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Detection of β-tubulin gene from benomyl sensitive isolates of *Colletotrichum gloeosporioides* causing anthracnose disease in mango

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Twenty six isolates of *Colletotrichum gloeosporioides* from anthracnose infected mango fruits were isolated from different places of Tamil Nadu, India and these isolates were identified as *C. gloeosporioides* by Internal transcribed spacer (ITS) and species specific (CgInt) primers. The sensitivity of *C. gloeosporioides* isolates to benomyl fungicide were evaluated at five different concentrations *viz.,* 0.5, 1, 2, 5, 10 mg/l through poisoned food technique and were expressed very less to highly sensitiveness to the fungicide. All the twenty six isolates were able to grow at the lowest concentration of 0.5 mg/l benomyl amended medium and the average mycelial growth was 70.70 mm at even days after inoculation. At the highest concentration of 10 mg/l benomyl, only two isolates viz., MCG 7 and 16 were able to grow with the mycelial diameter of 12.00 and 18.00 mm, respectively and 100% inhibition was found in the remaining isolates. In addition, benzimidazole sensitive and resistance β - tubulin gene sequences of TUB 1 and TUB2 were amplified from the benomyl sensitive isolates of *C. gloeosporioides*. The results indicated the differential resistance or sensitivity to benomyl fungicide against *C. gloeosporioides* and thereby allowed to identify the variability and diversity of the isolates on regional basis.

Key words: Mango*, C. gloeosporioides*, benomyl sensitive, β - tubulin gene.

INTRODUCTION

Many diseases are affecting mango, of which anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Sacc is the most devastating disease and major constraint in production and export of mango. Several fungicides have been used to manage the mango anthracnose as pre- and post-harvest sprays, including benzimidazoles, triazoles, strobilurins, acid amides, dithiocarbamates and heterocyclic compounds (Fei and Wang, 2004). Hot benomyl dips have been reported for effective control of anthracnose in harvested mango fruit (Kim et al., 2007). Benomyl has been used in agriculture for approximately 30 years and numerous cases of

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resistance have been reported, including *Colletotrichum* spp. on various crops (Whiteside, 1980; Picinini, 1994; Hewitt, 1998). Benzimidazoles are specific inhibitors of microtubule assembly that act by binding to their heterodimeric subunit (Davidse, 1986). Several studies have demonstrated that benzimidazole resistance is associated with point mutations in codon 198 or 200 of the β-tubulin gene in various fungi, including *Venturia inaequalis*, *Botrytis cinerea*, *Monilinia fructicola*, *C. gloeosporioides* and *Mycosphaerella fijiensis* (Ma et al., 2003; Peres et al., 2004; Chung et al., 2006; Kim et al., 2007). Other point mutations in the β-tubulin gene (Jung et al., 1992; Cooley and Caten, 1993) as well as in the putative leucine zipper protein CaBEN1 (Nakaune and Nakano, 2007) have also been associated with resistance to benzimidazoles. Maymon et al. (2006) reported that sequence analyses of the β-tubulin genes, *TUB1* and *TUB2*, of five sensitive and five resistant representative isolates of *C. gloeosporioides* from *Limonium* spp. revealed that the benomyl resistant isolates had an alanine substitute instead of a glutamic acid at position 198 in *TUB2*. Benzimidazole resistant isolates of *C. gloeosporioides* from different crops have been identified, but the molecular characteristics of these resistant isolates are still not well defined (Pei, 1981; Tsai et al., 2006). The molecular identification and monitoring of benzimidazole resistant fungi has been recognized as a useful tool in the study of fungicide resistance (Ma et al., 2003; Canas-Gutierrez et al., 2006). Housekeeping genes including the nuclear β-tubulin genes, which show higher DNA sequence variability, are more extensively used to develop diagnostics for fungi, (Fraaije et al., 2001; Mostert et al., 2006; Aroca et al., 2008). Partial β-tubulin sequences from *C. gloeosporioides* isolates of mango were amplified (Albertini et al., 1999; Yarden and Katan, 1993) as a molecular tool for the identification of benzimidazole-resistant isolates of *C. gloeosporioides*. Sanders et al. (2000) evaluated 158 isolates of *C. gloeosporioides* and reported, 17.7% were resistant to benomyl with 8.5% highly resistant due to mutations in the β - tubulin gene using this information, *C. gloeosporioides* isolates from mango fruits collected in India were evaluated for benomyl sensitivity and mutations in the β - tubulin gene in the present study.

MATERIALS AND METHODS

C. gloeosporioides from mango

Twenty six isolates of *C. gloeosporioides* collected from different districts of Tamil Nadu, India were evaluated in the study (Table 1). Their pathogenicity, variability in virulence was previously reported by Archana et al. (2014).

Sensitivity of *C. gloeosporioides* to benzimidazole fungicide – benomyl

Each isolate was cultured on Potato Dextrose Agar (PDA) medium in petri plates at 28±2°C for three days, after that mycelial disc (3 mm diameter) were cut from colony margins and a single disc was transferred to the centre of Petri plate containing PDA medium amended with 0.5, 1, 2, 5 and 10 mg/ml of the active ingredient of benomyl fungicide, which was added to PDA medium after autoclaving. Test cultures were incubated in dark at 28±2°C and then mycelial growth was measured 11 days after inoculation (Chung et al., 2010) and control plate was maintained by growing the isolates in the Petri plate containing PDA medium alone.

PCR based detection and diagnosis of *C. gloeosporioides* isolates using internal transcribed spacer (ITS) and CgInt primers

Genomic DNA for *C. gloeosporioides* isolates were extracted from the mycelial mat by Cetyl trimethyl ammonium bromide (CTAB) method as described by Knapp and Chandlee (1996). The ITS1-5.8S-ITS2 region of ribosomal DNA was amplified with ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') oligonucleotide primers. The amplification was performed in 50 µl reaction containing 1.5 units of *Taq* DNA polymerase (Qiagen, Germany), 1x polymerase chain reaction (PCR) buffer, 200 µM each dNTP, 0.2 µM each primer and 100 ng of template DNA followed by the thermal cycler reaction of 30 cycles (0.5 min at 94°C, 0.5 min at 56°C and 2 min at 72°C) with the final step at 72°C for 7 min (Tosa et al., 2004).

PCR amplification using the oligonucleotide primers ITS 4 (TCCTCCGCTTATTGATATGC) and CgInt (GGCCTCCCGCCTCCGGGCGG) were used for species identification. PCR reaction were as described above with amplification as follows: denaturation at 95°C for 5 min, 30 cycles of 30 s. at 95°C, 30 s at 48°C and 90 s at 72°C and a final extension of 10 min at 72°C (Maymon et al., 2006) in an Eppendorf gradient thermal cycler.

Analysis of benomyl resistance of *C. gloeosporioides* isolates using partial sequence of β-tubulin gene

PCR amplification of β-tubulin gene (*TUB2* and *TUB2B*) fragments were carried out using generic primer GENC (5′-GAGGAATTCCCAGACCGTATGATG- 3′) and TUB2 (5′-GACATCCTTCATAGCG-3′) to identify the benomyl resistant isolates (Koenraadt et al., 1992) and primer GENC and TUB2B (5′-GAC(A/G)TCCTTCAT(A/G)GCGA-3′) to identify benomyl sensitive isolates (Panaccione and Hanau, 1990). *TUB1*β-tubulin gene was amplified using the primer GENC (5′GAGGAATTCCCAGACCGTATGATG-3′) and TUB 1C (5′-TCAATCTGCTTGGTCGACACCTT-3′) to identify the resistant isolates.

Reactions prepared as above and PCR reactions were performed with 60 s of denaturation at 94°C; followed by 35 cycles consisting of 30 sec at 94°C, 30 sec at 50°C and 60 sec at 72°C; and a final extension period of 10 min at 72°C.

Agarose gel electrophoresis

Amplification products for the above 5 evaluations were separated in 1.2% (w/v) agarose gel in1x TAE buffer (0.4 M Tris, 0.2 M acetic acid, 10mM EDTA; pH 8.4) containing 0.5 µg/ml ethidium bromide. Electrophoresis was carried out at 85 V and the gel was documented using an Alpha Imager (Alpha Innotech Corporation, San Leandro, CA, USA). The sizes of the PCR products were determined by comparing with standard 100 bp or 1 kb molecular marker (Bangalore Genei Pvt. Ltd., Bangalore, India).

Table 1. Characterization of *C. gloeosporioides* isolates based on cultural characters in PDA medium.

Isolates	Variety	Place of collection	Colony colour	Pigmentation	Condia (µm)	
					Length	Width
MCG 1	Neelum	Rajapalayam	Dull White	-	11.34^{a}	6.23^{m}
MCG 2	Mallika	Krishnagiri	White	-	15.55^{kl}	5.30^{hi}
MCG 3	Himayuddin	Dharmapuri	white	-	13.33^{fg}	4.30^{b}
MCG 4	Raspuri	Krishnagiri	White	-	16.28^{m}	5.77^{jk}
MCG 5	Mulgoa	Palani	Blackish white	Black	11.76^{ab}	6.01^{l}
MCG 6	Nadusalai	Coimbatore	Dull white	-	12.45^{d}	5.87^{kl}
MCG 7	Himayuddin	Periyakulam	White	-	13.99^{h}	4.50^{cd}
MCG 8	Banganapalli	Tenkasi	Blackish white	Black	15.05^{j}	5.20^{h}
MCG 9	Mulgoa	Paiyur	Blackish white	Black	12.78^{de}	6.30^{m}
MCG 10	Alphonso	Tirunelveli	Blackish white	Black	11.87^{bc}	5.77^{jk}
MCG 11	Alphonso	Paiyur	Blackish white	Black	16.33^{m}	4.30^{b}
MCG 12	Kalapad	Nagercoil	Dull white	-	15.34^{jk}	5.80^{jk}
MCG 13	Senthuram	Trichy	White	-	13.74^{gh}	4.87^{fg}
MCG 14	Mulgoa	Erode	Blackish white	Black	16.01^{lm}	4.70^{ef}
MCG 15	Alphonso	Thiruvallur	White	-	12.75^{de}	5.20^{h}
MCG 16	Neelum	Theni	White	-	11.93^{bc}	4.34^{bc}
MCG 17	Neelum	Thanjavur	Blackish white	Black	12.56^{d}	4.60^{de}
MCG 18	Banganapalli	Kanyakumari	Black	Dark black	13.78^{gh}	5.46^{i}
MCG 19	Senthuram	Namakkal	Purple	-	15.98^{lm}	5.67^{j}
MCG 20	Neelum	Salem	Salmond orange	-	15.02^{j}	4.77^{ef}
MCG 21	Banglora	Dharmapuri	Yellowish white	Yellow	12.34^{cd}	4.10^{a}
MCG 22	Karuneelum	Paiyur	White	-	13.32^{fg}	4.80^{f}
MCG 23	Banglora	Nagapattinam	Blackish White	Black	14.48^{i}	4.30^{b}
MCG 24	Neelum	Madurai	white	-	15.32^{jk}	5.01^{g}
MCG 25	Neelum	Kalakad	Dull white	-	11.56^{ab}	4.72^{ef}
MCG 26	Mulgoa	Kayathar	White	-	13.08^{ef}	4.87^{fg}

Mean of three replications; in a column, means followed by a common letter are not significantly different at 5% level by DMRT.

Statistical analysis

The data were statistically analyzed using the IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, Philippines (Gomez and Gomez, 1984). Data were subjected to analysis of variance (ANOVA) at two significant levels ($P< 0.05$ and $P< 0.01$) and means were compared by Duncan's Multiple Range Test (DMRT).

RESULTS

Molecular detection of *C. gloeosporioides* isolates using ITS and CgInt primers

PCR amplification using universal primers of ITS 1 and ITS 4 produced the expected 560 bp fragment, confirming that 26 isolates belonged to the genus *Colletotrichum* (Figure 1). PCR amplification with *Cg*Int and ITS 4 species specific primers produced the expected 450 bp amplicon confirming the isolates were *C. gloeosporioides* (Figure 2).

Screening of *C. gloeosporioides* against benzimidazole fungicide – benomyl

The sensitivity of *C. gloeosporioides* isolates to the fungicide benomyl was evaluated based on the method of poisoned food technique at five different concentrations viz., 0.5, 1.0, 2.0, 5.0 and 10.0 mg/l (Figure 3). The growth of mycelia on the benomyl amended PDA medium was directly correlated to the sensitiveness of the isolates benomyl. Based on this, twenty six *C. gloeosporioides* isolates were designated as susceptible, moderately resistant and resistant to the fungicide benomyl. At the lowest concentration of 0.5 mg/l benomyl amended medium, all the twenty six isolates were able to grow and the diameter of the mycelium was measured eleven days after inoculation. The isolate MCG 21 produced the least growth of 27.23 mm followed by MCG 22(33.24 mm) and

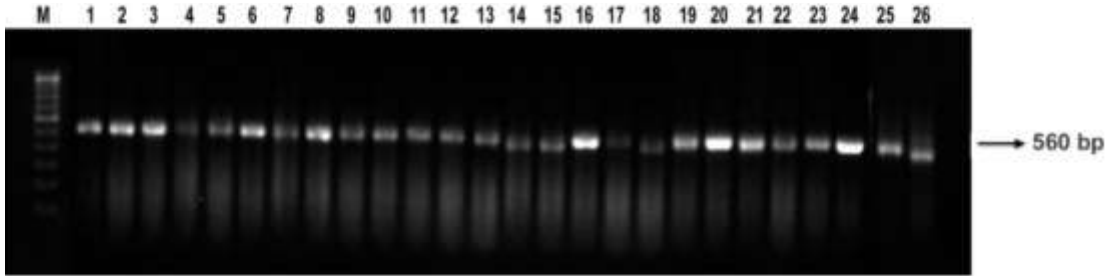

Figure 1. PCR amplification of ITS fragments of *Colletotrichum* isolates from Mango fruits infected by anthracnose disease, (M – Marker (100 bp); 1 – 26: MCG 1 – MCG 26 isolates of *Colletotrichum).*

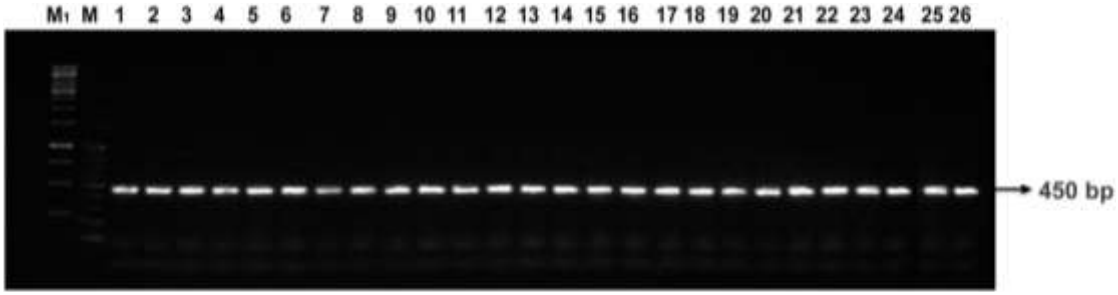

Figure 2. PCR amplification of *Colletotrichum* isolates using specific primer (CgInt + ITS 4), **(**M1 – Marker (1 kb); M – Marker (100 bp); 1 – 26: MCG 1 – MCG 26 isolates of mango anthracnose).

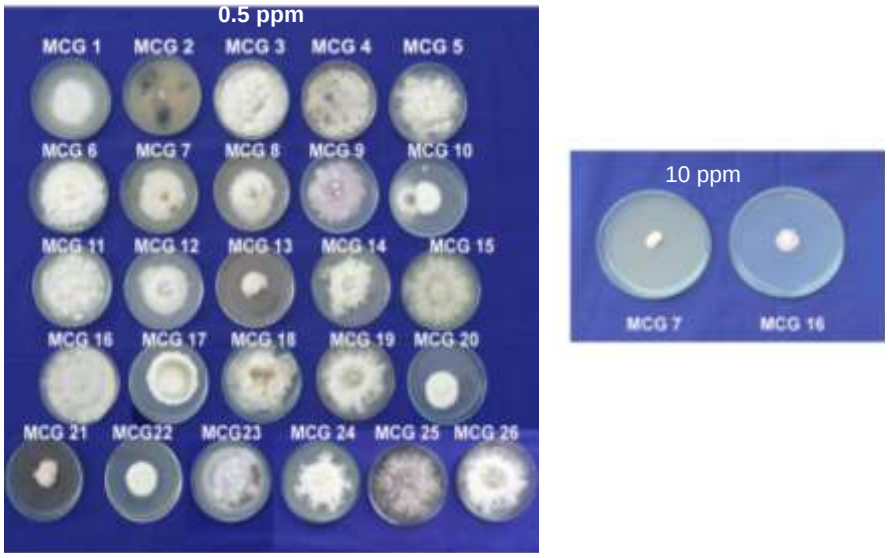

Figure 3. Sensitivity of *C. gloeosporioides* isolates to benomyl fungicides (Poisoned food method using PDA medium amended with different concentration of benomyl to evaluate the sensitivity of *C. gloeosporioides*).

MCG 13 (33.72 mm) which were significantly different from other isolates. The mycelial growth of 90 mm was observed in the isolates of MCG 3, 16, 23 and 26 at 0.5 mg/l concentration (Table 2). At the concentration of 1 mg/l of benomyl, the percent inhibition over control was higher in the isolate MCG 21 accounting 73.16%, which was on par with the isolates MCG 10 (71.60%) and MCG 22 (71.21%). Least inhibition of 3.06% was observed in

Table 2. Sensitivity of *C. gloeosporioides* isolates from mango to benomyl fungicide.

Isolates	Diameter of mycelial growth (mm) in benomyl amended PDA medium and in control plate					
	0.5 mg/l	1 mg/l	2 mg/l	5 mg/l	10 mg/l	Control
MCG 1	60.12^{e}	60.13^{e}	45.14^{l}	21.00^{jk}	0.00^{a}	90.00^{a}
MCG 2	62.34^{ef}	65.45^{fgh}	43.56^{k}	20.32^{ijk}	0.00^{a}	90.00^{a}
MCG 3	90.00^{m}	90.00^{o}	60.33^{p}	25.09^{m}	0.00^{a}	90.00^{a}
MCG 4	88.56^{lm}	87.25^{n}	55.55^{n}	22.12^{l}	0.00^{a}	90.00^{a}
MCG 5	77.34^{k}	67.56^{hij}	31.57^{e}	16.90^{fg}	0.00^{a}	90.00^{a}
MCG 6	88.10^{lm}	66.67^{ghi}	39.14^{hi}	19.32^{h}	0.00^{a}	90.00^{a}
MCG 7	63.00^{fg}	35.13^{c}	28.18^{d}	20.04^{hi}	12.00^{b}	90.00^{a}
MCG 8	65.03^{gh}	68.23^{ij}	47.62^{m}	17.31^{g}	0.00^{a}	90.00^{a}
MCG 9	74.12^{j}	69.33^{j}	54.34^{n}	21.16^{k}	0.00^{a}	90.00^{a}
MCG 10	45.45^{c}	25.56^{a}	18.54^{b}	10.05^{b}	0.00^{a}	90.00^{a}
MCG 11	88.26^{lm}	77.10^{kl}	37.16^{g}	13.45^{e}	0.00^{a}	90.00^{a}
MCG 12	63.16^{fg}	64.11^{f}	33.88^{f}	11.01^{c}	0.00^{a}	90.00^{a}
MCG 13	33.72^{b}	30.31^{b}	17.17^{b}	9.32^{b}	0.00^{a}	90.00^{a}
MCG 14	75.41^{jk}	65.09^{fg}	38.37^{gh}	20.19^{hij}	0.00^{a}	90.00^{a}
MCG 15	88.23^{lm}	79.87^{m}	40.37^{i}	21.17^{k}	0.00^{a}	90.00^{a}
MCG 16	90.00^{m}	85.44^{n}	41.82^{j}	30.41^{n}	18.00^{b}	90.00^{a}
MCG 17	68.00^{i}	55.33^{d}	28.17^{d}	12.36^{d}	0.00^{a}	90.00^{a}
MCG 18	87.08^{l}	75.23^{k}	37.77^{gh}	16.23^{f}	0.00^{a}	90.00^{a}
MCG 19	88.09^{lm}	76.12^{kl}	55.24^{n}	21.15^{k}	0.00^{a}	90.00^{a}
MCG 20	48.14 ^{d}	78.35^{lm}	57.31^{o}	20.00^{hi}	0.00^{a}	90.00^{a}
MCG 21	27.23^{a}	24.16^{a}	13.15^{a}	8.33^{a}	0.00^{a}	90.00^{a}
MCG 22	33.24^{b}	25.91^{a}	17.16^{b}	10.13^{b}	0.00^{a}	90.00^{a}
MCG 23	90.00^{m}	87.18^{n}	61.42^{p}	31.43^{o}	0.00^{a}	90.00^{a}
MCG 24	65.80^{hi}	54.53^{d}	26.34^{c}	14.04^{e}	0.00^{a}	90.00^{a}
MCG 25	87.73^{lm}	76.52^{kl}	31.35^{e}	13.22^{e}	0.00^{a}	90.00^{a}
MCG 26	90.00^{m}	85.14^{n}	43.36^{k}	22.16^{l}	0.00^{a}	90.00^{a}

*Mean of three replications; in a column, means followed by a common letter are not significantly different at 5% level by DMRT;

the isolate MCG 4. In addition, at the concentration of 2 mg/l the percent inhibition of mycelial growth was ranged from 31.76 to 85.39% and at the concentration of 5 mg/l it ranges from 65.08 to 90.74%. At the highest concentration of 10 mg/l, only two isolates viz., MCG 7 and MCG 16 were able to grow in benomyl poisoned petriplate with the mycelial diameter of 12.00 and 18.00 mm possessing 86.67 and 80.00% inhibition over control, respectively (Figure 4) among 26 isolates. By comparing the mycelial growth of *C. gloeosporioides* isolates at five different concentrations tested, it was concluded that the isolate MCG 21 was considered sensitive; MCG 7 and MCG 16 were resistant to benomyl fungicide.

PCR amplification of β-tubulin gene from *C. gloeosporioides*

In all the twenty six isolates of *C. gloeosporioides,* partial β – tubulin gene sequences of TUB 2 and TUB 1 were amplified by the appropriate primers from genomic DNA (Figure 5).

The results from PCR amplification, showed that the primer pairs of GENC and TUB2 amplified the resistant allele of TUB 2, (563bp fragment) amplified in ten isolates (MCG 3, MCG 7, MCG 8, MCG 11, MCG 12, MCG 13, MCG 16, MCG 19, MCG 20 and MCG 21) (Figure 5a). The sensitive allele of TUB 2 (563bp fragment) was amplified from nine isolates (MCG 2, MCG 3, MCG 6, MCG 8, MCG 19, MCG 20, MCG 21, MCG 24 and MCG 25) (Figure 5b). In addition, another β-tubulin resistant allele of TUB 1 (506bp fragment) was amplified by the primer pair of GENC and TUB1C from four isolates (MCG 19, MCG 20, MCG 24 and MCG 25) (Figure 5c). Results from this study have confirmed most of the isolates used as benzimidazole-resistant, which were collected from anthracnose infected mango fruits of different places.

Nucleotide sequence accession number

The sequence obtained in this study was assigned

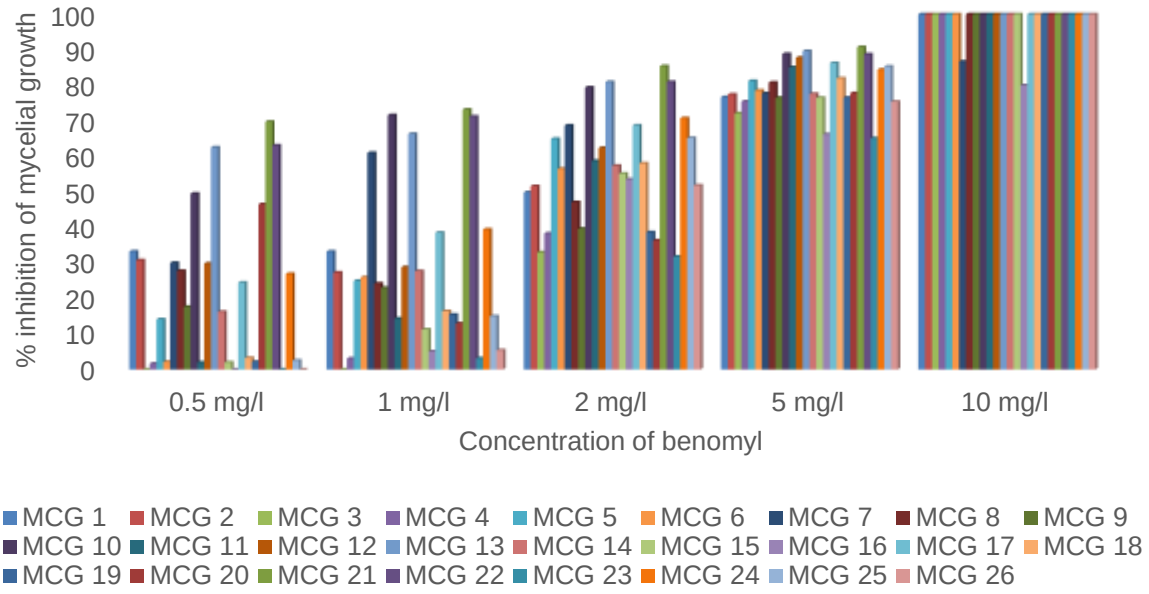

Figure 4. Efficiency of benomyl fungicide against *C. gloeosporioides* isolates *in vitro* analysis.

GenBank accession number of β-tubulin resistance gene isolates MCG 11-KJ470629.1 and MCG 16-KJ462468.1

DISCUSSION

The fungus *C. gloeosporioides* is known to be highly variable in nature. All twenty six isolates of the present study were identified as *C. gloeosporioides* based on the symptoms on host, substrate, conidial size and shape, shape of appressoria, growth rate in culture, colour of cultures as reported by von Arx (1970) and Sutton (1980). The traditional method of relying on cultural and morphological grounds for identification of *C. gloeosporioides*is variable rarely standardized and tends to mislead, since it is subjective in nature. 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 provide very accurate quantitative data required for control and quarantine decisions. Hence, the present study was resorted into exploitation of ITS and *Cg*Int derived primers for identification. The 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 attractive loci of PCR-based detection assays since they are readily accessible using universal primers (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 all the twenty six isolates and further confirmed to the group of *Colletotrichum.* The results confirmed the findings of Kamle et al. (2013) in amplification of ITS region of *C. gloeosporioides* from mango yielded 560 bp amplicon. Further, identification of ITS regions of nuclear rDNA has been good targets for differentiation and phylogenetic analysis of fungi (Dunne et al., 2002). In the present research, a sensitive PCR-based species-specific designed primer pair of *Cg*Int and ITS 4 was used with the aim to detect *C. gloeosporioides* of mango anthracnose pathogen from the infected fruit tissue, which amplified the DNA at 450 bp in all isolates. These results were supported by the earlier findings for the taxonomic identification of *Colletotrichum* species which have been used to distinguish between *Colletotrichum acutatum*, *C. gloeosporioides* and *Colletotrichum fragariae* (Forster and Adaskaveg, 1999; Urena-Padilla et al., 2002). Similarly, Sakinah et al. (2013) reported that, 38 *Colletotrichum* isolates from banana were identified as *C. gloeosporioides* (35) and *Colletotrichum musae* (3) through the amplification of ITS regions and β-tubulin gene using ITS 4 and 5 and Bt2a and Bt2b primers, respectively. Fungicide resistance is a key factor in limiting the efficacy and disease control strategy. The resistance may also be an important aid to our understanding, at a molecular level, of the fungicidal mechanism of action. In the present study, the sensitivity of *C. gloeosporioides* isolates to benomyl, a benzimidazole fungicide was determined, based on the mycelial growth at five different concentrations viz., 0.5, 1, 2, 5 and 10 mg/l. The twenty six isolates were

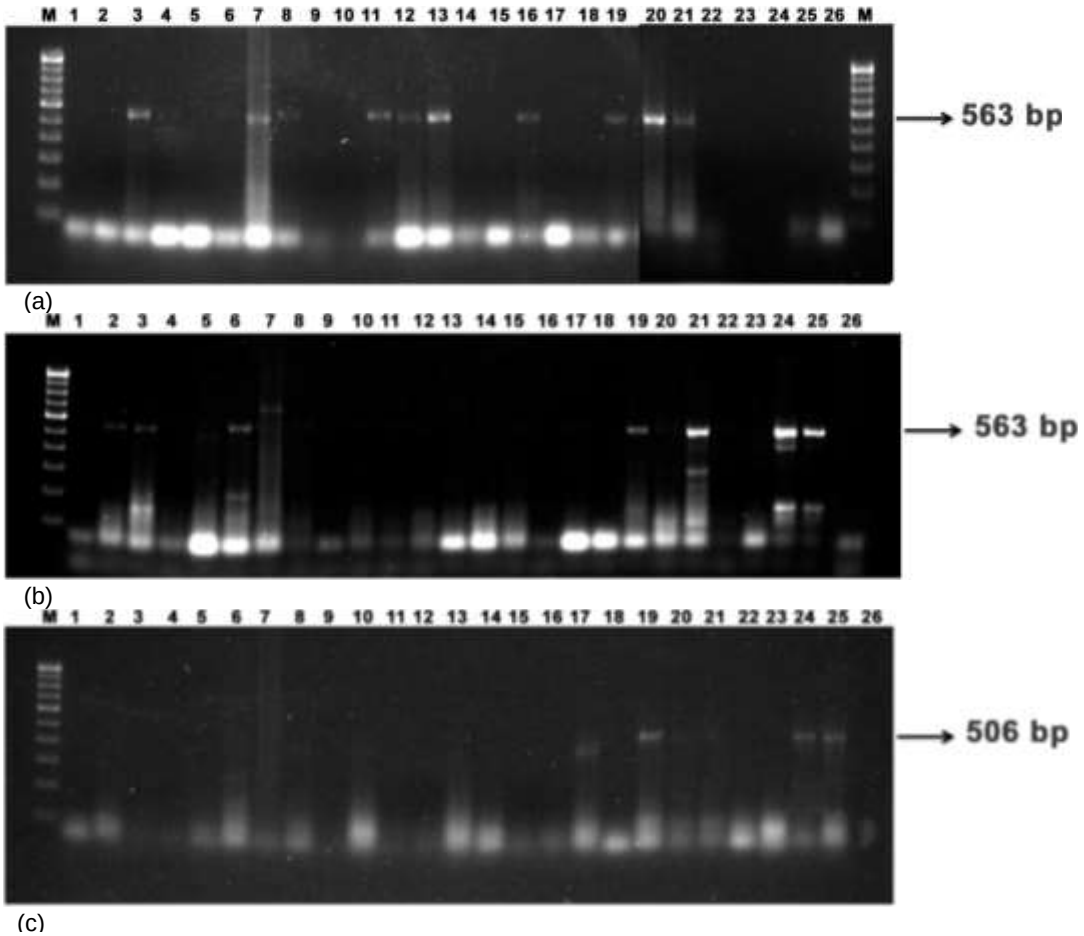

Figure 5. PCR amplification of partial β-tubulin sequence from benomyl resistant and sensitive isolates of *C. gloeosporioides,* (a. β-tubulin gene (*TUB2*) - b*enomyl resistant gene; b. β-tubulin gene (TUB2) - benomyl sensitive gene; c.*β-tubulin gene*TUB1* – benomyl resistant gene).

categorized as three viz., susceptible, moderately resistant and resistant, since significant variation was observed among the isolates in respect to sensitivity. In aqueous solution benomyl is rapidly hydrolysed to methyl benzimidazole – 2 – carbamate and this is probably the active fungi toxicant against pathogen. At the highest concentration of 10 mg/l benomyl, only isolates MCG 7 and MCG 16 were able to grow eleven days after inoculation, which confirmed their sensitiveness of the remaining isolates to benomyl. This might be due to benomyl does not inhibit the spore germination and conidial production but acts primarily by slowing the mycelial growth before or after infection (Peres et al., 2002).

Based on this, isolate MCG 21 was considered as sensitive MCG 5 as moderately resistant and MCG 16 as resistant to benomyl fungicide. This varied reaction obtained in the present study are similar to the findings of Chung et al. (2010), which demonstrated the sensitivity of thirty one *C. gloeosporioides* isolates from mango and strawberry to benzimidazole fungicides viz., benomyl, carbendazim and thiabendazole. In that, they reported that seventeen isolates of *C. gloeosporioides* were grouped as sensitive, two isolates were moderately resistant, nine isolates were resistant and three isolates were classified as highly resistant based on the colony diameter on PDA medium amended with benomyl at 10, 100 and 500 mg/ml concentration. It confirms the earlier reports of Sariah (1989), where the growth of sensitive isolates of *Colletotrichum capsici* were completely inhibited at 2.5 µg/ml benomyl while the resistant isolates grew on agar containing 1000 µg/ml fungicide. Peres et al. (2004) reported that mycelial growth of sensitive isolates was completely inhibited at 1.0 µg/ml of benomyl, whereas resistant isolates grew even at 10 µg/ml of benomyl. Similarly, Joshi et al. (2013) reported that, out of 30 isolates of *C*. *gloeosporioides*, isolate Cg 42 from mandarin orange was found to be insensitive to Benomyl with mean IC50 value of 318.85 µg/ml; while Cg 41 and Cg 43 was highly sensitive to Benomyl. In addition

Nalumpang et al. (2010) reported the sensitivity of *C. gloeosporioides* isolates from mango to carbendazim fungicide at various concentrations of 0.1, 1, 10, 100, 500 and 1,000 mg/l. They found that, among 59 isolates of *C. gloeosporioides*, 49 isolates were highly resistant (HR) phenotypes, 4 isolates were sensitive (S) phenotypes, none showed weakly resistance (WR) and moderately resistance (MR) phenotypes to carbendazim.

Benzimidazole fungicides act by inhibition of tubulin biosynthesis (Davidse, 1973), due to the mutations of β-tubulin gene which has been related to specific amino acid substitutions at several distinct regions within the ß-tubulin molecule (Fujimura et al., 1992). All the twenty six isolates of the current study were used to identify the presence of resistance and susceptible β-tubulin genes. Most often benzimidazole tolerance is due to mutations in the β -tubulin gene which reduce benzimidazole binding (Cooley and Caten, 1993; Reijo et al., 1994). These mutations can be used to rapidly identify tolerant strains with nucleic acid-based methods (Luck and Gillings, 1995).

Further, rapid identification of tolerant strains can aid in determining the fungicide resistance management policies. In the present research, partial β - tubulin gene sequences were amplified by the appropriate primers to the DNA fragment for TUB 2 and TUB 1 gene. Isolates MCG 3, MCG 7, MCG 8, MCG 11, MCG 12, MCG 13, MCG 16, MCG 19, MCG 20, MCG 21 had showed amplification product at 563 bp for benomyl resistant gene while the isolates of MCG 2, MCG 3, MCG 6, MCG 19, MCG 21, MCG 24, MCG 25 produced amplification for the primers of β-tubulin sensitive gene. In addition, alternative gene TUB 1 β-tubulin was amplified from four isolates viz., MCG 19, MCG 20, MCG 24 and MCG 25 at 506 bp. Peres et al. (2004) found that eight isolates of *C. gloeosporioides* were amplified by the portion of β – tubulin gene, with primer TB2R and TB2L.

Resistance to benzimidazole fungicides has been reported in many filamentous fungi, whereby the fungicide is unable to bind to a mutated β-tubulin protein containing an altered amino acid sequence (Davidse and Flach, 1977). Several studies have demonstrated that sequence analysis of the β-tubulin gene of benomyl-resistant fungi revealed that, resistance almost always is due to point mutations in codon 198 or 200 of the β-tubulin gene. Amino acid substitutions at these two codon positions result in different benomyl-resistance phenotypes (Albertini et al., 1999; Yarden and Katan, 1993).

Conclusion

The study reveals that a rapid benomyl tolerance screening could be necessary for examining the potential of different fungicide resistance. Pathogen *C. gloeosporioides* responsible for anthracnose in mango is composed of both benomyl-resistant and sensitive populations that are genetically distinct, which might be due to a single β-tubulin gene existing within the fungal genome; however, in others, an additional gene may be present that is divergent in sequence.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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