RESEARCH ARTICLE

Anthracnose disease of walnut (*Juglans regia*) in Kashmir valley

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ABSTRACT: Walnut anthracnose is one of the most important disease of walnut (*Juglans regia* L.) worldwide. In earlier stages of the disease development, spots are circular light brown slightly sunken which later on turned dark brown in colour with irregular margins. Maximum disease development in the field was observed during August and spots attained maximum size of 27-29 mm. The disease symptoms on the twigs initially appeared as dark brown oval lesions which later on turned black in colour with irregular margins attained maximum size of 26-27 mm. The pathogen produced white glabrous raised colony, which later turn creamy white to grayish white with well-defined lobate margins and concentric zones. Acervulus (113.48-190.49µm) appears in concentric rings having hyaline guttulated, crescent conidia (23.58 × 10.13µm). Maximum mycelia growth was achieved on corn meal agar, whereas potato dextrose agar proved to be best media for sporulation of isolated pathogen (*M. juglandis*). The fungus requires incubation period of 14-18 days to produce symptoms on potted walnut seedlings.

Keywords: Anthracnose, disease development, incubation period, *Juglans regia*, mycelial growth

Walnut (*Juglans regia* L.) is economically an important dry fruit crop which belongs to family *Juglandaceae*. In India, walnut is grown in Jammu and Kashmir, Arunachal Pradesh, Himachal Pradesh and Uttarakhand. In J&K, Walnut is grown in Badrawah, Poonch, Kupwara, Baramulla, Bandipora, Ganderbal, Budgam, Srinagar, Anantnag and other hilly areas occupying an area of 83,219 ha with an annual production of 20,873 tonnes (Anonymous, 2012). Jammu and Kashmir State has attained a special place in the international trade of walnuts contributing about 98 per cent of the total production in India (Sharma, 2012). Its cultivation plays a significant role in the economy of the farmers living in hilly and backward areas, where economic condition of the people is extremely fragile. Exports of walnut from Jammu and Kashmir State earns substantial foreign exchange up to 231.63 crore (Anonymous, 2012).

Although the agro-climatic conditions of Kashmir valley are congenial for cultivation of the walnut, yet its productivity has been relatively low compared to other parts of the world owing to many biotic and abiotic factors that inflict huge loss to walnut industry. Among the major biotic factors, the important fungal diseases include walnut anthracnose (*Marssonina juglandis* (Lib.) Magnus), root and crown rot [*Phytophthora cactorum* (Lebert and Cohn) Schrot], branch wilt [*Hendersonula toruloidea* Nattras], ringspot [*Ascochyta juglandis* Blotshausen], downy leaf spot [*Microstroma juglandis* (Berenger) sacc.], heart rot [*Polyporus squamosa* Huds.

ex Fr.] Fr.], powdery mildew [*Phyllactinia guttata* (Wallr. ex Fr.) Lev.; *Microsphaera extensa* (Cke and Peck)] besides stem canker and die back diseases caused by fungi like *Cytosperma leucosperma* (Pers. ex Fr.) Fr., *Nectria galligena* (Bres.), *Fusarium solani* (Mart. Sacc), etc (Anonymous, 2013). Amongst them, anthracnose is the wide spread foliar disease of *Juglans* spp. and the fungus attacks leaves, nuts and shoots of the current season growth (Belisario *et al.*, 2008). Symptoms develop on the on leaves and fruits as irregular necrotic areas that are often surrounded by small chlorotic halos. The disease causes premature defoliation, slows down plant growth, reduced quantity and quality of nut crops, thereby resulting in huge economic loss in the walnut cultivation regions of the world (Belisario *et al.*, 2001; Van Sambeek, 2003; Kalkisim, 2012).

MATERIALS AND METHODS

Symptomatology and microscopic examination of the fungus on the host

Detailed studies on the development of walnut anthracnose symptoms were carried out on three walnut plants selected out in the walnut orchard at Shalimar campus grown under natural epiphytotic conditions. Four branches bearing leaves and twigs selected random were tagged and were kept under constant observation throughout the growing season. Observations were recorded for the first appearance of disease symptoms and different stages of its development like size, shape

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and colour of the lesions. The diseased leaves and twigs collected from the orchards were further examined in the laboratory for the associated pathogen by teasing the diseased portion and observed under microscope. The leaves and twigs were also kept in moist chamber at 21°C for 36 hours to get the acervuli bulged. These bulged acervuli were lifted with the help of teasing needle under stereoscopic microscope and temporary mounts in cotton blue, lactophenol and distilled water were prepared. Acervuli and conidia were examined for their colour, shape, size and septation.

Morphological characteristics of the pathogen

The morphological characters of the pathogen were studied on both host as well as culture in a laboratory. Semi permanent slides were prepared from 14 days old culture stained with cotton blue in lacto phenol. The slides were examined under microscope (40 ×) with respect to following characters of the causal organism.

Colony: Colour, shape and mycelia growth: Colour, breadth, number of septa and branching, Acervuli: Colour, shape and size and Conidia: Colour, shape, size and number septa were studied.

Pathogenicity test

To ascertain whether the fungus isolated was primary cause of the disease, three sets of Pathogenicity tests

were conducted on one year walnut seedling. Three sets of plants with each set comprising of three plants were kept in 40 cm diameter pots containing sterilized soil. The pots were kept in artificially created humid chambers and maintained at room temperature. High humidity inside the chamber was maintained by timely irrigation and spraying the leaves with sterilized water. The plants were constantly observed for 7 days to rule out any latent infection prior to inoculation. One set of plants was given injuries with the help of sterilized teasing needle while as another set kept uninjured. Inoculation were made by spraying spore suspension (1×10^5 spores per ml) from young culture of *Marsonina juglandis* on the abaxial and adaxial surface of both injured and uninjured leaves with the help of atomizer. The injured and uninjured plants of third set were sprayed with sterilized water and served as a check. These plants were maintained for three weeks and were examined daily for symptom development. The symptoms were microscopically examined for presence of acervuli and conidia. The pathogenicity was confirmed after satisfying Koch's postulate.

Effect of media on growth and sporulation of fungus

The growth of the fungus was studied on different solid media viz., Potato dextrose agar, Richards, Czapeks (Dox) agar, Corn meal agar, Malt extract agar and Oatmeal agar. 30 ml of each sterilized media was poured into sterilized Petri plates under aseptic conditions.

Table 1. Symptomatological development of Anthracnose disease of walnut on leaves

Date of observation (Weekly)	Lesion on Leaf				
	Diameter (mm)		Shape	Colour	Other characters
	Range	Average			
April					
26	-	-	-	-	Disease free
May					
04	0.5-1.0	0.7	Circular	Light brown	Slightly sunken spots on leaves
12	1.0-2.5	1.5	-do-	-do-	-do-
20	2.5-6.0	3.8	-do-	-do-	Spots develop whitish centre
28	6.0-7.0	6.3	-do-	-do-	Circular dark brown spots on fruits
June					
06	7.0-8.0	7.4	-do-	-do-	Development of acervuli in concentric rings on undersurface of leaves
14	8.0 -10	8.9	-do-	Brown to dark brown	-do-
22	10-12	11.1	Circular to irregular	-do-	-do-
30	14-17	15.1	-do-	-do-	-do-
July					
07	17-19	18.0	-do-	-do-	-do-
15	19- 20	19.4	-do-	-do-	Yellow halos around leaf spots on ventral side.
23	20-22	20.8	Mostly irregular	-do-	Numerous Spots start coaleascing
31	22- 24	22.2	-do-	-do-	Formation of irregular Patches
August					
08	24-25	24.1	-do-	-do-	Chlorosis of highly infected leaves
16	25-26	25.2	-do-	-do-	-do-
24	26-27	26.5	-do-	-do-	Major portion of leaf gets involved
31	27-29	28.0	-do-	-do-	Defoliation

Inoculations were made with culture bits of 3mm diameter from actively growing fungus culture. Each treatment was replicated thrice in completely randomized design (CRD). The inoculated Petri plates were incubated at 21°C for 30 days. The radial growth of the pathogen in all the three replications from each medium was recorded and average of the three replications was taken as final observation. The sporulation was studied, by thoroughly homogenizing a 3mm mycelial disc in 5ml of sterilized distilled water. The spore suspension thus obtained was used for counting the number of spores with the help of haemocytometer.

RESULTS AND DISCUSSION

Periodical observations on symptoms development were recorded at an interval of seven days. On the leaves disease was first noticed in the 1st week of May and reached at its peak during late August (Table 1). Disease symptoms initially appeared as small, scattered, slightly sunken light brown spots ranging from 0.5-1.0 mm with an average of 0.7 mm in size (Fig. 1a). In the last week of May circular dark brown spots with whitish center were observed. In the 1st week of June black acervuli forming concentric rings appeared undersurface

of the leaves and size of the lesions ranged from 7.0-8.0 mm in diameter with an average of 7.4 mm. Later on lesions turned brown to dark brown in colour with irregular margins and size ranged from 10-12 mm with an average of 11.1 mm in the 3rd week of June (Fig. 1 b,c,e). The characteristic yellow halos were observed around the leaf lesion in the second week of July (Fig. 1d). The lesion size increased to 22-24 mm with average of 22.2 mm because of coalescing many lesions and large irregular patches of lesions were observed in the last week of July which was followed by chlorosis in the first week of August, that ultimately resulted in defoliation in the last week of August (Fig. 1 f,h). On twigs the disease was first noticed in the 1st week of May and reached at its peak during August (Table 2). Initial disease symptoms initially appeared as small, dark brown lesions ranging from 1.0 to 1.5 mm with an average of 1.2 mm in size (Fig. 2a). Later the lesions turned sunken in the second week of May and the size ranged from 1.5 to 2.5 mm in diameter with an average of 2.2 mm (Fig. 2b). Black acervuli developed on the lesions during the second week of June. Coalescing of lesions was observed in first week of August, attaining size of 25-26 mm with an average of 25.1 mm which was followed by necrosis of the twigs in the third week of August. This was followed

Fig. 1. Characteristic symptoms of walnut anthracnose on leaves (a) Initial sunken light brown lesions; (b) Circular to irregular dark brown lesions; (c) Lesions with whitish center (d) Yellow halos on ventral side; (e) Acervuli on undersurface of lesion; (f) Formation of large necrotic patches; (g) Necrotic patches on fruits; (h) Defoliation

Table 2. Symptomatological development of Anthracnose disease of walnut on twigs

Date of observation	Lesion on twig				
	Diameter(mm)		Shape	Colour	Other characteristics
	Range	Average			
April 26	-	-	-	-	Disease free
May 04	1-1.5	1.2	Oval	Dark brown	-
12	1.5-2.5	2.2	-do-	-do-	Sunken
20	2.5-2.9	2.6	-do-	-do-	-do-
28	2.5-4.5	3.4	-do-	-do-	-do-
June 06	4.5-7.5	5.7	-do-	-do-	-do-
14	7.5-8.0	7.7	-do-	-do-	Black pin point fruiting bodies(Acervuli)
22	8.0- 11.5	9.2	-do-	-do-	-do-
30	11.5-13.0	12.2	Oval to Irregular	Black	-do-
July 07	13- 14.5	13.7	-do-	-do-	-do-
15	14.5-17.0	15.7	-do-	-do-	-do-
23	17.0- 19.0	18.0	-do-	-do-	-do-
31	19.0-22.0	20.3	-do-	-do-	-do-
August 08	22.0-23.0	22.2	-do-	-do-	Coalescing of lesions
16	23.0- 25.0	24.0	-do-	-do-	-do-
24	25.0-26.0	25.1	-do-	-do-	Necrosis of twigs
31	26.0-27.0	26.5	-do-	-do-	Severely infected twigs dry up

Fig. 2. Characteristic symptoms of walnut anthracnose on twigs (a) Initial dark brown lesion; (b) Sunken lesion with whitish centre; (c) Coalescing of lesions; (d) Formation of large necrotic patch; (e) Necrosis of leaf and twig

Table 3. Morphological characters of *Marssonina juglandis* Magnus causing Anthracnose disease of walnut

Propagule type		Colour	In culture	On host	Shape/structure	Septation
Colony	15 days old	White	10-12.5 mm	-	Circular, whitish glabrous aerial tufts of mycelium in centre	-
	30 days old	Creamy white	23-24.4 mm	-	Circular, whitish glabrous aerial tufts of mycelium in centre, surrounded by flat white mass.	-
	40 days old	Greyish white	44.5-45 mm	-	Development of concentric zones, with well defined lobate margins, and radially furrowed.	-
Hyphae (width) µm)		Hyaline	2.5-3 (2.72)*	2-3 (2.41)*	Smooth and branched	Septate
Conidia µm)		Hyaline	20.26-32.42 × 8.10-12.15 (23.58×10.13)*	14.12-24.42 × 2.5-3.0 (17.82×2.26)*	Crescent, guttulated, bicelled	One septa
Acervuli im)		Pitch dark	113.48-190.49 (157.91)*	105-182 (141.76)*	Discoid, in concentric rings	-

*Figures in parenthesis are average values

by drying up of severely infected twigs (Fig. 2c,d,e,f). Similar type of symptoms on leaves and twigs caused by *M. juglandis* were also observed by earlier workers (Belisario, 2002).

The morphological characters of the pathogen were studied both on host as well as from culture and are presented in (Table 3). Microscopic observations of the pathogen on host revealed that the hyphae was branched, septate, smooth and hyaline measuring 2-3 μm in width with an average of 2.41 μm . Conidia were crescent shaped, hyaline, guttulated, one septum measuring 14.12-24.42 $\times$ 2.5-3.0 μm with an average of 17.82 $\times$ 2.26 μm . Fruiting bodies (acervuli) were sub-cuticular, discoid, pitch dark in colour and measured 105-182 μm with an average of 141.76 μm . The Petri plates inoculated with fungus, were critically observed for colony characters and the growth behaviour (Table 3). After 15 days of incubation at 21 $\pm$ 1 $^{\circ}\text{C}$, the colony appeared as circular whitish glabrous aerial mycelia tufts measuring (10-12.5 mm dia) (Fig. 4a), which later turned creamy white, surrounded by flat white mass (23-24.4 mm dia) after 30 days (Fig. 4b), and finally grayish white, with appearance of characteristic concentric zones, well defined lobate margins and radial furrows (44.5-45.0 mm dia) were observed after 45 days of incubation (Fig. 4c). Microscopic observations revealed that the pathogen produced branched, septate, smooth and hyaline hyphae measuring 2.5-3.0 μm in width with an average of 2.72 μm (Fig. 4d). Acervuli developed in concentric rings were discoid, pitch dark in colour measuring 113.48-190.49 μm in size with an average of 157.91 μm (Fig. 4 e, f). Conidia were crescent shaped, hyaline, guttulated with one septate measuring 20.26-32.42 $\times$ 8.10-12.15 μm with an average of 23.58 $\times$ 10.13 μm (Fig. 4h). On the basis of the morphological characters, pathogenicity test and comparison with authentic description, the causal pathogen of Anthracnose disease of walnut was identified

as *Marssonina juglandis* (Lib.) Magnus. The above morphological characters of the pathogen resemble with the description given by Sogonov *et al.* (2008), Dastjerdi *et al.* (2009) and Saremi and Amiri (2010).

Observations regarding the pathogenicity of the test fungus revealed that the initiation of typical symptoms of the disease 14 days after inoculation on injured leaves and twigs of potted plants. However, in uninjured leaves, the symptoms developed 18 days after inoculation. Re-isolation from infected leaves and twigs yielded the typical culture of the fungus, thus proved the Koch's postulates (Fig. 3a,b).

Several reports indicate that microorganisms differ in their nutritional requirements. The average radial growth (cm) of the pathogen after 30 days of incubation are presented in Table 4. The maximum growth on corn meal agar (6.11cm) followed by oat meal agar (2.75 cm) and potato dextrose agar (2.43 cm). There was no significant difference between oat meal agar and potato dextrose agar and also between malt extract agar (1.78 cm) and Czapek (Dox) agar (1.42 cm). Minimum growth (0.99 cm) was observed on Richard's Media. The data

Fig. 3. Establishment of pathogenicity of *Marssonina juglandis* on walnut seedling

Fig. 4. Morphological characters of *Marssonina juglandis*(Lib.) Magnus at 400x (a) 15 days old white colony; (b) 30 days old creamy white colony; (c) 45 days old grayish white colony; (d) Mycelium; (e) Acervuli in culture; (f) Acervuli in concentric ring; (g) Conidial ooze in culture; (h) Hyaline conidia

Fig. 5. Effect of different media on growth and sporulation of *Marssonina juglandis* at 21±1°C after 30 days of incubation

Table 4. Effect of different media on growth and sporulation of *Marssonina juglandis* at 21 ±1°C after 30 days of incubation

Media	Radial Mycelial growth (cm)	Sporulation (10 ⁶ /mm ²)
Corn Meal Agar	6.11a	0.45
Oat Meal Agar	2.75b	0.81
Potato Dextrose Agar	2.43bc	1.79
Malt Extract Agar	1.78cd	0.30
Czapek (Dox) Agar	1.42de	0.45
Richards	0.99ef	1.10
CD (p=0.05)	0.775	

further reveals that sporulation was maximum on potato dextrose agar media 1.79×10^6 spores per mm² followed by Richard's (1.10×10^6 spores mm²). Sporulation of 0.45×10^6 per mm² was recorded on both corn meal agar and Czapek (Dox) agar medium. Minimum sporulation was recorded on malt extract Agar (0.30×10^6 mm²). These results are in agreement with Nadroo (2006) and Salahi *et al.* (2009) who observed oat meal agar and potato dextrose agar as best medium for growth and sporulation, respectively.

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