Mycopath (2017) 15(2): 79-85 **Research Article**

Geographical distribution and morpho-molecular characterization of pre-harvest gray mold of strawberry in Punjab, Pakistan

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Abstract

In Pakistan, strawberry (*Fragaria ananassa* Duch.) is emerging as an economically important commercial fruit crop. Gray mold or *Botrytis* fruit rot of strawberry is widely distributed and economically important disease infecting the strawberry fruit at both pre- and post-harvest stages. To assess the percent disease prevalence and incidence, a comprehensive survey was carried out in 8 strawberry growing districts of Punjab *viz.* Rawalpindi, Sargodha, Gujranwala, Sialkot, Narowal, Sheikhupura, Lahore and Multan during crop seasons of 2014-15 and 2015-16. Disease prevalence and incidence ranges were found to be 87.5%-100% and 29.3%-48.13%, respectively in the studied areas. The highest disease incidence was recorded in Narowal district (48.13%) followed by Gujranwala (46.36%) and Sialkot (45.97%), whereas the lowest was seen in Rawalpindi (29.3%). Characterization of 61 isolates recovered from the study areas revealed that pure colonies of the fungus were cottony white to grayish in color. Fungus produced obovoid or ellipsoid conidia with 11-16 × 5-8 µm size and conidiophores ranged from 735 to 2805 µm along with production of dark brown to black sclerotia measured 2-7 mm. A total of 8 representative and highly virulent isolates one from each district were subjected to DNA amplified using inter transcribed spacer regions ITS (ITS1, 5.8S, ITS2) and submitted to GenBank database of NCBI with accession numbers MF959755 to MF959762, respectively. Sequences revealed 99 to 100% genetic similarity with *Botrytis cinerea* reference isolates. Phylogenetic analysis was done by constructing evolutionary tree with previously published sequences of *B. cinerea* in NCBI from different parts of world. Analysis of evolutionary tree highlighted three diverse sub trees. This is the first ever detailed study of its kind to know the status of the disease along with its distribution and characterization on this relatively new crop in Punjab, Pakistan.

Keywords: *Botrytis cinerea*, Gray mold, Strawberry, Incidence, Morpho-molecular characterization, Prevalence, Punjab, Pakistan

Introduction

Strawberry is one of the most widely grown small fruit crops in the world. Strawberries are rich in B-complex group, Vitamin C, minerals like potassium, manganese, copper, iron and iodine and serves as good source of dietary fibers and antioxidants (Basu *et al.,* 2014). Its world production has exceeded 8 million tons in 2016, where USA produces about 27% of the total production (FAO, 2016). It is an emerging fruit crop in Pakistan cultivated on approximately 1500 hectares (Mehmood *et al.,* 2017). *Botrytis cinerea* Pers. (teleomorph: *Botryotinia fuckeliana*) is a phytopathogenic fungus which causes gray mold/*Botrytis* fruit rot (BFR) on many economically important vegetable and fruit crops (Beever and Weeds, 2004). The disease causes severe pre-harvest and post-harvest losses. Attack of *B. cinerea* results up to 100% fruit yield loss. Worldwide, it causes annual losses of $10 billion to $100 billion (Boddy, 2016). The disease affects fruit in the field resulting in severe pre-harvest losses by causing rotting in green and red mature fruit which reduces crop yield and cause severe economic losses (Sutton, 1998; Williamson *et al.,* 2007). Typical symptoms on strawberry fruits were recorded as the affected tissue was covered with a thin layer of grayish white to dark gray mycelium, which later became fuzzy and rot enveloped the entire fruit. Prevalence of disease followed by high incidence results in direct losses in terms of fruit rotting.

Botrytis genus classification is largely based on cultural and morphological characteristics (Hennebert, 1973). Morphological characters are important aspects to determine the biology of pathogens but variability in growth, spore size and shape and pathogenicity has been reported in genus Botrytis. These features help us in recognition of *Botrytis* species and Staats *et al.* (2007) showed that molecular studies confirm traditional identification method. Characteristics such as colony color, texture, conidium size etc. are useful in describing some species (Beever and Weeds, 2004). Morphological identification is not highly accurate and to reduce chances of ambiguity, Polymerase

Chain Reaction (PCR) assay and nucleotide sequences of Internal Transcribed Spacers (ITS) region has been developed (Baffi *et al.*, 2012). Recently, DNA-based number of molecular approaches have been used widely to identify fungi, including *Botrytis* species.

Plenty of information regarding prevalence and incidence of the disease in various strawberry producing countries of the world is available but no data is there about Pakistan, Therefore, the aim of this study was to determine the disease prevalence and incidence during pre-harvest stage by conducting first ever extensive survey in Punjab and identifying the pathogen using cultural and morpho-molecular tools. This study will provide basis for the development of integrated disease management strategies in future.

Materials and Methods

Survey and collection of diseased samples

During the crop years 2014-15 and 2015-2016, a survey was conducted to collected rotted strawberry fruits exhibiting gray mold symptoms from 61 fields across eight important strawberry producing districts (Rawalpindi, Sargodha, Gujranwala, Sialkot, Narowal, Sheikhupura, Lahore and Multan) of Punjab province (31.1704° N, 72.7097° E) as shown in Figure 1. Prevalence and Incidence percentages were determined by using following formulae**.**

$$\text{Disease Prevalence Percentage} = \frac{\textit{Locations showing disese}}{\textit{Total locations examined}} \times 100$$

$$\text{Disease Incidence Percentage} = \frac{\textit{Number of infected plants}}{\textit{Total plants examined}} \times 100$$

Isolation, Cultural and Morphological Identification

Portions of diseased tissue from strawberry fruit were excised and subjected to sterilization with 2% sodium hypochlorite. Washing with sterile distilled water was done to remove excessive sodium hypochlorite and pieces were dried on double layer of sterilized filter papers. The pieces were placed on petri dishes having artificial growth media and incubated for 5-7 days at 25 °C ± 2 °C. The isolates were purified by using hyphal tip method (Tutte, 1969). Cultural and morphological characteristics *i.e.*, colony color, texture, shape and size of conidiophore were recorded after 5-7 days of incubation.

Pathogenicity tests

For pathogenicity tests, spore suspension of 10^5 spores/ml was adjusted by using haemocytometer and surface sterilized healthy strawberry fruits were inoculated with suspension. Fruits inoculated with sterile distilled water served as negative control. Sterile Petri dishes were aligned with wet blotter paper and inoculated fruits were transferred to on them. Petri dishes were incubated at 25± 2 °C. Re-isolation of pathogen was carried out and compared with original inoculum to fulfilled Koch's postulates (Johnston and Booth, 1983).

Molecular characterization

Eight highly virulent and representative fungal isolates were subjected to molecular characterization. Total genomic DNA was extracted by using PrepMan DNA extraction kit. Molecular diversity was studied through PCR technique. Two primers, ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') were used to amplify the internal transcribed spacer (ITS) regions of fungal pathogen (White *et al.*, 1990). The PCR test was done in 50 µL reaction comprising of 1 x PCR buffer, 0.2 mM of dNTPs, 5units of Taq polymerase, 20 ng of template DNA, 10pmols of each primer (ITS1 and ITS4) and 1.5 mM of $MgCl_2$. Amplification of both primers was carried out at 94 °C for 5 min for initial denaturation, followed by 30 cycles of denature at 94 °C, annealing at 56 °C and extension at 72 °C for 2 min with a final extension at 72 °C for 10 min. Two percent (w/v) agarose gel was used to run and visualize PCR products.

Nucleotide sequencing and sequence analysis

Amplified and positive PCR products after gel isolation were subjected to purification by following standard protocols of Gene JET PCR purification kit. Purified products were sequenced form DNA facility Iowa State University, USA. Obtained sequences were aligned with the help of BioEdit software. Final sequences were blasted on NCBI BLAST database and submitted in GenBank database of NCBI to get accession numbers. For construction of evolutionary tree, already published ITS sequences of *B. cinerea* from different countries of the world were taken from NCBI public database. Maximum composite likelihood method were used to compute evolutionary distances with the help of Molecular Evolutionary for Genetic Analysis (MEGA) version 6.0 (Tamura *et al.*, 2004).

Results

Geographical distribution and disease assessment

B. cinerea is a cosmopolitan plant pathogen with very broad host range. The fungus causes disease particularly under moist weather and optimum temperature conditions Gray mold appeared both on green as well as ripened fruits as shown in Fig. 2 a & b. Disease was recorded in the all of the survey districts and locations at differential ranges. The data suggested that overall disease prevalence had very high ranges from 87.5% to 100% that signifies gray mold infection on

strawberry as shown in Fig. 3. Maximum disease prevalence (100%) was recorded during crop season 2014-15 and 2015-16 in all surveyed districts. Maximum prevalence (100%) was recorded in Rawalpindi, Narowal, Lahore, Sheikhpura, Multan and Islamabad, whereas 87.50% was in Gujranwala. Minimum prevalence of 75% was recorded in both Sargodha and Sialkot districts.

Data on disease incidence was taken from each surveyed locations in each district (Fig. 4). From various locations visited across Punjab, Narowal showed the highest percentage of disease incidence ranging from 47.38 % to 48.88% during 2014-15 and 2015-16 respectively with an average of 48.13% followed by Gujranwala (47% and 45.71% with average of 46.36%), Sialkot (43.33% and 48.60% with average of 45.97%), Sargodha (45% and 46.36% with average of 44.10%). The minimum percentage of disease incidence was seen in Rawalpindi, ranging from 28.60% and 30% with an average of 29.30%. All other districts of Punjab showed the incidence of gray mold from 41.69% to 43.75% (Fig. 4).

Cultural and morphological characterization

From total recovered isolates, 61 were identified positive during pathogenic testing. All 61 isolates were subjected to cultural and morphological characterization. Fungal colonies on media were found to be cloudy white and grayish in color with abundant conidia and conidiophores were produced after 5-7 days of incubation. Conidiophores with an inflated basal cell colored hyaline to pale brown and measuring 730-2805 µm in length. The conidia were sub-hyaline, pale brown and light brown in color having ellipsoid or ovoid shape. Conidia measured 11–16×5-8 µm. After about 10-15 days, the fungus formed numerous hard, irregular, dark brown to black sclerotia ranging from 2-7 mm. Reading were average of 5 structures per isolate. The morphological characteristics of the identified isolates are summarized in Table 1.

Nucleotide sequence analysis

Nucleotide sequences of eight fungal isolates were submitted and accession numbers obtained respective to each one as shown in Table 2. All studied isolates were shared maximum 99-100% genetic homology. For accurate confirmation of pathogen at species level, reference sequences of *B. cinerea* reported from different regions of world were compared with isolated from current study. Evolutionary tree computed of *B. cinerea* along with reference isolates clearly indicates three different sub-trees, where *B. sinoallii* was used as outgroup. The ITS sequences of isolates shared genetic homology in sequences of ITS regions. Due to maximum genetic homology within the Botrytis specie, each sub-tree comprised of different isolates reported form different regions of the world. The sub-trees were comprised of same specie. Sub-tree one constitutes of five isolates *viz.* BRRP7, BRSG18, and BRMT68 that showed homology with reference isolates, CAU831, IPBCC.16.1358 and MHGNU F114 respectively. Similar clustering was found in sub-tree three, whereas in sub-tree two, both isolates (BRSP57 & BRLH60) from current study showed high genetic homology among them. Isolates form current study although showed varation among them due to differnces in nucleotides that results in clustring in different under sub-tress (Fig. 4).

Discussion

Gray mold showed significant varation in terms of prevalence and incidence in the study areas. Disease causal agent *B. cinerea* has broad host range and presence of more than one host at a same time resulted in high inoculum. Highest disease incidence recorded in Narowal and Gujranwala Districts may be attributed to occurrence of high average temperature in these districts during the period of crop survey (25 °C to 34 °C), accompanied by high relative humidity (>70%) resulted by rain spells and cloudy weather which may favors disease development. The least disease incidence (29.30 %) was observed in Rawalpindi which might be associated with low average temperature during the survey season *i.e.* 20 to 26 °C. Many previous research studies from different parts of world were found to be in accordance with current study. Study conducted by Ellis and Grove (1982) and Card (2005) indicated that pre-harvest yield losses could exceed 50% when environmental conditions were favorable for disease development and could be up to 90% in severe cases. According to Paulus *et al.* (1990) the incidence of grey mold, has been linked to both i.e. temperature and high humidity favors disease condition in the field. Similarly, Braun and Sutton (1988) and Jarvis (1964) indicated that high humidity and temperature induced maximum disease incidence. In wet seasons on unsprayed plants, 80-90% losses of flowers and fruit can occur. Association amongst temperature variation, rainfall intensity, surveying time, cropping pattern and the dispersal of inoculum are potential reasons for different levels of disease severity (Dodd *et al.*, 1992). In our study it was observed that high temperature along with random rainfalls during the growing season led to the high incidence levels in Narowal, Gujranwala and Sialkot.

In this study, isolates of *B. cinerea* showed variation in terms of cultural and morphological aspects. These differences were found to be due to geographical distribution of the studies isolates. A number of previous studies also used cultural and morphological characters for tentative identification of *B. cinerea*, whereas these characters were seen to be closely related to those described by earlier workers (Ellis and Waller 1974; Jarvis, 1977; Yu *et*

al., 2011; Ortuno *et al.*, 2011).

Hoist-Jensen *et al.* (1998) reported that ITS sequences are useful in separating *Botrytis* from the other genera in Sclerotiniaceae. The ITS sequence of all eight isolates showed shared genetic similarity with *B. cinerea* and observed to have diversity among isolates in nucleotide sequences of ITS regions that's leads to be the main reasons of generation of three different sub-trees. The sub-trees were comprised of single *Botrytis* specie and exhibiting maximum 99-100% genetic homology reference isolate. The deviation in nucleotide sequences may be credited for the diverse geographical distribution of these isolates. In this study, cultural and morphological characterization of the fungal isolates as *B. cinerea* were also confirmed by using molecular tools. Correct identification of the pathogen causing postharvest disease is central to selecting an appropriate disease control strategy. Important genera of anamorphic postharvest pathogens like *Penicillium*, *Aspergillus*, *Botrytis* and *Fusarium* etc. According to a review, *B. cinerea* ranked second in a list of the top 10 fungal plant pathogens based on scientific and economic importance worldwide (Dean *et al.*, 2012).

Mostly small farmers are engaged in strawberry production of having higher revenue for better livelihood. The presence of gray mold is currently a dilemma to small scale farmers possibly due to improper attention paid by them to effectively manage this disease and unexpected change in weather conditions which demands alternate environment friendly integrated disease management strategies.

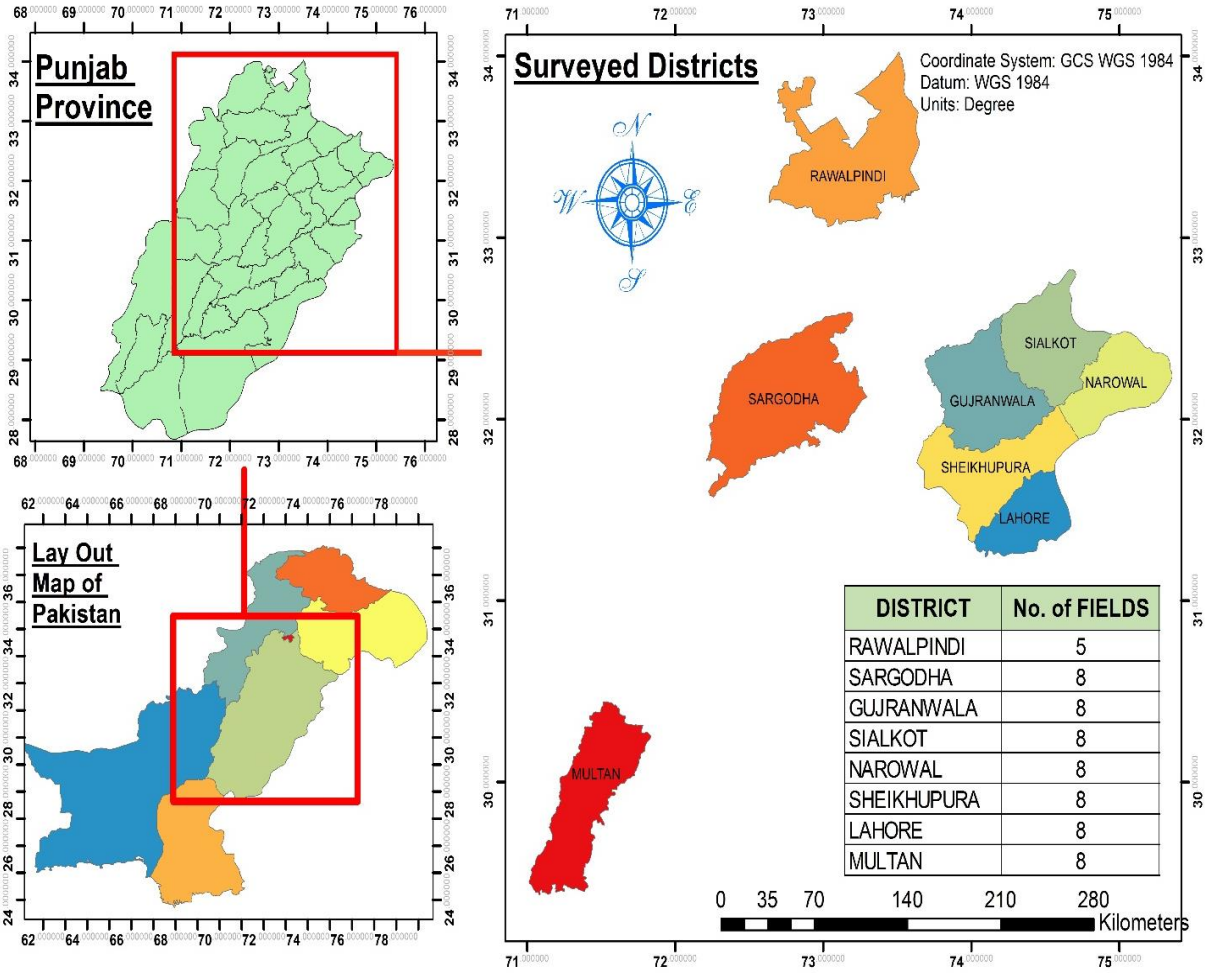

Fig. 1. Surveyed districts across Punjab for gray mold of strawberry during 2014-15 and 2015-16.

(a) (b)

Fig. 2. (a) Gray mold infection on green fruit, (b) Gray mold infection on mature fruit.

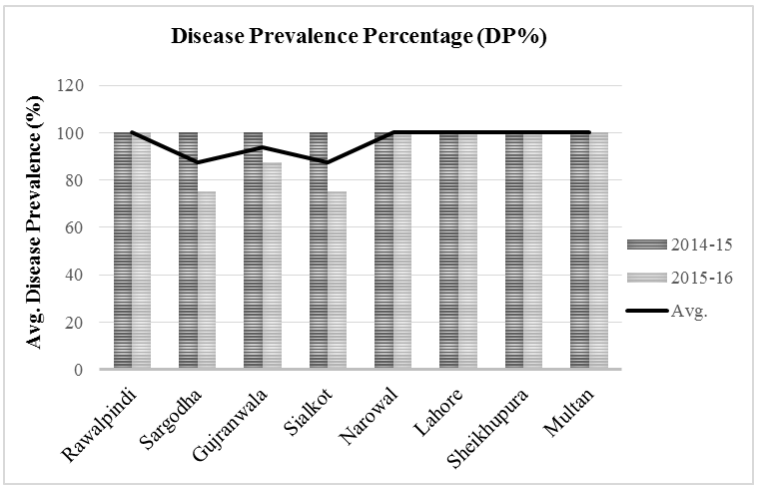

Fig. 3. Disease Incidence Percentage of Gray mold/ *Botrytis* Fruit Rot in the Punjab during crop season 2014 15 and 2015-16 (Black line shown average of both years).

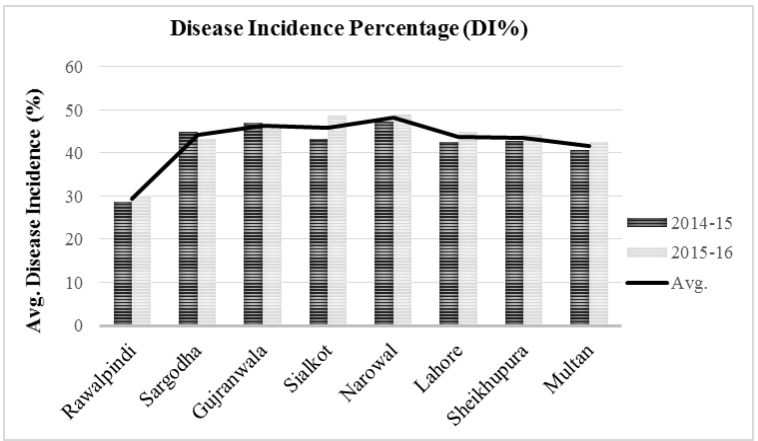

Fig. 4. Disease Incidence Percentage of Gray mold/ *Botrytis* Fruit Rot in the Punjab during crop season 2014-15 and 2015-16 (Black line shown average of both years).

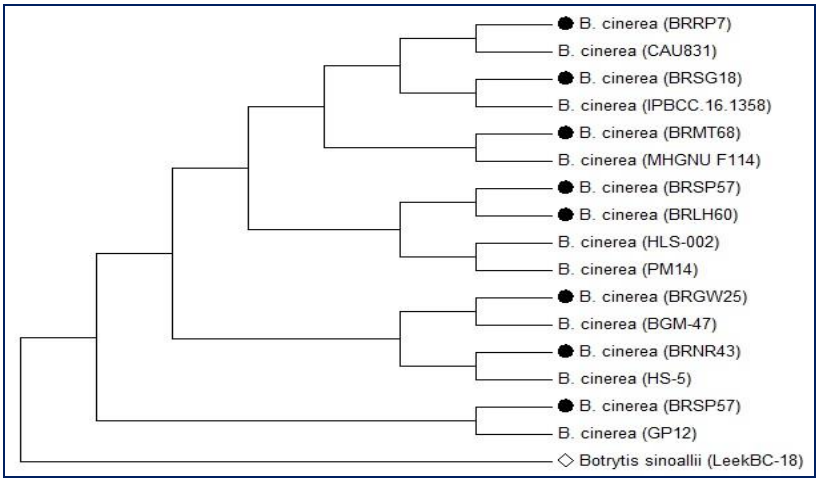

Fig. 4. Phylogenetic analysis of *Botrytis cinerea* isolates using Maximum Likelihood method based on ITS-5.8S rDNA (Isolates obtained from this study were marked with black circles, all other were reference isolates)

Table 1: Cultural and morphological characteristics of the gray mold fungus isolated from strawberry.

Characteristics		Current study Isolates	*B. cinerea*[1]	*B. cinerea*[2]
Colony	Color	Cloudy white or grayish	Gray to grayish brown	White to grayish brown
Conidia	Shape	Obovoid or Ellipsoid	Ellipsoid or ovoid	Lemon or ovoid
	Size (µm)	11-16 × 5-8	6.8-18.0 × 4-10	4.9-15.0 × 7.0-9.0
	Color	Light to pale brown, Sub-hyaline	Pale brown	Colorless to Light brown
Conidiophore	Size	730-2805	315-500	200-600
Sclerotia	Shape	Flat or Irregular	Flat or Irregular	Irregular
	Size (mm)	2-7mm	-	2-6.0
	Color	Dark brown or black	Black	Black-brown

Described by Ellis and Waller (1974); described by Yu *et al.* (2011).

Table 2: Details of *B. cinerea* isolates used in the phylogenetic analysis.

Isolates	District	Host	NCBI ID	Accession no.	Reference accession no.
BRRP7	Rawalpindi	Strawberry	NAS-BC1	MF959755	KX061437
BRSG18	Sargodha	Strawberry	NAS-BC2	MF959756	KF533013
BRGW25	Gujranwala	Strawberry	NAS-BC3	MF959757	KX783612
BRSK32	Sialkot	Strawberry	NAS-BC4	MF959758	MF925713
BRNR43	Narowal	Strawberry	NAS-BC5	MF959759	KT266232
BRSP57	Sheikhupura	Strawberry	NAS-BC6	MF959760	KX766413
BRLH60	Lahore	Strawberry	NAS-BC7	MF959761	KJ744343
BRMT68	Multan	Strawberry	NAS-BC8	MF959762	KU234690

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