Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(3): 4709-4712
Received: 27-03-2019
Accepted: 29-04-2019

S Priyadharshini
Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

A Kamalakannan
Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

V Paranidharan
Professor, Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Correspondence
V Paranidharan
Professor, Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

First characterization report of *Botrytis cinerea* infecting Tuberose in India

S Priyadharshini, A Kamalakannan and V Paranidharan

Abstract
Spot and blight (SAB) is an emerging disease infecting tuberose crop incited by *Botrytis cinerea* was newly identified in Tamil Nadu to cause heavy yield loss in tuberose production. In this study, the pathogen *Botrytis cinerea* isolates were subjected to morphological, physiological and molecular characterization. Four different isolates were isolated from tuberose growing areas of Tamil Nadu and identified as *Botrytis cinerea* on the basis of phenotype and genotype characterization. Morphologically the isolates BC1, BC2, BC3 and BC4 were observed to produce dull white, white, white and brown coloured, septate mycelium with conidiophore bearing oval to ellipsoid shaped conidia respectively on PDA medium at 20 °C. The Koch`s postulates proved the virulence and pathogenicity of all the isolates under glasshouse condition. The virulent isolates were confirmed as *Botrytis cinerea* by sequencing 18S-28S rRNA genes. The overall study was the first report on characterization of *Botrytis cinerea* infecting tuberose crop in India.

Keywords: Tuberose, *Botrytis cinerea*, morphological characterization, pathogenicity, molecular characterization

Introduction
Flowers have become an integral part of the human life. Floriculture is an emerging industry in India gaining high export potential and profitability. Among the flower crops, tuberose (*Polianthes tuberosa* L.), belongs to the member of Amaryllidaceae family originated from Mexico commonly referred to as lily in the Indian market. Tuberose occupies a selective and special position among commercial flowers due to its wide adaptability to varied climate and soil. Morocco, France, Hawaii, South Africa, India and China are the major producers of Tuberose (Ahmad *et al*., 2009) [2]. The flower is valued much by the aesthetic world for their beauty and fragrance. Due to its immense export potential, cultivation of tuberose is increasing day by day. Tuberose is prone to lot of pest and disease attacks thereby losing the market value. Among the fungal diseases, *Botrytis* spot and blight is the newly emerging disease of tuberose affecting foliar parts of the plant and in severe stages, it affects the floral parts and causing them to rot.

Botrytis is a polyphagus necrotrophic fungal pathogen. The genus *Botrytis* is highly diverse, with numerous species identified that differ in terms of their biology, ecology, morphological features and host range (Elad *et al*., 2004) [8]. Based on the extent of damage they cause, *Botrytis cinerea* ranks second most important fungal pathogen next to *Magnoporthe oryzae* (Dean *et al*., 2012) [6]. It has a wide host range covering more than 200 host plants. It can infect crops growing in temperate to sub- tropical areas. It frames its place among the most important and most destructive post-harvest pathogens. It causes damage to fruits, vegetables and ornamental crops not only in field or storage but also during transport (Jarvis, 1977) [9].

Materials and methods
Survey and isolation of *Botrytis cinerea*
A random survey was conducted in tuberose growing areas of Coimbatore and Erode districts of Tamil Nadu. Leaves exhibiting typical symptoms of spot and blight were collected in paper bags and brought to the laboratory, Tamil Nadu Agricultural University, Coimbatore. Further the samples were stored at 4 °C for further experiments.

The infected portion from the infected leaves was cut into bits along with a small portion of healthy leaf using sterile scalpel. These bits were surface sterilized with one percent sodium hypochlorite for one minute and were washed thrice with sterile distilled water and placed in Potato Dextrose Agar medium and incubated at 20 °C for 5 days. The pure cultures were obtained by single hyphal tip method after three days of incubation. The pure culture plates were incubated for about 7 to 10 days and stored at 4 °C (Zhou *et al*., 2014) [12].

Morphological characterization
Seven days old cultures were used for the phenotypic characterization of the isolated fungi. The isolates were observed for mycelial characters, colony morphology, sclerotial production and sporulation. Mycelial type, conidiophores and conidia were observed under microscope (Acero, 2006) [1].

Pathogenicity test
Pathogenicity test was carried out to prove Koch's postulate using spore suspension spray method. Culture plate was incubated at 15 °C for abundant sporulation of the pathogen. The sterile distilled water was poured into the plate having well sporulated *Botrytis* culture. The spores were collected by scrapping it with scalpel, the total number of spores were counted using haemocytometer. The spore suspension containing 4.2 x 10^4 conidia ml^{-1} were sprayed onto the healthy plant using atomizer. The inoculated plants were incubated at 20 °C and 90 % RH. The plants were incubated up to 7 to 10 days for symptom development (Dhyani *et al*., 2012) [7].

Molecular confirmation
The mycelium was collected from seven days old cultures of *Botrytis cinerea* in Potato Dextrose Broth and grounded to a fine powder using liquid nitrogen in a pestle and mortar. Genomic DNA was extracted by using CTAB method (Allen *et al*., 2006) [4]. The extracted DNA was stored at -20 °C for further use. The molecular confirmation was done by Polymerase Chain Reaction (PCR) using 18S rRNA universal primers ITS 1 (TCCGTAGCTGAACCTGCCG) and ITS 4 (TCCTCCGCTTATTGATATGC) (White *et al*., 1990) [11]. The PCR products were resolved by electrophoresis on 1.0 per cent agarose gel in 1X TAE buffer at 90 Mev for 1.5 hours. The gel was documented to identify the amplicon size of bands amplified. The amplified PCR product was sequenced by sanger dideoxy sequencing method at Barcode biosciences, Karnataka.

Results
Collection of infected samples
Survey conducted in Tuberose growing areas of Tamil Nadu revealed that disease incidence to be very high during winter and rainy season when the temperature falls below 20 °C. The occurrence of the disease was high in velliangadu areas of Coimbatore district with mean PDI of about 28 %.The symptoms appeared as water soaked elliptical spots on leaves and the severely infected leaves showed blighted appearance. In flowers the symptoms appeared as water soaked spots, covered fully with grey fuzzy growth of the fungus **(Fig 1).**

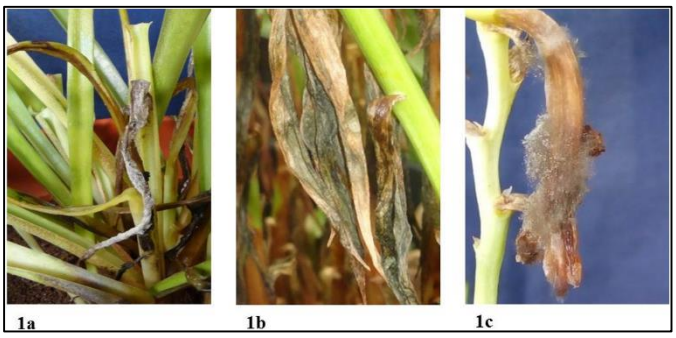


Fig 1: Symptoms of *Botrytis* spot and blight disease a) infection of pathogen on leaves b) severe infection on leaves c) Infected flower part showing typical rotting symptom

Isolation of *Botrytis* spp. from infected leaf samples
Four isolates were collected from two districts (Coimbatore and Erode) of Tamil Nadu. The isolates were named as Bc1 (Sathyamangalam), Bc2 (Bhavanisagar), Bc3 (Karamadai) and Bc4 (Velliangadu).

Morphological characterization of *Botrytis cinerea*
The microscopic observations revealed that all the four isolates exhibited significant difference in mycelial pattern with variation in their growth characters (Table 1) and (Fig 2). The observation on sclerotial production depicted that the sclerotia were produced when the when the fully grown cultures were exposed to stress condition when incubated at the temperature of about 35 °C for about five to six days. (Table 2) and (Fig 3). Histopathological characters were also observed from the seven days old cultures of all the four isolates (Fig 4).

Table 1: Mycelial characters and colony morphology of *Botrytis cinerea* isolated from two districts of Tamil Nadu

Isolates	Colour of the colony	Mycelium type	Growth characters	Sporulation
Bc1	Dull white	Irregular warty mycelium	Covers 9cm plate in 10 days	–
Bc2	White	Fluffy mycelium	Covers full plate in 7 days	+
Bc3	White	Concentric radial mycelium	Covers full plate in 6 days	+
Bc4	Brown	Powdery mycelium	Covers full plate in 5 days	+

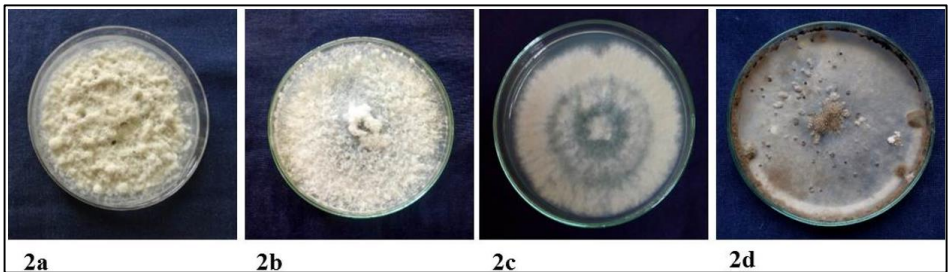


Fig 2: Mycelial characters and colony morphology of four isolates of *Botrytis cinerea*. a) Bc1 b) Bc2 c) Bc3 & d) Bc4

Table 2: Sclerotial pattern of *Botrytis cinerea* isolates

Isolates	Sclerotial type	Numbers per petri plate	Size
Bc1	Bulged sclerotia embedded inside mycelium	10- 15	Large
Bc2	Minute small sclerotia on the surface formed all over the plate	30- 40	Small
Bc3	Prominent black sclerotia at center	8- 10	Medium
Bc4	Irregular sclerotia formed random all over the plate	15 – 25	Medium

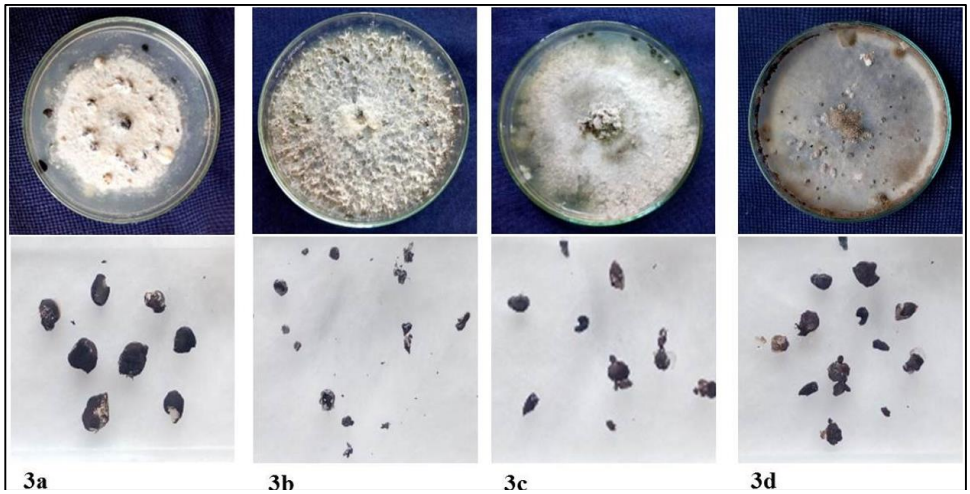


Fig 3: Sclerotial pattern of *Botrytis cinerea* isolates a) Bc1, b) Bc2, c) Bc 3 & d) Bc4

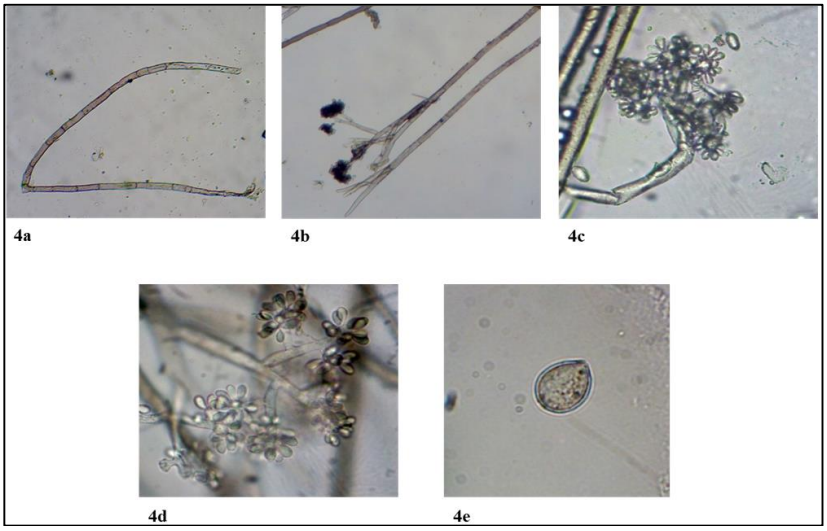


Fig 4: Histopathological observations of *Botrytis cinerea* a) Hyaline to brown coloured septate mycelium, b) Conidiophore dichotomously branched bearing conidia, c-d) Grapes bunch like conidia, e) Individual conidia ellipsoid to oval with hilum at the tip

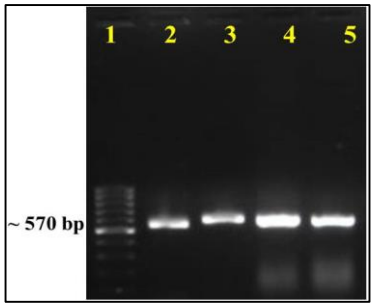
Pathogenicity test

The pathogenicity test in BOD chamber under *in vitro* condition revealed that at 7th day of inoculation, the symptoms appeared as water soaked spots on the leaves and in later stages the leaves were covered by fuzzy mycelium. Later at 10th day, flowers too got infected and covered with grey fuzzy mycelium. Finally the flowers got rotten and dead (Fig 5). The pathogen was re-isolated from the symptomatized plant parts expressing typical symptoms and the characters of the isolated pathogen was similar to the inoculated pathogen.

Fig 5: Pathogenicity test of *Botrytis cinerea* in tuberose. a) Pathogenicity of inoculated leaf b) Pathogenicity of the inoculated flower c) Complete rotting of flowers covered with grey mycelium

Molecular Characterization of *Botrytis cinerea* using ITS 1 and ITS 4 primers:

The pathogen was characterized molecularly by using ITS 1 and ITS 4 primers amplifying 18S-28S rRNA gene fragment of *B. cinerea* at 570 bp (approx), when visualized under UV light and the same was documented (Fig 6). The fragments of all the four isolates were was sequenced and submitted in NCBI database. The nucleotide sequence of 18S-28S rRNA gene shows 99.80 per cent similarity with *B. cinerea* strain submitted in NCBI (KT224806).



Lane1: 100 bp ladder, Lane2: BC1,
Lane3: BC2, Lane4: BC3 Lane5: BC4

Fig 6: PCR amplification of 18S-28S rRNA gene of *B. cinerea*

Discussion

During survey, four different isolates were isolated from tuberose growing areas of Coimbatore and Erode districts. The higher incidence of disease in winter season is correlated with high relative humidity 95 % and low temperature 20 °C favorable for the growth of pathogen. The pathogen was

isolated in PDA medium from the leaves exhibiting typical symptoms. The culture was purified by single spore isolation technique. The pathogen initially produced white coloured mycelium, later turned greyish brown. The hyphae were hyaline to brown coloured, septate in nature. Conidiophores of *Botrytis cinerea* arises from the hyphal mass, producing alternate branches. At the terminal end, conidia were in clusters which appeared as grapes bunch. The individual conidium was hyaline, oval to ellipsoid or globose in shape, having hilum at the tip. These characteristics were in accordance with the microscopic observation of *Botrytis cinerea* by Acero (2006) [1] where the pathogen produced conidiophores that branched alternatively producing grape bunch like oval shaped conidia. Similar findings were observed by Ahmed *et al*., (2007) [3] isolated *Botrytis cinerea* pathogen causing grey mold in chickpea plant. The pathogenicity test revealed that the infection takes place when the spore suspension was sprayed on the plants maintained in growth chamber at 20 °C with relative humidity of about 95 per cent for about 4 to 5 days. The pathogenicity test revealed that the isolate B4 was virulent among all the four isolates under temperature of 20 °C and relative humidity of about 90 per cent. The results were similar to that of the results obtained by Dhyani *et al*., (2012) [7]. They reported appearance of symptoms takes place, when the conidial suspension of *Botrytis* pathogen was sprayed on to the lilium plants and were kept in humidity chamber at 20 °C for 2 to 3 days. They also re-isolated the pathogen from the inoculated disease expressing plants. Similar findings were also obtained by Borges *et al.,* 2014 [5] while evaluating the effect of grey mold infection on age and height of tomato plant. The conidial suspension of pathogen were used to cause infection in plants.

For molecular characterization, the universal primers ITS 1 and ITS 4 were used to amplify 18S-28S rRNA of *Botrytis cinerea*. The amplicon size of about 570 bp were observed when the PCR products were visualized under UV in 1.2 per cent agarose gel. Similar ITS 1 and ITS 4 primer pairs were used by Kamaruzzaman *et al*., (2017) [10] for characterization of *Botrytis cinerea* isolated from cucumber plant. They also used the universal primers ITS 1 and ITS 4 primer pairs for amplification of 18S-28S rRNA gene fragment of *B. cinerea* in cucumber crop. Thus the study aims in overall characterization of *Botrytis* pathogen infecting tuberose in India, which helps in further management of disease in future prospects.

References

1. Acero FJF. Application of proteomics to the characterization of mechanisms pathogenicity in *Botrytis cinerea*. Use and evaluation of new fungicides. Ph.D thesis 2006, The University of Cadiz, Spain.
2. Ahmad I, Ahmad T, Asif M, Saleem M, Akram A. Effect of bulb size on growth, flowering and bulbils production of tuberose. Sar. J Agric. 2009; 25(3):391-397.
3. Ahmed AU, Pande S, Basandrai AK, Kishore GK, Rao JN. Variation in isolates of *Botrytis cinerea* causing Botrytis gray mold in chickpea. Ban. J Agril. Res. 2007; 32(1):135-143.
4. Allen G, Flores-Vergara M, Krasynanski S, Kumar S, Thompson WA. Modified protocol for rapid DNA isolation from plant tissues using cetyl trimethyl ammonium bromide. Nature Protocols. 2006; 1:2320-2325.
5. Borges AV, Saraiva RM, Maffia LA. Key factors to inoculate *Botrytis cinerea* in tomato plants. Summa Phytopathologica. 2014; 40(3):221-225.
6. Dean R, Vankan JA, Pretorius ZA, Hammond-kosack KE, Di pietro A, Spanu PD *et al*. The Top 10 fungal pathogens in molecular plant pathology. Molecular Plant Pathology. 2012; 13:414-430.
7. Dhyani A, Nautiyal BP, Nautiyal MC, Rivera MC, Prasad D, KP Singh. First report of *Botrytis cinerea* on *Lilium polyphyllum*, a critically endangered herb in Uttarakhand, India. Int. J of Exp. Bot. 2012; 81:157-159.
8. Elad Y, Williamson B, Tudzynski P, Delen N. *Botrytis* biology, pathology and control. Kluwer Academic Publishers, Dordrecht (Springer). 2004, 416.
9. Jarvis WR. *Botryotinia* and *Botrytis* species: Taxonomy, Physiology and Pathogenicity. A Guide to the Literature 1977, Monograph No. 15, Canada Department of Agriculture, Ottawa, Canada.
10. Kamaruzzaman M, Bhuiyan AA, Faruque MO. Isolation and molecular characterization of a wild type *B. cinerea* from infected bottle gourd (*Lagenaria sicerari*) in China. J of Adv. in Microbiol. 2018; 7:1-10.
11. White TJ, Bruns T, Lee S, Taylor T. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, In: PCr Protocols: a Guide to Methods and Applications, Academic Press, San Diego.1990, 315-322.
12. Zhou YJ, Zhang J. Morphological and phylogenetic identification of *Botrytis sinoviticola,* a novel cryptic species causing gray mold disease of table grapes (*Vitis vinifera*) in China. Mycologia. 2014, 13-32.