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Studies on Leaf Spot of Pomegranate (*Punica granatum* L.) caused by *Cercospora punicae* Henn.

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ABSTRACT: Pomegranate is a commercially growing fruit crop affected by several diseases of which leaf spot is an important disease hindering productivity. The objective of this study was Isolation, identification, cultural and Physiological characteristics of the *C. punicae*.*C. punicae* was isolated from typical lesions on leaf by standard tissue culture technique and single spore isolation technique after that, identified as *Cercospora punicae* by comparing with original descriptions. Pathogenicity was established by proving Koch's postulates. Cultural studies revealed that among different culture media, highest radial growth (90.00mm)was observed on potato dextrose agar (PDA) followed by oat meal agar (OMA, 89.66 mm),while profuse sporulation was observed on oat meal agar and also found sexual perithecium fruiting bodies of *Mycospherella punicae* along with ascospores on corn meal agar. The conidia are colourless, straight, needle shape with multiseptate, obclavate. Physiological studies revealed that 25°C was best temperature with pH 6.0 was best for the growth and sporulation of *C. punicae*. At temperature of 30°C with highest radial growth (79.33 mm) and dry mycelial weight (283.23 mg) was recorded and good sporulation at 25 °C was noticed. Optimum pH for the growth of pathogen is 6.5 to 7.0.

Keywords: Leaf spot, pomegranate, *Cercospora punicae.*

INTRODUCTION

Pomegranate is commercial growing fruit crop of India, is one among the major fruit crops of arid zone. In India, it is regarded as a "vital cash crop", grown in an area of 261 thousand ha with the production of 2315 thousand MT during 2019-20 (Anon., 2020). Among the different states growing pomegranate, Maharashtra is the largest producer occupying 2/3rd of total area (*i.e.* 147.91 thousand ha area and 1789.46 thousand MT production (Anon., 2020) in the country followed by Karnataka, Andhra Pradesh, Gujarat and Rajasthan. Karnataka accounts 19,000 ha area and 2.04 lakh tons' production with an average productivity of 10.74 ha^{-1} in 2019-20 (Anon., 2020).

Pomegranate fruit crop is declining now days due to many fungal and bacterial diseases *viz.*, *Ceratocystis fimbriata*, *Cercospora punicae*, *Xanthomonas axonopodis* pv. *punicae*, *Alternaria alternata* and *Colletotrichum gloeosporioides* causes leaf and fruit spots, followed by wilt and bacterial blight. Among the fungal diseases, *Cercospora* leaf spot of pomegranate caused by *Cercospora punicae* Henn. Which cause 10 to 15 per cent fruit loss during survey was conducted in southern Karnataka and also reduce the quality of fruits. *Cercospora* leaf spot disease of pomegranate is one of the main factors for low productivity. Leaf spot of pomegranate is spread at faster rate within the field of pomegranate during rainy season and severe infection causes the leaves turn yellow and fall prematurely, spots on leaves and fruits reduce the yield and quality of fruits which are unfit for marketing and also get lower prize. The *Cercospora* sp. affected plant shows small, dark brown spots on leaves, flowers and fruits that are initially circular but eventually become irregular. At more severe infection causes the leaves to turn yellow and fall prematurely. Spots on leaves and fruits lead to reduced yield and quality of the fruit.

The cercospora leaf and fruit spot of pomegranate was first recorded in Japan by Hennings in 1906 (Chupp, 1953). Subsequently, it was reported from Texas in 1909, and the causal agent was identified as *Cercospora lythracearum* (Westcott, 1971).

Hennings (1906) observed the leaf spot disease caused by *Cercospora punicae* on pomegranate as circular to irregular, amphigenous, grey centre with blackish brown margin.

Chupp (1953) described leaf spots are circular to somewhat angular, dark reddish brown to almost black with a diffused yellow halo and size varies from 0.5 to 5 mm in diameter.

Hong *et al.* (2014) observed the Leaf lesions were angular to irregular, initially greyish white to tan, later brown to dark brown and mostly surrounded with yellow haloes. Under continuous moist conditions, lesions became grey and cottony due to heavy sporulation of the causal fungus.

Prasad *et al.* (2014) described the symptoms of leaf spot on fenugreek caused by *C. traversiana.* As the infection is progressed, producing greynecrotic areas on the leaves which are often surrounded by yellow halo.

Avila *et al.* (2020) described the symptoms of cercospora leaf spot (CLS), leaden-grey areas that develop from the presence of characteristic lead-black olivaceous asexual fruiting bodies. Heavy infection leads to high defoliation, mainly in the inner part of the canopy.

Andrade *et al.* (2021) noticed cercospora leaf spot, an important disease in coffee caused by the fungus *Cercospora coffeicola*, it has appeared as two distinct symptoms on leaves which are 'Brown Eye Spot' (BES) and the 'Black Spot' (BS) in field conditions.

Now it has been considered a major problem because of its occurrence and spreading nature results from heavy losses to pomegranate growers.

Hence, the management of leaf and fruit spot of *Cercospora* disease of pomegranate is of major concern to the growers, wherever pomegranate is cultivated. Major practices like the use of disease-free planting material, orchard sanitation, pruning of diseased branches and application of fungicides aids in checking the spread of the disease.

MATERIAL AND METHODS

A. Collection and isolation of the pathogen

Infected leaves and fruits showing characteristic symptoms were collected and isolated by following standard tissue isolation techniques.

The infected leaf portions along with some healthy parts were cut into small pieces or bits and were surface sterilized with mercury chloride 0.1 per cent solution for a 15 seconds and then these infected bits of leaves were washed in sterilized distilled water for three times to remove the traces of mercuric chloride and later aseptically transferred on to sterile Petri plate, with Potato Dextrose Agar (PDA) and Oat meal agar (OMA) in a triangular manner with the help of sterile forceps and were incubated at room temperature (27±1^{o}C) for about one week and observed for fungal growth and sporulation.

B. Morphological identification of Cercospora pathogen

Pathogen *Cercospora punicae* identified on the bases of morphological characteristics on OMA medium. The colonies were whitish in colour and white to smoky white mycelium. Produced the septate, straight or needle shape, slightly curved conidia. Conidiophores were dark brown, solitary, multi septate.

C. Proving the pathogenicity

The healthy pomegranate plants were selected and washed thoroughly with tap water, and wiped using moist cotton swab. The inoculum suspension from 15 days old culture was prepared in potato dextrose broth with 1×10^{6} spores/ml and used for spraying. Branches were covered with polythene bags for 48 hr. to ensure successful penetration of the pathogen into the tissue. Similarly control plants were sprayed with sterile distilled water for comparison.

The polythene bags removed after 2 days and observation were taken regularly for the appearance and development of symptoms. After appearance of disease symptoms re-isolation was made from the diseased tissue of artificially infected plants. The obtained culture was compared with original culture to confirm the identity of the fungus and subsequent confirmation of Koch's postulates.

D. Cultural characters of C. punicae on different solid media

The cultural characters of *C. punicae* studied on eleven different non synthetic/semi-synthetic and synthetic solid media.

Twenty ml of each sterilized and cooled medium was poured aseptically into sterilized Petriplate. Five mm disc of the *C. punicae* was taken from actively growing culture with the aid of cork borer and a disc was placed at the centre on different culture media and Petridish and then incubated at 27 ± 1 ^{o}C for 15 days. Each of this experiment was replicated thrice and observations regarding cultural characters such as the colour, diameter and pigmentation of colony was recorded.

Effect of temperature on the growth of *C. punicae.* The fungal growth was tested at different temperature *viz.*, 15, 20, 25, 30 and 35^{o}C with three replications were maintained. Twenty-five ml of potato dextrose broth (PDB) was added into 100 ml of conical flask. The flasks were allowed to cool after sterilization. Later, the flasks and plates were inoculated with 5 mm disc of fungus which was collected from 12 days old culture and incubated at respective temperatures. The mycelial mat in PDB was harvested by filtering through Whatsman No.1 filter paper of 9 cm diameter and dried. The dry mycelial weight was recorded and also diameter of growth of the fungus was measured and results were analysed statistically.

Effect of pH on the growth of *C. punicae.* The liquid medium used in this study was potato dextrose broth. Hydrogen ion (pH) concentration of the media was determined by using pH meter. Adjustment of pH was done using 0.1 N alkali (Sodium hydroxide) or 0.1 N acid (Hydrochloric acid). Reaction of liquid media was adjusted to required pH *viz*., 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. Twenty-five mL of the medium was added to 100 ml conical flask and sterilized at 1.1 kgcm^{-2} pressure for 20 minutes at 121 °C. Each treatment was replicated thrice. To each flask, 5mm fungal disc was inoculated aseptically and incubated at 27 ± 1 ^{o}C for 12 days. The ideal pH for growth of the fungus was determined by harvesting mycelial mat that was filtered through Whatman filter paper and dry mycelial weight (mg) was recorded.

RESULTS

Symptomatology. *Cercospora* leaf spot produced typical symptoms as small, dark brown spots on leaves/fruits that are initially circular but eventually become irregular as they grow. On the leaves, the lesions are dark, reddish brown to almost black and show faint halo. The spots on fruit resemble bacterial spot, but they are darker and various size, without crack, no stickness and the twigs dries off and die.

A: spots on leaves, B: Dark colour spots on fruit, C: Pure culture of *C. punicae,* D: Mycelia growth on media.

Fig. 1. Symptoms on leaf and fruits and colon of *C. punicae.*

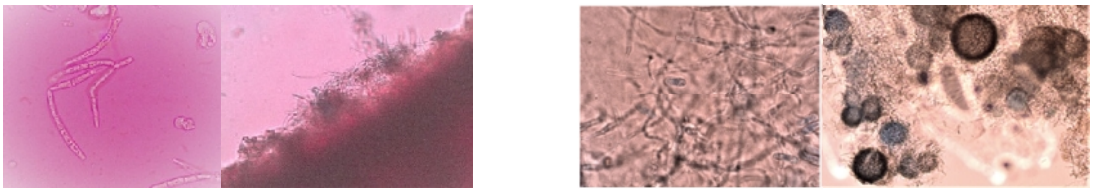
More severe infection causes the leaves to turn yellow and fall prematurely. Spots on leaves decrease yield and also quality of the fruit (Fig. 1).

Isolation and identification of pathogen. Infected leaves and fruits showing characteristic symptoms were collected and isolation was made by following standard tissue isolation method under aseptic conditions (Fig. 1).

Morphological identification and Pathogenicity proving of *C. punicae*

Pathogen *Cercospora punicae* identified on the basis of morphological characteristics on OMA medium. The colonies were whitish in colour and white to smoky white mycelium. Produced the septate, straight or needle shape, slightly curved conidia (Fig. 2). Conidiophores were dark brown, solitary, multi septate. The perfect stage of the pathogen *i.e. Mycospherella punicae* which produces as cospores are the sexual spores and fruiting body is perithecia, a cup like structure having small opening. Similar types of record were found by Park *et al.* (2020). They noticed that the *Cercospora* colonies on the PDA were pale pinkish to light grey, with cottony aerial mycelium, Conidia were hyaline, acicular to cylindrical, truncate to sub truncate at the base, 3–17-septate, Conidiophores were fasciculate, olivaceous brown, paler toward the apex, straight to slightly curved, 3–15-septate.

Sautua *et al*. (2020) observed the Cercospora on soyabean, the conidia were hyaline, solitary, straight, needle-shaped, truncate at the base, acute at the tip, uniform in width, indistinctly septate. Conidiophores were dark brown, thick-walled, solitary, straight or geniculate, uniform in width, multi-septate.



Emerging of conidia of *C. punicae* from mycelia

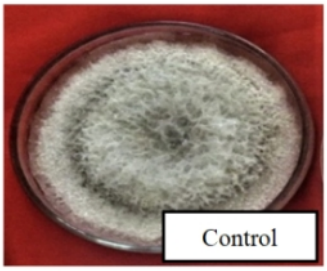
Perithecium fruiting body of *Mycospherella punicae* produced in the medium with two celled ascospores

Fig. 2: Microscopic observation septate mycelium and sexual spore.

Pathogenicity: Fungus was isolated from infected pomegranate leaves and pure culture was obtained by single spore isolation method and used for pathogenecity test.

The pure culture of *C. punicae* was artificially inoculated to healthy pomegranate plants by spraying of conidial suspension (10^6 conidia per ml). The symptoms of the disease appeared on 21 days after inoculation of mycelia. Initially, the minute reddish brown spots appeared on the leaf which turned later as light brown spots with narrow reddish brown margin and light yellow halo. No symptoms were observed on twigs or petioles *etc*. (Fig. 3). The microscopic characters of re isolated fungus were same as recorded in the original culture of the test fungus and colony characteristics of both the cultures were same. The symptoms produced by the *C. punicae* were compared in accordance with, Sharma (2018) observed the disease symptoms both on leaves and fruits of pomegranate plant. The leaf spotswere circular to angular, dark reddish brown to almost black with diffused yellow halo. Andrade *et al.* (2021) noticed cercospora leaf spot, an important disease in coffee caused by the fungus *Cercospora coffeicola*, can appear as two distinct symptoms on leaves which are 'brown eye spot' (BES) and the 'black spot' (BS) in field conditions.

(A) Symptom expression on inoculated leaf



(B) Re-isolation of *C.punicae* from infected leaf

Fig. 3. Proving pathogenicity of *Cercospora* leaf spot on pomegranate caused by *C. punicae.*

Cultural characteristics of *C. punicae* on different solid media. The growth of *C. punicae* was studied on eleven different solid culture media. The radial growth, colony characters and sporulation of the fungus were recorded when the maximum growth was attained on any one of the tested media. The effect of different culture media on the growth of fungi was significantly different. Maximum radial growth of *C. punicae* was recorded on potato dextrose agar (90.00 mm) which was on par with oatmeal agar (89.66 mm), Potato carrot agar (89.10 mm) which was followed by Richards's agar (88.18 mm), V8 juice agar (87.61mm), Martin Rose Bengal agar (86.66 mm), Sabouraud dextrose agar (86.41 mm), Czapek's Dox agar (86.25 mm), Corn meal agar (82.81 mm), Malt extract agar (65.58 mm and the least radial growth was recorded in water agar (34.18 mm) (Fig. 4, Table 1).

The colour of mycelium varied from white to light grey colour and the mycelial growth varied from regular fluffy raised to sparsely raised irregular. Good sporulation was recorded in oatmeal agar and poor sporulation was recorded on water agar media.

Similar type of results recorded by Nega *et al.* (2016) with highest mean colony diameter of *Cercospora zeae-maydis* on potato dextrose agar (57.2 mm) however least mean mycelial growth of 53.1mm was recorded on Malt extract agar (MEA).

Kallideen (2020) reported that oat meal agar was the best agar medium and that 25°C was the optimal temperature for the growth of *Cercospora* sp. on artificial media.

Table 1: Effect of different cultural media for the growth of *Cercospora punicae* .

Sr. No.	Different culture media	Radial growth (mm)*	Colony colour	Margin of colony	Texture of colony	Growth nature	Pigmentation
1.	Potato dextrose agar	90.00(71.57)	White	Regular smooth	Cotton	Aerial, raised	White
2.	Oat meal agar	89.66(70.73)	Smoky white	Regular smooth	Cotton	Aerial, raised	Light grey
3.	Potato carrot agar	89.10(69.77)	Smoky white	Regular smooth	Cotton	Partially raised	Light grey
4.	Richard's agar	88.18(69.54)	Light grey	Regular smooth	Velvety	Aerial	Grey
5.	V_8-juice agar	87.61(68.15)	White	Regular smooth	Cotton	Surface growth	Light grey
6.	Martin rose bengal agar	86.66(64.98)	Light grey	Irregular sparse	Cotton	Aerial, raised	Greyish
7.	Sabouraud dextrose agar	86.41 (64.6)	Smoky white	Regular smooth	Cotton	Aerial	Light grey
8.	Czapek's Dox agar	86.25 (63.51)	Smoky grey	Irregular sparse	Velvety	Aerial, fluffy raised	Dark grey
9.	Corn meal agar	82.81 (61.39)	White orange	Irregular sparse	Cotton	Surface growth	Orange
10.	Malt extract agar	65.58 (50.38)	White	Regular smooth	Cotton	Partially raised	Creamy white
11.	Water agar	34.18 (33.08)	Dull white	-	-	Sparsely	-
SEm ±		0.081					
CD @ 1%		0.238					

*Figures in parenthesis are arc sine transformed values

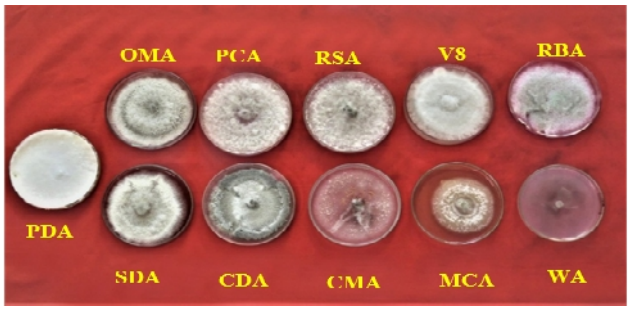


Fig. 4. Growth of *C.punicae* on different cultural media.

Effect of temperature on *C. punicae* on solid culture media. The experiment was done to know the optimum temperature for growth of *C. punicae.* For this study, different temperature levels *viz.*, 15, 20, 25, 30 and 35 °C were tested and results are presented in Table 2 and Fig. 5.

The growth of *C. punicae* was gradually increased from 20 to 30 °C and later it was decreased at increasing temperature. The growth differences seen in all temperatures were statistically significant from each other. The temperature of 25°C was significantly superior to other temperature levels by recording the maximum radial growth (90 mm) followed by 30°C (86.91 mm), 20°C (57.91 mm) and 35°C (14.75 mm) pathogen growth not seen at 15°C (0.00 mm). The colour of mycelia was white at all temperature. In accordance with Sharma (2018) recorded maximum growth of *C. punicae* at a temperature of 25±1°C.

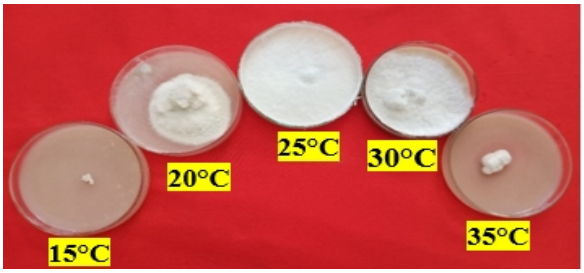


Fig. 5. Effect of temperature on *C. punicae* on solid culture media.

Effect of temperature on *C. punicae* in liquid media. This experiment was conducted to know best temperature for growth of *C. punicae* by using potato dextrose broth at different temperature levels *viz.*, 15, 20, 25, 30 and 35 °C. Among them maximum dry mycelial weight was observed at 25 °C.

Dry mycelial weight of *C. punicae* was significantly influenced by different temperature levels tested. Significantly higher dry mycelial weight of the fungus was observed at temperature 25°C (360.00 mg) which was followed by temperature level of 30°C (283.00 mg), 20°C (197.00 mg) and 35°C (29.00 mg). There was no growth of the fungus at 15°C. The results were presented in Table 2 and Fig. 6.

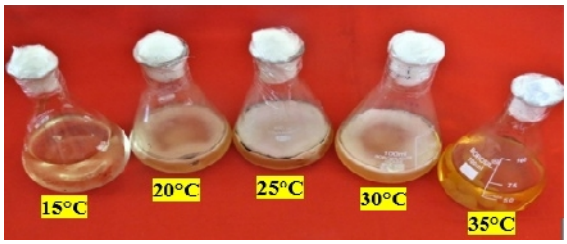


Fig. 6. Effect of temperature on *C. punicae* in liquid media.

Table 2: Effect of temperature on growth of *C. punicae.*

Sr. No.	Temperature (°C)	Potato dextrose broth	Potato dextrose agar		
		Dry mycelial weight (mg)*	Radial growth (mm)*	Mycelial colour	Type of growth
1.	15	0.00	00.00(0.00)	No growth	No growth
2.	20	197. 00	57.91(49.55)	Smoky white	Fluffy raised, irregular
3.	25	360.00	90.00(71.57)	White	Flat, regular
4.	30	283.00	86.91(68.79)	Smoky white	Fluffy raised, irregular
5.	35	29.00	14.75(22.58)	White	Raised, irregular
Sem ±		0.011	0.115		
CD @ 1%		0.048	0.367		

*Figures in parenthesis are arc sine transformed values.

Effect of pH on the growth of *C. punicae* in liquid media. The study was carried out to know the optimum Ph required for the growth of *C. punicae*, dry mycelial noted at different pH levels *viz.*, 4, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5, results shown in Table 3 and Fig. 7.

The highest dry mycelial weight of *C. punicae* was recorded at the pH of 6.0 (240.56 mg) which is on par with the pH 6.5 (210.71 mg) and these are followed by pH 7.00 (200.50 mg), pH 7.5 (160.88 mg), pH 5.5 (118.50 mg), 5.0 (115.75 mg), 4.5 (110.05), 4.0 (99.98 mg) 8.0 (89.78 mg) and the growth was not seen at 8.5 pH (0.00 mg).

The results obtained are in accordance with Sharma (2018) reported the optimum pH of *C. punicae* growth *in vitro* was 6.0 to 6.5pH.

Table 3: Effect of pH on growth of *C. punicae* in potato dextrose broth medium.

Sr. No.	pH level	Dry mycelial weight (mg)*
1.	4.0	99.98
2.	4.5	110.05
3.	5.0	115.75
4.	5.5	118.50
5.	6.0	240.56
6.	6.5	210.71
7.	7.0	200.50
8.	7.5	160.88
9.	8.0	89.78
10.	8.5	0.00
SEm ±		0.111
CD @ 1%		0.330

Fig. 7. Effect of pH on growth of *C. punicae* on potato dextrose broth.

DISCUSSION

Cercospora leaf spot pathogen was isolated by using affected leaves of pomegranate plant, then it was identified as *Cercospora punicae* based on morphological characters and proved by pathogenicity test. *C. punicae* produced septate mycelium, having colony characters *viz.*, initially white and gradually turned to smoky white colour. It produced straight to slightly curved or needle shaped 3 to 5 celled septate conidia. The perithecial fruiting bodies with two celled as cospores were observed in oat meal agar. Pathogenicity was proved by spraying spore suspension of *C. punicae* on to punctured healthy pomegranate leaves. The *Cercospora* leaf spot showed brown to dark brown spot with faint halo symptoms on leaves after 21 days of inoculation. The maximum radial mycelial growth was observed at 25°C on PDA (90 mm) and highest dry mycelial weight (360.00 mg) was recorded on potato dextrose broth. The growth of *C. punicae* was tested at different pH level ranging from 4 to 8.5. The dry mycelial weight of pathogen was highest at pH 6 (240.56 mg). Among the eleven different cultural media tested, the growth of *C. punicae* showed highest radial growth (90.00 mm) which was recorded on PDA and sporulation was seen on oat meal agar (OMA). The *Cercospora* produced fruiting bodies when isolated on both oat meal agar and corn meal agar. Similar types of results were compared with, Sharma (2018) observed the disease symptoms both on leaves and fruits of pomegranate plant. The leaf spots were circular to angular, dark reddish brown to almost black with diffused yellow halo. The lesions on the fruit were small but conspicuous dark brown, circular initially becoming unequal irregular blotches. These blotches covered a considerable proportion of the surface of the fruit which turned light to dark brown in colour at 25°C temperature and pH of 5 to 6 on PDA.

Future line of work, molecular confirmation of cercospora leaf spot pathogen *C. punicae* is required for the further studies. Development of Integrated Disease Management (IDM) strategies for management of *C. punicae.*

CONCLUSION

This studies revealed that leaf spot of pomegranate plant disease was caused by *Cercospora punicae.* The pathogen has well grown at temperature of 25°C and oat meal agar (OMA) was the best media for sporulation.

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Conflict of Interest. None.

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