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Morphological and Cultural Variability of *C. gleosporioides* Causing Leaf and Fruit Spot of Pomegranate

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ABSTRACT: In India anthracnose, caused by *C. gloeosporioides* is the second most important disease of pomegranate next to bacterial blight commonly referred as oily spot. Recently, the pathogen showed high variability and development of fungicide-resistant strains. Therefore, present study was undertaken to study the morphological and cultural variability. All the isolates were identified as *C. gloeosporioides* on the basis of their morphology. The pathogen exhibited high level of variability and all the 16 isolates were found to be pathogenic and produced diseased symptoms within one week of inoculation. The mean colony diameter of all the isolates ranged from 85.33 mm to 89.67 mm. The colony colour of the isolates varied from grey, white and black. The reverse colony showed black and white colony colour. Sporulation was categorized as heavy to low with and spore size ranged from 9.146µm to 13.67µm. The isolates differed in their ability to produce symptoms. On the basis of their virulence, the isolates PCg 7, 8 and 14 were found to be very aggressive while PCg 5, 6 and 13 were moderately aggressive and the rest of the isolates were less aggressive.

Keywords: Anthracnose, *C. gloeosporioides*, PCg, morphologival and cultural variability.

INTRODUCTION

Fruit sector is the major constraint in the agriculture of India. It is a profitable deal amongst all the farming activities as it provides good employment and leads to economic upliftment of the farmers. It is drought tolerant and can even tolerate moderate frost; it can also be grown in dry areas. Pomegranate has a high medicinal value. No other fruit have such high medicinal value as compared to that of Pomegranate. The Annual production of Pomegranate in our country is 13.45 MT with Maharashtra being the leading state in both area and production. In India, anthracnose caused by *C. gloeosporioides* is the second most important disease of pomegranate next to bacterial blight commonly known as oily spot. It damages foliage and creates severe problems in nurseries and young orchards when crowded and moist (Kumar and Rani 2010). It can also reduce flower set, leading in yield declines. Anthracnose signs can be found on the leaves, twigs, petioles, flower groups (panicles), and fruits. Lesions on leaves begin as tiny, angular brown to black spots and can enlarge to cause extensive dead areas. During dry conditions, the lesions may fall out of the leaves.

The first signs of disease are small black or dark-brown spots that can enlarge, coalesce, and kill the flowers before they produce fruits, significantly decreasing yield. Petioles, twigs, and stalks are also affected, developing the characteristic black, expanding lesions seen on fruits, leaves, and flowers. Anthracnose-affected ripe fruits get sunken, pronounced decay spots that are dark brown to black before or after harvesting. Premature fruit drop page from branches is possible. Fruit can rot extensively as a result of the fruit spots coalescing and ultimately penetrating deeply into the fruit, which happens frequently. Until the fruit ripens, the majority of infections in green produce are latent and essentially invisible. Thus, fruits that seem healthy at harvest can quickly exhibit serious anthracnose signs as they ripen. Fruits that are infected when they are mature spread the fungus into storage, resulting in significant loss during storage, transportation, and marketing (Haggag, 2010). The fungal pathotype determines how

severe the disease is on the host plants. The variability amongst the pathogen makes it difficult to classify and manage. The pathogen exhibits high level of variability with reference to its radial growth, colony characters, sporulation, spore size and aggressivity. Hence, the study on morphological and cultural variability of the pathogen was undertaken, so that the pathogen can be easily identified and the appropriate management strategies can be undertaken.

MATERIAL AND METHODS

Collection and isolation of *Colletotrichum gloeosporioides*

The leaf and fruit samples exhibiting typical symptoms of anthracnose were collected from 9 districts of M.P.K.V. jurisdiction *viz.* Ahmadnagar, Dhule, Jalgaon, Kolhapur, Nashik, Pune, Sangli, Satara and Solapur.

Table 1: Disease samples collected from different locations of Maharashtra were designated as follows.

Isolcate No's	Village	Taluka	District
Pcg 1	Loni	Rahata	Ahmadnagar
Pcg 2	Vilad	Rahuri	Ahmadnagar
Pcg 3	Kolhar	Rahuri	Ahmadnagar
Pcg 4	Bhabareshwar	Rahata	Ahmadnagar
Pcg 5	Kopargaon	Kopargaon	Ahmadnagar
Pcg 6	Pravranagar	Rahata	Ahmadnagar
Pcg 7	Pathri	Sinner	Nashik
Pcg 8	Satana	Satana	Nashik
Pcg 9	Sri krushna nursery	Khatau	Satara
Pcg 10	Shrushti nursery	Khatau	Satara
Pcg 11	Chikhli	Mohol	Solapur
Pcg 12	Kasbe	Miraj	Sangli
Pcg 13	Belhe	Karveer	Kolhapur
Pcg 14	Shinban	Sakri	Dhule
Pcg 15	Chalisgaon	Chalisgaon	Jalgaon
Pcg 16	Shirur	Shirur	Pune

The infected portion of fruits with characteristic symptoms was cut into small bits. These bits were surface sterilized by 0.1% $HgCl_2$ and washed thrice with sterile distilled water and dried on sterile blotting paper. The bits were then inoculated on PDA media. Further, the plates were incubated at 28°C for 7 days. After 7 days, the colonies so formed were separated and purified on PDA medium. The fungal isolates were maintained on PDA slants at 5°C. The isolates sub cultured once in three months to maintain their viability.

Pathogenicity: Under laboratory conditions, the pathogenicity was proved by detached fruit technique (DFT). The fruits with desired age were collected with long stalk (3-4 inches). Before inoculation, the fruits were washed with tap water, air dried, surface disinfected with 0.1% mercuric chloride solution one minute followed by thorough but gentle rinsing with sterilized water for three times to remove the traces of disinfectant. Thereafter, the fruits were kept on flask or beaker (100ml) by inserting the fruit stalk in sterilized water, which is previously filled in them. Sterilized polythene bags were used to cover the fruits. It was done to provide 24 hours pre-inoculation incubation of fruits as suggested by Manandhar *et al.* (1995). Next day the bags were removed and inoculation was made at the site of fruit. The combination of two inoculation method was used to have maximum infection of disease. Micro-droplet inoculation technique (MDIT) with spore suspension having 105 conidia ml^{-1}. The mycelial discs were placed in inverted position and covered with small cotton swab to provide moisture for conidial germination and infection. This was then incubated at 27°C and 70-80% humidity for seven days.

Isolation and identification:

Cultural and morphological study of *Colletotrichum gloeosporioides*: The isolate of *Colletotrichum gloeosporioides* was grown on PDA medium for the cultural and morphological study. Morphological characters (colony radial growth, colony colour, colony reverse, pigmentation, zonation and nature of growing margin) were recorded after 7 days of inoculation with the help of image analyzer in each replicate (Talhinhas *et al.,* 2005). Slides were prepared from 10 days old culture and number of spores, presence of conidial masses, setae were measured with haemocytometer. Radical growth of the fungus was measured after 7 days.

Aggresitivity: The fruits were washed with distilled water and then with 0.1% $HgCl_2$ and then three times with sterilized distilled water. The spore suspension of all isolates was sprayed on fruits and which were previously injured by rubbing carborundum powder. The fruits were kept at 27 ± 2ºC for 10 days. Fruits sprayed with distilled sterilized water were treated as control. The aggressivity of isolates was calculated on the basis of days taken for initiation of symptoms, development of acervulus and lesion size.

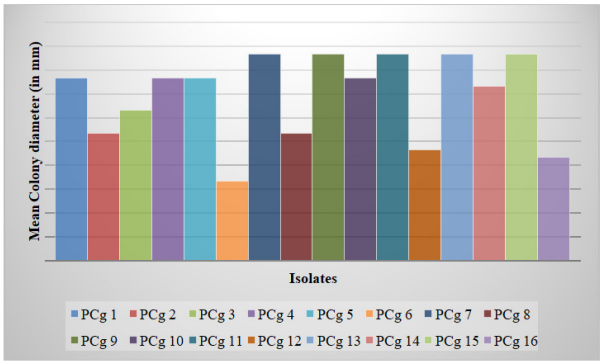
RESULTS

Morphological characteristics of *Colletotrichum gleosporioides*: The pathogen showed high variability

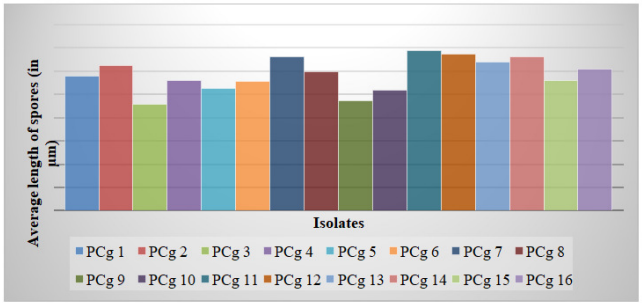
with respect to its morphological characterstics, cultural parameters and virulence.

Radial growth and Mean colony diameter: The mycelial growth of 16 different isolates of *Collectotrichum gleosporioides* was measured 7days after inoculation from purified culture grown on PDA media. The mean colony diameter showed no significant difference (*i.e*., they were at par). It ranged from 85.33 mm to 89.67mm (Table 2 and Fig. 1). The results were in conformity with the findings of Ekabote (1994); Jayalakshmi (2010) but differ with the findings Akthar (2000); Sudhakar (2000); Prashanth (2007); Rani and Murthy (2004); Ashutosh *et al*. (2012).

Colony Colour: The colony colour of isolates varied from white, grey and black. The isolate PCg1 and 10 produced white colonies while isolates PCg 5, 11 and 15 produced grey colonies. The isolate PCg 7 had complete black colony and isolate PCg 2 had dark grey colony. Some isolates produced two coloured colonies. The isolate PCg 3, 4, 8, 9, 12, 13, 14 had grey and white colony, PCg6 had colony of white centre and grey colour in the margin. The isolate PCg 16 produced grey and black colony (Table 2, Fig. 2a). The results are in collaboration to the findings of Irwin and Cameron (1978) who reported the existence of morphological variable pathogens in *C. gloeosporioides.* Yang (2011); Thomidis and Exadaktylou (2011); *Rahimlou et al.* (2014) *found that* the mycelium was white- grey turning olive green over time, and produced oval to cylindrical, hyaline, unicellular, aseptate conidia. These findings are in conformity with the results obtained by Singh (2011); Hasabnis (1984) in respect of mycelial colour. Further, the results are similar to the findings of Thakare (1991); Hande (2001) which are regarded as the general characteristics of mycelium.



(a)



(b)

Fig. 1. (a) Mean mycelial growth after 7 days on PDA media (b) Average spore size of isolates.

Colony reverse: The reverse colony morphology was examined in order to identify the concentric rings and zones produced by the acervuli. But, none of the isolates produced acervuli in the culture. Almost all the isolates had black centre and white margin except isolate PCg 7 had complete black colony and PCg 8 which produced black and orange colony (Table 2, Fig. 2b).

Mycelium Growth Pattern: The margin of the isolates PCg 1, 3, 6, 7, 8, 10, 12, 13, 15, 16 was found to be regular while that of isolates PCg 1, 4, 5, 9, 14 was irregular, when they were inoculated on PDA media (Table 2, Fig. 2a, b).

Sporulation: The isolates showed considerable difference in terms of sporulation. The isolates were divided into 5 groups on the basis of sporulation. Out of the 16 different isolates, the isolate PCg 6, 10 and 14 produced very less spores while the isolates PCg 3 and 9 had low sporulation. The isolates PCg 2, 11, 12, 13 had medium sporulation and isolates PCg 1, 4, 5, 7, 15, 16 had heavy sporulation. The average spore size of the isolates varied from 9.146 µm to 13.798 µm in length. The average conidial size of PCg 11(13.798 µm) was largest while that of PCg 3 (9.146 µm) was shortest (Table 2, Fig. 2).

The conclusions of Ekabote (1994); Jayalakshmi (2010) supported the results, however those of Akthar (2000); Sudhakar (2000); Prashanth (2007); Rani and Murthy (2004); Ashutosh *et al.* (2012).

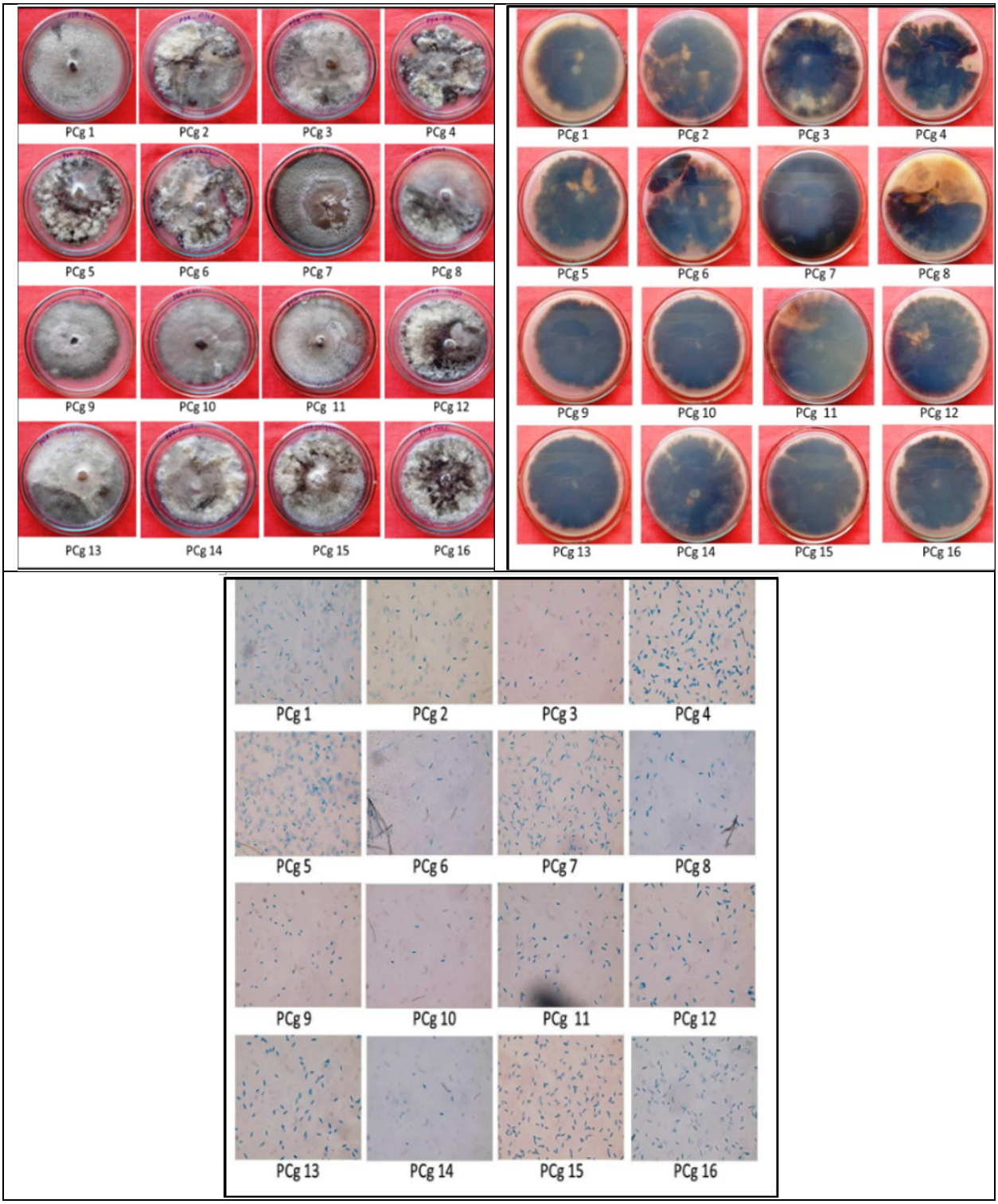


Fig. 2. (a) Colony morphology of PCg isolates on PDA media; **(b)** Reverse colony morphology of PCg isolates on PDA media; **(c)** Spores of PCg isolates observed under 40x.

Table 2: Morphological variations in isolates of *Colletotrichum gloeosporioides.*

Sr. No.	Colony characters			Mean colony diameter (in mm)	Sporulation and spore size (length)
	Front	Back	Growth pattern		
Pcg 1	White	Black centre and white margin	Circular	88.67	Heavy, 11.63 µm
Pcg 2	Dark grey	Black centre and white margin	Irregular	86.33	Medium, 12.5 µm
Pcg 3	Grey and white	Black centre and white margin	Circular	87.33	Low, 9.146 µm
Pcg 4	Grey and white	Black centre and white margin	Irregular	88.67	Heavy, 11.23 µm
Pcg 5	Grey	Black centre and white margin	Irregular	88.67	Heavy, 10.55 µm
Pcg 6	White centre and grey colour	Black centre and white margin	Circular	84.33	Very Low 11.14 µm
Pcg 7	Complete black	Complete black	Circular	89.67	Heavy, 13.25 µm
Pcg 8	Grey and white	Black and orange	Circular	86.33	Very Low, 11.98 µm
Pcg 9	Grey and white	Black centre and white margin	Irregular	89.67	Low 9.45 µm
Pcg 10	White	Black centre and white margin	Circular	88.67	Very low 10.413 µm
Pcg 11	Grey	Black centre and white margin	Circular	89.67	Medium 13.798 µm
Pcg 12	Grey and white	Black centre and white margin	Circular	85.67	Medium 13.466 µm
Pcg 13	Grey and white	Black centre and white margin	Circular	89.67	Medium 12.798 µm
Pcg 14	Grey and white	Black centre and white margin	Irregular	88.33	Very low 13.236 µm
Pcg 15	Grey	Black centre and white margin	Circular	89.67	Heavy 11.23 µm
Pcg 16	Grey and white	Black centre and white margin	Circular	85.33	Heavy 12.21 µm
SE	NA	NA	NA	8.04	NA
CD	NA	NA	NA	NS	NA

Pathogenicity and Aggressivity: The isolates even varied with respect to their aggresivity to cause the disease. The isolates PCg 7, 8 and 14 were found to be very aggressive while PCg 5, 6 and 13 were moderately aggressive and PCg 1, 2, 3, 4, 5, 6, 9, 10, 11, 12, 15 and 16 were less aggressive (Table 3, Fig. 3 and 4). All the isolates could produce acervuli on the inoculated fruits except PCg 1, 2, 5, 9, 10, 11, 13 and 15. In addition to this, Joshi *et al.* (2014) assessed the severity of disease (aggressivity of pathogen) by evaluating 11 different pomegranate varieties against 6 isolates of *C. gloeosporioides* and Isolate Cg 86 was found to be virulent.

Table 3: Evaluation of Aggressiveness of PCg isolates.

Isolates	Initiation of Symptoms	Average Lesion size (in mm)	Formation of acervulus	Aggressiveness
Pcg 1	After 8 days	19.33	No	Low
Pcg 2	After 8 days	20.56	No	Low
Pcg 3	After 8 days	20.98	Yes	Low
Pcg 4	After 8 days	21.67	Yes	Low
Pcg 5	After 6 days	15.5	No	Moderate
Pcg 6	After 7 days	22.24	Yes	Moderate
Pcg 7	After 4 days	27.66	Yes	High
Pcg 8	After 5 days	26.97	Yes	High
Pcg 9	After 8 days	19.3	No	Low
Pcg 10	After 8 days	16.5	No	Low
Pcg 11	After 8 days	18.9	No	Low
Pcg 12	After 8 days	21.6	Yes	Low
Pcg 13	After 6 days	22.7	No	Moderate
Pcg 14	After 5 days	25.7	Yes	High
Pcg 15	After 8 days	17.3	No	Low
Pcg 16	After 8 days	22.5	Yes	Low

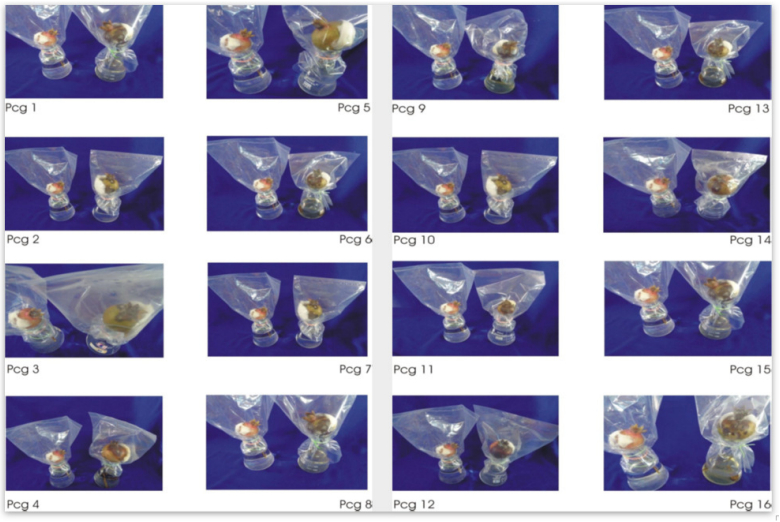


Fig. 3. Pathogenicity studies of *Colletotrichum gloeosporioides* isolates.

Fig. 4. Aggressiveness studies of *Colletotrichum gloeosporioides* isolates

CONCLUSIONS

Colletotrichum gleosporioides exhibits a wide range of morphological variability within the isolates. Therefore, there was a need to study these parameters, so as to ease the classification, identification and management of the pathogen. Despite of isolates being of same species, the isolates show remarkable differences with respect to colonial morphology (grey to white), sporulation (heavy to low), spore size (ranging from 9.146 µm to 13.798 µm) and aggressiveness. The variability within the same species may be due to environmental conditions prevailing in the location of the isolate from where it is collected.

FUTURE SCOPE

The variation in colony colour and mycelia growth of different isolates even in the same media can be argued that variation in the isolates may be inherent and hence the variation can be correlated with the virulence and aggressivity of isolates. The mutation can be the reason for this variability which might increase the susceptibility of host to the pathogen and therefore morphological and physiological characters can be used as preliminary test to study the mutation, variability, susceptibitlity or resistance to fungicides and aggressivity of pathogen towards hosts.

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Conflict of Interest. None.

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