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CULTURAL CHARACTERIZATION OF *COLLETOTRICHUM GLOEOSPORIOIDES,* THE CAUSAL AGENT OF CITRUS DIEBACK

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A B S T R A C T

Citrus dieback caused by *Colletotrichum gloeosporioides*, is one of the major constrains in citrus production in Pakistan. To manage this disease, a comprehensive study of its pathogen is important. The present study is therefore conducted to investigate the effect of different culture media such as potato dextrose agar (PDA), bean extract agar (BEA), malt and yeast extract agar (MYEA) and five pH levels 5.0, 5.5, 6.0, 6.5 along with unamended control on growth and sporulation of the fungus. Culture characters of the fungus varied according to culture media. It forms fluffy and dense culture on PDA media while flat and sparse colonies on BEA and MYEA media. Colony diameter of the fungus was also significantly different for culture media. It was measured from underside of the petri plates, using a transparent scale. Measurement was made along two perpendicular lines and their mean was recorded. The highest mean colony diameter (5.98cm) was observed on MYEA, followed by BEA (5.88cm) and the lowest (5.72cm) on PDA. Conversely, higher mycelium weight (429.2 mg) of the fungus was achieved on PDA medium, followed by BEA (397.4 mg) and the lowest on MYEA (351.8 mg). Moreover, pH level 6.5 showed highest colony diameter (6.038cm). Highly significant variations among the culture media or pH levels and or the interaction of the two were observed for spore concentration. The greatest spore concentration (705.6 thousands/ml) was achieved on MYEA medium followed BEA and PDA. Similarly, highest spore concentration (1346 thousands spores/ml) was achieved at pH 6.0 on MYEA medium. In other media, the pH did not show significant differences. The mean values of spore concentrations of *C. gloeosporioides* at different pH level were however, significantly different with the highest at pH 6.0 (501.3) and the lowest at pH 5.5 (124).

Keywords: Citrus dieback, culture media, characterization, sporulation.

INTRODUCTION

Citrus is one of the most important fruit crops of Pakistan, sharing nearly 40 % of its total fruit produced (Atif *et al.,* 2015). Sargodha, Faisalabad, Sahiwal, Multan, Khanewal, Dargi, Haripur and Dir are major citrus producing districts of Pakistan (Tahir, 2014). However, its production is adversely affected by several fungal diseases including dieback, wilt, root rot and sooty mold (Anjum and javaid, 2005). Citrus dieback caused by *Colletotrichum gloeosporioides*, is the most serious disease of citrus fruit (Javed *et al.,* 2007). *Colletotrichum gloeosporioides* exhibited white, grey and orange to pink grey colour in cultural colony while the conidia are hyaline and cylindrical with rounded ends (Chowdappa *et al.,* 2012). The fungus affect leaves, twigs, flowers and fruits of the tree. On matured leaves, green spots that later turn brown are produced at the margin, tip or rarely near the midrib. Dark colored pustules developed in these spots, may show pink spore masses during moist weather. The affected twigs start dying from tip to

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downward, giving silvery white appearance studded with small black dots constituting the fruiting bodies of the pathogen (Timmer, 2000). Fruit decay may also occur, when the disease progress to them (Silva *et al.,* 2014).

The endemic nature of the disease in Pakistan accounts for its significant role in reducing both the quality and quantity of its produce, which necessitate its proper control. In order to manage the disease, it is important to make comprehensive studies of its fungal pathogen (Javed *et al.,* 2007). One of the basic requirements for successful growth of the fungus is to study *in- vitro* conditions that favor its maximum growth and sporulation.

The fungus grows efficiently on various synthetic and semi synthetic medium (Mishra and Tripathi, 2015). The composition of different culture media has a direct influence on growth and sporulation of fungus (Shin *et al.,* 2000; Kumara and Rawal, 2008). Selection of nutrient-balanced medium is therefore, vital for proper growth of fungal pathogens (Hilton, 1999). Sadahkar. (2000) found five different nutrient media including potato dextrose agar, Brown،s agar, Richard's agar, Sabouraud،s agar and oat meal agar good for mycelial growth of the fungus. Similarly, media containing starch and peptone is considered good for its growth (Ashutosh *et al.,* 2012). Moreover, pH of the medium also effect fungal growth and sporulation. A pH range 5.5-7.0 is found efficient for growth and development of the pathogen (Ghuffar *et al.,* 2017).

The present study is therefore, designed to test the effect of different culture media and pH levels on growth and sporulation of the fungus.

MATERIALS AND METHODS

Isolation and Identification of *Colletotrichum gloeosporioides:* An extensive survey of citrus orchards in district Peshawar, was made to collect disease samples. Infected twigs with characteristic disease symptoms were cut into 1cm size, surface disinfected with 0.1% mercuric chloride for 15-20 seconds, washed with three changes of sterilized distilled water, placed on potato dextrose agar medium @ 3 pieces per plate and incubated at 25°C for seven days. The fungus was identified based on the morphology of hypha, spores and acervuli according to Agron. (2009), Ellis. (2009) and Weir *et al.,* (2012).

Preparation of different culture media and adjustment of pH levels: Three different culture media such as potato dextrose agar (PDA), bean extract agar (BEA), malt and yeast extract agar (MYEA) and five pH levels 5.0, 5.5, 6.0, 6.5 along with unamended control were used in the experiment with six replications to study their effect on colony diameter and spore concentration of *C. gloeosporioides.*

Potato Dextrose Agar (PDA) medium was prepared using the ingredients given in (Table 1), Potato tubers were washed thoroughly, peeled with sharp knife, cut into pieces and boiled in 1 liter distilled water for 30 minutes. The boiled potatoes were filtered through double layered muslin cloth. After cooling, dextrose and agar were added in the filtrate and final volume was adjusted to one liter. For BEA medium, bean (250 g) were boiled in 1000 ml distilled water for one hour, cooled and filtered through double layered muslin cloth. An amount of 20 g of agar were added in the filtrate and final volume was made up to one liter. MYEA medium was prepared by thoroughly mixing its ingredients (Table-1) in 1000 ml sterilized distilled water and heat the suspension gently to dissolve the ingredients.

Table 1. Ingredients used for preparation of different culture media (1000 ml) for the growth and sporulation of *Colletotrichum gloeosporioides*

Culture medium	Ingredients	Amount used
Potato Dextrose Agar (PDA) medium	Potato Glucose Agar	250 g 20 g 20 g
Bean Extract Agar (BEA)	Bean Agar	250 g 20 g
Malt Yeast Extract Agar	Malt extract Potassium dihydrogen phosphate (KH_2PO_4) Agar Yeast extract Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	3 g 0.5 g 20 g 2 g 0.5 g

Different pH levels was adjusted using pH meter, before all the media were autoclaved and poured in pre sterilized petri plates. After it cooling, medium in each petri plates was inoculated with 3 mm diameter plug from actively growing margins of 7- day old culture of *C. gloeosporioides.*

Data recording regarding colony diameter (cm), dry mycelial weight (mg) and spore concentration/ml (thousands): Measurement of colony diameter was made after 7 days of inoculation. Fungal radial growth was measured from the underside of the petri plates with the help of a measuring scale. Measurement was made along two perpendicular lines and their mean was recorded. Data on colony characters such as pattern of mycelium growth, colour, texture, margins, pigmentation and zonation was also recorded for all culture media.

Total mycelial mat from three petri plates of each treatment were harvested, blended with 100 ml distilled water and filtered through Whatman filter paper of a known weight. Filter paper along with mycelial mat were dried and weighed. Dry mycelial weight was measured by using following formula.

$$\text{Dry mycelial weight} = \text{Total wt. of filter paper along with mycelia} - \text{Initial wt. of filter paper}$$

The spore concentration in each treatment was determined by blending three plates of each medium containing fungus culture with sterilized distilled water for 2-3 seconds, sieving the suspension through 3- fold muslin cloth and counting the number of spore under the microscope using a haemocytometer.

DATA ANALYSIS

Data on colony diameter and spore concentration were subjected to analysis of variance and least significant difference (LSD) test using statistical package STATISTIX 8.1.

RESULTS

Data on colony characters such as pattern of mycelium growth, colour, texture, margins, pigmentation and zonation are presented in table 2. Surprisingly, culture characters of the fungus varied on different culture media. The fungus forms fluffy and dense culture on PDA media, while the same fungus produced flat and sparsed colonies on BEA and MYEA media (Figure 1).

Table 2. Cultural characteristics of *Colletotrichum gloeosporioides* at different culture media.

Culture Medium	Colour	Texture	Margins	Zonation	Pigmentation
PDA	Icy White	Fluffy and dense	Regular	Yes	No
BEA	White	Flat and sparse	Regular	Yes	Yellow
MYEA	White	Flat and very sparse	Regular	Yes	Orange

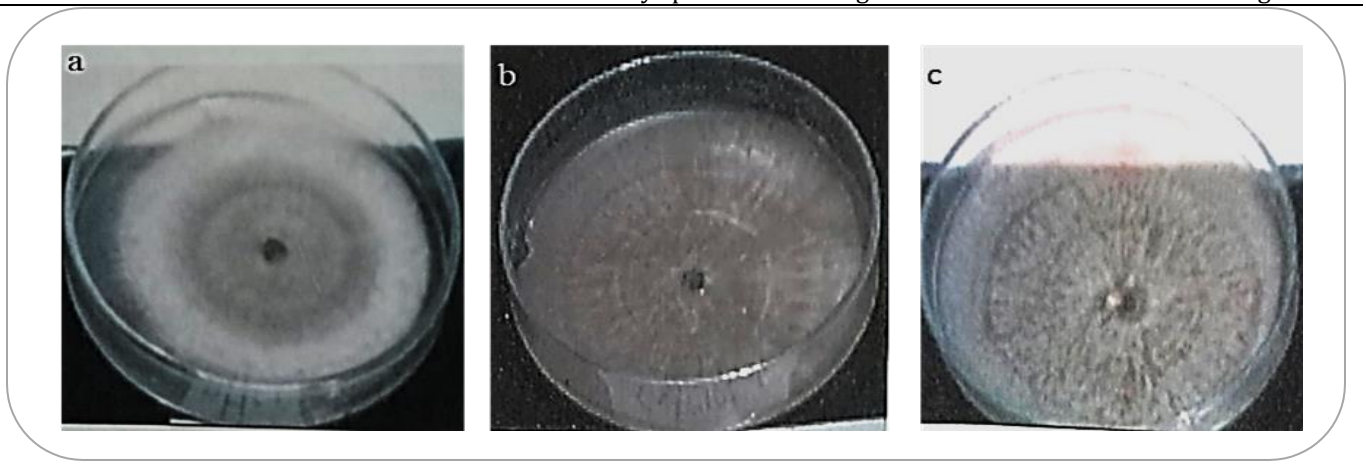

Figure 1. Colonies of *Colletotrichum gloeosporioides* at potato dextrose agar PDA (a), bean extract agar BEA (b) and malt and yeast extract agar MYEA (c) media.

Effect of different culture media and pH levels on colony diameter of *Colletotrichum gloeosporioides:* The culture media were significantly different (P= 0.05) from one another in colony diameter of *C. gloeosporioides.* The highest mean colony diameter (5.98cm) was observed on MYEA, followed by BEA (5.88cm) and the lowest (5.72cm) on PDA (Table 3). The highest pH level (6.5) showed highest colony diameter (6.038cm) which was significantly different from that of control (5.664cm). However, differences of the former with other pH levels were non-significant. Similarly, the combined effect of culture media and pH on colony diameter of *C. gloeosporioides* was non-significant.

Table 3. Average colony diameter (cm) of *C. gloeosporioides* as affected by different culture media and pH levels

Culture Medium	pH Levels					Mean
	Control	5.0	5.5	6.0	6.5	
PDA	5.457	5.733	5.633	5.713	6.088	5.725 B
BEA	5.693	5.840	5.932	5.938	6.027	5.886 AB
MYEA	5.843	6.192	6.027	5.862	5.998	5.984 A
Mean	5.664 B	5.922 A	5.864 AB	5.838 AB	6.038 A	

Effect of different culture media and pH levels on dry mycelial weight (mg) of *Colletotrichum gloeosporioides:* The effect of culture media on dry mycelial weight of the fungus were significantly different (P= 0.05) however, non-significant for pH levels and their interactive effect (Table 4). Higher mycelium weight (429.2 mg) was achieved on PDA medium, followed by BEA (397.4 mg) and the lowest on MYEA (351.8 mg).

Table 4. Average dry mycelial weight (mg) of *C. gloeosporioides* as affected by different culture media and pH levels.

Culture Medium	pH Levels					Mean
	Control	5.0	5.5	6.0	6.5	
PDA	386	420	415	455	470	429.2 A
BEA	350	371	409	423	434	397.4 AB
MYEA	319	321	350	379	390	351.8 B
Mean	351.67	370.67	391.34	419	431.34	

Effect of different culture media and pH levels on sporulation of *Colletotrichum gloeosporioides:* Data regarding spore concentration are presented in table 5. Statistical analysis of the data showed highly significant variations among the culture media or pH levels and or the interaction of the two. The greatest spore concentration (705.6 thousands/ml) was achieved on MYEA medium followed BEA and PDA media. There was no significant difference between BEA and PDA media in terms of spore concentration. However, significant difference occurred at different pH levels of MYEA medium with highest spore concentration (1346 thousands spores/ml) at pH 6.0. In other culture media, the pH levels did not show significant differences. The overall mean values of spore concentrations of *C. gloeosporioides* at different pH level were however, significantly different with the highest at pH 6.0 (501.3) and the lowest at pH 5.5 (124)

Table 5. Average spore concentration/ml (thousands) of *C. gloeosporioides* as affected by different culture media and pH levels.

Culture medium	pH levels					Mean
	Control	5.0	5.5	6.0	6.5	
PDA	14 A	90 A	66 A	4 A	154 A	65.5 A
BEA	108 A	90 A	30 A	154 A	84 A	93.2 A
MYEA	676 BC	734 CD	276 A	1346 E	496 B	705.6 B
	266 B	304.68 C	124 A	501.3 C	244.67 A	

Means followed by the same letters within a column/row are non-significantly different at 5% level of significance.

DISCUSSION

Cultural media are the source of carbohydrate, proteins, lipids and other necessary elements (Shin *et al*., 2000). Selection of nutrient-balanced medium is therefore, vital for proper growth of fungal pathogens (Hilton, 1999). Similarly, pH of the medium may also optimize growth conditions for fungal growth. In this study the effect of culture media and pH levels were investigated for growth and sporulation of *Colletotrichum gloeosporioides,* the causal agent of citrus dieback.

Among the tested media maximum colony diameter and spore concentration was recorded on MYEA media followed by BEA and PDA. Surprisingly, maximum dry mycelial weight was observed on PDA medium followed by BEA and MYEA. These findings are in strong agreement with Vega *et al.* (2003) who suggested that nutritionally poor media triggers sporulation earlier by shortening the vegetative growth phase of the fungus. Moreover, our cultural character study also strengthens this idea as more dense and fluffy growths of the fungus were recorded on PDA media while it is sparse and very sparse on BEA and MYEA media respectively. Probably the nutrients being supplied by MYEA medium were poorly utilized for the mycelial growth rather than sporulation. Thus this medium can be used for production of inoculum of the fungus in future studies. PDA medium, is however, best for growth of the fungus. Similar results were also obtained by Amarjit *et al.* (2006) and Tasiwal and Benagi. (2009).

The values on colony diameter were non significantly different from one another in terms of the interaction of culture media with pH levels. This suggested that the pH levels being tested did not affect the growth of the fungus as they provide acidic medium, favorable for the fungus in majority of cases. However, when the pH was more (about pH 7.0), in the unamended control, colony diameter decreased due to change in pH level. These findings are parallel with the work of Ghuffar *et al.,* (2017) who find maximum fungus growth in the range of PH 5.0- 6.0. However it decreased at pH 7.0. Similarly more acidic medium having pH 3 and 4 didn't support fungus growth. The same pH range was also reported suitable by Chandra *et al.* (2004).

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Contribution of Authors:

Azra Nadeem	:	Provided the conceptual framework of the study and developed the manuscript
Saeedullah	:	Performed lab work
Robina Karim	:	Analyzed the data
Amna Fareed	:	Helped in manuscript development
Faizan	:	Recorded all the data