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OPTIMIZATION OF CULTURE CONDITIONS FOR MYCELIAL GROWTH AND SPORULATION OF *MYROTHECIUM RORIDUM*

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ABSTRACT

Culture and nutrition conditions of *Myrothecium roridum* Tode were optimized by conducting a series of interlined experiments on a growth medium, temperature, pH, and photoperiod. In contrast, relation of culture age with virulence was measured by fungal development on young leaves of bitter melon. The physiological response was measured on colony radial growth and spore production. Among the six test growth media, i.e., nutrient agar (NA), potato dextrose agar (PDA), Czapek-Dox agar (CDA), glucose agar (GA), malt extract agar (MEA), and bitter melon agar (BMA), the highest radial growth (77 mm) and the highest number of spores (239×10^6 spores/ml) were observed on PDA. Incubation temperature was evaluated between a range of 15-40 °C, and the highest colony growth (87 mm) was observed at 30 °C, whereas the highest spore production (315×10^6 spores/ml) was at 35 °C. Different pH levels, i.e., 5, 5.5, 6, 6.5, 7, and 7.5, were optimized, and the highest colony growth (87 mm) and spore production (504×10^6 spores/ml) was recorded at pH 5.0. Impact of photoperiod was studied, and the highest mycelial growth (88 mm) and maximum spore production (524×10^6 spores/ml) was observed at 16/8 h alternate light and dark period. It was concluded that the optimum conditions for mycelia growth and spore production was pH 5.0-6.0 and at 30 ± 2 °C in PDA with 16/8 h alternate light and dark photoperiod.

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INTRODUCTION

Myrothecium roridum is a facultative parasite with a large number (around 263 plant species) of plant hosts, including vegetables, fruits, and ornamental plants (Murakami and Shirata, 2005; Silva and Meyer, 2006). It is widely distributed in the world's hot and humid tropical climates and has been recognized as a serious damaging agent to the leaf, crown, and fruit of the plant. An increasing number of reports of *Myrothecium* spp. attacking on several plant species worldwide indicate that pathogen is under continuous evolutionary changes

and expanding its pathogenicity to other plant families. If favorable conditions persist, it has a potential threat to result in an epidemic. The distribution frequency of *Myrothecium* conidia is affected by soil surface temperature so that mono cropping culture, field sanitation, and rain contribute to disease epidemics (Fish *et al.*, 2012). During the last decade, many first reports of *M. roridum* causing Myrothecium leaf spot have been published from countries with diverse climatic conditions (Seebold *et al.*, 2005; Mangandi *et al.*, 2007; Mmbaga *et al.*, 2010; Zhao *et al.*, 2010; Talukdar

and Prasad, 2013; Kwon *et al.*, 2014; Han *et al.*, 2014). It has also been reported to form an endophytic association in *Pinus albicaulis* (Worapong, 2009). In Pakistan, the *Myrothecium* leaf spot (MLS) disease of bitter melon was first reported in 1988 at Faisalabad district of Punjab province (Ali *et al.*, 1988). Later on, it was found associated with seeds and isolated frequently from rotted and un-germinated seeds of cucurbits during seed health testing (Shakir and Mirza, 1992; Sultana and Ghaffar, 2007). According to the official reports of the year 2007 of pest warning, Punjab Agriculture Department, it has found distributed throughout the bitter melon growing areas in Punjab, Pakistan.

The virulence factor of fungi can be predicted based on germination, radial growth, sporulation. The nutritional process from the host influences the growth and colonization of fungal pathogens, reflecting the severity of infection. The role of implication of fungal nutritional physiology attributes in management strategies was investigated by several researchers (Shah *et al.*, 2005; Hussain *et al.*, 2010). The relation between source of nutrition and production of secondary metabolite toxins has been demonstrated for plant pathogenic fungi (Howlett, 2006). The development of models based on fungal physiological responses may aid in generating information for incorporation in disease management approaches (Wilson and Talbot, 2009). However, there is the scarcity of information on relationship between physiological attributes and virulence of *M. roridum* on bitter melon. Therefore in the present investigation, different nutrition and cultural conditions were employed to find optimum conditions to harvest virulent cultures of the fungus so that reliable investigations for onward studies on disease management.

MATERIALS AND METHODS

Studies were conducted at Seed and Post-harvest Pathology Lab, Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan. The single test spore *Myrothecium roridum* (strain Mr 10: Accession number FCBP 1155) isolated from bitter melon plant was exposed to a wide range of growth media, incubation temperature and period, pH and photoperiod. The pathogen's physiological requirements were optimized through experimental layout and practiced using completely randomized design (CRD).

To evaluate the nutrients provisions of the pathogen,

different culture media i.e. nutrient agar (NA; beef extract, 3.0 g; peptone, 5.0 g; agar, 15.0 g; pH, 6.8), potato dextrose agar (PDA; potato infusions, 4.0 g; dextrose, 20.0 g; agar, 15.0 g, pH, 5.6), Czapek-Dox agar (CDA; sucrose, 30.0 g; sodium nitrate, 2.0 g; dipotassium phosphate, 1.0 g; magnesium sulphate, 0.5 g; potassium chloride, 0.5 g; ferrous sulphate, 0.01 g; agar 15.0 g, pH, 7.3), glucose agar (GA; glucose, 20.0 g; agar, 15.0 g), malt extract agar (MEA; malt extract, 20.0 g; agar, 15.0 g) and bitter melon agar (BGA; bitter melon fresh leaves, 20.0 g; agar, 15.0 g) media were used. Under aseptic conditions, Pyrex glass Petri plates (90 mm) containing test media were inoculated on the center with a 5 mm disc of 5 days old culture of the *M. roridum* and incubated at 25 °C. Mycelia growth was measured at 72 hours till the maximum growth was achieved on a treatment. The most suitable medium yielding maximum colony growth and the number of spore/ml was used for onward studies.

The temperature was optimized for *M. roridum* by plating the fungus (5 mm plug) on the most suitable selected medium, incubating at 15, 20, 25, 30, 35, and 40 °C for ten days. The response of the fungus was measured by mycelial growth and the number of spore/ml. Optimum pH for the maximum growth of the *M. roridum* was studied on most suitable medium incubated at best-selected temperature for ten days. The optimum pH level was made by adjusting pH of the medium adjusted to 5, 5.5, 6, 6.5, 7, and 7.5 by HCl and NaOH before autoclaving. Data were recorded for the maximum colony growth and the number of spore/ml. The -selected medium, pH, and temperature were employed for the impact of photoperiod on the growth of *M. roridum*. The test Petri plates were incubated under continuous darkness, continuous light, and 16/8 hrs. dark and light conditions for ten days. Optimization was measured on colony growth and the number of spore/ml.

Fresh leaves of a commercial variety of bitter melon (Jaunpuri) were placed on plane agar medium. A 3 mm plug from an actively growing colony of 2 to 14-day old culture multiplied under best-selected conditions was placed on the center of the leaf and incubated at 25 ± 2°C in 12hrs alternate light and darkness. The observations were recorded at 12 hrs interval for infection development stages (latent period to disease severity by observing various stages of infection initiation, development, and colonization) in marked 2 cm² area along with spore density.

All the treatments replicated thrice, and each replication has five plates. Replicates mean were used to calculate standard deviation and standard error. Data was analyzed by analysis of variance followed by Tukey's HSD test using SPSS v 15.0.

RESULTS

Among the test growth media, the highest radial growth (77 mm) was observed on PDA, whereas on BGA, MEA, GA, and CDA colony diameter was 68, 64, 56, 43, respectively (Figure 1). The least radial growth 37 mm was recorded on NA medium. But spore production trend was not in line with radial growth development. Among all tested growth media, when grown on PDA,

the highest number of spores (239×10^6 spores/ml) were counted, while on GA, the least number of spores (49×10^6 spores/ml) were observed.

Temperature optimization studies were conducted on PDA, which proved the best growth medium among all tested media. The role of temperature seemed to be more dominant towards the radial growth and spore production. The highest colony growth (87 mm) was observed at 30 °C, whereas highest spore production (315×10^6 spores/ml) was at 35 °C (Figure 2). Colony growth was 46, 84, and 73 mm at 20, 25, and 35 °C, respectively. Least colony growth (28 mm) and minimum spore production (39×10^6 spores/ml) was recorded at 15 °C.

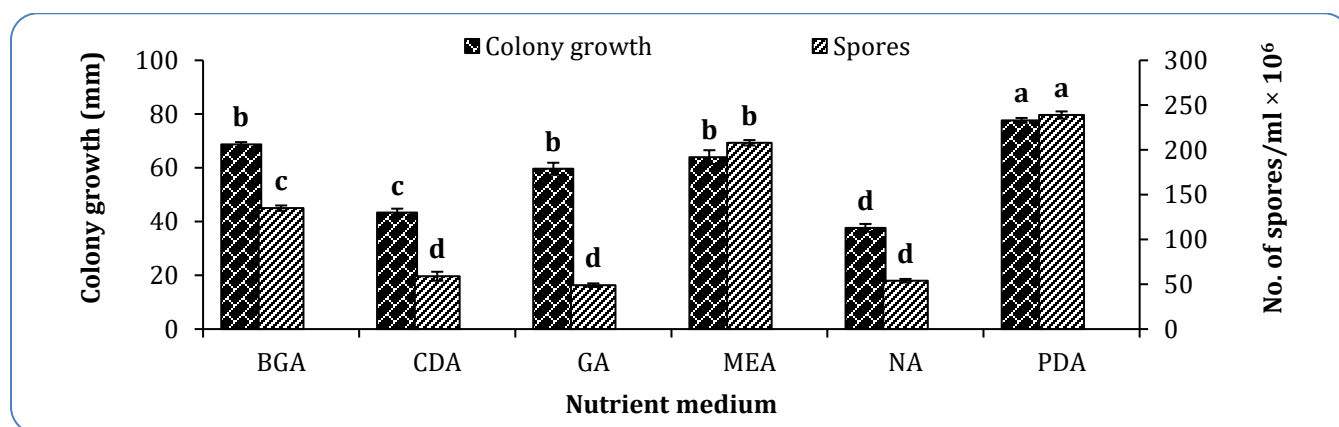


Figure 1. Effect of nutrient medium on colony growth and sporulation of *Myrothecium roridum*. The vertical bars illustrate the standard error of means. Values with different letters on top of bar show a significant difference ($P \leq 0.05$) as determined by Tukey's HSD test. Primary Y axis= colony growth (mm); Secondary Y axis= number of spores/ mL; NA; nutrient agar, PDA; potato dextrose agar, CDA; czapek-dox agar, MEA; malt extract agar, BGA; bitter gourd agar.

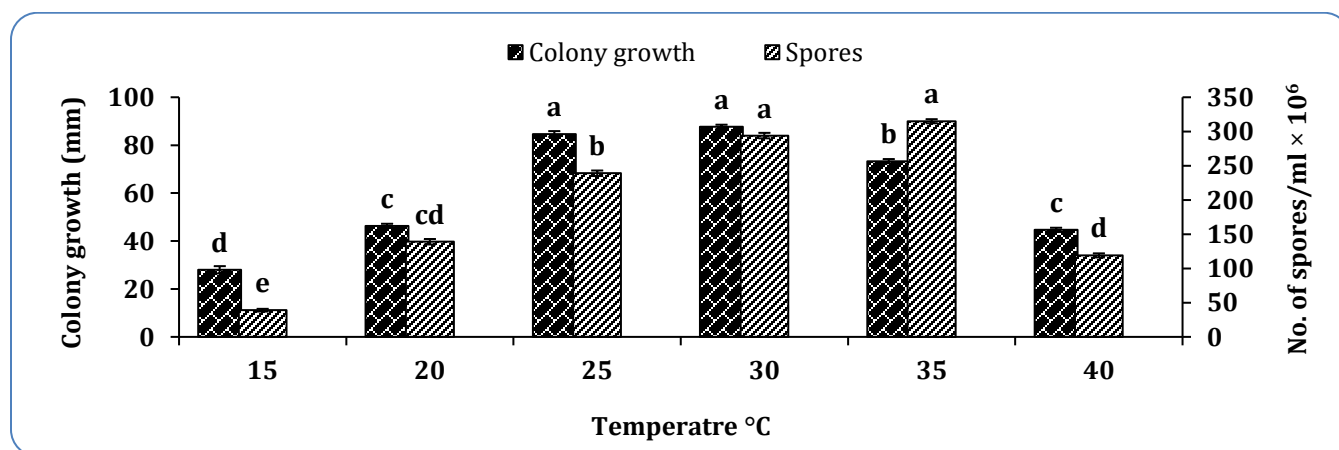


Figure 2. Effect of different temperatures on colony growth and sporulation of *Myrothecium roridum* was measured using PDA medium. The vertical bars illustrate the standard error of means. Values with different letters on top of the bar show a significant difference ($P \leq 0.05$) as determined by Tukey's HSD test. Primary Y axis= colony growth (mm); Secondary Y axis= number of spores/ml.

Studies for optimization of growth medium pH were conducted at pH 5, 5.5, 6, 6.5, 7, and 7.5 levels on PDA medium and incubated at 30 °C, which was best-growing temperature for *M. roridum*. A decreasing trend in colony growth and spore production was observed with an increase in pH level. The highest colony growth (87 mm) was observed at pH 5 and least (45 mm) at pH 7.5, whereas 5.5, 6.0, 6.5, and 7.0 exhibited intermediate level of growth with 74, 71, 62, and 54 mm colony diameter, respectively (Figure 3). The highest spore production (504×10^6 spores/ml) was recorded at pH

5.0, whereas minimum spore production (119×10^6 spores/ml) was at pH 7.5.

Photoperiod optimization studies were conducted at 24h light, 24h dark, and 16/8 h alternate light and dark periods and incubated at 30 °C. The highest mycelial growth (88 mm) was observed at 16/8 h alternate light and dark period; whereas least was observed at 24h light (Figure 4). The maximum spore production (524×10^6 spores/ml) at 16/8 h alternate light and dark period and minimum (268×10^6 spores/ml) at 24h dark.

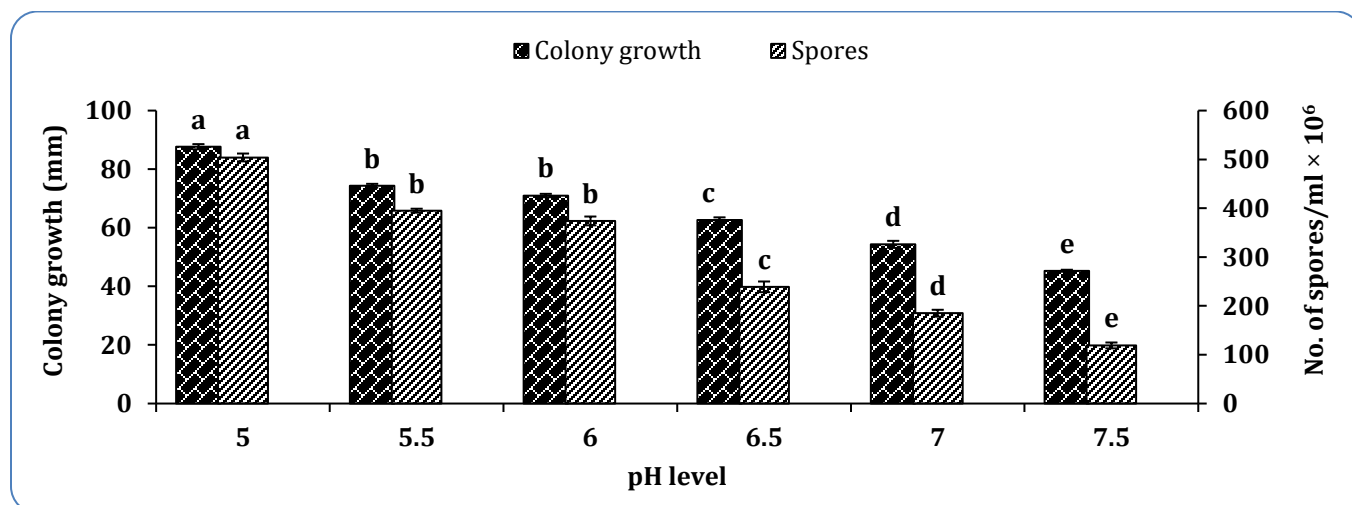


Figure 3. Effect of pH on colony growth and sporulation of *Myrothecium roridum* was measured using PDA medium. The vertical bars illustrate the standard error of means. Values with different letters on top of bar show a significant difference ($P \leq 0.05$) as determined by Tukey's HSD test. Primary Y axis= colony growth (mm); Secondary Y axis= number of spores/ml.

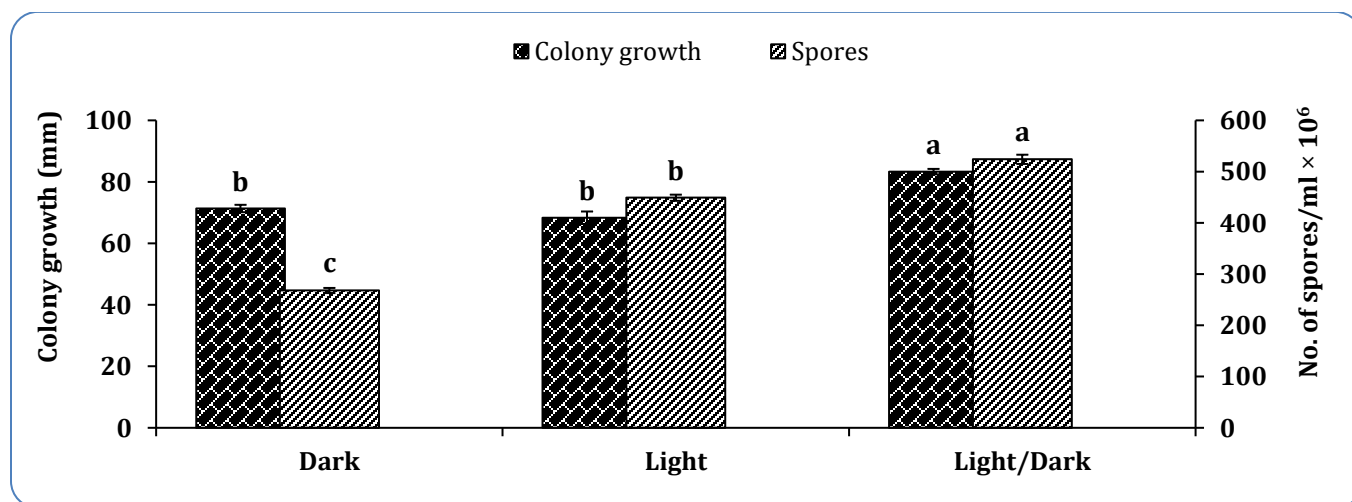


Figure 4. Effect of photoperiod on colony growth and sporulation of *Myrothecium roridum*. The vertical bars illustrate the standard error of means. Values with different letters on top of bar show a significant difference ($P \leq 0.05$) as determined by Tukey's HSD test. Primary Y axis= colony growth (mm); Secondary Y axis= number of spores/ml.

The relationship between virulence and age of the culture was evaluated by inoculating 2-10 day old culture on detached leaves. The investigations were conducted by placing young leaves of bitter gourd on plain agar medium and incubated at 25 ± 2 °C to cover 2 cm area. A 3 mm plug of 2-10 day old culture developed on optimum nutrient medium, temperature, pH, and light/darkness conditions. The virulence was

measured based on infection development, conidia, and sporodochia production on 2cm² area of the leaf (Figure 5).

According to the data recorded at 12 h interval, 5-6 days old culture proved the most virulent as it covered 2cm² prescribed area of the leaf in 82 h whereas least growth rate was observed for 2 day old culture which covered prescribed 2cm² leaf areas in 126 h.

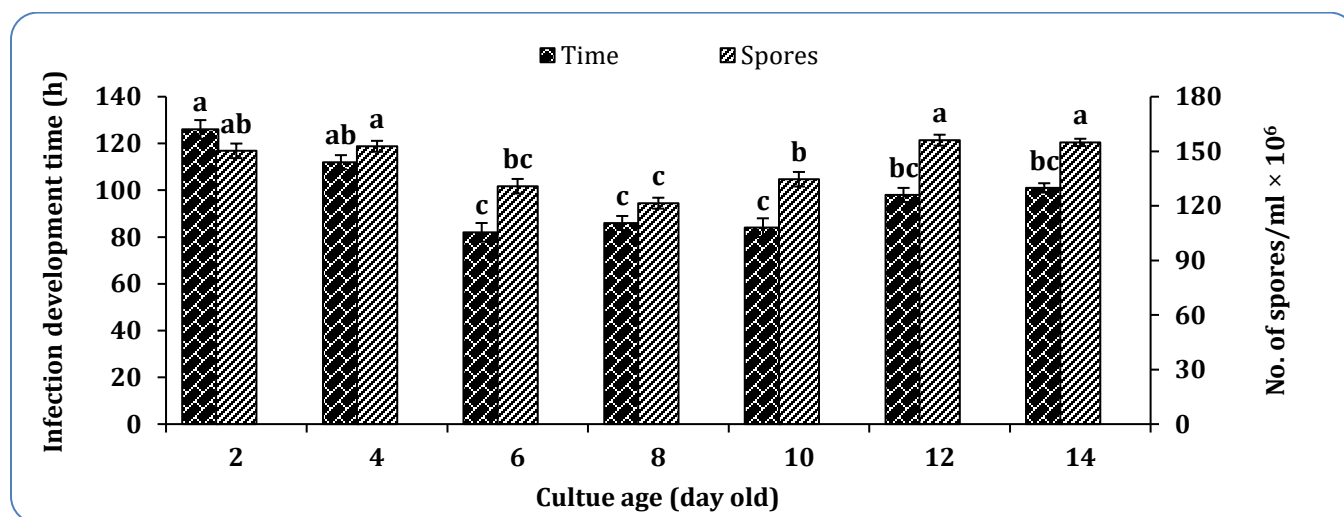


Figure 5. Evaluation of culture age on infection development and spore production of *Myrothecium roridum* on bitter gourd leaves. The vertical bars illustrate the standard error of means. Values with different letters on top of bar show a significant difference ($P \leq 0.05$) as determined by Tukey's HSD test. Primary Y axis= colony growth (mm); Secondary Y axis= number of spores/ml.

DISCUSSION

Compatibility of virulence attribute of plant pathogenic fungi with growth medium and incubating conditions are essential for conducting investigations on disease management strategies. Temperature and nutrient availability plays a crucial role in inducing the diseases caused by different microorganisms. Suleiman *et al.* (2011) established that nutrient and growth conditions' effect on fungal physiology affects its pathological association with the host. Physiological attributes influencing colony growth and subsequently conidia and sporodochia production of *M. roridum* were analyzed. Among the test growth media, PDA medium was best to support the mycelia growth, followed by BGA medium. The difference in fungal colony diameter was found significant at $P < 0.05$. In present studies, Nutrient agar medium exhibited the least number of spore production whereas Okunowo *et al.* (2010) showed a lower number of spore production in Czapek-Dox agar medium. The presence of chloride ions in Czapek-Dox medium was

the suggested reason for lesser spore development, and same might be true for nutrient agar medium (Okunowo *et al.*, 2010). High temperatures (28-30 °C) and humidity trigger the rapid mycelial growth and sporulation of *M. roridum*. These findings are in line with previous studies by Talukdar and Dantre (2013). Abrashev *et al.* (2013) reported that exposure of *Aspergillus niger* at 30 °C+ effects on morphological character by decreasing the size of hyphal elements and increasing "active length" mycelia septation.

However no such effect on mycelial structure was observed in *M. roridum*. Different pH levels in fungal growth medium also influence the fungal mycelia growth significantly. The highest radial growth was observed at pH 5.0 followed by pH 5.5 with an 8h dark period, whereas sporodochia production per 5 mm² was highest at 35 °C. Different studies confirmed that pH levels between 5.0 and 6.5 are suitable for maximum radial growth of *M. roridum* (Okunowo *et al.*, 2010; Talukdar and Dantre, 2013). It is evident that PDA with pH levels

5.0-6.0 proved the best growth medium with 16/8 h light and dark periods at 30 °C. Pathogenic microorganisms have a complex signaling and adaptability determinants of virulence and host infection (Waugh, 2000). Mycelia growth is not a reliable tool for measuring the virulence of the pathogen. From the present investigations, it is evident that virulence directly relates to spore production. Aggressive behavior was measured by taking 3 mm plug from the periphery of the culture, raised on the optimum growth conditions, placed on young leaves of bitter melon to cover 2 cm² area. Fungus remained dormant for 24 hrs. but covered 2 cm area in 3-5 days. The key difference observed in 1-4 day old culture and 5-10 day old culture was conidia and sporodochia production. Infection development pattern for 7-8 and 9-10 day old culture was not significantly different than 5-6 day old culture. The present investigations revealed that the virulence factor is more related to spore production than the radial growth of the colony. These findings might help understand the physiology of the pathogen that could lead to developing proper management strategies for the disease.

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CONFLICT OF INTEREST

There is no conflict of interest among authors.

AUTHORS CONTRIBUTIONS

S. N. Khan and G. Mohy-Ud-Din, designed and supervised the study, S. Naz and S. Farooq, conducted research experiments, analyzed the data and wrote the manuscript. M. Iqbal, M. Idrees S. Mehboob and H. M. Riaz contributed in designing of study and editing of manuscript. All authors have revised and approved the final manuscript.

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