International Journal of Agricultural Technology 2016 Vol. 12(2): 337-348
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Two New Bottle Gourd Fruit Rot Causing Pathogens from Sub-Himalayan West Bengal

Saha, A.[1], Das, S.[1], Chakraborty, P.[1], Saha, B.[1], Saha, D.[2] and Saha, A.[1*]

[1]Department of Botany, University of North Bengal, Siliguri-734013, India; [2]Department of Biotechnology, University of North Bengal, Siliguri-734013, India.

Saha, A., Das, S., Chakraborty, P., Saha, B., Saha, D. and Saha, A. (2016). Two new bottle gourd fruit rot causing pathogens from sub-himalayan west Bengal. International Journal of Agricultural Technology 12(2):337-348.

Abstract *Colletotrichum* sp. and *Fusarium* sp. are the two major fungi that cause bottle gourd fruit rot. Typical fruit symptoms include hard, sunken, and dark brown to black patches gradually covering entirely the whole fruit. Pathogen isolates were obtained from diseased bottle gourd fruits, on PDA medium forming a white to gray colonies. The conidia of *Colletotrichum* sp. were hyaline in color, cylindrical with both apices rounded. On the other hand the conidia of *Fusarium* sp. were also hyaline, beaked and having transverse septa. Pathogenicity tests with representative isolates were conducted on symptomless fresh bottle gourd fruits. Both the tested fungal pathogen caused fruit rot on bottle gourd fruit. Koch's postulates were fulfilled by reisolation of the pathogens from the test fruits. PCR analysis (using universal primer set ITS1/ ITS4) of genomic DNA from the pathogens and bioinformatic analysis further confirmed presence of the pathogens. These two fungi as fruit rot causing pathogens in bottle gourd are being reported for the first time.

Keywords: Fruit-rot, *Lagenaria siceraria*, *Colletotrichum*, *Fusarium*.

Introduction

Bottle gourd [*Lagenaria siceraria* (Molina) Standl.] is an annual herbaceous climber of family Cucurbitaceae. Bottle gourd fruits are used as vegetable. Leaves and twigs are also used as leafy vegetable. Woody rind (exocarp) of mature and dry fruits is used mostly for manufacture of containers, musical instruments, fishing floats etc. (Heiser, 1979). Currently bottle gourd is cultivated widely in India, China, Srilanka and Bangladesh, for its edible parts and also for its medicinal application (Prajapati *et al*., 2010). Bottle gourd is rich in minerals (potassium and calcium) and vitamins (vitamin A and B). It also contains various biologically active constituents including flavonoids, saponins, triterpenes. Thus, bottle gourd is considered as an important source of

***Corresponding author:** Saha, A.; **Email:** asahanbu@yahoo.co.in

pharmaceuticals for mankind and is advised for treatment of diseases like diabetes, jaundice, ulcers, hepatic disorders, cardiac disorders, and for blood pressure control (Prajapati *et al*., 2010 and Milind *et al.,* 2011).

Like many other plants, Bottle gourd is also vulnerable to fungal attacks. Specially, the fruits are susceptible because of their high moisture content. The fruits are decomposed easily following fungal attack. Fruit rot is very common in the cultivated fields. The cultivation of bottle gourd is affected by different pathogens including fungi, bacteria and viruses. Among the pathogens fungi such as *Cercospora citrulina*, *Alternaria cucumerina* has been reported to cause major problems by producing leaf spot and leaf blight disease and reducing in fruit yield (Maheshwari *et al.,* 2013). The occurance of southern blight caused by *Sclerotium rolfsii* on bottle gourd has also been reported in South Carolina (Ling *et al*., 2008).

In the present study area (sub-Himalyan West Bengal) approximately 50 hectares of land is under cultivation of Bottle gourd. Both premature and mature fruits are prone to attack by fungi. Fruit rot is very common in this area, rendering large amount of crop loss every year. Due to importance of Bottle gourd as vegetable and due to substantial crop loss, there is an urgent need for investigation of its causal pathogens and also for their control. Hence, the present work has been taken into consideration with an objective to identify the underlying microbial pathogens and also to know their mode of infection to combat the disease.

Materials and methods

Sample collection

Twenty three rotten fruit samples of bottle gourd were randomly collected from several bottle gourd cultivated areas of sub-Himalyan West Bengal, India. The samples were collected in separate sterilized zip-pack and were appropriately labelled and brought to the Molecular Plant Pathology Laboratory of University of North Bengal and stored at 4^0C for further works.

Screening of fungal pathogens

The bottle gourd fruits with symptoms of fruit rot were cut with a sterilized blade and the dissected portions were surface sterilized with 0.01% mercuric chloride ($HgCl_2$) for 1 minute. The pieces were then washed with sterilized distilled water for four consecutive times to remove traces of $HgCl_2$ left behind. The surface sterilized rotten portions were then cut into 2mm² small

pieces with heat sterilized blade in aseptic condition and transferred into potato dextrose agar (PDA) slants. The tubes were then maintained in an incubator at 28±2^{o}C for 5 days as proposed by Thiyam and Sharma (2013).

Verification of Koch's postulates

Koch's postulation was done for each of the fungal isolate for verification of its ability to induce the disease in fresh healthy fruits. The whole experiment was done *in vivo* in the present study area. Firstly some healthy growing fruits were selected on the cultivation area and were then surface sterilized with 75% ethanol. Then 4-5 scratches were made on the fruit using a sterilized scalpel. The scratches were then inoculated with conidial suspension made from each fungal isolates. In control sets similar scratches were made but mounted with sterile distilled water. The inoculated fruits as well as control fruits were then covered separately with sterilized polythene bags with small holes on it for the passage of air and to resist insect attack.

Suitable medium for growth and conidia production of the pathogens

Two different commonly used sterilized solidified media viz. Potato dextrose agar (PDA) and Oat meal agar (OMA) and one natural medium known as *Lagenaria* dextrose agar (LDA) prepared using extracts of bottle gourd, carrot, potato, dextrose and agar as required. Mycelial discs (5mm) of each isolate were placed on plates of three different media in triplicates and incubated at 28±2^{o}C for three days. The colony diameter and abundance of conidia formation were recorded after 2 days interval up to 6 days.

Morphology and physiology of the pathogens

A small portion of the fungal cultures were mounted into glass slide using lactophenol-cotton blue and are then observed under compound light microscope. Detailed morphological properties of the fungi such as septation of hyphae, type and shape of spore, etc were observed and were recorded as proposed by Amadi *et al.* (2014). For confirmation of identification one sample of a pathogen was sent to Indian Type Culture collection (ITCC) situated at IARI, New Delhi.

Isolation of fungal DNA

The cultures were grown in 25 ml of potato dextrose broth (PDB) and incubated as stationary culture for 7 d at 28±2°C. Mycelium was harvested by filtering through Whatman No.1 filter paper, washed twice with distilled water, dried and ground to a fine powder in liquid nitrogen using sterile mortar and pestle. DNA of both *Colletotrichum* and *Fusarium* isolates were extracted using CTAB method (Dellaporta *et al.*, 1983) modified by Sharma *et al.* (2003). Plant

tissue (1g $5ml^{-1}$) was homogenized with 2% CTAB-DNA extraction buffer of 60˚C for 1hr and mixed with chloroform-isoamylalcohol (24:1) and then centrifuged. Aqueous phase was subjected to precipitation by using 0.6 volume of isopropanol and precipitates were washed with 70% ethyl alcohol and dried overnight. DNA was treated with RNase and was dissolved in T E buffer [pH 8] and stored at -20˚C for further use. All extracted DNAs were diluted 10-fold in sterile distilled deionised water and quantified by use of ethidium bromide fluorescence.

Polymerase chain reaction (PCR) and sequencing

The ITS regions 1 and 4, including 18S, 5.8S and 28S rDNA were amplified by using universal primers ITS 1 (5`-TCCGTAGGTGAACCTGCGG-3`) and ITS 4 (5`-TCCTCCGCTTATTGATATGC-3`). Amplification was performed in a 25 µl reaction volume containing 20 ng genomic DNA from each isolate, 3U Taq DNA polymerase, 2.5 mM $MgCl_2$, dNTPs (2.5 mM each) and 1 µM each of the forward and reverse primers in 10X Taq buffer B (Genei, Bangalore). It was subjected to initial denaturation of 2 min at 94 ˚C followed by 35 cycles of 1 min at 94˚C, 1 min at 55 ˚C and 2 min at 72 ˚C, ending with a final extension of 10 min at 72 ˚C. The amplification was carried out using a Gene Amp 2400 thermal cycler PCR system (Perkin Elmer). After PCR amplified DNA fragment was purified using Genei Quick PCR purification Kit (Genei, Bangalore). The sequencing was done from automated DNA sequencing service from Xcelris Genomics Ltd, Bangalore. Sequencing was done in both directions using ITS 1 forward and ITS 4 reverse primers.

Phylogenetic analysis

The sequence data were analyzed using BLAST at the NCBI website (http://www.ncbi.nlm.nih.gov/). The sequence data from three amplified PCR products were assembled and analyzed using CLUSTAL W from MEGA 6.0 version software. The sequence was submitted to GenBank with proper annotations. The accessions of GenBank were also received (Accession numbers KC355249 for *Fusarium* isolate F/A/1 and KR263845 for *Colletotrichum* isolate F/A/2). The sequences were compared with equivalent sequences from a range of other crop infecting fungus present in GenBank. Multiple sequence alignment was carried out using the software CLUSTAL W in MEGA 6.0 version (Tamura *et al*., 2013). An evolutionary tree was constructed using Neighbor-Joining method (Saitou and Nei 1997). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2013).

Results

Survey, disease incidence and symptomatology

Disease symptoms were commonly observed on both mature and immature green fruits in the field. Immature fruits when infected produced yellowish brown superficial discoloration that developed into dark brown to black patches sometimes almost covering the fruits. Lesions may also appear on petioles and in such cases the young fruits fall from the climber. Symptoms on mature fruits appeared as black colored hard and sunken areas that gradually develop into large water-soaked areas and ultimately leading to spoilage of the whole fruit (Figure 1). The severely infected plants bear no fruits. Fruit rot incidence was found to vary from location to location. On an average 59% fruit rots were recorded in the sub-Himalayan foot hill region of West Bengal but 42% fruit rots were recorded in distant areas from foot hills. These data indicated that incidence of fruit rot was high in Sub-Himalayan region of West Bengal.

Figure 1. Field study and collection of rotten fruits from bottle gourd field. a. Picture showing fruit rot in premature fruit; b. Picture showing fruit rot in mature fruit.

Establishment of the two isolates as pathogens following Koch's postulates

After isolation of fungal pathogen from the infected fruit samples it was necessary to test the Koch's postulates for all the fungal isolates. Detailed

procedure of 'Koch's postulates' have been presented in the Materials and methods. Results of Koch's postulates indicated that among the 23 isolates, two isolates (F/A/1 and F/A/2) were pathogenic to bottle gourd. This was due to in all the cases of re-isolation, isolate F/A/1 and F/A/2 were consistently found to be associated with the infection of the fruit.

Mycelial growth and sporulation of two isolates in different solid media

In order to evaluate the vegetative growth and sporulation of the two pathogenic isolates in solid media, three different media viz., Potato dextrose agar (PDA), Oat meal agar (OMA) and one natural medium known as *Lagenaria* dextrose agar (LDA) prepared using extracts of bottle gourd, carrot, potato, dextrose and agar agar. After 6 days of incubation in PDA, radial growth of fungi (F/A/1) mycelia was 73.36 mm in diameter and in case of fungi (F/A/2) it was 90 mm covering the whole plate. Both the fungi showed moderate level of sporulation (conidia formation) after 6 days of incubation in PDA. In OMA after 6 days of incubation the radial growth of mycelia of F/A/1 and F/A/2 respectively were 54.29 mm and 66.66 mm in diameter. OMA was found to be best medium for sporulation of both the fungi. Radial growths of F/A/1 and F/A/2 mycelia in LDA were 69.18 mm and 89.66 mm respectively, after 6 days of incubation moderate sporulation was observed in case of both the fungi. From the Table 1 and Fig 2, it was evident that, growth of both the pathogens was best in PDA but sporulation was best in OMA.

Tableure 1. Mycelia growth and sporulation of *Colletotrichum gloeosporioides* and *Fusarium pallidoroceum*

***Colletotrichum gloeosporioides* (Isolate code F/A/1)**						
Growth Media	**Days of incubation**					
	2		**4**		**6**	
	Growth	**Sporulation**	**Growth**	**Sporulation**	**Growth**	**Sporulation**
PDA	9.83	-	48.87	-	73.36	+
OMA	9.11	-	35.92	+	54.29	++
LDA	9.67	-	43.25	-	69.18	+
***Fusarium pallidoroceum* (Isolate code F/A/2)**						
Growth Media	**Days of incubation**					
	2		**4**		**6**	
	Growth	**Sporulation**	**Growth**	**Sporulation**	**Growth**	**Sporulation**
PDA	11.96	-	77.66	-	90.00	+
OMA	9.88	-	53.32	+	66.66	++
LDA	10.66	-	76.11	+	89.66	+

PDA= Potato dextrose agar; OMA = Oat meal agar; LDA = Lagenaria dextrose agar

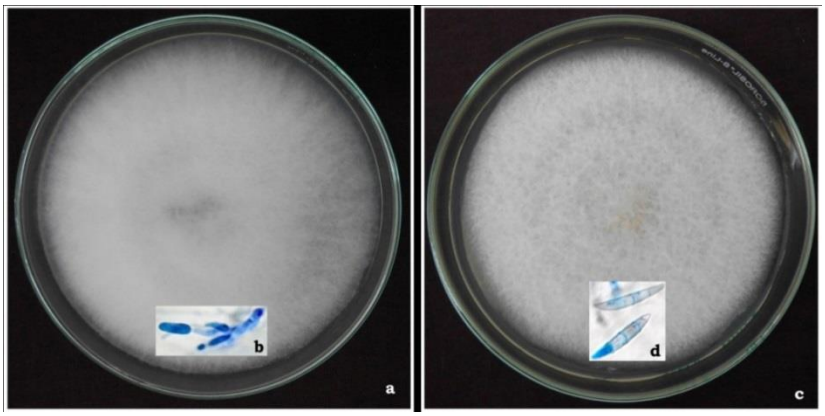

Figure 2. a. Mycelian mat of *Colletotrichum gleosporioides*; b. Spores of *Colletotrichum gleosporioides*; c. Mycelian mat of *Fusarium pallidoroceum*; d. Spores of *Fusarium pallidoroceum*

Morphology and identification

For microscopic observations, mycelia of both the fungi were taken in microscopic slides from pure culture and stained with cotton-blue in lacto-phenol. The slides were mounted with cover glass, sealed and observed under microscope. Immature mycelia of F/A/1 were hyaline in color. The conidia were cylindrical with both apices rounded. The conidial sizes varied from 6.95-14.76 × 4.23-7.52µm. The mycelia of F/A/2 were also hyaline in color. Conidia of the fungus were beaked and having transverse septa. Conidia were produced from simple septate conidiophores in simple or branched acropelal chains. The length and breadth of mature conidia were 10-40 µm and 6-12 µm respectively. The diameter of the mature hyphae ranged between 3-5 µm. Based on the morphological characteristics the two fungi F/A/1 and were identified as *Colletotrichum* sp. and *Fusarium* sp. respectively (Fig 2). One of the fungi (F/A/2) was sent to Indian Type Culture collection (ITCC) situated at IARI, New Delhi, for species level confirmation which was identified as *Fusarium pallidoroseum* (Identification no. 9486.14).

Identification of fungi by polymerase chain reaction (PCR)

The fungi associated with fruit rot were detected by PCR using ITS specific primers, ITS 1/ ITS 4. Total two fungi established as pathogen by Koch's postulation were further identified by polymerase chain reaction (Fig 3). All the samples showed positive PCR amplification and an expected ~500 nucleotide long sequences containing partial sequences of 18S ribosomal RNA gene and 28S ribosomal RNA gene, and complete sequences of internal transcribed spacer-1, 5.8S ribosomal RNA gene and internal transcribed spacer-2.

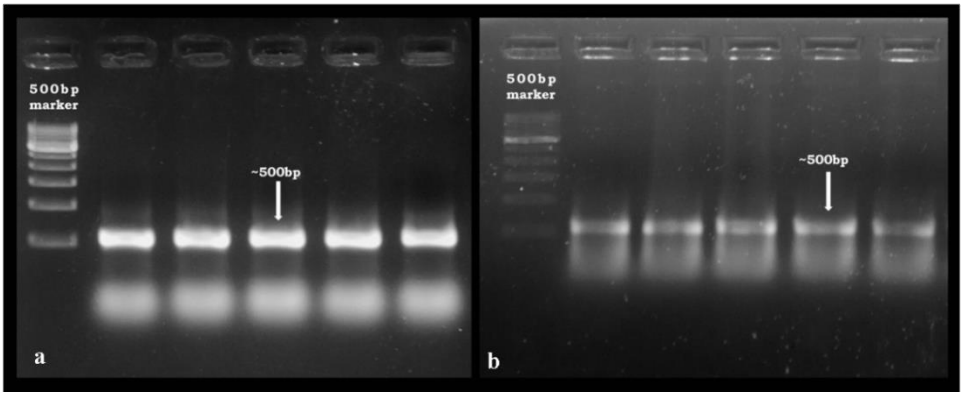

Figure 3. Agarose gel electrophoresis of PCR amplified ITS regions: (a) F/A/1 and (b) F/A/2.

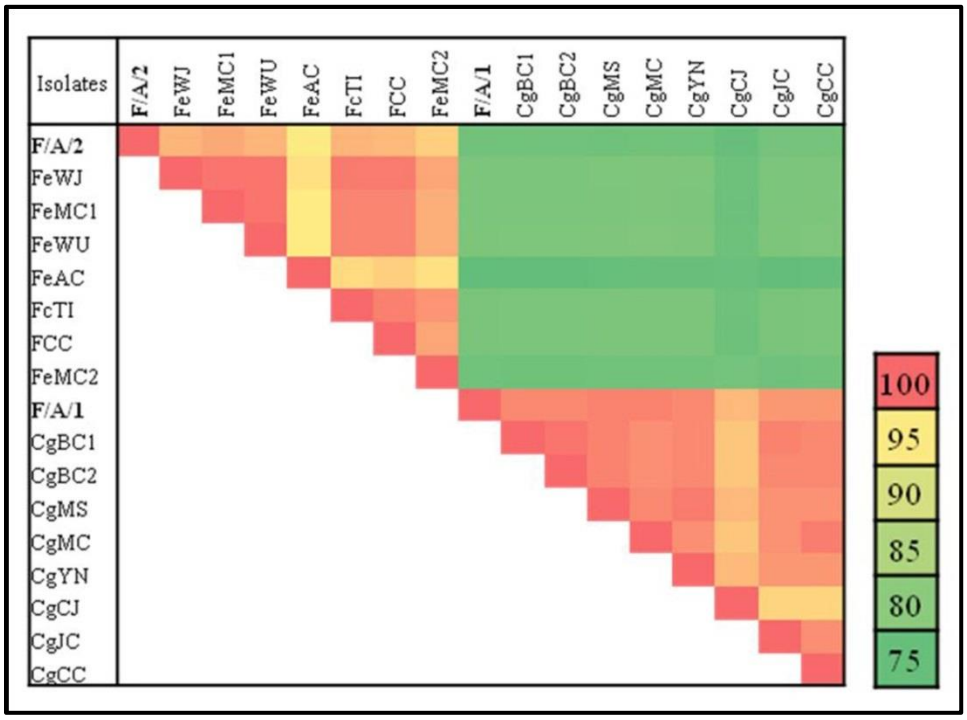

Figure 4. Nucleotide sequence identity matrix of the isolates (*C. gloeosporioides* and *Fusarium* sp.) of the present study following 18s rDNA sequence analysis with that of some other pathogenic *C. gloeosporioides* and *Fusarium* sp.

Analysis of fungal nucleotide sequence associated with fruit rot

The sequence analysis showed that the present fungi F/A/1 shared 96-100% nt identity with other fruit rot causing *Fusarium* sp., and F/A/2 shared

95-100% nt identity with other *Colletotrichium gloeosporioides* species causing fruit rot. But these two fungi shared 74-79% nt identity among each other and 79-99% nt identities with other fungi of genbank used for phylogenetic analysis in the present study (Fig 4). The phylogenetic analysis showed overall two groups amongst all the isolates analyzed (Fig.5). The isolates F/A/1 (Acc. No. KC355249), grouped into one clade (GR-1) i.e., with *Colletotrichum* spp. and F/A/2 (Acc. No. KR263845) into another clade (GR-2) i.e., *Fusarium* spp. But, in GR-1 present isolate F/A/1 pair with *Colletotrichum gloeosporioides* (Acc. No. AB981196) infecting Cinnamon (*Cinnamomum verum*) causing anthracnose in Japan with 93% bootstrap value and 97% nt identity. In clade GR-2, present isolate F/A/2 pair with *Fusarium equiseti* (Acc. No. KF515650) infecting *Avicennia marina* causing Black Stems in China with 94% bootstrap value and 95% nt identity (Fig 5).

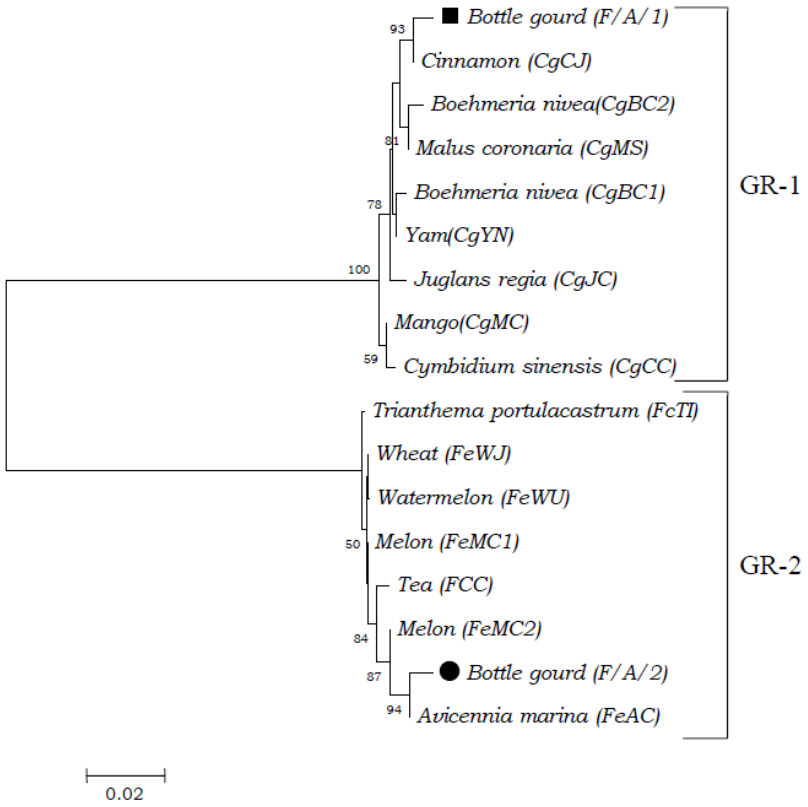

Figure 5. Phylogenetic tree was generated using neighbor joining method, showing the relationship of present fungal pathogenic isolates from bottle gourd (*C. gleosporioides* and *F. pallidoroceum*) with some other pathogenic *C. gleosporioides* and *Fusarium* sp. published in genbank.

Discussion

Fresh Fruits are rich and easy source of different types of minerals, vitamins and other essential nutritional components. Fruits also contain huge amount of water. Because of this fruits are very much prone to fungal attack. Worldwide the cultivation of different types of fruits is a major challenge due to presence of high water content. Some of such fruits are tomato, watermelon, grapes, cucumber, bottle gourd etc. Two most important fruit rot pathogens reported all over the world are *Fusarium* and *Colletotrichum* spp.

In India *Colletotrichum capsici* the casual fungus of die back/fruit rot, causes heavy losses (upto 60%) in chilli (*Capsicum annum* L.) production (Kaur *et al.*, 2011). In Serbia, four different species of *Colletotrichum* i.e *Colletotrichum gloeosporioides, Colletotrichum acutatum, Colletotrichum coccodes,* and *Colletotrichum dematium* were reported as the causal pathogens responsible the occurrence of anthracnose on tomato (Zivkovic *et al*., 2010). In Brazil various fruits like mango, guava, papaya, avocado etc. were reported to be susceptible to anthracnose caused by *Colletotrichum gloeosporioides.* In case of subtropical crops such as apple, grape, peach etc. the disease is caused by *C. acutatum* (Peres *et al.,* 2002). In 2009 Masayahit *et al.,* reported the occurrence of anthracnose on dragon fruit in Malysia caused by *Colletotrichum gloeosporioides.* In 2008 Miles and Schilder reported that *Colletotrichum acutatum* causes anthracnose fruit rot disease in blueberries which is the most common and widespread disease of blueberries in the United States. They also reported pre-harvest fruit losses of 10 to 20 percent and post-harvest losses of up to 100 percent. Zhang *et al*. in 2014 reported the occurance of anthracnose disease on *Trichosanthes kirilowii* in China, caused by *Colletotrichum gloeosporioides.* In Serbia, *Fusarium oxysporum* has been reported as the most important species of pathogen causing fruit rot and also wilt in tomato by rendering root and basal stem deterioration (Ignjatov *et al.,* 2012). Some species of *Fusarium* i.e. *Fusarium semitectum, Fusarium oxysporium, Fusarium moniliforme* (Hawa *et al*., 2010) and *Fusarium solani* (Rita *et al*., 2013) were also found to cause wilt and stem rot in dragon fruit.

In all of the above reports the identification of the fungus were done by cultural characteristics of the fungus, microscopic structural properties and molecular identification methods. For molecular identification two universal conserved primers from ribosomal DNA gene i.e ITS1 and ITS4 were used. In some cases species-specific primers derived from ITS1 and ITS4 were also used.

Bottle gourd is a major horticultural crop of sub-Himalayan West Bengal (also popularly known as North Bengal). In this area bottle gourd is

predominately grown for its fruits. In the present study it is found that bottle gourd fruits are very vulnerable to fungal attack causing fruit rot in both mature and pre-mature phases of the fruits. In the present study around 42%-59% of yield loss has been experienced.

From 23 rotten fruit samples 7 different pure fungal cultures were obtained. Identification of the fungi on the basis of morphological characteristics, as well as molecular characteristics (in some cases) was performed. All the sixteen isolates from bottle gourd fruit rot samples were subjected to pathogenicity test. Among them two fungal cultures were able to render re-occurrence of the fruit disease symptoms successfully. Thus, from this result it is evident that isolate F/A/1 and F/A/2 causing fruit rot disease in bottle gourd in the present study area (Sub-Himalayan West Bengal) is being caused either by a particular species of *Colletotrichum* (*C. gloeosporioides*) or by a particular species of *Fusarium* (*F. pallidoroceum*) or by both synergistically. As there is no report of the above mentioned two fungi as pathogen of *Lagenaria siceraria*, hence, it is also a new host report of the two fungi such as *C. gloeosporioides* and *F. pallidoroceum.*

Acknowledgement

This work was supported by the University Grants commission, New Delhi under BSR Grant (Ref No. 1585/R-2013 dated 25-03-2013) to Arnab saha.

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(Received: 3 February 2016, accepted: 28 February 2016)