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Isolation and Characterization of the Incitant of Leaf Spot of Turmeric and *in-vitro* Efficacy of Native Isolate of Endophytic Bacteria

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Abstract

Turmeric, *Curcuma longa* L. is an important commercial spice crop cultivated in Meghalaya covering 2,649 ha area with 16,497 MT productions. However, the turmeric cultivation is severely affected by leaf spot disease limiting its yield. So, the present study was conducted to identify the pathogen associated with leaf spot disease of turmeric as well as to check the efficiency of bacterial endophytes in managing the disease. Based on the morphological and cultural studies, six isolates of *Colletotrichum gloeosporioides* were identified as the causal organism of leaf spot disease of turmeric. The isolates on PDA medium produced white to grey fluffy (raised/ flat) cottony culture with serrated margin. All the isolates produced dark brown acervuli and globular conidia with oil globules inside. Five bacterial endophytes *viz*., BE 1, BE 222, M1W1, NGB21 and SVC 11 were tested against *C. gloeosporioides* by using dual culture assay. They were able to inhibit the mycelial growth of *C. gloeosporioides* in the range of 35.82-68.11%. The highest percent inhibition in dual culture assay was recorded in the isolate NGB 21 (68.11%) followed by isolate BE1 (59.89%).

Keywords: *Colletotrichum*, *Curcuma longa* L., Endophytes, Leaf spot, Meghalaya, Turmeric

Introduction

Turmeric, *Curcuma longa* L., is a well-known rhizomatous spice crop from the family Zingiberaceae. The turmeric is native to South-East Asia. In India, turmeric has been grown and used extensively since ancient days for medicinal, religious, culinary as well as cosmetic purposes. In Ayurveda, it is mentioned to cure sore throat, gall bladder stones and other stomach complaints. It can also be used as natural antiseptic and has anti-inflammatory, carminative and anti-helminthic properties (Pruthi, 1976). In the world, turmeric is widely known as 'Indian Saffron' as well as Golden Spice of Life'.

India dominates the international turmeric market in production, consumption and export. The country has an area of 2.38 lakh hectares under turmeric cultivation with 11.33 lac MT productions. The Meghalaya state has a cultivation area of 2,649 hectares and a 16,497 MT of turmeric annually (Anonymous, 2019). 80% of turmeric produced annually is used in domestic consumption and rest 20% is exported from the country. In 2019 alone, India has exported turmeric worth 194.35 billion US dollars approximately (Shahbandeh, 2020).

Turmeric is highly susceptible to various soil-borne and foliar diseases. Among foliar diseases, leaf spot caused by *Colletotrichum* spp. imparts maximum damage to the crop and limits its yield (Rao *et al.*, 1993). Mc Rae (1917) first reported that the leaf spot of turmeric is caused by *C. capsici* from Tamil Nadu state of India. It was later found that *C. gloeosporioides* is also causing the leaf spot disease with same symptoms (Patel *et al.*, 2005; Chawda *et al.*, 2012). On the severely infected plants (*i.e.*, > 50%), the disease can cause a yield loss of 25.83-62.12% in fresh weight of rhizomes and 42.10-62.10% loss in dry weight of rhizomes (Hudge and Ghugul, 2010).

Article History

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In Meghalaya, there is a vast potentiality of turmeric cultivation but turmeric cultivation in the state suffers severely from the leaf spot disease. Therefore, it is important to identify the pathogen(s) responsible for turmeric leaf spot disease and to check the effectiveness of endophytes in managing the disease. So, the present study was conducted to identify the leaf spot causing pathogen of turmeric on the basis of morphological and cultural characteristics and its *in-vitro* management using bacterial endophytes.

Materials and Methods

Isolation and Identification of the Pathogen

For isolation of the pathogen, naturally infected turmeric leaves were collected from farmer's field from different locations of Meghalaya. The leaves were first observed under microscope for preliminary identification of the pathogen. The leaves with typical leaf spot symptom were cut into small bits containing both diseased and healthy portions together. The bits were then surface sterilized by dipping in 1% sodium hypochlorite solution for about 30 seconds to 1 minute followed by 3-4 times of serial washing with sterile distilled water to remove the traces of chemical. The surface sterilized bits were then air dried in sterilized blotting paper. The bits were then aseptically transferred to Petri plates containing PDA as the main isolation medium by using sterilized inoculation needle. The plates were then incubated at 28±2 °C for getting fungal growth. A loopful of fungal mycelium from the pure culture were observed under compound microscope and identified by comparing with the available literature.

Pathogenicity Test

Pathogenicity test was conducted by surface sterilising the healthy turmeric leaves with 1% sodium hypochlorite solution and then washed with sterile distilled water for 3 to 4 times. The surface sterilized leaves were then air dried and pin pricked to make wounds. 10 µl of 10 days old conidial suspension was inoculated into the pin pricked leaves. Inoculated leaves were kept in humid chamber at 28±2 °C for 7 days (Adhipathi *et al.*, 2013).

The symptoms that manifested on artificially inoculated leaves were meticulously compared to those of naturally infected plants. The organism was re-isolated from the leaves that had been artificially inoculated, and the resulting culture was compared to the original.

In-vitro Management using Bacterial Endophytes

Efficacy of the collected endophytes against the pathogen was evaluated by using dual culture assay (Dennis and Webster, 1971). In this method, a disc of one-week old fungal culture was placed in the centre of PDA plate. The plates were allowed to incubate for 24 hrs at 28±1 °C. After one day of incubation, 24 hours old bacterial cultures were streaked parallel on either side of the fungal disc 2.5 cm away from the disc followed by incubation at 28±1 °C for 7 days. Control plates were maintained by inoculation of the fungal disc in the middle of the PDA plate followed by 7 days incubation at 28±1 °C. Three replications were maintained for each treatment. Plates were observed on the basis of the growth of the radial hyphae and were compared with the control plate. The efficacy of tested endophytes was expressed as percent inhibition over the control which was calculated by the formula given by Vincent (1947).

Radial growth Inhibition (%),

$$I = \frac{C - T}{C} \times 100$$

Where,

I = percent inhibition;
C = Radial growth of the fungus in control (cm);
T = Radial growth of the fungus in treatment (cm).

Results and Discussion

Isolation and Identification of the Causal Organism

The leaf spot appears as elliptical to oblong brown coloured spot surrounded by yellow halo. In the centre of the spot black coloured acervuli appeared as dotted concentric ring (Figure 1a). The organism associated with the leaf spot disease was isolated from the infected leaves of turmeric following standard tissue isolation method. The organism in potato dextrose agar medium produced white to grey culture with fluffy (flat/ raised) cottony mycelial growth with zonation and serrated margin (Figure 1c). The colour varied on reverse side of the Petri plate from yellowish white to pinkish white to olivaceous grey to grey in colour (Figure 1d). Than *et al.* (2008) in their research observed a variation of greyish white to dark grey culture colour of *C. gloeosporioides* with distinct zonation which produced globular conidia with more than two oil globules inside. Wang *et al.* (2020) reported that the cultures of *C. gloeosporioides* on PDA produced aerial milky to pale grey to grey colonies with a variation of olivaceous grey to dark grey on reverse side of the plate.

Dark brown coloured acervuli with setae were produced on PDA plates after 10 days of inoculation at 28±2 °C (Figure 1f). The diameter of acervulus was observed in the range of 54.19-94.23 µm. This finding on the diameter of acervulus is in accordance with Akhtar *et al.* (2009) who reported that the diameter of acervuli produced by *C. gloeosporioides* varied from 10-30 µm, however sometimes they coalesced up to 120 µm. Likewise, Sharma (2019) found that the isolates of *C. gloeosporioides* produced acervuli in PDA which varied from 55.28-95.80 µm in diameter.

The conidial size of six isolates of the pathogen can be ranged from [7.9 - (10.5) - 12.1] µm to [7.27 - (8.34) - 10] µm in length and [3.1 - (3.46) - 4.15] µm to [1.97 - (2.58) - 3.29] µm. The conidia were non-septate, straight cylindrical in shape and carried oil globules (Figure 1e). Gautam (2014) mentioned that the size and shape of *C. gloeosporioides* vary widely with respect to the host plant and its area of origin. He stated that the shape of conidia may vary from ovoid to oblong to slightly curved to dumbbell which are hyaline in colour. He further stated that the size of conidia in average varies from 10-15 µm in length and 5-7 µm in breadth. Prabakar *et al.* (2005) found that the conidia size varies from 8.3 × 2.0 µm to 27.4 × 6.6 µm.

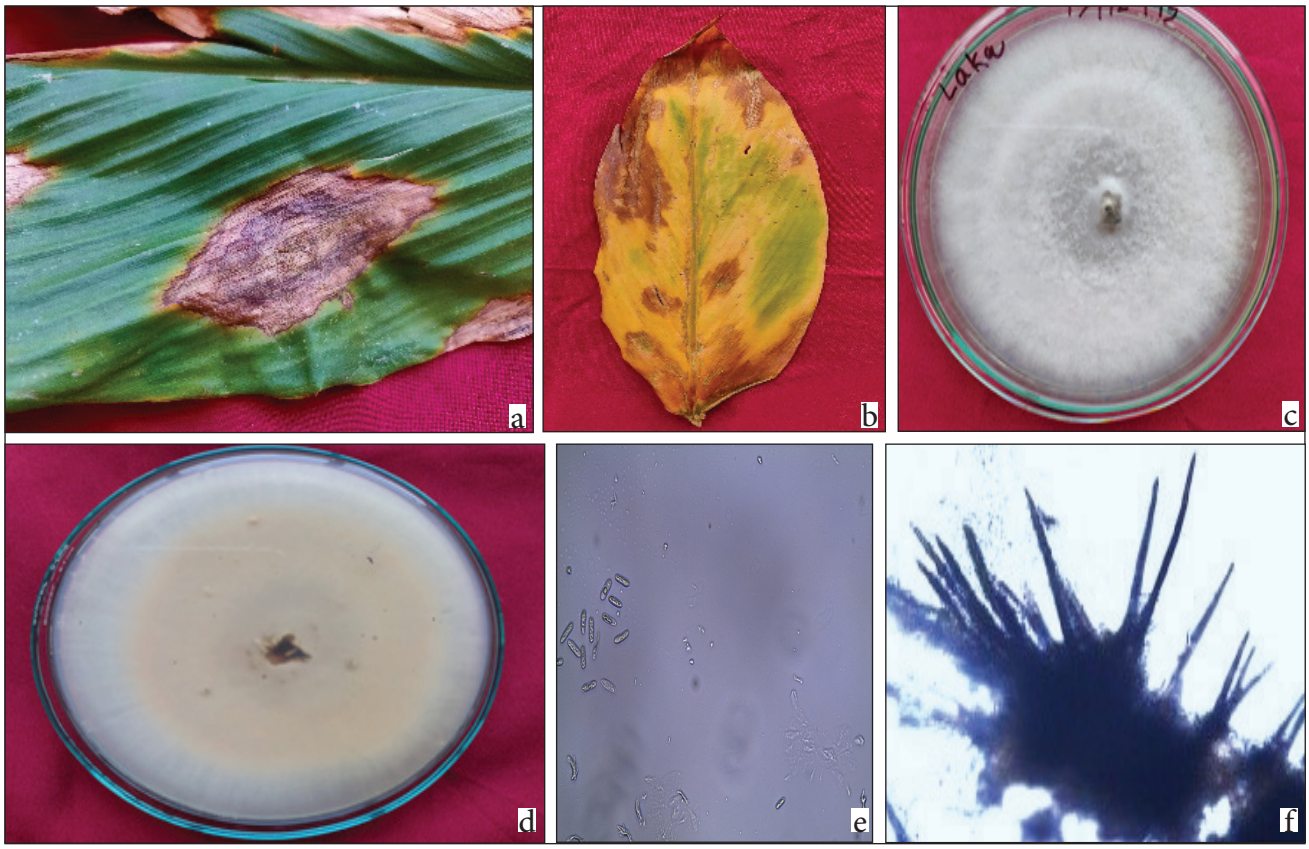

Figure 1: Isolation, identification and pathogenicity of *C. gloeosporioides viz*. (a) Fresh turmeric leaf with leaf spot symptom; (b) Symptoms produced on healthy turmeric leaf after artificial inoculation of the pathogen; (c-d) Pure culture of *C. gloeosporioides* on PDA medium (front and reverse); (e) Cylindrical conidia with oil globules formed on PDA plate; (f) Acervuli with setae

The growth of the fungi after 7 days of incubation at 28±2 °C after 10 days of incubation lies in the range of 76-90 mm. This is in accordance with Dev (2017) who reported that *C. gloeosporioides* on PDA after ten days of incubation produced 86 mm radial growth. Papade *et al.* (2019) in their study on morphological character of *C. gloeosporioides* isolated from different hosts found the radial growth in the range of 72-89 mm in the isolates on PDA after eight days of incubation.

Based on the comparison of morphological and cultural characteristics of the pathogen in the present study with the earlier reports, the pathogen was identified as *Colletotrichum gloeosporioides.*

Pathogenicity Test

After 4 to 5 days of inoculation of the conidial suspension into the surface sterilized and pinpricked healthy turmeric leaves, minute spot appeared, which was reddish brown in colour. The spots gradually increased in size with a yellow halo. After seven days of incubation the inoculated leaves produced slightly sunken and dark brown to chocolate-coloured elliptical lesions. The lesions were surrounded by bright coloured yellow halo (Figure 1b). The results of pathogenicity test are in accordance with Patel (2005) where *C. gloeosporioides* on inoculation to detached healthy turmeric leaves produced large irregular and depressed dark chocolate-coloured lesions surrounded by a bright yellow halo.

In-vitro Management using Bacterial Endophytes

Five bacterial endophytes *viz.*, BE 1, BE 222, M1W1, NGB 21 and SVC 11 were tested *in vitro* against the pathogen by using dual culture assay. They were able to inhibit the mycelial growth of *C. gloeosporioides* in the range of 35.82-68.11% (Table 1). The present study revealed that NGB 21 had the highest percent inhibition *i.e.*, 68.11% followed by BE 1 (59.89%), SVC 11 (49.88%), BE 222 (45.65%) and M1W1 (35.82%). Tasiwal *et al.* (2009) tested the antagonistic activity of biocontrol agents against *C. gloeosporioides.*

Table 1: Percent inhibition of the radial growth of *C. gloeosporioides* over control by the bacterial endophytes in dual culture assay

Sl. No.	Endophyte isolate code	Inhibition (%)
1	BE 1	59.89*
2	BE 222	44.65
3	M1W1	35.82
4	NGB 21	68.11
5	SVC 11	49.88
	Control	0.00
	SED	3.36
	CD (0.05%)	7.32

*Mean of three replications, F-test at 5% significant level.

They reported 50.97% and 42.87% inhibition of the radial growth of the pathogen by *Bacillus subtilis* and *Pseudomonas fluorescens* respectively. Ann *et al.* (2015) mentioned that the three isolates of Bacillus species *viz.,* CBF, YCA0098 and YCA5593 were able to inhibit the mycelial growth (50.1%, 45.9% and 44.7% respectively) and spore germination of *C. gloeosporioides* causing anthracnose of black pepper. Earlier, efficacy of native biocontrol agent has been found to be effective for the management of *C. gloeosporioides* causing fruit rot of bhoot jolokia (Senapoty *et al.*, 2019).

Conclusion

The present study showed that, the isolates of *C. gloeosporioides* causing leaf spot of turmeric varied in morphological as well as pathogenic ability from one another. Out of seven tested endophytes, NGB 21 and BE 1 were found highly effective against the most virulent isolates of *C. gloeosporioides*. The results of present study pave the way for further works on biological management of *C. gloeosporioides* with the effective endophytic flora in organic ecosystem.

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