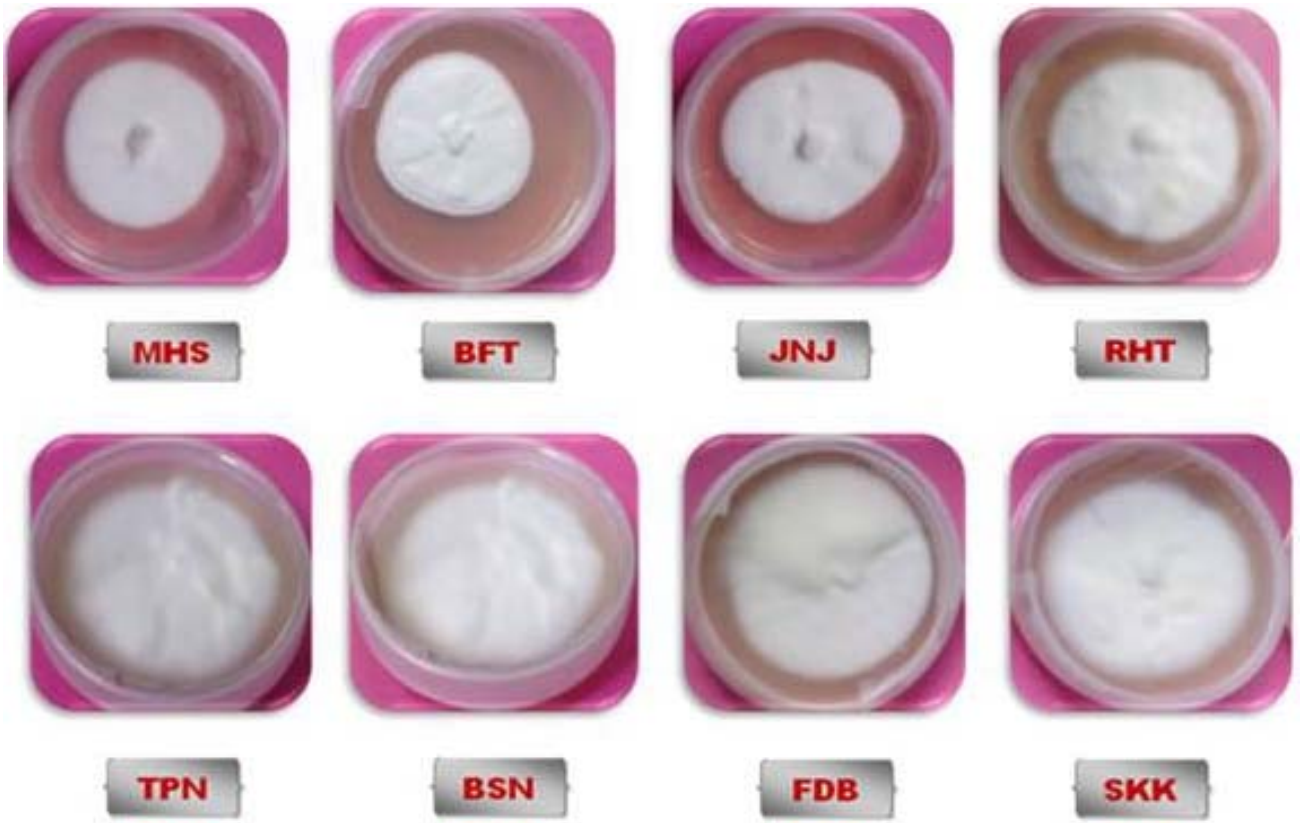

Fig. 2. Morphological characteristics of *Verticillium fungicola* isolates on PDA media

features regarding radial growth, colony pattern, pigmentation and size of conidia of different isolates of wet bubble disease of button mushroom. Our findings also supported by various researchers (Sujata and Sai, 2010; Raghuwanshi, *et al.,* 2013). They also performed significant studies by collecting different isolates of various pathogens, which were responsible for various degree of aggressiveness when infected the host.

CONCLUSION

Morphologically all the eight isolates of *V. fungicola* showed variability in radial growth, colony pattern, pigmentation and conidial size. Three isolates *i.e.* MHS, BFT and JNJ showed uneven pattern with less feathery growth, while rest of the isolates had even and profuse feathery growth. All isolates had whitish colony with light yellow to dark yellow pigmentation underside. Isolates namely BSN, TPN and FDB were fast growing that completed 45 mm radial growth within 12 days, while three isolates SKK, RHT and JNJ fall in medium category as it took fifteen days to complete 45 mm radial growth. While two isolates MHS and BFT were slow growing took seventeen days for 45mm radial growth. The size of conidia also varied among different isolates and fast growing (BSN) isolate had large size (3.0-7.9 × 1.1-2.5 µm) conidia.

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