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Identification of the Fungal Pathogen that Causes Strawberry Anthracnose in Bangladesh and Evaluation of *In Vitro* Fungicide Activity

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This study was conducted to identify the *Colletotrichum* species causing anthracnose disease of strawberry in Balgladesh and to evaluate *in vitro* activity of commercial fungicides it. Based on morphological and cultural characteristics, all 22 isolates were identified as *Colletotrichum gloeosporioides*. They developed white or glittery colonies with grey to dark grey reverse colony colors and they produced cylindrical conidia. The efficacy of five commercial fungicides, Bavistin DF, Dithane M-45, Sulcox 50 WP, Corzim 50 WP and Rovral 50 WP, were tested against the fungus. Bavistin inhibited radial growth completely and was followed in efficacy by Dithane M-45. In Bavistin DF treated media, the fungus did not produce conidia. The percent inhibition of radial growth of the fungus was increased with the increasing concentrations of fungicide.

KEYWORDS : Anthracnose, Bavistin, *Colletotrichum gloeosporioides*, Fungicide, Strawberry

Strawberry (*Fragaria ananassa* Duch Family Rosaceae) (Sadhu and Chattopadhyay, 2001) are native to temperate zones but grow well in subtropical areas. In Bangladesh, it is becoming more popular and its importance as a commercial product is increasing. There is little information about the diseases of strawberry and pest control measures in Bangladesh. Therefore, it is important to identify the fungal diseases of strawberry and to test the effectiveness of commercial fungicides to reduce production loss. Strawberry anthracnose was first described in 1931 by Brooks, who named the causal organism *Colletotrichum fragariae* (Brooks, 1931). He initially concluded that anthracnose was primarily a disease of stolons but later reported that the pathogen occasionally attacks petioles in field conditions. (Brooks, 1935) later reported that the pathogen also caused rhizome rot and wilt. (Howard *et al*., 1983) described a black leaf spot phase of strawberry that was caused by *C. gloesporioides* (= *C. fragariae*) which is often found in association with anthracnose symptoms on stolons and petioles. Anthracnose is responsible for major losses of strawberry production worldwide (Howard *et al*., 1992). Fruit rot and flower blight are the common symptoms in fruiting fields, whereas lesions on stolons, petioles and leaves are particularly damaging in nurseries (Freeman and Katan, 1997). Therefore, the present study was conducted to identify the fungi associated with anthracnose disease of strawberry based on morphological and cultural characteristics and to evaluate the *in vitro* efficacy of five commercial fungicides against the fungi.

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Materials and Methods

Isolation and identification of fungi. A total of 350 samples of infected tissues were collected from strawberry plants from the Rajshahi District of Bangladesh (Table 2). The fungus was isolated from infected strawberry leaves and petioles following the standard procedures of (Agostini and Timmer, 1992). Tissue was cut from the advancing margin of the lesion, surface sterilized with 0.1% sodium hypochlorite solution and washed in three three times in sterile distilled water. The sterilized tissue was dried on sterile filter paper on a clean bench, plated on potato dextrose agar (PDA) and incubated at 28°C for 10 days. Mycelia from pure cultures were examined under dissecting and compound microscopes and identified by comparing their morphological and cultural characteristics with published descriptions (Brooks, 1931; Howard *et al*., 1983; Smith *et al*., 1990).

Pathogenecity of *Colletotrichum gloeosporioides*. Conidia were harvested from 7-day-old cultures by flooding Petri dishes with 10 *ml* of sterile distilled water and dislodging conidia by softly scraping colonies with a sterilized, narrow edged glass slide. Aqueous conidial suspensions were filtered through sterile cheese cloth to remove mycelia. Conidial suspensions were adjusted with sterilized water to a concentration of 10^3 conidia/*ml* and sprayed onto 6 healthy, detached strawberry leaves. Six healthy, detached leaves were simultaneously sprayed with sterilized distilled water without conidia to serve as controls.

Response of *Colletotrichum gloeosporioides* to fungicides. Five commercial fungicides, Dithane M-45 (Manganese ethylene-bis-dithiocarbamate), Corzim 50 WP (Carbondazim), Bavistin DF (2-Benzimidazolecarbamic acid, methyl ester), Sulcox 50 WP (Copper oxychloride) and Rovral 50 WP (3-(3,5-dichlorophenyl)-N-(1-methyl ethyl) -2,4 dioxo-1-imidazolidine carboxamide), were evaluated *in vitro* using the poison food technique (Adams and Wong, 1991). Fungicide suspensions of 500 $\mu g/ml$, 1000 $\mu g/ml$, 1500 $\mu g/ml$, and 2000 $\mu g/ml$ were prepared by dissolving requisite quantities of each fungicide in autoclaved and cooled PDA just before pouring into Petri dishes. Twenty ml of fungicide-amended media was poured into each 9 cm sterilized Petri dish. Three replicates were performed for each concentration of each fungicide. Medium without fungicide served as a control. Mycelial discs (5 mm) were cut from 7-days-old cultures using a cork borer, placed in the centre of each Petri dish after solidification of PDA and incubated at 28°C. Percent inhibition of growth of *Colletotrichum gloeosporioides* was recorded using the following formula:

$$\% \text{ inhibition} = \frac{X - Y}{X} \times 100$$

Where, X = radius of fungal mycelia on control plate and Y = radius of fungal mycelia on fungicide-treated plate

Sporulation test. Sporulation was measured after 10 days of incubation on media amended with different concentrations of fungicide. Five mm mycelia discs were cut randomly from four different areas and were macerated in 1 *ml* of sterilized distilled water. A 0.1 *ml* aliquot of the resulting suspension was placed on a clean glass slide and the number of spores was counted under low power of a compound microscope using a haemocytometer (Islam *et al*., 2003).

Statistical analysis. The percent inhibition of radial growth was subjected to arcsine transformation. Analysis of variance was performed and the means were separated using Least Significance Different Test at $p < 0.05$ (Zaman *et al*., 1982). Statistical analysis was done whenever required.

Results and Discussion

Isolation and identification of fungi. Characteristic symptoms of anthracnose in infected strawberry plants are usually black leaf spots, about 1~3 mm in diameter, although the spots may also remain light gray (Fig. 1A). Leaves can become heavily spotted without dying. In cases of severe infection, dark and sunken lesions also developed on the petioles (Fig. 1B) and stolons, which resulted in leaf mortality. In ripening fruit, round, sunken and watery lesions developed, which were usually dark brown or black in color (Fig. 1C). Reddish brown streaking developed in the crowns of wilted plants.

Acervuli with setae were produced (Fig. 1H) after three weeks of incubation at 28°C on PDA plates. Acervuli were 10 to 30 μm in diameter, with 0~2 setae, often the acervuli coalseced up to 120 μm in diameter. Setae were 24 to 80 μm in length and 4 to 6 μm in diameter, with 1~3 transverse septa.

Conidial sizes of the 22 isolates were compared and the mean for each isolate ranged from 12.9 to 16.5 μm long and 4.4 to 6.5 μm wide (Table 1). The size and shape of conidia from these isolates fit the description for conidia of *C. gloeosporioides* (anamorph = *G. cingulata*) (Smith and Black, 1990). (Smith and Black, 1990) reported that the principal pathogen responsible for anthracnose disease of strawberry are *C. acutatum*, *C. fragariae* and *C. gloeosporioides* but perithecia are produced only by *C. gloesporioides* (Table 1). Our fungi formed typical perithecia in culture and differed from the other two species of *Colletotrichum* (Smith and Black, 1990). We concluded that our fungi were *C. gloeosporioides* because the length, width and other morphological characteristics of the conidia match published descriptions.

Table 1. Comparison of conidial characteristics of *Colletotrichum gloesporioides, C. acutatum* and *C. fragariae*

Fungal descriptions	Conidial morphology of the fungus			Ascigerous state
	Shape	Length (μm)	Width (μm)	
Isolated pathogen from strawberry	Unicellular, hyaline, smooth, oblong, and dambel shaped	12.9~16.5	4.4~6.5	+
C. gloesporioides Sutton (1992)	Straight, cylindrical, apex obtuse, base truncate.	11~17.5	3~6	+
C. acutatum	Hyaline, straight and usually pointed end.	8.5~16.5	2.5~4	–
C. fragaria Smith *et al*., 1990	Narrower at one end and have a slightly pointed morphology.	12.4~15.0	4.4~5.2	–
Howard *et al*., 1983	Cylindrical or boat shaped	17.2~18	6.2~6.5	–
Brooks *et al*., 1931	Cylindrical or boat shaped	16.4	4.8	–

+ = Described, – = Not described

Table 2. Characteristic symptoms of various phases of anthracnose of strawberry and characteristics of the causal fungi on PDA

Name of the isolate	Infection phase	Symptoms	Characteristics on PDA plates		
			Colony color	Setae	Conidial shape
SL001	Black leaf spot	The spot are about 1-2mm in diameter and are usually black but may remain light gery.	white	–	Cylindrical
SL002			white	–	Cylindrical
SL003			white	–	Cylindrical
SL214			white	–	Cylindrical
SP-223		Dark, sunken lesions can girdle the petiols and stolons.	white	–	Cylindrical
SP001			white	–	Cylindrical
SP002			white	–	Cylindrical
SP003			white	–	Cylindrical
SR001			Orange white	+	Cylindrical
SR002			Orange white	+	Cylindrical
SR003			Orange white	+	Cylindrical
SC225	Crown rot	Radish brown discoloration on the internal crown	white	+	Cylindrical
SC217			white	_	Cylindrical
SC228			white	+	Cylindrical
SI001	Fruit rot	Blighted flowers turn brown and remain attached to the plant.	white	+	Cylindrical
SI002			white	+	Cylindrical
SI003			white	+	Cylindrical
SF001		Dark sunken lesion developed on the fruit.	white	+	Cylindrical
SF002			white	+	Cylindrical
SF003			white	+	Cylindrical
SS01			white	+	Cylindrical
SS02			white	+	Cylindrical

The conidia that developed on the acervuli of all the 22 isolates were hyaline, cylindrical and single celled. Isolates SR001, SC225, SC217, SC228, SI001, SF001, SS001 and SS002 produced setae in culture, while the remaining isolates did not (Table 2). These results deviated from the results of (Smith and Black, 1990). They reported that *C. gloeosporidies* does not produce setae in culture but that *C. fragariae* does. The acervuli of *C. gloesporioides* is setae or glabrous (Mordue, 1971). All the 22 isolates were produced cylindrical conidia and white to dark brown colonies, which fits the result of (Smith and Black 1990).

The growth of the fungi after 7 days of incubation at 28°C on PDA plates was 7.5 to 9 cm. According to (Smith and Black 1990), *C. gloeosporioides* can be easily differentiated from *C. acutatum* and *C. fragariae* by their growth on PDA plates. The greatest difference in growth occurred at 32°C, where the average diameter of 5-day-old *C. acutatum* was 13 mm, compared to 69 mm for *C. fragariae* and 63 mm for *C. gloeosporioides*.

Colony characteristics on PDA plates were also observed. The texture was loose, the top surface was smooth, acervuli were setose or glabrous in culture and colony color was white in early stages but turned grayish in later stages. These observations support the results of (Azad *et al.*, 2005). Based on the mode of conidiogenesis, morphology, cultural characteristics and growth rates, we identified the fungi as *C. gloeosporioides*.

Pathogenecity of *Colletotrichum gloeosporioides*. Watery, irregular blotches and pale brown spots 1 to 3 mm in diameter appeared on leaves 3 to 5 days after inoculation. Tissues with lesions began to rot and/or blight when lesions enlarged and coalesced 6 to 8 days after inoculation (Fig. 1D and 1E). Control leaves had no symptoms. *C. gloeosporidies* was consistently re-isolated from diseased leaves. Our results demonstrate that isolates were pathogenic to strawberry.

Response of *Colletotrichum gloeosporioides* to fungicides. A progressive increase in percent inhibition of radial growth of the pathogen was observed with the increase in concentration of the fungicides (Table 3). Bavistin DF was the most effective fungicide in this experiment. Growth of the fungus was totally checked using all four concentrations of Bavistin DF. (Meah and Khan 1986) used Bavistin DF, Rovral WP, Dithane M-45 and Topsin M to test the *in vitro* control of *C. gloesporioides*, the cause of fruit rot of guava. They observed that Bavistin DF (0.2%), Roval WP (0.2%) and Topsin M (0.2%) were effective in inhibiting mycelial growth. (Alam *et al.*, 2000) found that Bavistin DF, Dithane M-45 and Tilt 250 EC were the most effective fungicides of *Bipolaris sorokiniana* at 500 *μg/ml* to 2,500 *μg/ml* and 1/10 to 1/1000 *ml* (Ahmed *et al.*, 1991) evaluated eight fungicides and observed that Dithane M-45 gives the best control of anthracnose (*Colletotrichum gloeosporioides*),

Table 3. Percent inhibition of radial growth and mean sporulation of *Colletotrichum gloesporioides* at different fungicide concentrations

	Inhibition percentage at different concentration (*µg/ml*)				Mean sporulation after 10 days of incubation at different concentration (*µg/ml*)			
Fungicide	500	1000	1500	2000	500	1000	1500	2000
Dithane M-45	56.94b	62.29b	72.17b	81.22b	++	+	+	+
Sulcox 50 WP	25.07d	28.15c	30.4cd	39.91cd	+++	+++	+++	++
Corzim 50WP	31.89c	22.26d	39.23c	43.34c	+++	+++	++	++
Bavistin DF	100a	100a	100a	100a	–	–	–	–
Rovral 50WP	31.06c	28.12c	44.69c	50.82c	++	+++	++	+
Lsd	2.70	1.51	2.20	1.89				

+ = Poor sporulation, ++ = Good sporulation, +++ = Abundant sporulation, – = No sporulation

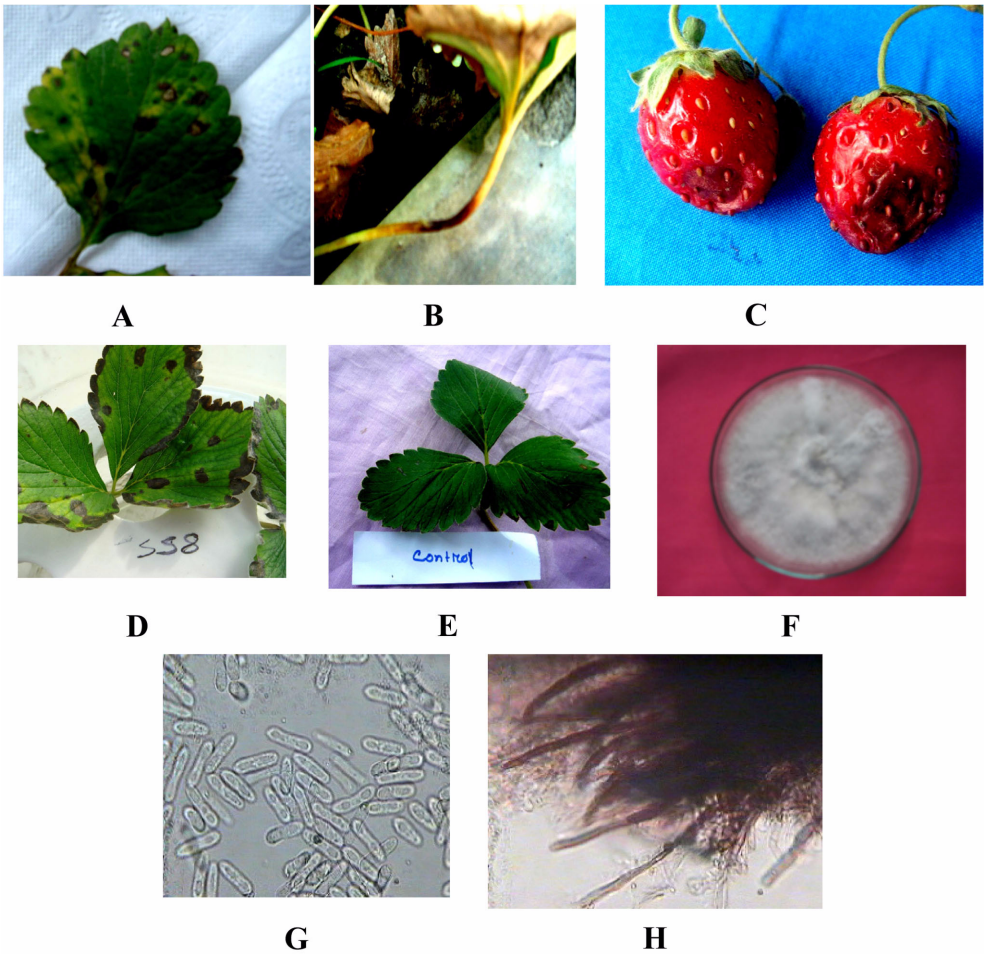

Fig. 1. Isolation, identification, and pathogenecity of *Colletotrichum gloesporioides*. A~C, Symptoms of anthracnose disease of strawberry; D~F, Symptoms reproduced by inoculation of strawberry with *C. gloeosporidies*; F, Colony of *C. gloeosporidies*; G, Conidia formed on PDA plate at 28°C; H, Acervuli with setae.

followed by Bordeaux mixture. Our results agree with these earlier findings. We found that Bavistin DF was highly effective and that Dithane M-45. Rovral WP, Corzim WP and Sulcox WP were less effective fungicides against *C. gloesporioides*.

Sporulation test. No spores were formed when the fungus was grown on media containing Bavistin. However, the fungus produced spores on PDA medium amended with the other fungicides. (Alam *et al.*, 2000) reported that 100% germination inhibition occurred with the application of Bavistin and Thiovit after 15 minutes and 25 minutes of immersion in 0.25% Cupravit. With the increase in the fungicide concentration and the immersion period, the inhibition of conidial germination of this fungus also increased.

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