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Characterisation of pathogens associated with die-back and twig blight of almond (*Prunus amygdalus* Batsch.)

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Abstract
Die-back and twig blight are the most important diseases of almond (*Prunus amygdalus* Batsch.) plantation world wide. The pathogens associated with die-back and twig blight diseases were isolated and pathogenicity established on two year old potted almond saplings budded with cultivar "Waris". Based on morphological characters, pathogenicity test and comparison with the authentic descriptions the pathogens were identified as *Diplodia seriata* De Not. associated with die-back and *Cryptosporiopsis* sp. associated with twig blight. The fungus isolated from die-back almond twigs on potato dextrose agar medium exhibited cottony and floccose growth. The white colour of the fungal colony finally changed to grayish or dark grayish. The pycnidia embedded in culture medium were globose and black in colour. Conidia mostly pigmented while as till within the pycnidium were, oval, ellipsoidal to cylindrical, aseptate, few developing one transverse median septa. The pathogen associated with twig blight of almond produce fluffy, lanose to loose colony with raised centre without any oppressed margins. The older colony appears compact with little cottony growth but showed oppressed margins. Acervuli were round to elongate with irregular opening and chocolate brown to dark black in colour. Conidiophores were absent, and conidia were one celled oval to ellipsoidal in shape with one end round and the other slightly tapered, hyaline to slightly greenish-brown in colour.

Keywords: colony, conidia, die-back, pathogenicity and twig blight

Introduction
Almond (*Prunus amygdalus* Batsch.), belonging to family Rosaceae, is one of the most important nut crop cultivated in temperate regions of the world. The probable origin of almond is believed to be the Mediterranean region (Ladizinsky, 1999) [13]. Almond possesses wide spread popularity and is even considered as golden crop of California (Rogers, 1974) [21]. Its cultivation is mainly confined to the countries lying between 36º and 45ºN latitude (Rugini and Monastra, 1991) [22]. World shelled almond production has increased many fold from 1034 metric tonnes in 1995 to 2065 metric tonnes in 2007 (Ahmad and Verma, 2009) [2]. The total world production of almond was estimated 2.51 million metric tonnes in 2010 (FAOSAT, 2013) [9]. Almonds are the healthiest and most nutritious nuts of all, considered as a well-balanced cholesterol free food. The medicinal benefits of almond include anti-inflammation, immunity boosting, anti-hepatotoxicity, improved complexion, and improved movement of food through colon and prevention of cancer (Ahmad and Verma, 2009) [2]. In India, almond is mainly grown in the state of Jammu & Kashmir (J&K) and Himachal Pradesh (H.P.) over an area of 23.81 thousand hectares, yielding 11.47 thousand metric tonnes (Kumar, 2010) [12]. However, commercial cultivation of this nut fruit is mainly confined to the Kashmir Valley. In the Valley, it is mainly grown in district Budgam, Pulwama, Shopian, Gandarbal and other hilly areas occupying an area of 15.93 thousand hectares with a total production of 8.21 thousand MT (Anonymous, 2014) [3]. Although the almond tree is native to the Mediterranean region, this beautiful tree has adopted to the climate of Kashmir. In spite of favorable environmental conditions for almond cultivation, the tree is attacked by various diseases which include Die-back and twig blight is of prime importance (Puttoo, 1992; Adaskaveg *et al.*, 1998; Gramaje *et al.*, 2012; Olmo *et al.*, 2016) [20, 1, 11, 17]. During the present few years, almond orchards of the valley have been facing a serious threat due to die-back and twig blight diseases.

Materials and Methods
The present investigations were conducted in the Division of Plant Pathology, SKUAST-K, Shalimar Campus.

Pathogenicity test

The pathogenicity test of the pathogen(s) isolated from both the diseases was performed as per the technique employed by Milholland (1972) [16] on healthy two years old potted almond saplings budded with cultivar 'Waris'. The apparently healthy potted saplings were transferred to the polyethylene chambers in the 1st week of March 2014 and immediately sprayed with copper-oxychloride @ 0.3 per cent to exclude any harbouring pathogen(s). Pots were kept in diffused sunlight under polyethylene chambers especially designed for the purpose and humidity was maintained by timely irrigating the pots and intermittently spraying with sterilized distilled water.

Two sets of healthy potted saplings were selected and inoculated with 7 days old mycelial bits of isolated fungal pathogen (s). In first set the selected twigs from potted saplings were first headed back. The cut surface was surface sterilized with 70 per cent ethanol and 1mm thick mycelia plug of die-back causing pathogen was put on it. While as in second set 5mm vertical and horizontal incisions of "T" shape were made on the selected twigs after surface sterilizing the site with 70 per cent ethanol and 1mm thick mycelia plug of twig blight causing pathogen was put on it. The inoculated incisions were covered with moistened absorbent cotton to maintain proper moisture. Un-inoculated twigs covered with moistened cotton served as control. All the potted plants were kept in polyethylene chambers and sprayed with sterilized distilled water as and when required till the development of typical symptoms. Re-isolations of pathogens from artificially inoculated twigs were carried out and the resultant cultures were compared with the original ones to satisfy Koch's postulates.

Morpho-cultural characteristics of isolated pathogens

The morphological characteristics of the causal pathogens associated with die-back and twig blight diseases were studied both on host (*in vivo*) as well as after culturing on medium (*in vitro*).

In culture

The important characteristics studied were as under:

a) Cultural characters

i) Nature of growth of colony
ii) Nature of pigmentation if any

b) Morphological characters

Colony	:	Colour, shape and margins
Mycelium	:	Colour, septation and width
Fruiting body	:	Colour, shape and size
Conidiogenous cell	:	Colour, shape, size and septation
Conidia	:	Colour, shape, size and septation

On host

The infected twigs with fruiting structure initials were cut off along with some healthy portion, kept in humid chamber at room temperature (20±1°C) in the laboratory and observed for mycelial colour, size and septation after 72 hours. The observations regarding fungal fructifications were taken after 3 days. The swollen fruiting structures on twigs were observed under stereoscopic microscope for their type, shape and colour. Temporary and semi-permanent slides, prepared from fruiting structures of incubated twigs, were examined under microscope for various morphological features.

Identification of the pathogen (s)

The pathogens were identified on the bases of morphological characters, pathogenicity and comparison with authentic descriptions given by different authors.

Results and Discussion

The pathogens associated with die-back and twig blight diseases of almond were identified on the bases of morphological and cultural characters and compared with authentic descriptions for identification. The fungus isolated from die-backed diseased twigs on potato dextrose agar medium produced loose, cottony and floccose fungal colonies with dark grey aerial mycelium. The hyphae were branched, smooth, thick walled, septate and dark brown in colour. The pycnidia were globose, thick walled, without any papille, dark brown to black in colour. The conidiophores reduced to conidiogenous cells were hyaline, thin walled and cylindrical in shape producing a single apical conidium. Conidia mostly pigmented while still within the pycnidium, were ovoid or ellipsoidal to cylindrical with obtuse apex and truncate to rounded base, aseptate, while a few developed transverse median septa. However, the size of different thallus parts viz. pycnidia and pycnidiospores were larger when grown in culture than that obtained directly from host (Table 1; Plate 1). The sexual stage of the fungus reported elsewhere, was neither observed on host nor in culture, hence the identification was confirmed on the basis of asexual stage and cultural characters only. Denman *et al.* (2000) [7] also reported the rear occurrence of telomorphic stage of the fungus in nature. Based on morphological characters both on host as well as in culture and comparison with literature, the fungus responsible for die-back disease of almond was identified as *Diplodia seriata* De Not. The characteristic features of the isolated fungus were compared with the authentic descriptions given by Punithalingam and Waller (1973) [19], Shoemarker (1964) [23], Laundon (1973) [14], Slippers *et al.* (2004) [24] and Phillips *et al.* (2007) [18] with which these characters fully corroborate. Since the perfect state of the fungus neither developed in culture nor was it obtained on host during the period of study, indicating that under existing environmental conditions the fungus grows and reproduces asexually. However, the fungus has been found to reproduce sexually and the perfect state identified as *Botryosphaeria obtusa* (Schw.) Shoemarker.

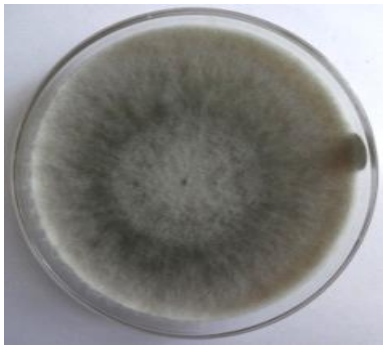
Table 1: Morpho-cultural chracteristics of *Diplodia seriata* De Not. Causing die-back disease of almond (*Prunus amygdalus* Bastsch.)

Thallus part	Shape and growth characters	Colour	Size*	Septation
On host				
Hypha	Hyphae branched, thick walled, smooth	Yellowish brown to Dark brown	2.5-3.6 µm (Av. 2.9 µm) width	Septate
Pycnidium	Globose, solitary or botryose, thick walled, ostiolated, immersed in host tissue, partially becoming erumpent at maturity	Dark brown to black	150-238 µm (Av. 215.34 µm)	-
Conidiogenous	Thin walled, swollen at base, cylindrical,	Hyaline	6.00-10.23 × 2.55-	-

cell	producing single apical conidium		5.00 µm (Av. 8.15 × 4.20 µm)	
Conidia	Ovoid or ellipsoidal, with rounded apex and truncate or rounded base	Initially hyaline, later turning yellowish and finally brown	17.32-29.42 × 8.95-12.41 µm (Av. 24.65 × 10.98 µm)	Initially aseptate, later on developing one transverse median septum
In culture				
Colony	Initially cottony and floccose, becoming loose and fluffy with dense aerial mycelium with no clear margins, pycnidia formed after 13-14 days	Initially hyaline changing to grayish and finally to grayish black	-	-
Hypha	Hyphae smooth, thick walled, branched, hyphal segments short	Hyaline to dark brown	2.8-4.0 µm (Av. 3.4 µm) width	Septate
Pycnidium	Partially embedded in culture medium, becoming erumpent, scattered, globose, thick walled, ostiolate without papilla	Black	162-265 µm (Av. 223.51 µm)	-
Conidiogenous cell	Thin walled, smooth, swollen at base, cylindrical, producing single apical conidium	Hyaline	7.28-10.46 × 2.80-5.25 µm (Av. 8.70 × 5.00 µm)	-
Conidia	Ellipsoidal to cylindrical, with rounded apex and truncate or rounded base	Hyaline changing to yellowish and finally to dark brown	18.93-30.00 × 9.12-13.35 µm (Av. 25.15 × 11.42 µm)	Initially aseptate, later on developing one transverse median septum

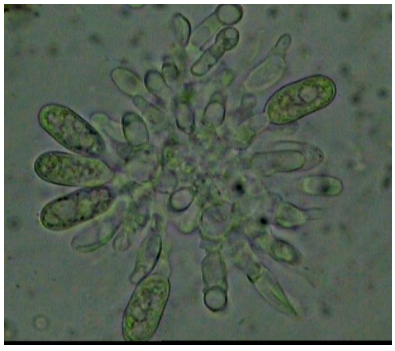
*Figures based on mean of 50 microscopic observations at 40X

Three days old white cottony fungal colony grown on PDA

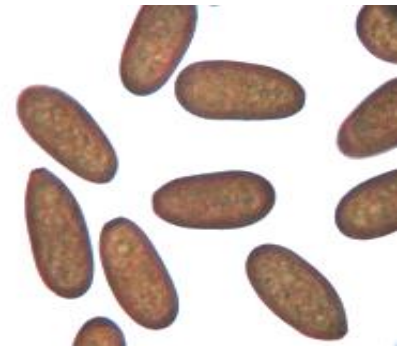


Seven days old grayish black fungal colony colony grown on PDA

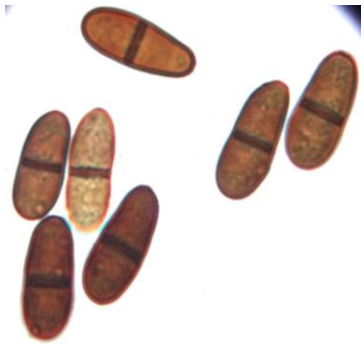
Pycnidial formation in culture medium (PDA)



Conidiogenous cells with developing conidia



Completely melonized conidia from culture



Conidia with transvers median septa from culture

Plate 1: Morpho-cultural characteristics of *Diplodia seriata* De Not.

The pathogenic behavior of isolated fungus associated with die-back disease of almond has been established following Koch's postulates on two years old potted almond saplings budded with cultivar "Waris". The initial symptoms characterized by yellow to brown discoloration appeared after 16-18 days of inoculation near the inoculation site. The discoloration showed down-ward elongation ultimately leading to chlorosis and necrosis. Olmo *et al.* (2016) [17] also observed prominent disease symptoms on almond twigs after 3-4 weeks of inoculation with *D. seriata*. Brooks and Ferrin (1994) [6] reported rapid die-back and twig death in summer inoculations with *B. dothidea.* Re-isolations from the diseased twigs yielded original inoculants repeatedly, thus satisfying Koch's postulates.

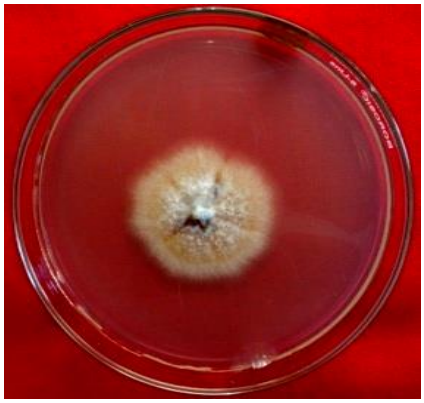
The fungus isolated from blighted almond twigs produced readily furrowed, compacted, grayish black fungal colony with scanty aerial mycelium. Dugan (1993) [8] also observed similar cultural characteristics for apple anthracanose caused by *Cryptosporiopsis curvispora.* The hyphae were septate, smooth, thin walled and brown in colour. Acervuli produced in cultural medium were seprate or confluent submerged in cultural medium, chocolate brown to black in colour and lacking setae. The conidia were released as brown ooze single celled, oval to ellipsoidal in shape with one end round and the other slightly tapered, hyaline to slightly greenish-brown in colour (Table 2; Plate 2). The sexual stage of the fungus was neither developed on host nor in culture, hence the identification was confirmed on the basis of asexual stage and cultural characters only. Based on morphological and cultural characters both on host and in culture and the literature reviewed, the fungus responsible for twig blight disease of almond was identified as *Cryptosporiopsis* sp. The characteristic features of the isolated fungus were compared with the authentic descriptions given by Wilkinson (1945) [26], Sutton (1980) [25], Lele *et al.* (1981) [15], Gene *et al.* (1990) [10] and Beig (2006) [5] with which these characters fully corroborate. Since the perfect state of the fungus neither developed in culture nor was it obtained on host during the period of study, indicating that under existing environmental conditions the fungus grows and reproduces asexually. However, the fungus has been found to reproduce sexually and the perfect state identified as *Pezicula carticola* (Edgerton) Nannf.

Table 2: Morpho-cultural characteristics of *Cryptosporiopsis* sp. causing twig blight disease of almond (*Prunus amygdalus* Bastsch.)

Thallus part	Shape and growth characters	Colour	Size*	Septation
On host				
Hypha	Smooth, thin walled and branched	Hyaline to brown	1.34-4.37 µm (Av.3.0 µm) width	Septate
Acervuli	Pin heads, solitary, mostly elongated with irregular openings	Dark chocolate brown with purplish tinge	147.84-369.60 X 136.52-182.40 µm (Av. 274.35 X 158.54 µm)	-
Conidia	Oval to ellipsoidal, one end round and other slightly tapered	Hyaline to slightly greenish	4.9-9.4 X 2.2-2.7 µm (Av. 7.4 X 2.4 µm)	-
In culture				
Colony	Initially cottony, loose with no margins. However it turns into a compressed colony with appressed margins. Colony raised at centre and sometimes radially furrowed. Acervuli formed after 6-7 days	Initially pure white which turns dirty white and then to grayish black. Reverse side olive green with dark centre	-	-
Hypha	Smooth, thin walled, straight and branched	Hyaline to yellowish green	1.46-4.64 µm (Av.3.2 µm) width	septate
Acervuli	Spherical to irregular, solitary or in groups, irregularly situated or sometimes formed in rings. Acervuli initially semi hard turn into coal tar like drops which harden and become dome shaped	Initially dirty white, then light brown and finally dark black	194.38-398.96 X 156.44-194.80 µm (Av. 292.12-169.25 µm)	-
Conidia	Oval to ellipsoidal, one end round while other slightly tapered	Hyaline-yellowish	5.4-10.0 X 2.0-2.6 µm (Av. 7.7- 2.5 µm)	-

*Figures based on mean of50 microscopic observations at 40X

Pathogenic behavior of isolated pathogen associated with twig blight has been established on two years old almond saplings budded with cultivar "Waris". Initial symptoms (wilting of leaves near inoculation site) appear 9-10 days after inoculation. The peculiar symptoms of twig blight on inoculated twigs were down-ward necrotic extension, clinging of wilted leaves and girdling of inoculated twigs. The observed symptoms on inoculated twigs were in conformity with those of Beig (2003) [4] who observed the appearance of initial disease symptoms on almond twigs inoculated with *Cryptosporiopsis* sp. within 9-10 days of inoculation. Re-isolation from the diseased twigs yielded original inoculants repeatedly, thus satisfying Koch's postulates.

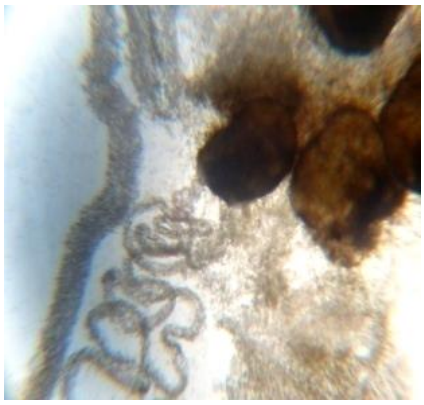


Seven days old dirty white to brown fungal colony grown on PDA

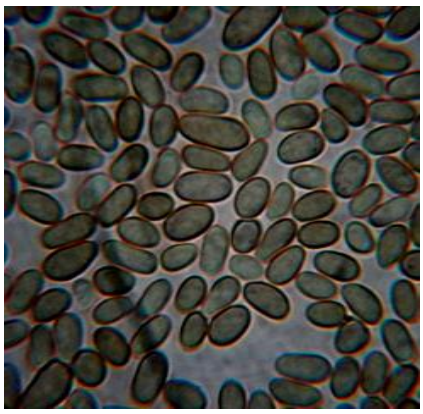
Twenty-one days old grayish black fungal colony grown on PDA

Acervuli formation in circle

Spherical to irregular, Semi hard acervuli on PDA



Conidia releaseing pattern from acervuli



Oval to ellipsoidal conidia from culture

Plate 2: Morpho-cultural characteristics of *Cryptosporyopsis* sp.

Conclusion

In light of present investigations, it can be concluded that die-back and twig blight are economically important disease of almond is prevalent in the almond growing areas of the valley and is a potential threat to almond plantation. The disease is initiated by fungus *Diplodia seriata* De Not. and *Cryptosporiopsis* sp.

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