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First Report of Needle Blight on Blue Pine (*Pinus wallichiana*) and Aleppo Pine (*P. halepensis*), Caused by *Lophodermium macci*, from Asia

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Abstract: Blue pine (*Pinus wallichiana*), the dominant species of pine widely grown in the Himalayan region including Jammu and Kashmir State, has been found affected by needle cast disease. Recently during forest survey for needle blight diseases conducted in the year 2010 a new fungal pathogen, *Lophodermium macci* Sokolski and Berube, was identified for the first time from Asia with the help of morphological characterization and ITS-1 DNA sequencing technique. A free hand section in lactic acid fuchsin was used for morphological characterization. For molecular identification DNA was isolated from hysterothecia formed on the infected needles. The fungus, previously recorded on *Pinus strobus,* has been noticed for the first time on Blue pine (*Pinus wallichiana*) and Aleppo pine (*P. halepensis*). The pathogen *L. macci* is closely (98%) related to *L. pini-excelsae* and *L. nitens*. The conidiomata of *L. macci* which have not previously been documented were observed for the first time. The conidiomata were subepidermal dark brown to black in color. Black zone lines were only present when adjacent to other *Lophodermium* species. Since *Lophodermium macci* Sokolski and Berube has not so far been reported from any of the Asian countries so this forms the first report of this fungus from Asia. Further, the presence of *L. macci* on *Pinus wallichiana* and *P. halepensis* are new host records worldwide.

Key words: Asia, *Lophodermium macci*, needle blight, pine, *Pinus wallichiana, Pinus halepensis*

INTRODUCTION

Blue pine, *Pinus wallichiana* (Jack.), belongs to the genus *Pinus* and family Coniferae. It is an evergreen arbor of monotypic genus in family Pinaceae widely growing in Jammu and Kashmir State. The tree is abundantly found in moist and dry temperate forest types of Western and Central Himalayas from Kashmir to Bhutan at an altitudinal zonation of 1700-3700 mamsl (Lazarev *et al*., 2007).

Blue pine (*Pinus wallichiana*) and Allepo pine (*Pinus halepensis*), are often damaged by insects and pathogens (Huang *et al*., 2008). Among the diseases, Lophodermium needle blight is a serious disease incited by various *Lophodermium* species (Hou *et al*., 2006, 2009). At first, the symptoms appear as bar-like circular or sub-rounded yellowish-brown spots on needles. Then the needles turn yellow and wither, even may fall off in autumn. Extensive needle drop not only reduces the ornamental value of conifers, but also influences the tree growth (Xiaoming *et al*., 2010). Among all the needle blight diseases of pine Lophodermium needle blight is considered as a major constraint in its successful regeneration (Johnston, 2001; Stenstrom and Ihrmark, 2005; Luo *et al*., 2010). Initially the symptoms appear on needles as light green to yellow spots which later turn reddish brown surrounded by yellow margins (Xiaoming *et al*., 2010; Lilja *et al*., 2010). The affected needles turn straw coloured and fall off prematurely in autumn (Diwani and Millar, 1987; Barnard and Ash, 1998). Extensive needle drop not only reduces the ornamental value of conifers, but also influences the tree growth (Douce *et al*., 2002; Hanso and Drenkhan, 2011).

Lophodermium Chevall. is a large and complex genus in the family Rhytismataceae and includes more than 250 species of foliar inhabiting fungi (Johnston, 2001). *Lophodermium* species have a wide host spectrum, including families of gymnosperm and angiosperm (Luo *et al*., 2010). Genus *Lophodermium* has received much attention by forest pathologists because some species are important pathogens of conifers causing needle blight diseases (Dabker, 1932; Minter, 1981). Worldwide more than 25 species of *Lophodermia* have been reported on conifers (Minter, 1981, 1995; Sokolski *et al*., 2004; Hou *et al*., 2006, 2009). From Asia about 15 *Lophodermium* species have been reported

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(Sharma and Sharma, 1983; Bilgrami *et al*., 1991; Ojha *et al*., 2004). After consulting the related iteratures ofconifer-inhabiting *Lophodermium* spp. (Hou and Wang, 1995; Johnston, 2001; Johnston *et al*., 2003; Sokolski *et al*., 2004; Hou *et al*., 2006, 2009; Xiaoming *et al*., 2010) and comparing the pathogen on *P. wallichiana* with *L. pinastri*, *L. pini-execlsae* and *L. nitens*, it was found that there were great variations between the three species. The pathogen on *P. wallichiana* and *P. halepensis*, therefore, is identified as a new species of *Lophodermium* here. In the present study, a new species of *Lophodermium* isolated from the conifer needles, causing needle blight disease in *Pinus wallichiana* and *P. halepensis* is reported on the basis of conventional and molecular identification methods.

MATERIALS AND METHODS

Study area: In April 2010, Blue pine (*Pinus wallichiana*) and Allepo pine (*Pinus halepensis*) needles bearing typical needle cast (blight) symptoms were collected from conifer forests of Baramulla, Srinagar and Anantnag districts in Jammu and Kashmir State, India. The spores from ascomata formed on fallen needles of blue pine were used for the isolation of causal pathogen.

Morphological studies: For various morphological studies, the sections of different thickness of ascoma were made in water containing 0.1% (w/v) cotton blue in lactic acid as per Hou *et al*. (2009). The fungus was identified on the basis of cultural and morphological characters and characterized to species level using the keys of Minter (1981). The pathogenecity was established on two year old Blue pine and Allepo pine seedlings by using the perfect state spore of the fungus. The diseased needle specimens were preserved in the Mycology and Forest Pathology Laboratory, Division of Plant Pathology, SKUAST-K, Shalimar, Srinagar (J and K). For observing the outlines of ascomata and conidiomata, vertical sections were mounted in lactic acid or cotton blue with pretreatment in water. Gelatinous sheaths surrounding ascospores and paraphyses were observed in water or cotton blue in lactic acid. Ascospore contents were observed in water. Measurements for ascospores and paraphyses were made using material mounted in 5% KOH from 30 ascospores, asci and paraphyses for each specimen. The figures of external shapes and internal structures of fruiting bodies were drawn using the microscopic painting device (Magnus Live USB 2.0 camera; Magnus Pro 4).

Molecular methods: The molecular sequencing of the pathogen was carried out followed by Hou *et al*. (2009) Total genomic DNA was extracted from ascomata or mycelium as per Hou *et al*. (2009). New sequence for ITS rDNA region was obtained for species of *Lophodermium macci*. The primers ITS1 were chosen and PCR was performed in 25 μL reaction (White *et al*., 1990; Hou *et al*., 2009). The PCR products were sent to Capital Normal University Xisanthuanbeilu, Beijing China for purifying, sequencing and editing.

RESULTS

During the survey for needle cast/blight disease of blue pine in selected areas of Kashmir, several pine needles were noticed to have ascocarps without any transverse black zone lines, a characteristic symptom of needle cast caused by *Lophodermium macci*. The ascoma were mostly found on abaxial surface and rarely on adaxial surface of the needles. The anamorph (Fig. 1d, e) of the isolated pathogen was found to be *Leptrostroma* species. The identification of *L. macci* was subsequently confirmed by molecular sequencing technique using ITS-1 primer (Fig. 2) at Capital Normal University Xisanthuanbeilu, Beijing China. Comparison of morphological and molecular data with other species and ribosomal DNA sequence of isolates showed high level of genetic similarity (98% identity) for the internal transcribed spacer region of *L. pini-excelsae*.

Symptomatology of disease: The disease on needles initially appeared as yellowish spots which later turned brown with yellowish halos. The spots on needle later coalesced to give blighted appearance (Fig. 1a) and ultimately resulted in premature defoliation. The stereoscopic examination of blighted needles showed the presence of small pycnidia (spermatia) from June to July (Fig. 1d). The presence of conidiomata in this species was a conspicuous observation which has not so far been reported elsewhere on pines. Conidia were 8.34×1.60 μm in size (range, length 6.42-10.72 μm and width 1.09-1.93 μm) (Fig. 1e). The mature hysterothecia of fungus, singly or in chains with prominent slit in centre and surrounded by grayish-shiny perimeter line, were abundantly found on diseased needles from July to September (Fig. 1f). In field, the ascospores were discharged from early July to early October, with vigorous dispersal from late July to early September.

Cultural-morphological characterization: The ascoma on *Pinus wallichiana* and *P. halepensis* needles were initially sub-epidermal but a t maturity appeared bulged.

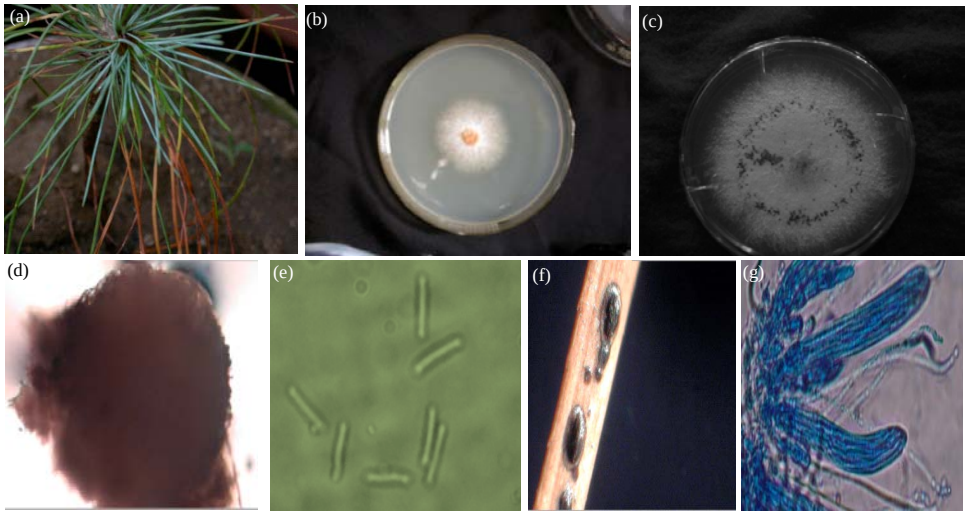

Fig. 1(a-g): (a) Blue pine needle showing needle cast disease symptoms; (b) culture of *Lophodermium macci* (c) pycnidial formation on culture plate; (d) Pycnidia of *L. macci;* (e) conidia, (f) hyserothecia and (g) acsi and paraphysis

GAAGAGACCCCCCTATTCTCCCCTATGTTTACCACACTTTGTTGCCTTGGCGCACTGCGCCAGCGGATCAAAACCCTTGAATCA
TTGCTGTCTGAGTACTATATAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG
CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGGGGGCATG
CCTGTTCGAGCGTCATTACAACCCTCAAGCTCTGCTTGGTATTGGGCTCGCCCTGTAGGGCTTGCCTCAAAGTCAGTGGCGGCT
ACCGTCCGACCTTCAGCGCAGTACTACTCGTCGCTGTTAGGGAGGGCCTACAACCCGCCATCACAACCCCCACTTTACAAGGTT
GACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGTCGGAGGAAGA

Fig. 2: Primer sequence used to amplify partial *Lophodermium macci* r-DNA ITS region

When picked these were observed to be deep-seated inside the plant tissue. Ascomata on both the sides of needles (more than 80% on adaxial side) were usually scattered and occasionally confluent. In surface view, ascomata were 500-900×300-500 μm, and clypeus 400-650×150-300 μm in size. The ascoma were mostly rounded with elliptical ends and occasionally acute or slightly irregular. Ascoma were shiny, black in centre for 1/3-1/2 (-2/3) surface area surrounded by grey or grey-brown margin with a conspicuous black perimeter line. The perimeter lining was moderately raised on the surface of the needle. The ascoma were opened by a single longitudinal slit. The lips of ascoma were inconspicuous under dissecting microscope but visible in thin sections under compound microscope. The lips were split extending 1/2–2/3rd length of ascoma.

The fungus was easy to isolate on malt extract agar (MEA), however good growth was observed on potato dextrose agar (PDA) after transfer. The colonies had irregular margins. The mycleium was creamish white with sparse growth and grew slowly. After 3-4 week, creamish buff pycnidial initials were observed in the form of concentric rings. The pycnidia later on turned black.

Paraphyses were rare, and when present were septate having hooked tips and measured 70-110×2 μm. Asci were 70-110×7-8 μm in size (Fig. 1g) and ascospores 55-80×2 μm, filiform, arranged in fascicles, sometimes helically coiled, slightly tapering toward base, hyaline and aseptate (Table 1). The discharged ascospores were covered with a gelatinous sheath of 2-3 μm thickness. Leptrostroma/conidiomata stage was present, mostly on adaxial side of needles, scattered to crowded, sometimes coalescing. In surface view conidiomata measured 500-750 (640)×350-630 (490) μm and conidia 8.34×1.60 μm in size (range, length 6.42-10.72 μm and width 1.09-1.93 μm). Zone lines were absent.

The fungus was easy to isolate on malt extract agar, however good growth was observed on potato dextrose agar after transfer. The colonies had irregular margins.

Table 1: Comparative morphological characteristics of *L. macci* and *L. pinastri*

Morphological characters	*L. macci*	*L. pinastri*
Zone lines	Absent	Present
Ascocarp position	Abaxial	All sides
Lips	Discrete, black	Visible, grey, red, orange, yellow
Lips	Visible	Visible
Basal wall	Poorly developed	Poorly developed
Clypeus	Partly subepidermal	Partly subepidermal
Ecology	Senesced needles	Senesced needles
Host	Haploxylon pines	Diploxylon pines
Paraphyses	Bent, swollen septate, very rare	Straight, unswollen
Ascocarp	500-900 (μm)	700-1200 (μm)
Ascus	70-110×7-8 (μm)	110-155×9.5-11.5 (μm)
Ascospores	55-80×2 (μm)	70-110×2 (μm)

The mycelium was whitish farinaceous to creamish with sparse growth and grew slowly (Fig. 1b). After 3-4 weeks, the creamish buff pycnidial initials were observed in the form of concentric rings (Fig. 1c). The pycnidia later on turned black.

Ecology: Lophodermium macci has been observed on both intact as well as on fallen needles (over-wintered in litter) of *P. wallichiana* (five-needle) and *P. halepensis* (two-needles) pines in Kashmir conifer forests.

Habitat: Collected from fallen needles in litter and on host (*P. wallichiana* and *P. halepensis*).

Known distribution: Canada and USA on *Pinus strobes*.

Molecular characterization of pathogen: The molecular sequencing of ITS region of DNA from the isolated fungus, using ITS-1 primer (Table 1) has been assigned accession number 37441 by the NCBI and sequence published under the same accession number. On the basis of morphological and molecular sequencing the pathogen has been identified as *Lophodermium macci* Sokolski and Berube, *hitherto* unrecorded from Asian continent. The taxon has recently been reported only from Canada and USA on *Pinus strobus* (Sokolski *et al*., 2004) so this is the first host record of *Lophodermium macci* on *Pinus wallichiana* and *Pinus halepensis*.

DISCUSSION

The *Lophodermium macci* Sokolski and Berube is an ascomycetous fungus which has so far been reported from USA and Canada only that too on only one pine species *Pinus strobus*, thus the present report is a new record from Asian continent. In Jammu and Kashmir the fungus causes needle blight disease in two pine species *Pinus wallichiana* (five-needle) and *P. halepensis* (two-needle) which is the new host-fungus interaction from the world. Only three species of *Lophodermium* have been reported from Jammu and Kashmir State (India) viz., *L. pinastri* and *L. pini-excelsae* on *Pinus wallichiana* and *L. picea* on *Abies pindrow* (Bagchee and Singh, 1960; Sharma and Sharma, 1983). A study of freehand sections of ascocarps mounted on lactic acid fuchsin (Hou *et al*., 2009) and use of identification keys of (Minter, 1981) led to the identification of pathogen as *Lophodermium macci* Sokolski and Berube.

Lophodermium macci belongs to the species complex whose members often are difficult to differentiate (Sokolski *et al*., 2004). *Lophodermium macci* is morphologically similar to *L. pinastri* and *L. pini-excelsae* However, they are different in many aspects: *L. macci* is characterized by the smaller proportions of the central black area in the external appearance and the central longitudinal split in the total length of ascoma, the covering stroma connecting to the basal stroma, and paraphysis tips which are gradually swollen or irregularly branched even suddenly swelling to globules. Besides, the anamorph of the present species is different from *L. pinastri* and *L. pini-excelsae*. In *L. macci*, conidiomata are flask to pear shaped and opened by a round ostiole on the top; its conidia are much longer (8.34×1.60 μm) and zone lines are absent. Whereas *L. pinastri* has the covering stroma not extending to the basal stroma, simple paraphysis tips and longer asci and ascospores (110-155 μm and 70-140 μm, respectively (Dabker, 1932; Minter, 1981), and elliptical to elongated elliptical conidiomata opened by a lateral split, shorter and thinner conidia 4.5-6.25×1.0 μm (Minter, 1981) and the abundant zone lines (Minter *et al*., 1978; Minter, 1980; Lin and Tang, 1988; Johnston, 2001). Comparisons of ascomata of *L. macci* with those of *L. pinastri, L. staleyi* and *L. pini-excelsae* were described by Minter (1981). The similarities among these species are the black clypeus with grey margin, a black perimeter line and subepidermal nature. The most useful characteristic for distinguishing *L. macci* from these species is the manner in which the ascocarp is embedded in the needle (Table 1). This is the principal criterion separating *L. pinastri* from *L. pini-excelsae* used by Minter *et al*. (1978) and Cannon and Minter (1986). In this case the difference is in the depth of embedded ascocarps. *Lophodermium macci* displaces two or three epidermal cells (rarely four) of the host on each side of the clypeus, while *L. pini excelsae* displaces more than four, usually five. The number of epidermal cells left on the bottom of the ascocarp is five and more for *L. pini-excelsae* and eight and more for *L. macci*. The species of *Lophodermium* on conifers have high host specificity at their generic level of

the hosts (Hou *et al*., 2009). Therefore, combining the morphology, host specificity and molecular analyses, the species of *Lophodermium* on *P. wallichiana* and *P. halepensis* is proposed as a new record from Asian continent.

CONCLUSION

Lophodermium macci, characterized by subepidermal black hysterothecia and surrounded by sharply defined greyish perimeter line, is a new report from Asia and its presence on *Pinus wallichiana* and *P. halepensis* are new host records worldwide. The conidiomata which have not previously been documented were observed for the first time. The conidiomata were subepidermal dark brown to black in colour. Black zone lines were only present when adjacent to other *Lophodermium* species.

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