Original Research Article

Molecular characterization of *Colletotrichum gloeosporioides* (*Glomerella cingulata*) causing anthracnose disease in Mango

Comment [ac1]: Mango Anthracnose Disease.

Abstract

Mango production in Tamil Nadu is known to be adversely affected by *C*. *gloeosporioides*. Fungal isolation was done using PDA medium in order to identify the disease's causative agent. A total of twenty-four *C. gloeosporioides* isolates were obtained from various Tamil Nadu mango-growing locations. The isolates were identified morphologically and molecularly. The microscopic features were used to identify *C. gloeosporioides*. The CTAB technique was employed to separate the DNA from the isolates of *C. gloeosporioides*. A total of 24 isolates were subjected to ITS region amplification; all of these isolates exhibited amplified products with a size range of 560 bp, indicating that they were *C. gloeosporioides* isolates. The MkCgF and MkCgR PCR primer combination was unique to the *C. gloeosporioides* species. It has been confirmed that all 24 of the *C. gloeosporioides* isolates are really C. gloeosporioides by amplifying the predicted size of 380 bp.

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Keywords : Mango, *C. gloeosporioides*, ITS, MkCgF/MkCgR

Introduction

Mango anthracnose is a worldwide disease that is extremely destructive to mangoes before and after harvest. The economic and scientific importance of postharvest damage from mango anthracnose disease is that it lowers fruit quality and shelf life, thereby influencing export quality standards (Kankam *et al.,* 2022). The *Colletotrichum* species complex is responsible for the fungal disease known as MAD. Weir et al. (2012) used morphological and molecular techniques to determine that roughly twenty-two species and one subspecies make up the *C. gloeosporioides* complex. However, several *C. gloeosporioides* isolates in various locations worldwide are characterized by characteristic black, expanding lesions on mango plant parts, including fruits, leaves, flowers, petioles, twigs, and stems (Tovar *et al.,* 2020).

Comment [ac3]: Post-harvest

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The most serious fungal disease limiting the production and export of mango fruits worldwide is mango anthracnose disease. The causal organism of mango anthracnose disease, C. gloeosporioides, is one of the most economically significant agents that might impact the way mango fruits are sorted, packaged, shipped, stored, and sold (Bhagwat et al., 2015). Mango losses due to anthracnose disease range from 30% to 60% annually, with 100% destruction possible under the right circumstances (Kamle and Kumar, 2016).

Oval or irregular dark to deep brown sunken point-sized flecks of varied sizes are the disease's symptoms, which are prominent on young leaves but can also appear on older leaves. These spots are distributed throughout the leaf surface. These dots grow into bigger lesions with an irregular or circular form and a crimson halo surrounding them as the leaf ages (Qin et al., 2019). Although it can happen at any point in the fruit's life cycle, mango anthracnose disease is most frequently found on immature fruits and during transportation and storage. The infection on bigger fruits could remain latent or inactive until the fruit ripens, at which point black, deep necrotic lesions emerge on the fruit peel and quickly enlarge. The younger fruits are either aborted or mummified (De Souza et al., 2013). Certain morphological characteristics, including mycelial growth, conidia size, colony color, texture, and the presence or absence of setae, have historically been used to diagnose mango anthracnose disease (Ashraful et al., 2017).

Molecular techniques are sensitive, fast, precise, and accurate in identifying the disease's causative organisms, which aids in the comprehension of the mechanisms behind disease progression as well as management (Kamle *et al*., 2013). According to Cannon et al. (2012), the ITS gene sequence is useful for identifying *Colletotrichum* spp., but it is unable to differentiate between species that are closely related.

Unfortunately, species categorization sometimes requires time-consuming and complex identification due to character overlap. Adopting efficient agricultural techniques as soon as feasible requires accurate identification. Since ITS has limitations, researchers have also looked at the differences between *Colletotrichum* species using additional genes, including actin, β-tubulin, chitin synthase, glyceraldehyde-3-phosphate dehydrogenase, calmodulin, and glutamine synthetase. various approaches have to be developed in order to recognize and distinguish between

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various fungus species. Plant samples that contain *Collectrotrichum* spp. may be effectively detected using species-specific molecular primers. The PCR approach offers a quick, easy, and dependable substitute for traditional techniques for identifying common fungal isolates. The use of molecular approaches to identify *C. gloeosporioides* is the study's goal.

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MATERIALS AND METHODS

Survey and assessment

During 2019 to 2021, a market survey was conducted across several regions in Tamil Nadu to determine the geographical distribution and severity of the anthracnose disease in mango. Mango fruits exhibiting brown to black sunken patches have been assessed according to standard grading by Prabakar *et al.* (2005) to determine the Percent Disease Index.

$$\text{Per cent Disease Index (PDI)} = \frac{\text{Sum of all rating} \times 100}{\text{Total number of fruits observed} \times \text{maximum grade used}}$$

List 1 : Description of Grade and category value

Description of Grade	Category value
No lesion	0
Small restricted lesions covering 1 to 25 % of fruit surface	1
Moderate Lesions covering 25.1 to 50 % of fruit surface	2
Coalescence of small lesions covering 50.1 to 75 % of fruit surface	3
Lesions covering more than 75 %	4

Isolation and Identification of *Colletotrichum gloeosporioides*.

Twenty-four *C. gloeosporioides* isolates were obtained from fruits infected with anthracnose. Small tissue sections separated from both healthy and diseased fruit tissue. The tissue pieces are surface sterilized with 70% ethanol, then exposed to sodium hypochlorite for 60 seconds. The tissue pieces are allowed to air dry while being

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kept in a laminar hood made of sterilized tissue paper. The sterile tissue fragments were placed on plates that were filled with Potato Dextrose Agar medium to facilitate the development of mycelium. The tips of the mycelia hyphae were then transferred, together with an agar plug, to Petri plates that were filled with PDA and left to incubate. Twelve days following inoculation, the plates are covered by *C. gloeosporioides* mycelium (Ranjitham et al 2011).

Morphological characters of the isolates: Twenty-four isolates of *C. gloeosporioides* were grown individually on PDA medium for ten days in order to study the variation in cultural and morphological characters. Three replications were maintained for each isolate. Various cultural characters such as colour, diameter, zonation, pigmentation, sporulation and margin of colony on PDA medium were observed. The shape of the conidia, length and width of 10 spores were measured for each isolate using a Phase contrast microscope under 40 X magnifications.

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Molecular characterization of *C. gloeosporioides* isolates:

Genomic DNA was extracted from the mycelial mat of *C. gloeosporioides* isolates by Cetyl Trimethyl Ammonium Bromide (CTAB) method as described by Knapp . The ITS1- region of ribosomal DNA from twenty-four isolates of *C. gloeosporioides* was amplified with ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3')27 . In addition, PCR amplifications of ITS 4 primer (TCCTCCGCTTATTGATATGC) coupled with specific primer (MKCgF) (TTGCTTCGGCGGGTAGGGTC) and MKCgR (ACGCAAAGGAGGCTCCGGGA) were used to confirm as these isolates were of C. gloeosporioides

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Results

Market survey

A Market survey was conducted to assess the status of anthracnose disease incidence in major markets 10 districts of Tamil Nadu (Table 1.). The mean level of disease incidence was calculated based on the occurrence of anthracnose disease on locally available varieties of Alphonso, Banglora, Banganapalli, Mulgoa, Neelum,

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Nadusalai, Imampasand and Senthuram. From the survey it is inferred that maximum mean level of anthracnose disease incidence was recorded in a Theni district (65.5 PDI) followed by Krishnagiri district (62.08 PDI). Least mean level of disease incidence was recorded in Namakkal district (16.2) followed by Trichy district.

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Morphological diversity of *C. gloeosporioides*:

Twenty-four strains of *C. gloeosporioides* were collected from mango fruits of various kinds infected with anthracnose. A comparison was made between the morphological characteristics and colony development rate of twenty-four isolates in order to determine the differences in pathogenic fungi that cause anthracnose disease. Regardless of the variety from which they were acquired, distinctness in those features led to the formation of ten morphological groups, and those groups were connected with isolates of *C. gloeosporioides*. (Table 2; Fig. 1).

DNA Isolation and Molecular characterization

Although the 24 isolates were morphologically identified as *Colletotrichum gloeosporioides* to ensure the accuracy it is further subjected to molecular characterization through universal and species specific oligomers. The analysis resulted as follows, all the isolates produce amplicons at 560 bp and to confirm the species it is again involved in PCR with species specific oligomers and it yield amplicons at 380 bp. Since all the isolates are confirmed as *Colletotrichum gloeosporioides* in order to substantiate the confirmation, it is sequenced and analyzed in NCBI Blast Tool in which it shows the isolates has 95% similarity identity with *Colletotrichum gloeosporioides* NCBI data base with a accession number ON799265 and OR685106.

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Discussion

Post-harvest losses of mango might be due to various factors, in that fruit losses are severe due to fungal diseases, especially the anthracnose which contributed a considerable concern. (Dofuor *et al.,* 2023) reported that postharvest losses could go up to 100% depending on cultivars, locations, cultural practices and environment. Thus, complete survey is needed to assess the post-harvest losses in fruits. In the present study, a systematic market survey was conducted in ten different places of Tamil Nadu

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to record the severity of anthracnose disease of mango from the locally available varieties. From the results, the disease incidence was more in Theni, accounting the mean Per cent disease index of 65.5 , followed by Krishnagiri (62. 8 %). Considering the varieties, Neelum variety possesses higher disease index of 65.5 per cent followed by Malkowa (48 %) and least severity was recorded in Nadusalai (20.5) in vellore district and followed by banganapalli in Madurai distrct (21.65 %).

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The results are consistent with Maymon et al. (2006) findings, which showed that *Lasiodiplodia theobromae* was 1% common for anthracnose and stem end rot diseases caused by *C. gloeosporioides* among the five different domestic mango markets, while *Alternaria alternata* and *Aspergillus niger* were 80% common in Punjab. According to Sutton et al. (1980), morphological analysis of these *C. gloeosporioides* isolates showed a significant degree of variation. Diverse PDA medium variances were noted among the 10 groups that emerged from this investigation, according to the colony color Grey white, black, brownish black, grey, greysh black, grayish brown, grayish white, salmon, salmon grey, white. Across 24 isolates, 4 types of texture regular fluffy (9 isolates), dense (3 isolates), scanty (6 isolates) and sparse (6 isolates) were observed, regardless of the color of the colony with the pigmentation black and grey. The majority of these isolates produced acervuli that were black in color and sporulated at varying speeds. When cultivated on PDA, all isolates maintained the same basic growth pattern and colony type. However, there was some variation in the colony development, color, mycelium pigmentation, conidia size, and sporulation rate among the groups that were characterized. All twenty-four isolates had hyaline conidia with oil globules, ranging in size from µm in length and 2.8 to 5.50 µm in width. Previous research on *Colletotrichum's* taxonomy of most species was conducted using the differences in conidial size, shape, appressoria, and colony characteristics.

. The dynamics of disease and, by implication, the effectiveness of disease management techniques, such as the creation of disease-resistant cultivars, are significantly influenced by the diversity of pathogens. Based on mycelial growth, these 24 *C. gloeosporioides* isolates exhibit variability as well.

The fungus *C. gloeosporioides* is known to be highly variable in nature. Many morphological traits of the genus *Colletotrichum* are extremely plastic and variable and

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depend mostly on cultural and environmental conditions, which are rarely standardised (Sutton, 1992). Molecular systematics has been successfully used in studies of this problematic genus and has resulted in well-defined delineations of species (Cannon *et al.*, 2012;). The traditional method of relying on cultural and morphological grounds for identification is not dependable as the identification based on morphology and cultural grounds in many cases misleads since it is subjective in nature. The existence of various pathotypes in this fungus further makes it complicated for identification. Molecular genetic studies have provided useful data for clarifying the systematics of the genus *Colletotrichum* (Martinez *et al.,* 2002). The polymerase chain reaction (Mullis and Faloona, 1987) offers the opportunity to characterize fungal symbionts by amplification of specific sequences and can provide very accurate quantitative data required for control and quarantine decisions. Hence, the present study was resorted into exploitation of ITS and Mkcg derived primers for identification. The internal transcribed spacer (ITS) region contains two variable non coding regions that are nested within the rDNA repeat between the highly conserved small subunit, 5.8S and large subunit rRNA genes (Gardes and Bruns, 1993). The internal transcribed spacer regions (ITS1 and ITS4) within the nuclear ribosomal gene clusters are particularly attractive loci of PCR-based detection assays since they are readily accessible using universal primers. In fungi, the entire ITS region is often rely between 600 and 800 bp and can be amplified with universal primers that are complementary to sequences within the rRNA genes (White *et al.,* 1990). In the present study, ITS 1 and ITS 4 primers amplified a fragment of 560 bp corresponding to the region of 18S rDNA sequence in twenty four isolates of *Colletotrichum* and the result confirmed that all the twenty four isolates pertain to the group of *Colletotrichum.* The results confirmed the findings of Kamle *et al.* (2013b) where in they reported amplification of ITS region of *C. gloeosporioides* from mango yielded 560 bp amplicon. Similarly, many workers confirmed the pathogen by amplifying ITS region of *C. gloeosporioides* (Xiao *et al.,* 2004; Tussell *et al.,* 2008; Xie *et al.,* 2010). The findings of present study are supported by the statement by Sherriff *et al.* (1994) whereby the ITS region should provide opportunities for more rapid progress in taxonomic studies of the genus *Colletotrichum*. The ITS regions of nuclear rDNA have been good targets for the identification, differentiation and phylogenetic analysis of fungi using molecular techniques (Dunne *et al.,* 2002). For *Colletotrichum* species, it is common for single hosts to become infected by a single species or for multiple hosts to be infected by a single species of the pathogen (Freeman, 2000). The development of species-specific primers has provided a

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powerful tool for the detection of plant pathogens. In the present investigation, a sensitive PCR-based diagnostic assay was developed with the aim to detect *C. gloeosporioides* of mango anthracnose pathogen in infected fruit tissue using species-specific designed primer pair of Mkcg f and Mkcg r which amplified the DNA at 380 bp. The size of the amplification product (450 bp) obtained with species-specific primers was similar to previous reports of *C. gloeosporioides* (Kamle 2013).

Conclusion

In conclusion, the morphological characteristics and virulence index of each of the twenty-four *C. gloeosporioides* isolates vary. An efficient method for resolving the issue of fungal species identification and characterisation is the combination of phenotypic and molecular features. We demonstrated that reliable identification of the *Colletotrichum* species causing anthracnose disease in mangos may be accomplished through genetic analysis through ITS and Species specific primer.

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Table 1: Distribution of Mango anthracnose in different regions of Tamil Nadu

PLACE/ VARIETY	NEELUM	BANGALORA	BANGANAPALLI	IMAMPASAND	NADUSALAI	SENDHURAM	ALPHONSA	MALKOVA
Salem	54.5^{d} (47.58)	25.2^{c} (30.13)	40.2^{ab} (39.342)	-d	25.6^{c} (30.36)	23.6 ^{e} (29.06)	25.9^{e} (30.59)	22.1^{e} (28.04)
Coimbatore	45.2^{f} (42.44)	29.6^{b} (32.95)	35.3^{c} (36.45)	25.5^{c} (30.32)	22.1^{de} (28.04)	21.8^{e} (27.8)	33.8^{c} (35.54)	33.5^{bc} (35.35)
Namakkal	48.2^{ef} (43.96)	16.2^{e} (23.72)	33.3^{cd} (35.23)	-d	21.9^{ef} (27.89)	f	-g	31.5^{c} (34.13)
Trichy	49.2^{e} (44.54)	20.8^{d} (27.12)	30^{e} (33.20)	-d	19.6^{g} (26.27)	f	-g	33.3^{bc} (35.23)
Dindigul	56.1^{cd} (48.50)	25.9^{c} (30.59)	25.7^{f} (30.46)	-d	23.8^{cd} (29.19)	f	29.9^{d} (33.14)	35.6^{b} (36.62)
Madurai	59.5^{bc} (50.49)	30.5^{b} (33.51)	21.6^{g} (27.68)	-d	33.3^{b} (35.24)	35.5^{c} (36.56)	22.5^{f} (28.31)	32.15^{c} (34.53)
Theni	65.5^{a} (54.04)	38.4^{a} (38.28)	42.1^{a} (40.45)	-d	36.5^{a} (37.16)	41.2^{a} (39.92)	42^{a} (40.83)	46.52^{a} (43.00)
Dharmapuri	49.7^{e} (44.82)	31.5^{b} (34.13)	32d^{e} (39.34)	33.6^{b} (35.42)	31.8^{b} (34.32)	38.5^{b} (38.3)	36.5^{b} (37.16)	32.58^{c} (34.8)
Vellore	41.5^{g} (40.10)	25.2^{c} (30.13)	21.05^{g} (36.44)	-d	20^{fg} (26.55)	33.1^{d} (35.11)	-f	27.3^{d} (31.49)
Krishnagiri	62.8^{ab} (52.41)	39.6^{a} (38.98)	39.5^{b} (35.24)	41.2^{a} (39.92)	35.9^{a} (36.8)	40.5^{a} (39.52)	40.9^{a} (39.11)	48.5^{a} (44.13)

Mean of three replicaton
In a a column, means followed by a common letter are not significantly different at 5% level by DMRT
Values in paranthesis are arc sin transformed values

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Table 2 : Colony characters of *Colletotrichum gloeosporioides* isolates

Isolates	Colony colour	Texture	Pigmentation	Colony Diameter	Sporulation
Cgman1	Greyish White	Fluffy	—	8.2(16.63)^{b}	+
Cgman2	Greyish White	Fluffy	—	8.8(17.25)^{a}	+
Cgman3	Grey	Sparse	—	8(16.43)^{bc}	++
Cgman4	Grey	Fluffy	—	7.4(15.78)^{cdef}	+
Cgman5	White	Fluffy	—	8.9(17.3)^{a}	+
Cgman6	Salmon	Sparse	—	6.9(15.23)^{fgh}	++
Cgman7	Salmon	Sparse	—	6.8(15.11)^{fgh}	++
Cgman8	Brownish Black	Scanty	Black	6.3(14.53)^{hi}	+++
Cgman9	Brownish Black	Scanty	Black	5.9(14.05)^{i}	+++
Cgman10	White	Fluffy	Grey	9(17.46)^{a}	+
Cgman11	Salmon	Sparse	—	7.3(15.67)^{def}	++
Cgman12	Greyish Black	Scanty	Black	6.5(14.76)^{ghi}	+++
Cgman13	Salmon Grey	Fluffy	Black	8.9(17.35)^{a}	+
Cgman14	Greyish Brown	Scanty	Black	6.4(14.64)^{hi}	+++
Cgman15	Greyish White	Sparse	—	8.9(17.35)^{a}	++
Cgman16	Salmon	Sparse	—	7.4(15.78)^{cdef}	++
Cgman17	Black	Dense	Black	7.1(15.44)^{efg}	+
Cgman18	Grey	Fluffy	Black	7.8(16.21)^{bcd}	+
Cgman19	White	Fluffy	—	8.9(17.35)^{a}	+
Cgman20	Grey	Fluffy	—	7.8(16.21)^{bcd}	+
Cgman21	Greyish Brown	Scanty	Black	6.9(15.22)^{fgh}	+++
Cgman22	White	Dense	—	8.2(16.63)^{b}	+
Cgman23	Salmon Grey	Scanty	Black	5.9(14.05)^{i}	+++
Cgman 24	Black	Dense	Black	7.7(16.11)^{bcde}	+

Mean of three replicaton
In a a column, means followed by a common letter are not significantly different at 5% level by DMRT
SEd=0.2894 CD=0.7

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Fig 1 : Cultural characteristics of *Colletotrichum gloeosporioides* in PDA medium

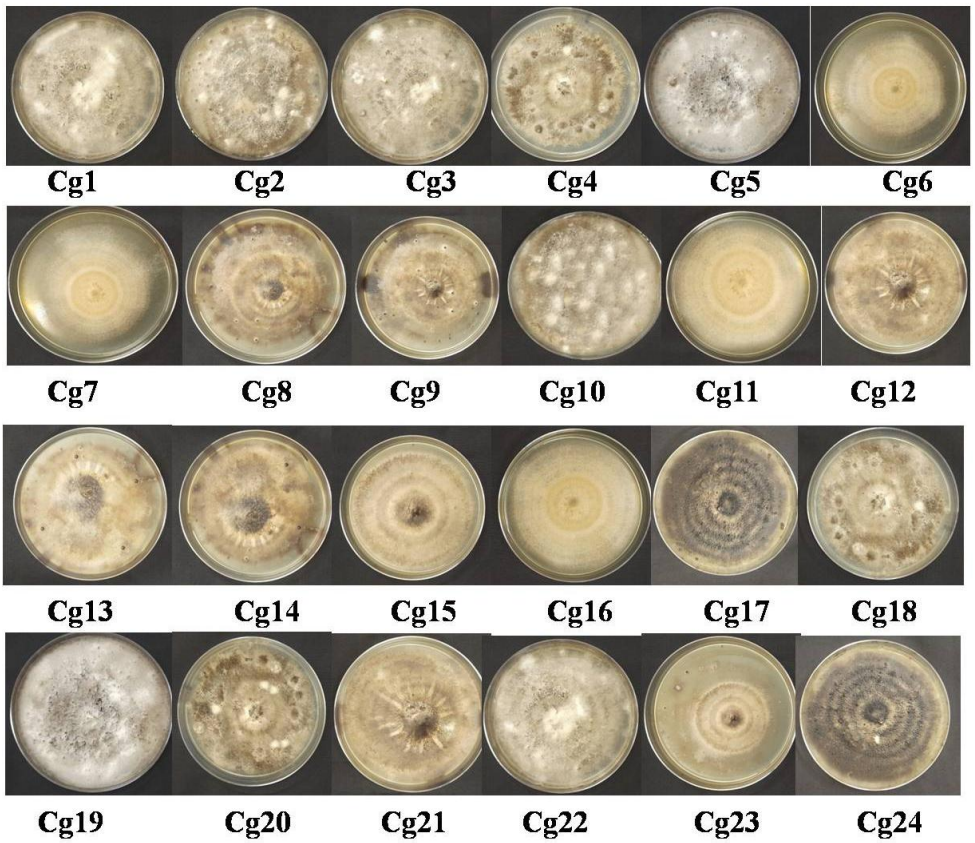

Table 3: Morphology characters of *Colletotrichum gloeosporioides* isolates

Isolates	Myceliu m	Colour	Conida	Length	Width	Oil globule s	Acervuli	Setae	Colour
Cgman1	Septate	Hyalin e	Cylindrical	14.8 ghihk (22.62)	3.6d (10.93)	Present	Present	Absent	Hyaline
Cgman2	Septate	Hyalin e	Cylindrical	16.2^{def} (23.73)	5.5a (13.56)	Present	Present	Absent	Hyaline
Cgman3	Septate	Hyalin e	Cylindrical	13^{m} (21.13)	2.9e (9.8)	Present	Present	Absent	Hyaline
Cgman4	Septate	Hyalin e	Cylindrical	13.8^{jklm} (21.80)	4.4c (12.10)	Present	Present	Absent	Hyaline
Cgman5	Septate	Hyalin e	Cylindrical	19.8^{a} (26.42)	5.5a (13.55)	Present	Present	Absent	Hyaline
Cgman6	Septate	Hyalin e	Cylindrical	13.7^{klm} (21.71)	4.5c (12.24)	Present	Present	Absent	Hyaline
Cgman7	Septate	Hyalin e	Cylindrical	17.8^{bc} (24.94)	2.8e (9.633)	Present	Present	Absent	Hyaline
Cgman8	Septate	Hyalin e	Cylindrical	13.8j^{klm} (21.8)	4.4c (12.10)	Present	Present	Absent	Hyaline
Cgman9	Septate	Hyalin e	Cylindrical	14.6^{hijkl} (22.45)	4.5c (12.24)	Present	Present	Absent	Hyaline
Cgman10	Septate	Hyalin e	Cylindrical	13.4l^{m} (21.46)	3.6d (10.93)	Present	Present	Absent	Hyaline
Cgman11	Septate	Hyalin e	Cylindrical	16.0^{efg} (23.57)	4.4d (12.10)	Present	Present	Absent	Hyaline
Cgman12	Septate	Hyalin e	Cylindrical	12.8^{m} (20.96)	4.5c (12.24)	Present	Present	Absent	Hyaline
Cgman13	Septate	Hyalin e	Cylindrical	15.2^{fghi} (22.94)	5.6a (13.68)	Present	Present	Absent	Hyaline
Cgman14	Septate	Hyalin e	Cylindrical	15.7^{efgh} (23.34)	4.4d (12.10)	Present	Present	Absent	Hyaline
Cgman15	Septate	Hyalin e	Cylindrical	15.0^{fghij} (22.78)	4.7bc (12.51)	Present	Present	Absent	Hyaline
Cgman16	Septate	Hyalin e	Cylindrical	18.1^{b} (25.17)	4.6bc (12.38)	Present	Present	Absent	Hyaline
Cgman17	Septate	Hyalin e	Cylindrical	16.7^{cde} (24.11)	3.0ab (9.97)	Present	Present	Absent	Hyaline
Cgman18	Septate	Hyalin e	Cylindrical	17.7^{bc} (24.87)	3.6d (10.93)	Present	Present	Absent	Hyaline
Cgman19	Septate	Hyalin e	Cylindrical	15.6^{efghi} (23.25)	4.3c (11.96)	Present	Present	Absent	Hyaline
Cgman20	Septate	Hyalin e	Cylindrical	15.2^{fghi} (22.93)	3.3de (10.46)	Present	Present	Absent	Hyaline

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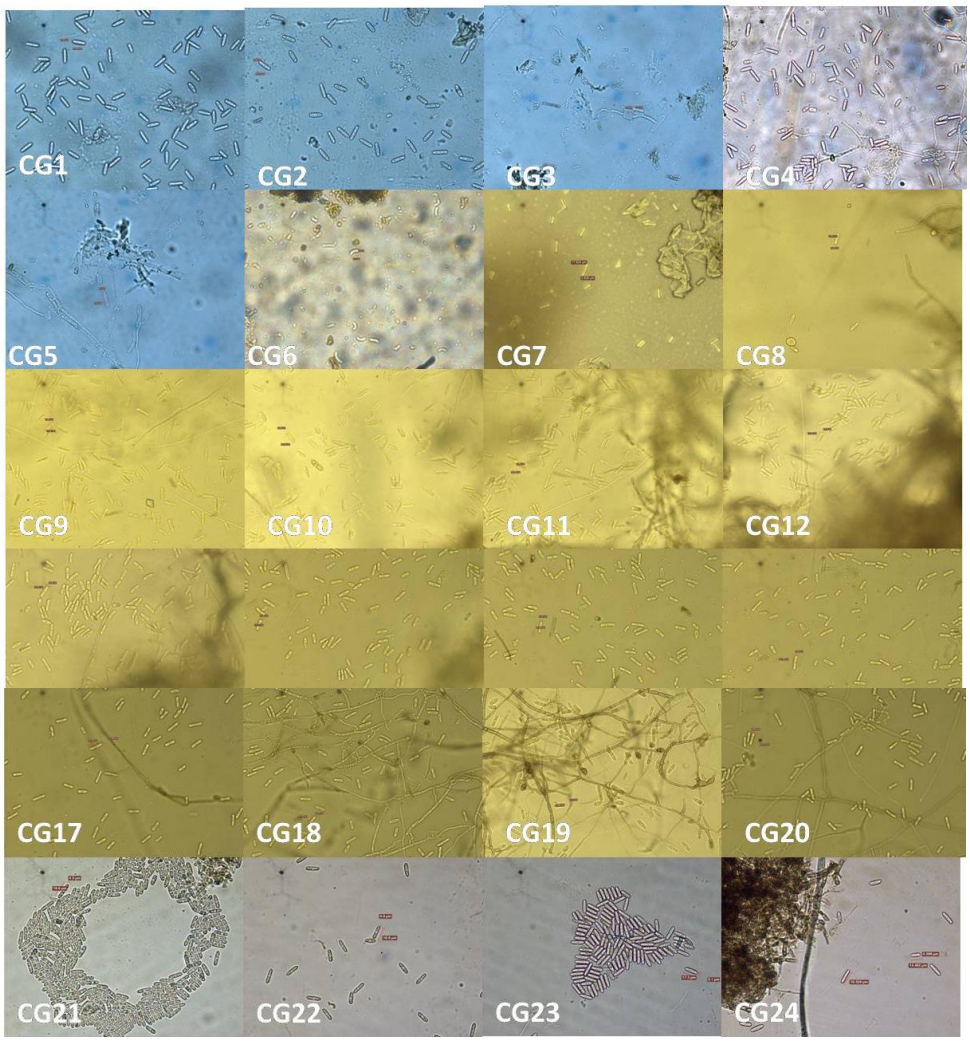
Cgman21	Septate	Hyaline	Cylindrical	19.6[a] (26.27)	4.4c (12.10)	Present	Present	Absent	Hyaline
Cgman22	Septate	Hyaline	Cylindrical	16.6[cde] (24.03)	4.6bc (12.38)	Present	Present	Absent	Hyaline
Cgman23	Septate	Hyaline	Cylindrical	17.3[bcd] (24.56)	5.1ab (13.0)	Present	Present	Absent	Hyaline
Cgman24	Septate	Hyaline	Cylindrical	14.4[cde] (22.295)	4.5c (12.24)	Present	Present	Absent	Hyaline

Mean of three replicaton
In a a column, means followed by a common letter are not significantly different at 5% level by DMRT
SEd=0.2669 CD=0.7
Fig 2 : Conidial characters of *Colletotrichum gloeosporioides isolates*



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