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RESEARCH ARTICLE

Morphological, cultural and physiological characterization of *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc., the cause of anthracnose of pomegranate (*Punica granatum* L.)

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ABSTRACT

The severity of pomegranate anthracnose was more in Bagalkot district (35.84%) followed by Koppal (27.22%), Bijapur (24.85%) and Raichur (18.14%) districts. The identity of the fungus was confirmed as *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc and deposited at NCFT, New Dehli. The isolates of *C. gloeosporioides* showed maximum growth on Potato dextrose agar and Richard's broth on 13 th day after incubation at 27±1°C. There was variability among eight isolates of *C. gloeosporioides* with respect to type of growth, mycelial colour, pigmentation, size of the spore and sporulation. The highest radial growth and sporulation of the fungus was recorded at 30 °C, with 100 per cent relative humidity and also light condition having 12 hours darkness alternated with 12 hours light.

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INTRODUCTION

Pomegranate (*Punica granatum* L.) is a commercially important fruit of both tropical and subtropical countries and belongs to the family Punicaceae. The crop is gaining more importance due to its adoptability to low temperature in winter, drought and salt tolerance. In the world, India ranks third in position with respect to area and production. However, the fruits are susceptible to various diseases caused by fungi, bacteria and physiological disorders. Among the various fungal diseases, anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. is one of the most serious disease of pomegranate worldwide. In India, anthracnose disease was first reported by McRae (1924).

Anthracnose causing *Colletotrichum gloeosporioides* isolates are very dynamic and exhibit high variation with respect to cultural, morphological and pathogenic characters. Hence, there is a need to understand the diversity of *C. gloeosporioides* isolates collected from different geographic regions and cultivars.

MATERIAL AND METHODS

The samples were collected during 2005 to 2007 by roving method of survey from various geographical locations of Northern Dry Zone of Karnataka by using Wheeler's formula (1969) for calculating per cent disease index (PDI). The collected samples were used for the repeated isolation of the fungus from the infected tissues of pomegranate by following the standard tissue isolation method. The cultures

obtained were identified based on the morphological characters of the mycelium and spore and test of pathogencity by pinprick method using detached fruit technique.

Growth phase of *C. gloeosporioides* on potato dextrose broth (PDB) :

The 60 ml of potato dextrose broth (PDB) was added into each of 150 ml conical flask and sterilized. The flasks were then inoculated with 5 mm disc of fungal culture and incubated at 27 ± 1°C for different intervals. Three flasks were harvested at a time, starting from the 3 day onwards up to 17th day by leaving a gap of 2 days between the two successive harvests. Each set of experiment was replicated thrice and they were incubated at 27 ± 1° C. The cultures were filtered through previously weighed Whatman No. 42 filter paper of 9 cm diameter, which were dried to a constant temperature at 60°C in an electric oven prior to filtration. The mycelial mat on the filter paper was thoroughly washed with sterile distilled water to get rid off the salts likely to be associated with the mycelial mass. The filter paper along with the mycelial mat were dried to a constant weight at 60°C and weighed immediately on an analytical balance. The difference between final and initial weight of filter discs was taken as the weight of the mycelia. The data were analyzed statistically.

Dry mycelial weight (mg) = Total weight of filter paper – Initial weight of filter paper along with mycelia

Growth phase of *C. gloeosporioides* on different solid and liquid media :

The cultural characters of single spore isolates of *C. gloeosporioides* were studied on synthetic *viz.*, Richard's agar, Czapeck's agar and Sabouraud's agar whereas for non or semi-synthetic media like PDA, Oatmeal agar, Malt extract agar and Host leaf extract solid and liquid media. The composition and preparation of the above mentioned synthetic and semi-synthetic media were obtained from Ainsworth and Bisby's 'Dictionary of the Fungi' by Hawksworth *et al*., (1983). Growth phase of *C. gloeosporioides* on different solid and liquid media were studied as per the procedure followed in growth phase of *C. gloeosporioides* on Potato dextrose broth (PDB) and variability in cultural characters such as the colony diameter, dry mycelial weight, colony colour, and sporulation were recorded. The sporulation was graded as follows :

Sr. No.	Score	Grade	Description (conidia/microscopic field [400 X])
			C. gloeosporioides
1.	++++	Excellent	> 75
2.	+++	Good	50-75
3.	++	Fair	25-50
4.	+	Poor	1-25
5.	-	No sporulation	---

The medium which supported the highest mycelial growth was used for to study the growth phase of eight *C. gloeosporioides* isolates with respect to variability in cultural characters such as the colony diameter, dry mycelial weight, colony colour, and sporulation.

Effect of temperature on the growth and sporulation of *C. gloeosporioides* :

The growth of fungus was tested at 15, 20, 25, 30 and 35° C. Twenty ml of Potato dextrose agar was poured into 90 mm diameter Petriplates. After solidification, 5 mm discs from actively growing culture were cut and inoculated to the solidified media containing Petriplates using cork borer and incubated for 13 days by keeping *C. gloeosporioides* in the incubator adjusted to required temperature levels. Each treatment was replicated thrice. After incubation period, radial growth and sporulation in solid media were recorded as described earlier.

Effect of relative humidity (RH) levels on growth and sporulation of *C. gloeosporioides* :

The growth of *C. gloeosporioides* was tested at different RH levels *viz.*, 60, 70, 80, 90 and 100 per cent. Twenty ml Potato dextrose agar was poured into 90 mm diameter petriplates. After solidification, 5 mm discs from actively growing culture were cut and inoculated into the solidified Petriplates and were incubated for 13 days in the desiccators. Different levels of RH were created by using different concentrations of sulphuric acid (H_2SO_4). The desiccators were kept at 27 ± 1° C with three replications. After incubation, radial growth and sporulation in solid media were recorded as described earlier.

Effect of light on growth and sporulation of *C. gloeosporioides:*

The effect of light on the growth of *C. gloeosporioides* was studied by exposing it on Potato dextrose agar to alternate cycle of 12 hours light followed by dark, 12 hours dark followed by light, continuous light and continuous darkness. Petriplates were inoculated with 5 mm disc taken from the periphery of 8 days old pure culture. Each treatment was replicated four times and incubated for 13 days. Colony diameter and colony characters were analyzed statistically.

RESULTS AND DISCUSSION

The result obtained from the present investigation are summarized below :

Collection, isolation and identification of *C. gloeosporioides:*

The severity of pomegranate anthracnose was more in Bagalkot district (35.84%) followed by Koppal (27.22%), Bijapur (24.85%) and Raichur (18.14%) districts during roving survey.

Eight different isolates were collected from different locations by roving method of survey. The pure culture of isolated fungus were obtained by single spore isolation technique and pathogencity proved by pinprick method using detached fruit technique. The study on morphological characteristics of isolates of *Colletotricum gloeosporioides* indicated, septate hyphe, conidiomata acervulli and hyaline conidiophore, conidia single celled, hyaline, straight, cylindrical, attenuated or rounded at both the ends, with one to two globules in the conidium. The conidia measured 10.9-20.6 µm in length and 4.39-6.65 µm in width. The description given agreed with Jefferies *et al*. (1990). Further, the identity of the fungus was confirmed by depositing the culture at National Centre for Fungal Taxonomy, New Delhi with identification No. 1285.07 to 1291.07. Among the isolates of *C. gloeosporioides*, a local isolates of Raichur was picked up for further studies. The growth phase of *C. gloeosporioides* study revealed that dry mycelial weight of fungus recorded gradual increase starting from 3 day (70.00 mg) and reached peak growth on 13th day (423.00 mg), while on 17th day it showed 373.33 mg dry mycelial weight and then started declining from 15th day (400.33 mg) onwards. The results obtained are presented in the Table 1 . The present studies are in conformity with Ekbote *et al*. (1997) and differs with Sudhakar (2000) who found that the maximum growth of fungus was attained on 14th day of incubation.

Cultural and morphological characteristics of *C. gloeosporioides* on different solid and liquid 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 data are presented in Table 2. Among the non or semi-synthetic media used for growth and sporulation of *C. gloeosporioides*, maximum growth (89.66 mm) and good sporulation of the fungus was recorded on PDA followed by Oatmeal agar, which recorded growth of 86.00 mm and fair sporulation. While least growth (49.83 mm) and poor sporulation was observed on host leaf extract medium. Present studies are in accordance to the better performance of *C. gloeosporioides* on PDA which may be

Table1: Effect of different incubation periods on growth of *C. gloeosporioides*

Sr. No.	Days after incubation	Dry mycelial weight (mg)#
1.	3	070.00
2.	5	120.66
3.	7	218.33
4.	9	263.00
5.	11	344.33
6.	13	423.00
7.	15	400.33
8.	17	373.33
S.Em±		1.107
C.D. at 1%		4.661

Mean of three replications

Table 2 : Cultural and morphological characteristics of *C. gloeosporioides* on different solid and liquid media

Sr. No.	Different media	Mycelium					Sporulation	Grade
		Radial growth (mm)#	Dry mycelial weight (mg)#	Colour	Type of growth	Pigmentation		
1.	Czapeck's agar	75.16	189.66	Pure white	Fluffy raised	White	+	Poor
2.	Malt extract agar	77.16	309.16	Light white	Sparse growth	Brown to black	+	Poor
3.	Host leaf extract agar	49.83	176.00	Light white	Sparse growth	Light orange	+	Poor
4.	Richard's agar	87.50	434.50	Pure white	Fluffy raised	White	++	Fair
5.	Oat meal agar	86.00	405.16	White	Fluffy raised	Reddish orange	++	Fair
6.	Sabouraud's agar	84.50	399.50	Light red	Fluffy raised	Pinkish orange	+	Poor
7.	Potato dextrose agar	89.66	420.83	White	Fluffy flat	Light pinkish	+++	Good
S.Em±		0.354	1.077					
C.D. at 1%		1.529	4.652					

Mean three replications

attributed to inherent complex nature of material supporting good fungal growth owing to provision of some additional nutrients as reported by Ekbote *et al*. (1997), Sudhakar (2000) and Akhtar (2000).

On synthetic media, *C. gloeosporioides* also differed with respect to growth and sporulation. Among the synthetic medium, maximum growth (87.50 mm) and fair sporulation were recorded on Richard's agar and least growth (75.16 mm) with poor sporulation was recorded on Czapeck's agar.

However, in case of broth, maximum dry mycelial weight (434.50 mg) was recorded in Richard's medium broth and least dry mycelial weight (176.00 mg) was recorded in host leaf extract broth after thirteen days of incubation as given in Table 2. The present studies are in accordance to Sudhakar (2000) who reported that maximum radial growth was recorded in five media (Sabouraud's agar, Richards's agar, Brown's agar, Potato dextrose agar and Oatmeal agar) and they did not differ significantly. The least colony growth was recorded in Asthana and Hawker's 'A' medium. Further, he reported that in liquid media, the maximum mycelial weight was recorded in Richards's broth (288.33 mg) and least in Asthana and Hawker's 'A' broth (166.66 mg) after 16 days of incubation.

Response of different isolates of *C. gloeosporioides* on Potato dextrose solid and liquid media :

Eight isolates were collected from different geographical locations and were grown in the universal media of Potato dextrose agar on both solid and liquid media. Maximum radial growth and dry mycelial weight were recorded in isolate Cgr-2 (90.00 mm) and Cgk-1 (434.33 mg), respectively. Least radial growth (40.00 mm) and dry mycelial weight (216.66 mg) were recorded in Cgs-1 isolate, while excellent sporulation was recorded in Cgb-1 isolates after 13 days of incubation as shown in Table 3. Earlier workers observed the difference in morphological characteristics like colony characters, average colony diameter, sporulation, average growth rate/ day and average number of mature acervuli/7 mm disc on same isolates on different media (Ekbote *et al*.,1997). Thus, it is opined that variability of these geographical isolates needs to be confirmed with molecular tools like RAPD and RFLP.

Maximum dry mycelial weight of *C. gloeosporioides* was obtained on Potato dextrose broth on 13th day after incubation. Similar results on dry mycelial weight of eight isolates of *C. gloeosporioides* was recorded on Potato dextrose broth. It was also observed that further increase in incubation periods resulted in decrease in mycelial growth in all the isolates. The eight isolates of *C. gloeosporioides* also showed diverse response in attaining the maximum growth.

Physiological characters of *C. gloeosporioides* :

Among the external factors, which influence the growth of fungi, temperature plays an important role. Temperature affects almost every function of fungi including the growth and sporulation. Temperature has profound effect on the vegetative and reproductive activities of the fungi. Effect of temperature on mycelial growth revealed that maximum growth (90.00 mm) was at 30^{o}C with increase in temperature to level of 35^{o}C, the mycelial growth was decreased and least mycelial growth (28.66 mm) was observed at 15^{o}C. However, the temperature at 20-30^{o}C was found to be optimum for the growth and sporulation of *C. gloeosporioides* as shown in Table 4. This is in agreement with the observation made on *Glomeralla cingulata*, *C. capsici* and *C. gloeosporioides* by various workers who noticed best growth of these fungi at 25-29^{o}C (Prasanna Kumar, 2001). Maximum sporulation of fungus

Table 3 : Cultural and morphological characteristics of eight isolates of *C. gloeosporioides* on Potato dextrose agar

Sr. No	Isolates	Radial growth (mm)#	Dry mycelial weight (mg)#	Characters of mycelium and spore				
				Mycelial colony	Pigmentation	Spore size (µm)	Sporulation	Grade
1.	Cgb-1	79.50	268.83	White raised	Light red	1.37-1.5×0.87-1.0	++++	Excellent
2.	Cgb-2	87.00	233.33	White suppressed	Light brown	2.00-2.5×0.75	+++	Good
3.	Cgk-1	85.00	434.33	White raised	White	2.25-2.50×0.90-1.00	++	Fair
4.	Cgk-2	80.83	422.50	White raised	White	2.1-2.2×0.8-0.90	++	Fair
5.	Cgk-3	83.00	427.66	Medium white suppressed	White	2.12-2.5×0.60-0.75	++	Fair
6.	Cgr-1	88.50	416.16	Medium white	White	2.0-2.1×0.75-0.90	+	Poor
7.	Cgr-2	90.00	418.33	Medium white	White	1.75-2.0×0.5-0.6	+	Poor
8.	Cgs-1	40.00	216.66	Cream, suppressed	Cream	2.1-2.5×1-1.25	+	Poor
S.Em±		1.055	2.221					
C.D. at 1%		4.443	9.351					

#Mean of three replications

was at 30° C and least sporulation was found at temperature 15° C, which is an important pathogenic character. The present results are also in accordance with Estrada *et al*. (2000).

Humidity is an important pre-condition for radial growth of fungi. The relative humidity of 100 per cent recorded maximum mycelial growth of 89.66 mm with good sporulation, whereas least radial growth was recorded at 60 per cent relative humidity (61.16 mm) with excellent sporulation as shown in Table 5. The present findings are in agreement with the results obtained by Dodd *et al.* (1991).

Light has a profound effect on growth and sporulation of fungus. The preliminary studies carried out in the present investigation with *C. gloeosporioides* indicated a maximum growth (82.75 mm) and sporulation (fair) when it was exposed to alternate light and dark condition (first 12 hours light alternate with 12 hours dark) at par with 12 hours dark and 12 hours light (79.50 mm) and fair sporulation. Least radial growth (55.25 mm) and poor sporulation were recorded when *C. gloeosporioides* was exposed to continuous light as shown in Table 6. When exposed to alternate light and darkness, it attained maximum radial growth which might be due to induction of certain metabolic process necessary for growth and sporulation of the fungus, which usually doesn't occur in continuous light. Similar experiments conducted by Mishra and Sirdhana (1980) observed in their studies that the diurnal light exposure favoured good growth and sporulation compared to continuous light.

Table 4 : Effect of temperature on growth and sporulation of *C. gloeosporioides* on Potato dextrose agar

Sr. No.	Temperature (°C)	Radial growth (mm)#	Mycelial colour	Sporulation	Grade
1.	15	28.66	Raised, brown to black	+	Poor
2.	20	68.00	Suppressed, orange	++	Fair
3.	25	73.33	Suppressed, light orange	+++	Good
4.	30	90.00	Suppressed, light white	++++	Excellent
5.	35	87.00	Suppressed, light red	++	Fair
S.Em±		1.267			
C.D.at 1%		6.013			

Mean of three replications

Table 5 : Effect of relative humidity (%) on growth and sporulation of *C. gloeosporioides* on Potato dextrose agar

Sr. No	Relative humidity (%)	Radial growth (mm)#	Mycelial colour	Sporulation	Grade
1.	60	61.16	Raised, white	++++	Excellent
2.	70	70.33	Medium, white	++	Fair
3.	80	76.33	White mix with light orange	+++	Good
4.	90	80.66	Medium white	+	Poor
5.	100	89.66	Light white, raised	+++	Good
S.Em±		1.438			
C.D.at 1%		6.822			

Mean of three replications

Table 6 : Effect of light on growth and sporulation of *C. gloeosporioides*

Sr. No	Treatments	Radial growth (mm)#	Mycelial colony	Spore size	Pigmentation	Sporulation	Grade
1.	12 hours alternate light/ dark	79.50	Medium growth	7.0-8.4×21.7-22.4	White	+	Poor
2.	12 hours alternate dark/ light	82.75	Suppressing growth	7.0-7.7×22.4-24.5	Slight red	++	Fair
3.	24 hours light	55.25	Good growth	7.7-8.4×29.4-31.5	Medium red	+	Poor
4.	24 hours dark	75.25	Very good growth	7.0-7.7×28.0-29.4	Slight white	+	Poor
S.Em±		0.891					
C.D.at 1%.		4.093					

Mean of four replications.

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