

# *Myrothecium verrucaria* causing needle blight disease on Blue pine (*Pinus wallichiana*): molecular characterization and host range

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**Abstract** Laboratory and nursery experiments were conducted to identify the causal agent of a needle blight of *Pinus wallichiana*, a species native to the Western Himalayas. The pathogen was identified as *Myrothecium verrucaria*, on the basis of morphological, cultural and molecular characterization. BLAST analysis of ITS sequences of the pathogen revealed maximum sequence identity of 99% with *M. verrucaria*. The sequence is the first of this fungus from *P. wallichiana*. Phylogenetic analysis grouped all *M. verrucaria* isolates in a single clade; *M. roridum* and *M. inundatum* clustered in separate clades. The pathogen grew optimally at  $25 \pm 1$  °C on oat meal agar, pH 5.5. Inoculation experiments with *M. verrucaria* demonstrated pathogenicity on *Pinus halepensis*, *Cedrus deodara* and *Cryptomeria japonica*, in addition to *Pinus wallichiana*.

**Keywords** Conifers · ITS sequencing · Needle blight · Physiology · Phylogenetic · *Pinus wallichiana*

## Introduction

The Western Himalayan subalpine conifer forests cover an area of 39,700 km<sup>2</sup> in India, Nepal, Pakistan and

Afghanistan and are dominated by Blue pine (*P. wallichiana*), Chilgoza pine (*Pinus gerardiana*), fir (*Abies pindrow*) and spruce (*Picea smithiana*) (Dar and Sundarapandian 2016). Blue pine, also known as Kail or Himalayan pine, generally grows at an elevation of 1500–3000 masl, up to 3400 masl in Bhutan (Farjon 2013). In India, it is widely distributed in Jammu & Kashmir, Himachal Pradesh, Garwal hills in the west and sporadically distributed in Arunachal Pradesh in the east (Ghimire et al. 2010). Several insect pests and diseases attack pine trees (Shaw et al. 2009) and among these needle blight is a serious disease which inflicts huge economic losses due to premature needle drop (Ahanger et al. 2011). Needle blight causes the death of tree under repeated infection (Bradshaw 2004; Kennelly 2013). Needle blight disease is incited by a number of pathogens which include *Diplodia pinea* (Diplodia tip blight), *Dothistroma septosporum* and *Dothistroma pini* (red band needle blight), *Lophodermium seditiosum* and *L. pinastri* (Lophodermium needle cast), *Ploioderma* spp. (Ploioderma needle cast), *Cercospora pini-densiflora* (Cercospora needle blight), *Myrothecium roridum* (Myrothecium needle blight), *Meloderma desmazierii* (Meloderma needle blight), and *Phoma eupyrene* (Phoma blight) (Ivory 1994; Barnes et al. 2004; Groenewald et al. 2007; Barnes et al. 2007; Brown and Webber 2008; La Porta et al. 2008; Ahanger et al. 2011). Among these, *Myrothecium* needle blight is a potent threat to pine trees and the disease appears as small, round or oval spots with light to dark brown margins in infected portions of needles which

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later dry and abscise from the seedlings and plantations.

The genus *Myrothecium*, first described in the eighteenth century, includes eight species and three new combinations, most of which are saprotrophs (Tulloch 1972). Only *M. roridum* Tode ex Fr. is an aggressive plant pathogen, while *M. verrucaria* (Alb. & Schwein.) Ditmaris [syn. *Gliocladium fimbriatum*, *Metarhizium glutinosum*, *Peziza verrucaria*] is regarded a weak pathogen (Farr et al. 2009). *M. roridum* has also been reported as an endophyte in *Pinus albicaulis* from Oregon USA (Worapong et al. 2009), while *Myrothecium* cf. *indicum* has been found in the phloem of *Pinus sylvestris* infested by *Tomicus piniperda* (Jankowiak and Kruek 2006). Both species are soil- and seed-borne pathogens which attack aerial parts of vegetable crops, fruit and ornamentals plants (Gazzoni and Yorinory 1996; Mendes et al. 1998; Murakami and Shirata 2005; Nasreen-Sultana and Ghaffar 2009). *M. roridum* has a wide host range in comparison to the narrow host range of *M. verrucaria* (Poltronieri et al. 2003; Murakami et al. 2005; Silva and Meyer 2006). *M. verrucaria* has shown high incidence on seeds of watermelon, bitter gourd and muskmelon (Belisario et al. 1999; Bharath et al. 2006) and has recently been reported to cause black spot on ginger in Japan (Yamazaki et al. 2014).

The dissemination of *M. roridum* and *M. verrucaria* occurs through rain or irrigation. These pathogens need specific physiological conditions for growth and sporulation, and generation of such information through *in vitro* studies is of paramount importance in devising effective management strategies. Therefore, the present investigation aimed to identify the causal pathogen of needle blight in Blue pine by morphological, cultural, molecular and pathological characterization and to study the physiological conditions required by the pathogen to grow and cause disease.

## Materials and methods

### Isolation of the pathogen

Diseased needles of *Pinus wallichiana* showing small, round or oval brown spots with dark brown margins were collected during surveys of conifer-dominated forests in Jammu and Kashmir (India). The needles were gently washed with sterilized water and kept in a humid

chamber for 24 h. These were then cut into small pieces, immersed for 1 min in 70% ethanol, surface sterilized for 5 min in sodium hypochlorite (4% active chlorine) and transferred to sterile filter paper to dry. Needles were examined under a stereoscopic microscope for fruiting structures. Tissue pieces with fruiting bodies were placed on Petri-dishes containing potato dextrose agar (PDA) and incubated for 7 days at  $25 \pm 1$  °C. Fungal cultures developing from these tissues/fruiting bodies were purified on PDA using the hyphal tip method (Tulloch 1972).

### Morphological studies

Morphological characterization of the isolates was conducted using light- and stereo-microscopes with incident light. The shape, size and colour of fungal colonies and mycelial growth were recorded on PDA and oat meal agar following incubation at  $25 \pm 1$  °C and compared with the literature (Fitton and Holliday 1970; Ellis 1971; Tulloch 1972; Domsch et al. 2007). The cultures were sub-cultured onto fresh PDA slants every 30 days. The identity of the fungus was confirmed by the Institute of Fungal Taxonomy, New Delhi (India). The diseased specimens were preserved in the herbarium of the Division of Plant Pathology, SKUAST-Kashmir, Shalimar, Srinagar, J&K (India).

### Cultural and physiological studies

To ascertain the best medium for growth, the fungus was inoculated onto five culture media Czapek Dox agar, malt extract agar, oat meal agar, yeast extract agar and potato dextrose agar (PDA) in Petri-dishes and incubated at  $25 \pm 1$  °C for 7 days. To determine the optimum temperature for growth, the fungus was grown on the most suitable medium (oat meal agar) and incubated at six temperatures: 10, 15, 20, 25, 30 and 35 °C. The mycelial radial growth was assessed after 10 days of incubation. For pH studies, the most suitable medium (oat meal broth) was first adjusted to the required pH: 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 by using 0.1 N NaOH or 0.1 N HCl (Patil et al. 2007). The 150 ml Erlenmeyer flasks containing 25 ml broth were inoculated with fresh fungal culture and incubated at  $25 \pm 1$  °C for 10 days. The mycelium in each flask was collected on pre-weighed filter papers and oven-dried at 60 °C for 6 h before estimating the dry biomass. In all the work, the medium was autoclaved at 15 psi for 20 min. Fungal

discs, 5 mm diameter, taken from 7 days old cultures, were used to inoculate culture media. All experiments were performed in a completely randomized design with each treatment replicated five times. Data were statistically analyzed using analysis of variance as per Gomez and Gomez (1984).

#### Molecular characterization and phylogeny of the pathogen

Pathogen identification was confirmed by molecular characterization based on 5.8S ribosomal DNA sequencing.

#### Genomic DNA extraction

The pathogen was first cultured on oat meal broth in 150 ml Erlenmeyer flasks for 10 days at  $25 \pm 1$  °C. The mycelium was harvested and filtered through a double layer of sterilized Whatman filter paper (Grade I), 150 mm diameter (Sigma-Aldrich, Merck), dried between two layers of filter paper in a laminar flow cabinet and stored at  $-80$  °C until further use. The total genomic DNA of the fungus was extracted using the CTAB (cetyltrimethyl ammonium bromide) method (Murray and Thompson 1980) and diluted to a final concentration of 20–25 ng/ $\mu$ l.

#### PCR based internal transcribed spacer (ITS) 5.8S sequencing and data analysis

The 5.8S rDNA was amplified using ITS1 and ITS4 primers (White et al. 1990). The PCR was carried out in 0.2 ml PCR tube with 25  $\mu$ l reaction volume containing 1X buffer (Fermentas), 1.5 mM  $MgCl_2$ , 0.2 mM dNTP

mix (Fermentas), 1 U Taq DNA polymerase (Fermentas), 40–50 ng DNA template, 0.4 pmol primers and 14.8  $\mu$ l sterilized distilled water. Amplification was performed using Whatman Biometra thermal cycler (T-Gradient, Göttingen, Germany) programmed for initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 2 min and a final extension at 72 °C for 10 min. The PCR products were electrophoresed to ensure successful amplification. The amplicon was lyophilized and sent for custom sequencing (Bioscience, Merck, New Delhi, India). The sequences were retrieved from chromatograms, trimmed and assembled using CLC Genomic Workbench version 7.5.1 (CLC bio, Aarhus N, Denmark). Sequence alignment and editing was performed with BioEdit Sequence Alignment Program (Altschul et al. 1990) and compared with sequences already available in databases using BLASTn (<http://www.ncbi.nlm.nih.gov/BLAST>). The consensus sequence was reconfirmed by comparing it with the original data output. The sequence was aligned using CLUSTALW software (Thompson et al. 1994) and submitted to GenBank, NCBI.

Phylogenetic analysis was performed by comparing the pathogen sequence with 32 sequences of *M. verrucaria*, six sequences of *M. roridum* and two sequences of *M. inundatum* (outgroup), retrieved from the NCBI GeneBank database (<http://www.ncbi.nlm.nih.gov/nucleotide>) (Table 1). All the nucleotide sequences were aligned using CLUSTAL X 1.8 multiple alignment programme (Thompson et al. 1997) and refined manually. The GENEDOC package ([www.psc.edu/biomed/genedoc/gdpf.html](http://www.psc.edu/biomed/genedoc/gdpf.html)) was used for formatting the sequences to make them compatible with the desired software. Alignment of sequences was also performed

**Table 1** The sequences of *Myrothecium* species, retrieved from the GeneBank (NCBI) database, used for phylogenetic analysis of *Myrothecium verrucaria*

Pathogen	No. of sequences	GenBank accession no.
<i>Myrothecium verrucaria</i>	32	AJ302003, EU927366, FJ235085, HM043804, HQ607996, HQ608048, HQ625520, JX501292, JN650593, AB778924, JF812340, GQ131886, KM215639, KC140223, KC140221, KC140225, AY303603, KC140222, KC140220, GU183129, EF017211, KF750592, KJ026703, JQ356542, KJ026704, HQ596904, KM246762, JN618368, HM358041, KC806230, KC140228 and JX077018
<i>M. roridum</i>	06	FJ231214, FJ914699, HM052823, HQ115647, JF724154 and KJ813720
<i>M. inundatum</i> (out group)	02	AY254152 and FJ797514

using CLUSTALW in MEGA software version 6.0 (Tamura et al. 2013). The distances were calculated using Kimura-2-parameter substitution model, and a tree constructed using the Neighbour Joining method. A bootstrap analysis was performed using 1000 re-samples of the data.

### Symptomatological studies

The symptomatological studies were conducted on two years old *P. wallichiana* randomly selected in the Forest Nursery, SKUAST-Kashmir, Shalimar, Srinagar (J&K). Selected seedlings (20) were monitored regularly from the 1st fortnight of February to the 1st fortnight of October in 2012 and 2013 for disease development. These seedlings were not sprayed (no biocide) throughout the growing season. Observations with respect to size, shape and colour of lesions were recorded from the initiation of disease symptoms at 10 days intervals. The fungal fructification, if any, on needles was monitored at 15 days interval.

### Inoculations

To confirm the pathogenic nature of fungus, Koch's postulates were fulfilled by inoculation of the fungus onto Blue pine plants of 2 years age. The inoculum was prepared by harvesting the conidial mass from 21 days old culture of the fungus grown on PDA. An aqueous conidial suspension containing  $1 \times 10^7$  conidia/ml was prepared following replicate haemocytometer counts. The conidial suspension was sprayed onto plants with an atomizer on three pine seedlings with wounded pine needles. Wounds were created by gently rubbing carbondum onto the pine needles of each plant before spraying the spore suspension (Quezado Duval et al. 2010). Another set of three pine seedlings were sprayed with the same conidial concentration without wounding pine needles for comparison. A third set of three plants was sprayed with sterile water as controls. To maintain high relative humidity conducive for disease development, each plant was covered with a polyethylene bag and incubated in a greenhouse at  $28 \pm 2$  °C for the first 2 days. Development of spots/symptoms was monitored daily for 15 days. The pathogen was re-isolated from the inoculated needles after infection.

### Inoculation experiment to study the host range

Inoculation of the pathogen was performed on potted plants (2 years age) of *P. wallichiana*, *P. halepensis*, *Cedrus deodara* and *Cryptomeria japonica*. Plants were raised in plastic pots (10 cm diameter) with a single plant in each pot. Inoculation, incubation and observations on symptom development on 8 replicate plants per species were recorded as described above. The plants were regularly monitored for 15 days for the appearance of disease symptoms. Disease severity was recorded using a 0–4 scale, based on Skilling and Nicholls (1974); wherein 0 = needles/whorls showing no disease symptoms; 1 = needles/whorls with 1–25% infection; 2 = needles/whorls with 26–50% infection; 3 = needles/whorls with 51–75% infection; 4 = needles/whorls with 76–100% infection. Disease intensity was calculated using the formula:

$$\text{Disease intensity}(\%) = \frac{\sum(n \times v)}{N \times 4} \times 100$$

Where  $\Sigma$  represents summation; n = Number of diseased needles/whorls in each category; v = Numerical value of each category; N = Number of needles/whorls examined; 4 = Maximum severity value.

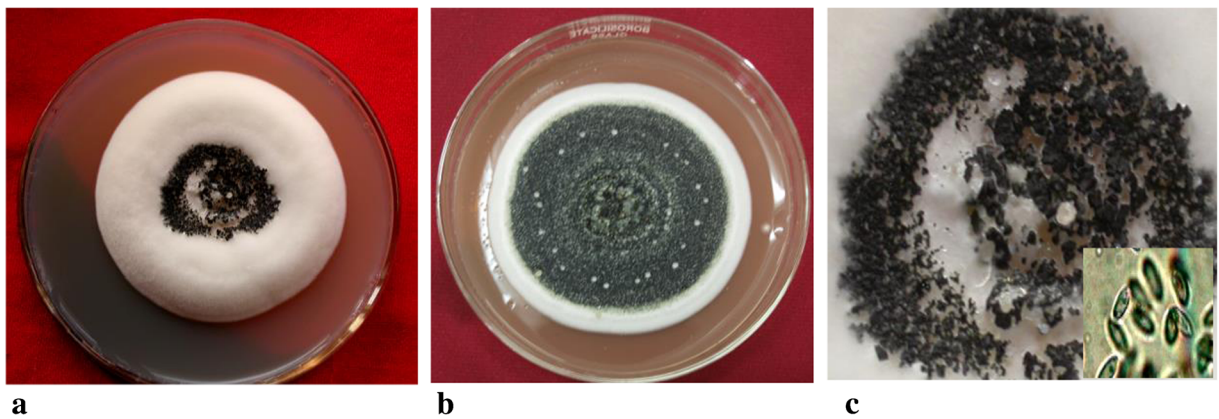
## Results and Discussion

### Isolation of the pathogen

The pathogen associated with needle blight was isolated from the diseased needles of *Pinus wallichiana* bearing peculiar symptoms and fungal fructifications. On PDA, the fungus grew 70–80 mm in diameter after 10 days of incubation at  $25 \pm 1$  °C. On the basis of symptomatology and morphological, cultural and molecular characterization, the pathogen was identified as *Myrothecium verrucaria* [(Alb. & Schwein) Ditmar, in Sturm, *Deutschl. Fl.*, 3 Abt. (Pilze Deutschl.) 1(1): 7 (1813)] (Fitton and Holliday 1970; Ellis 1971; Tulloch 1972; Domsch et al. 2007).

### Morphological and cultural studies

The purified culture initially produced white compact colonies. On PDA, the fungus produced creamy-white, floccose-dense colonies with irregular mycelial growth



**Fig. 1** Growth of *Myrothecium verrucaria* on **a** PDA, **b** Oat meal agar, **c** Black sporodochia in concentric pattern [inset showing spores]

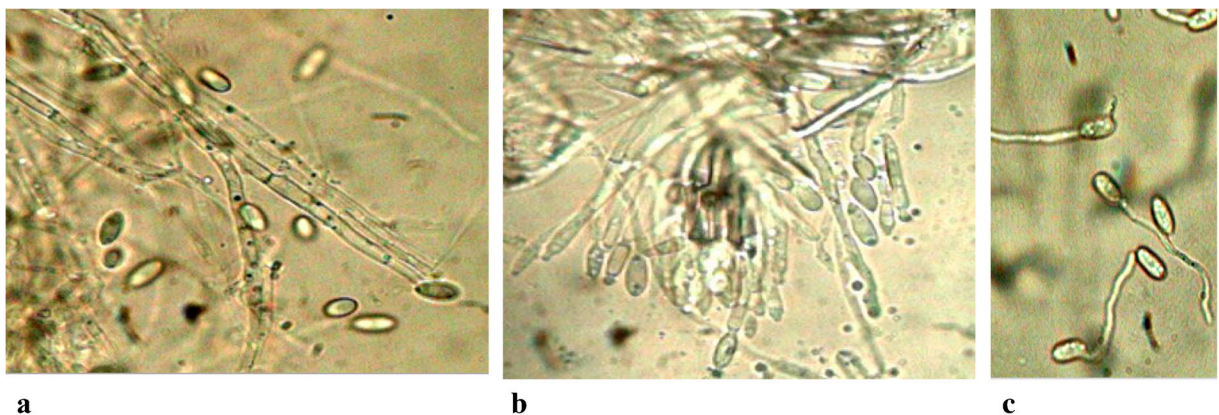
in which abundant distinct black sporodochia were formed in a concentric pattern (Fig. 1a–c) after 10–12 days of incubation at  $25 \pm 1$  °C. The mycelium on PDA was immersed, branched, septate and hyaline to pale brown. Sporodochia in culture released conidia morphologically similar to those observed on host. Ruptured sporodochia released septate conidia from acervular masses (Fig. 2a–c). Conidial size ranged from 5.3 to 8.8  $\mu\text{m}$  (mean 6.8  $\mu\text{m}$ ) in length and 2.8 to 5.2  $\mu\text{m}$  (mean 3.7  $\mu\text{m}$ ) in width. The phialides measured 8.0–11.5  $\mu\text{m}$  (mean 9.7) in length and 1.3–2.5  $\mu\text{m}$  (mean 2.0  $\mu\text{m}$ ) in width. Sporodochia on PDA appeared as light-green pin-head points which turned dark-green and black after 8–10 days. Stereomicroscopic examinations revealed the presence of phialides and acervuli. The conidia were ellipsoidal or cylindrical in shape with round ends, smooth, protuberant at top and truncated at bottom. Conidia were hyaline to light-green while conidial mass was green to black. Yamazaki et al. (2014) also noted that this

fungus formed white stellate colonies with a greenish black spore mass on PDA. The sporangia produced were fusiform, monocellular and  $7.5\text{--}5.0 \times 2.0\text{--}3.0$   $\mu\text{m}$  in size.

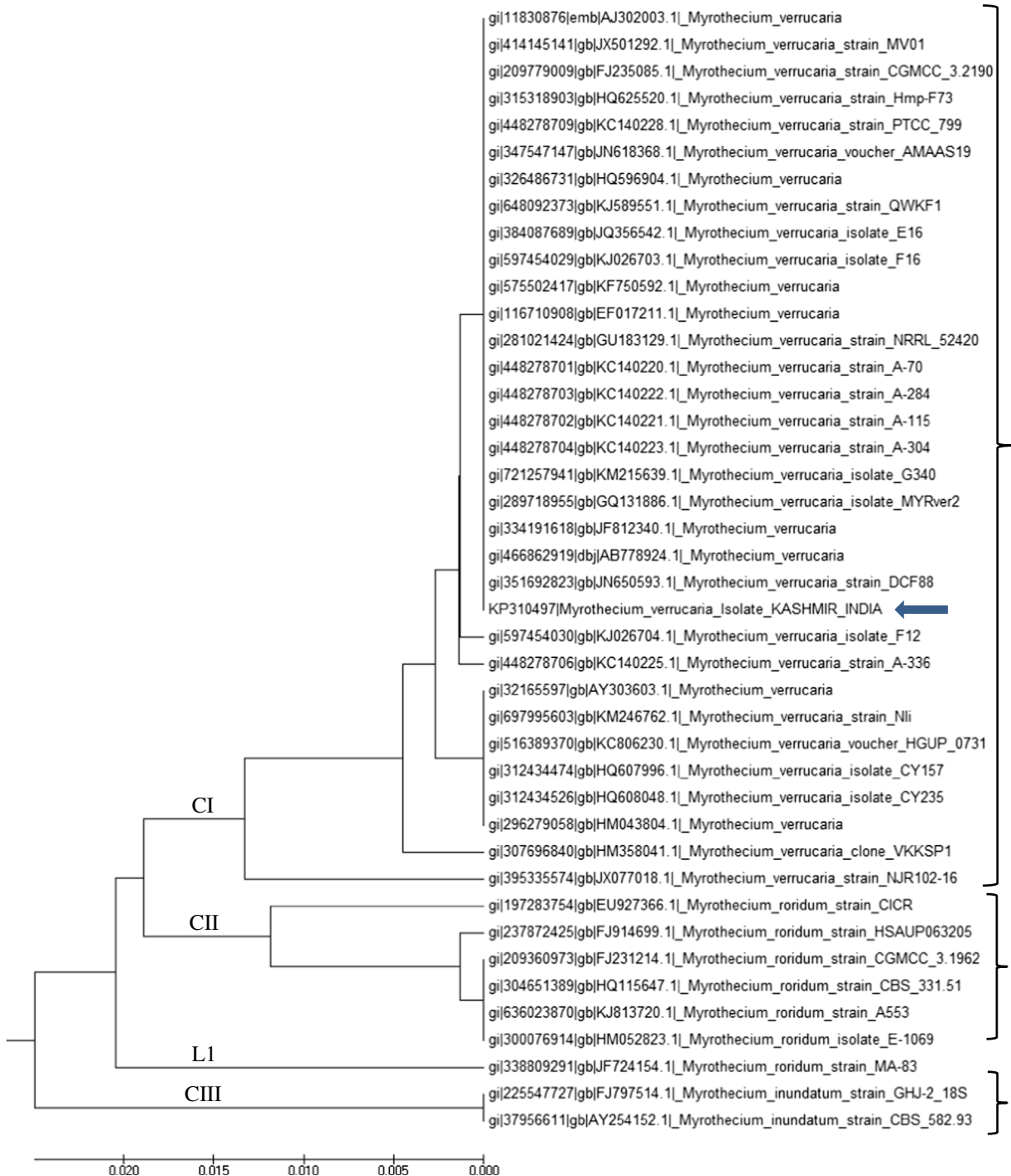
#### Molecular and phylogenetic analysis

The ITS based sequences of pathogen showed 99, 94 and 93% similarity with *Myrothecium verrucaria*, *M. roridum* and *M. inundatum*, respectively (Fig. 3). The sequence, submitted to GenBank NCBI under Accession No. KP310497, is the first sequence of *M. verrucaria* from *Pinus wallichiana* and has not been reported so far on this host worldwide. *M. verrucaria* causing needle blight on pines has been observed for the first time in India.

In phylogenetic analysis, the *M. verrucaria* sequence from the present study aligned with different sequences of *Myrothecium* species available in NCBI database. A phylogenetic tree was generated (Fig. 3) using the



**Fig. 2** Needle blight pathogen *Myrothecium verrucaria*, **a** Conidia and conidiophores (400 $\times$ ), **b** Sporodochia (50 $\times$ ), **c** Germinating spores

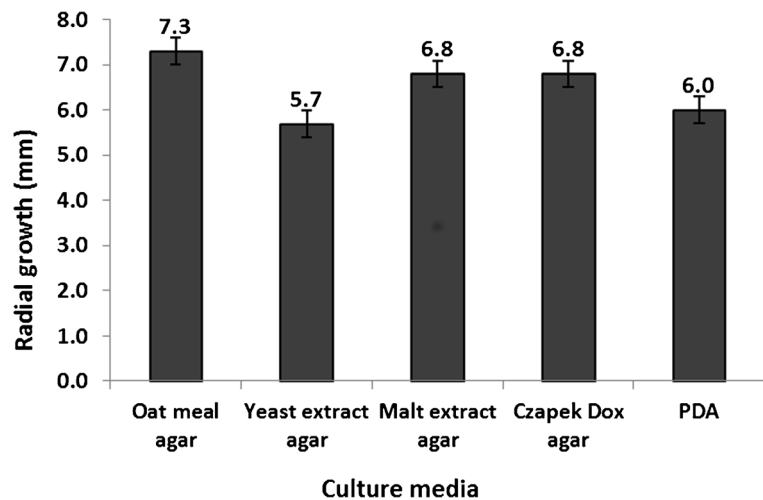


**Fig. 3** Phylogenetic tree generated using Kimura-2-parameter substitution model for the analysis of *Myrothecium verrucaria* isolates

Kimura-2-parameter substitution model and different taxa were clustered together in a bootstrap test with 1000 replicates. The phylogenetic analysis grouped all *M. verrucaria* sequences including the present one in a

single clade (CI). *M. roridum* (closely related) sequences were grouped in clade CII and *M. inundatum* (out group) sequences clustered separately in clade CIII, thereby indicating that ITS regions were highly conserved among

**Fig. 4** Effect of culture media on radial mycelial growth of *Myrothecium verrucaria* over 10 days



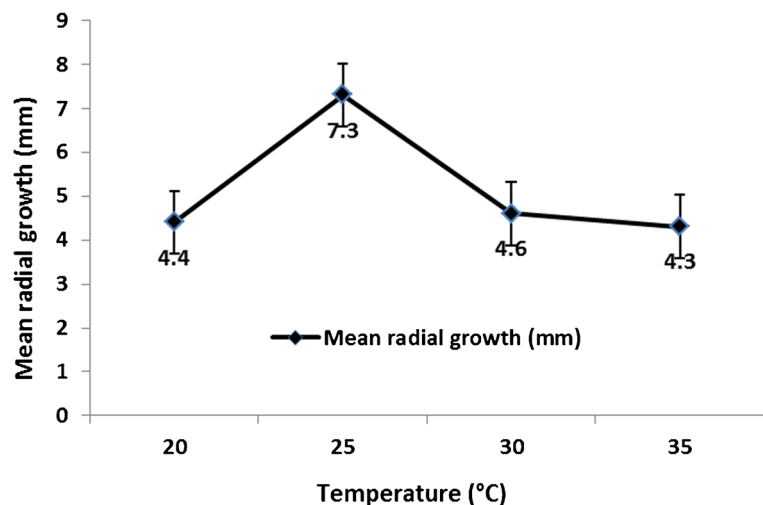
different sequences of *M. verrucaria* and other species; however, one sequence of *M. roridum* represented in a separate lineage (L1).

#### Cultural and physiological studies

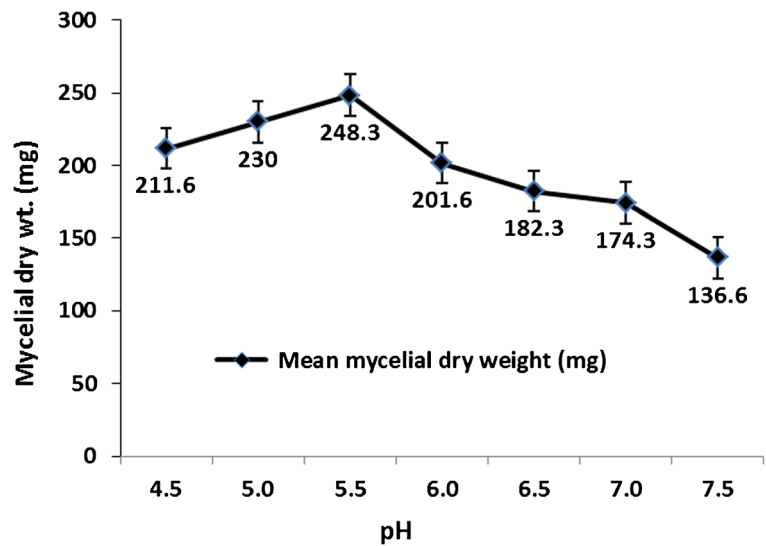
All the five culture-media tested supported the fungal growth with a significant variation (at  $P_{0.05}$ ) in radial mycelial growth recorded after 10 days of incubation. Oat meal agar supported the maximum mean radial growth 7.3 mm after 10 days incubation at  $25 \pm 1$  °C which was significantly greater than on other media ( $P_{0.05} = 0.193$ ). This was followed by malt extract agar and Czapek Dox agar on which growth was similar (Fig. 4). The least mycelial growth was observed on yeast extract agar. A progressive increase in radial mycelial growth was observed with increase in incubation

temperature up to  $25 \pm 1$  °C, beyond which growth rate declined (Fig. 5). The maximum radial growth of 7.3 mm was observed at  $25 \pm 1$  °C followed by  $30 \pm 1$  °C, thus indicating 25 °C as the optimum temperature for growth of the fungus ( $P_{0.05} = 0.593$ ). The least mycelial growth was observed at 20 and 35 °C. The pathogen showed growth over a wide pH range of 4.5 to 7.5 (Fig. 6). The maximum mycelial dry weight of 248 mg was recorded at pH 5.5 followed by pH 5.0, 4.5 and 4.0 ( $P_{0.05} = 10.10$ ). The least mycelial dry biomass was observed at pH 7.5. The findings are broadly in agreement with Okunowo et al. (2010) and Yamazaki et al. (2014) who reported that *M. verrucaria* grows on PDA at 10 to 35 °C with maximum growth at 30 °C. Chauhan and Suryanarayan (1970) observed 25 °C as the best temperature for growth and sporulation of *M. roridum*. Potato sucrose agar has been reported as

**Fig. 5** Effect of incubation temperature on radial mycelial growth of *Myrothecium verrucaria* over 10 days



**Fig. 6** Effect of pH on mycelial growth of *Myrothecium verrucaria* over 10 days

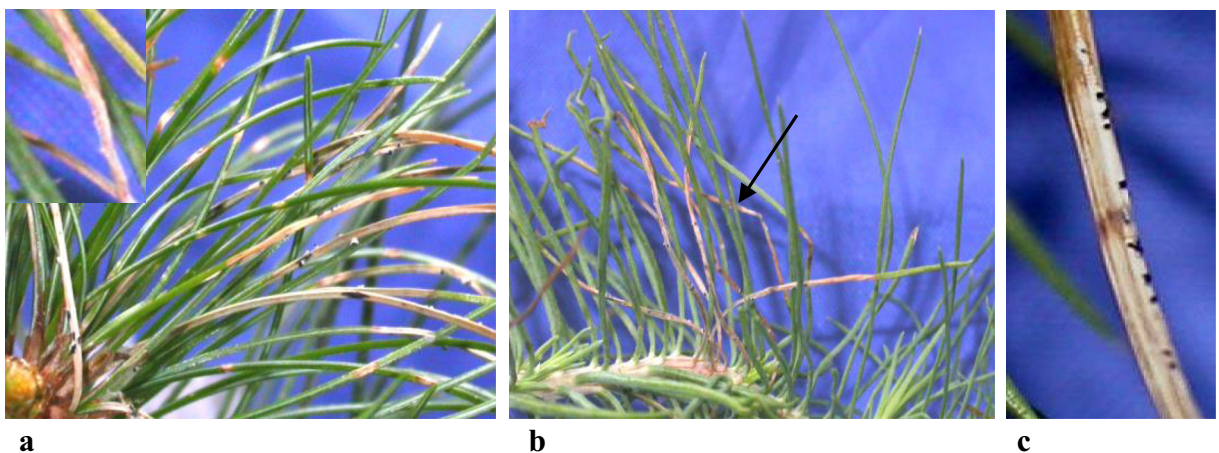


the most favourable medium for the growth of *M. verrucaria* and malt extract for sporulation (Okunowo et al. 2010). The pathogen showed the highest growth at pH 5.5–6.5 and lowest at pH 8.6; while the optimum temperature for conidial germination in acidified medium ranged from 20 to 28 °C and the optimum temperature for spore production was 30 °C (Chauhan and Suryanarayan 1970; Worapong et al. 2009; Okunowo et al. 2010).

#### Symptomatology

Symptoms of needle blight disease were first observed in March–April as slight whitish chlorotic lesions of 0.1 to 0.5 mm size on *Pinus wallichiana* needles (Fig. 7a).

During May to mid-June, these lesions enlarged to 1.0–1.8 mm in size and turned greenish-yellow in colour. Later on, the centre of these lesions turned straw to brown with dark brown margins of 0.8 mm width (Fig. 7b). An erumpent creamy fungal layer appeared on these brown spots with oval to irregular sporodochia in the 2nd fortnight of May. Stereoscopic examination of blighted needles revealed the presence of black acervular mass (Fig. 7c) containing conidia on these erumpent fructifications in the 1st fortnight of July. From mid July onwards, these lesions elongated parallel to the length of needles, with browning in both directions from the infection site and premature needle defoliation in severe cases. The pathogen is known to attack several plant species and generally



**Fig. 7** Needle blight in Blue pine (*Pinus wallichiana*) **a** White chlorotic spots, **b** Spots turn yellow **c** Blighted needles with black acervuli





**Fig. 8** Symptom expression of *Myrothecium verrucaria* on **a** *Cedrus deodara*, **b** *Pinus halepensis*, **c** *Cryptomeria japonica*

causes round dark-brown leaf spot in cucurbits, red clover, water melon and muskmelon, which at later stages coalesce to form blighted areas on the leaves (Cunfer et al. 1969; Leath and Kendall 1983; Belisario et al. 1999; Bharath et al. 2006; Nasreen-Sultana and Ghaffar 2009).

#### Inoculations

The pathogen produced straw brown lesions on the needles of Blue pine seedlings within two weeks of inoculation. The lesions gradually enlarged, coalesced and turned the entire needle brown to dark brown usually from the tip back. Severe infection resulted in premature defoliation. The pathogen was re-isolated from the infected needles thereby proving Koch's postulates. Based on the absence of any report on this pathogen attacking Blue pine (*Pinus wallichiana*) from the fungal flora of India or abroad (Bilgrami et al. 1991; Sarbhoy et al. 1996; Jammaludin et al. 2004; Nasreen-Sultana and Ghaffar 2009; Han et al. 2014), this is the first confirmation of the occurrence of this pathogen on Blue pine. The identity of fungus was confirmed by the Institute of Fungal Taxonomy, New Delhi (India) and culture deposited under Accession No. 2841.09.

#### Host range

*Myrothecium verrucaria* showed a wide host range. *Pinus wallichiana* and *P. halepensis* required a minimum of 12 days incubation period symptom expression. *Cryptomeria japonica* and *Cedrus deodara* took a minimum of 13 and 15 days incubation, respectively, for symptom expression. In cross-pathogenicity tests, in addition to its original host, the pathogen also infected

other hosts: *P. halepensis*, *C. deodara* and *C. japonica* when inoculated by foliar inoculation. The fungus produced concentric lesions on host needles (Fig. 8a and b). All the plant species tested, except *C. deodara*, enabled the development of sporodochia which were occasionally visible with the naked eye and concentrated mainly on the edges of the lesions. The variability in virulence observed in the present study is supported by Taneja et al. (1990). *M. verrucaria* isolated from leafy spurge infected 54 of the 62 plant species tested, proving its wide host range and can cause foliar spots on many cultivated plants (Yang and Jong 1995; Quezado Duval et al. 2010).

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#### Compliance with ethical standards

**Ethical statement** This research did not involve any human and/or animal participants.

**Conflict of interest** The authors declare that they don't have any conflict of interests.

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