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ISOLATION, IDENTIFICATION & PURIFICATION OF *MYROTHECIUM RORIDUM* CAUSING LEAF SPOT OF BAEL

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ABSTRACT

The present investigations were carried out during 2019-2021 at the main campus of university is located at Kumarganj on Ayodhya-Raebareli road, approximately 42 km away from Ayodhya. Geographically experiment site from where leaves were collected is situated at 26.541° N latitude, 81.832° E latitude and at an altitude of 113 meters from sea level in North Indo Gangetic plain. Bael crop has been hampered with a lot number of fungal diseases like root rot, leaf spot, dieback caused by *Fusarium solani*, *Myrothecium roridum* and *Alternaria alternate* respectively, out of which leaf spot caused by *Myrothecium roridum* have proved to be a prominent hindrance in its cultivation in nursery plants. The main problem with this fungus is that the symptom observed on bael leaves are morphologically much more similar with *A. Solani* because same symtpoms with necrosis around the leaves and the concentric ring formation creates a more confusion. After, inoculation of fungus and 24 hours of incubation at 25°C, the fungus began to proliferate. Single spore isolation was used to purify the culture, which was then kept on PDA medium for further research. Small, circular or irregular in shape, brown in colour, and chlorosis around the lesions were detected on leaves, however these spots grew larger and covered a larger area subsequently. Circular brown dots on the upper surface of the leaf were first noticed during the rainy season (July-August). Concentric rings and sporodochia grouped in a relatively concentric manner were common in older leaf spots (15-20 days following creation of new spots). The discharge of necrotic tissues caused a characteristic shot hole in the leaves at an advanced stage of disease progression.

Key words: Bael, botanicals, Myrothecium, fungicides

Introduction

Bael (*Aegle marmelos*) a fruit crop of family Rutaceae is one of the medicinally treasured tree species (Chanda, 2008) out of the 250,000 living terrestrial plant species on earth. Although bael being native to northern India, its being cultivated in different parts of world. Bael is one of the most appreciated plants used in ayurvedic medicine by the Indian and other South Asian inhabitants in ancient history (Jagetia and Baliga, 2004). The root is also a key component of "dasmula," an Ayurvedic remedy regarded to be a miraculous treatment for digestive problems, which contains 10 roots. There is antidiarrheal potential of chloroform extract of the root of *Aegle marmelos* (Correa) Linn (Mazumder *et al.,* 2006). Bael leaf powder can be used to treat bowel syndrome (Atal *et al.*, 2012). Making a variety of items with bael is therefore very profitable economically. *Aegle marmelos* commonly known as 'bilwa' or 'beal' is highly valued plant for its characteristic's aroma and medicinal value (Sampath *et.al.* 2012).

But it has been hampered with a lot number of fungal diseases like root rot, leaf spot, dieback caused by

Fusarium solani, Myrothecium roridum and *Alternaria alternate* respectively (Anonymous, 2016), out of which leaf spot caused by *Myrothecium roridum* have proved to be a prominent hindrance in its cultivation in nursery plants (Anonymous, 2020). The main problem with this fungus is that the symptom observed on bael leaves are morphologically much more similar with *A. Solani* because same symptoms with necrosis around the leaves and the concentric ring formation creates a more confusion. (Tulloch, 1972), There are eight known species of the genus Myrothecium, many of which are saprophytes found in soil. Necrosis on anthurium has reportedly been caused by *M. roridum* in Brazil (Quezado Duval *et al.,* 2010). When a host cell dies, this pathogen can still produce poisons and colonise the dead cell (Murakami *et al.,* 1999 & Murakami & Shirata, 2005).

The colony of PDA-cultured media was observed, the peripheral area was white and the central area was yellowish. When the fungus was incubated on the PDA for more than 30 days, black conidiomata developed. Sporodochia were sessile, and setae were absent. Images of the conidia were observed by phase-contrast microscopy and SEM. Conidia were 5~6 × 1~1.2 µm in size and cylindrical in shape with rounded ends similar to those of *M. roridum* (Quezado Duval *et al.,* 2010). The conidial mass was green in color despite staining with lactophenol blue solution.

The Pathogen

The casual organism Myrothecium roridum was isolated from infected plants showing typical symptoms of *Myrothecium* leaf spot and was characterized culturally and morphologically following standard protocols. *Myrothecium roridum* is a soil fungus and survives in this environment as a saprophyte in decaying plant tissues (Domsch *et al*., 2007). Despite its saprophytic nature, *Myrothecium* is able to cause diseases, mainly in the aerial parts of some plant species (Ahrazem *et al*., 2000; Domsch *et al*., 2007). It is a facultative parasite with a large number of plant hosts, including vegetables, fruits and ornamental plants (Murakami and Shirata, 2005). Considering the significance of the bael crop and severity of this fungal disease, our present study was done to identify the pathogen and study its cultural and morphological variability. Initial symptoms of the disease appear as small round or oval, brown spot with dark brown margin on leaves in the infected plant.

Isolation, purification & identification of test fungus

The infected leaves of Bael were cut into small pieces, surface sterilized with 0.1% sodium hypochloride (NaClO) solution followed by three washing with sterile distilled water and placing in moist chamber than after 1 to 2 days fungal mycelium growth was seen than finally small bits of fungus kept on the previously poured and solidified potato dextrose agar medium in Petri plates for isolation of the pathogen. The plates were incubated at 25°C in an incubator. The plates were observed after mycelial growth from the inoculated mycelium bits. Mycelial were then sub-cultured, purified by hyphal tip method and maintained culture on PDA slant & Petri plate kept on incubator at 25°C. All the growth characters were recorded and compared with the standard reports publish for confirmation. The fungus was isolated from symptomatic leaves, and its pathogenicity was confirmed. Based on the morphological characteristics and molecular analysis, the pathogen was identified as *Myrothecium roridum* Tode ex Fr. This is the first report of M. Roridum causing leaf spot on *A. Andraeanum* in China (Ben *et al.,* 2014).

Isolation, purification & identification of test fungi associated with disease appear at nursery stage.

On PDA slants, infected parts of diseased bael leaves were combined with some healthy portions to isolate the fungus. After 24 hours of incubation at 25°C, the fungus began to proliferate. Single spore isolation was used to purify the culture, which was then kept on PDA medium

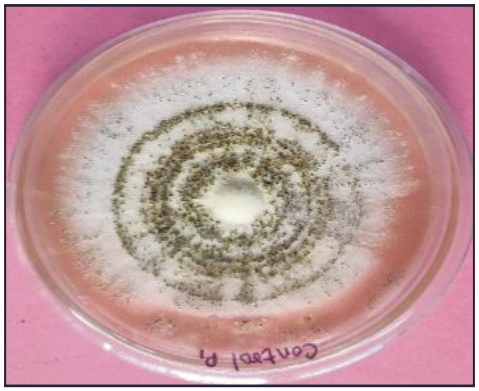

Fig. 1: Plate of Myrothecium.

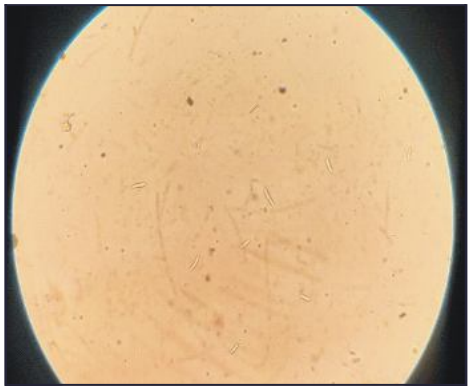

Fig. 2: Conidia.

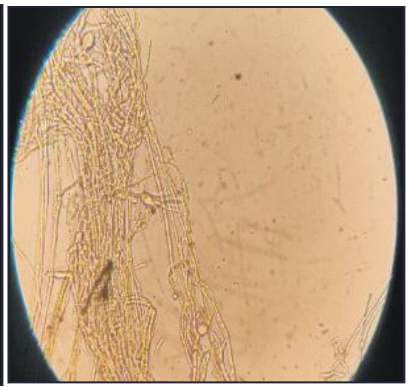

Fig. 3: Mycelium of Myrothecium

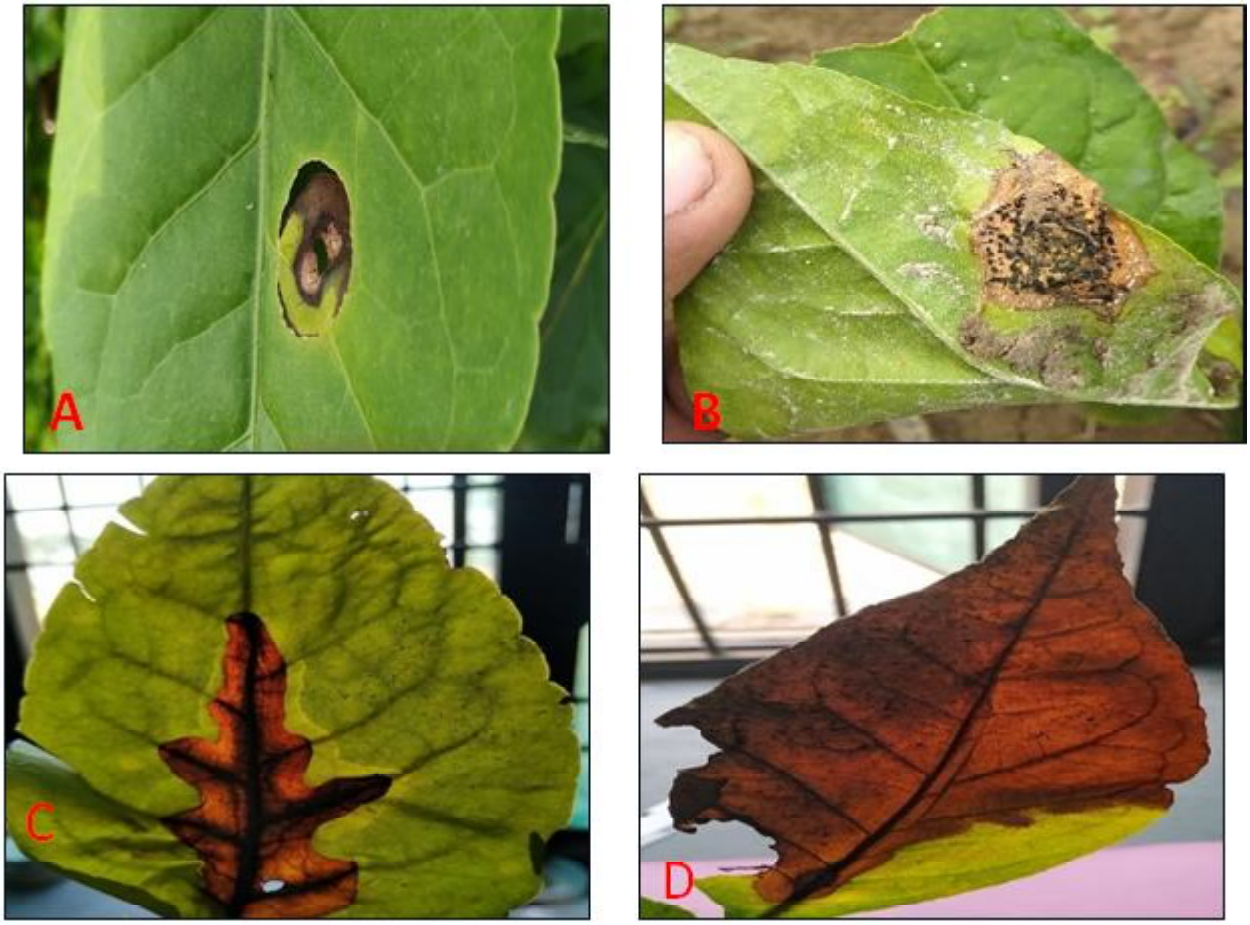

Fig. 4: (A) Myrothecium leaf spot of bael. (B) Sporodochia produce in lower surface of the Myrothecium infected leaves. (C) Myrothecium spot on mid rib of the leaves. (D) Myrothecium present on midrib covered whole surface of the leaves and produces necrosis symptom.

for further research. The recovered fungus was subsequently inoculated in Petri plates with PDA and identified on the basis of their cultural and morphological features as well as microscopic tests in the laboratory. Later it can be confirmed by ITCC (Indian Type Culture Collection), Division of Plant Pathology, IARI, New Delhi with Id. No. 9556.14 (*Myrothecium roridum*).

Symptoms of *Myrothecium roridum*

Small, circular or irregular in shape, brown in colour, and chlorosis around the lesions were detected on leaves, however these spots grew larger and covered a larger area subsequently. Circular brown dots on the upper surface of the leaf were first noticed during the rainy season (July-August). Concentric rings and sporodochia grouped in a relatively concentric manner were common in older leaf spots (15-20 days following creation of new spots) (Fig.3). The discharge of necrotic tissues caused a characteristic shot hole in the leaves at an advanced stage of disease progression. Mangandi *et al*. (2007) also discovered that the symptoms were similar to those of Myrothecium leaf spot, which has been reported on gardenia, begonia, and new guinea impatiens. On potato dextrose agar, isolates from lesions showed white, floccose colonies with sporodochia in dark green-to-black concentric rings. Singh (2008) also studied that the leaf spot of grapevine caused by *Myrothecium roridum* Tode ex Fr. Singh (2008) also looked at the *Myrothecium roridum* Tode ex Fr. leaf spot on grapevines. The symptoms emerge in the form of small, elongated lesions on the leaves, but they can also appear on petioles and fragile shoots. For the host range study, thirty plant species from nine distinct families were intentionally inoculated with *Myrothecium roridum* spore-cum-mycelial suspension.

Pathogenicity test of fungus

To demonstrate pathogenicity, Bael plants growing in pots were injected with *Myrothecium roridum* spore suspension using an atomizer sprayer during the overnight hours. After inoculation, typical signs occurred on the leaves after 7 days, which were comparable to those observed in natural settings. The pathogen was re-isolated, and the pathogen's cultural and morphological behaviour,

Fig. 5(A-C): Pathogenicity test fungus of *Myrothecium roridum.*

i.e., isolated from naturally infected and artificially injected bael plants, were similar. Koch's hypotheses were therefore proven. Mayer *et al.*, (2005) found that the pathogen could be isolated and grown on potato-dextrose-agar (PDA) media. The pathogenicity was tested on previously disinfested healthy bolls from Cotton cv. Fibermax 966(R6 vegetative stage). There were thirteen isolates of *M. roridum* examined, with eight from cotton and five from soybean. A fungal isolate (DUCC4002) was discovered by Known *et al.*, (2014) during an examination of microbes and pests in plant culture media from imported anthurium pots. The fungal isolate identified was Myrothecium roridum, based on morphological characters such as colony shape on potato dextrose agar, spore microstructures observed under light and scanning electron microscopy, and the results of phylogenetic analysis using an internal transcribed spacer rDNA sequence. The fungus could colonise and develop sporodochia on the inoculated leaves, according to pathogenicity testing on anthurium leaves. This is the first time *M. roridum* has been found in imported plant culture medium in Korea. Piyaboon *et al.*, (2016) reported that the fungal isolates were sub-cultured on PDA and incubated at 28°C, which is consistent with the current findings. The water hyacinth leaves and petioles were sprayed with 1 108 spores mL^{-1}, whereas the control treatment was treated with 10 mL distilled water. NYQB452 was not able to produce colonies with black concentric rings on PDA medium, which was consistent with the report of *M. roridum* on common bean. The amplified product was sequenced and identified with BLAST analysis in the Gene Bank database. The 624-bp amplicon (GenBank Accession No. MH050392) revealed 99 percent similarity to the previously published M. roridum, BBA 71015 (AJ302001) isolate from GenBank. The fungus that causes leaf spot on *Brassica oleracea* L. was identified as *M. roridum* Tode ex. based on morphological and molecular characterisation. To confirm pathogenicity, 10^6 conidia mL^{-1} were sprayed on the leaves of ten healthy cabbage cultivar Jingfeng No.1 seedlings, with ten seedlings treated with sterile water serving as controls.

Conclusion

All though the symptom of *Myrothecium* leaf spot is almost similar to *Alternaria* leaf spot so detection of pathogen on field is very confusing as both the pathogen develop necrotic region as well as concentric ring. The only variable symptom which can be seen different from *Alternaria* is *Myrothecium* produces black sclerotia around the concentric rings after few days leaf.

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Declarations

Conflict of interest: All authors declare that they have no conflict of interest.

Ethical approval: Not applicable.

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