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MOLECULAR IDENTIFICATION AND PATHOGENIC POTENTIAL OF *BOTRYTIS CINEREA* ISOLATES CAUSING FRUIT BLIGHT OF CUCUMBER UNDER PROTECTIVE GREENHOUSE IN EGYPT

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Abstract

Survey of fruits blight (grey mould disease) on fruits of cucumber plants cultivations during growing winter season 2016 - 2017 in protective plastic houses of some Governorates in Egypt indicated that fruits blight is the most epidemic foliar disease of cucumber. *Botrytis cinerea* was the common fungi of diseased fruits at El-Beheira followed by El- Gharbeia Governorates respectively. Pathogenicity test of fungal isolates revealed that *Botrytis cinerea* isolates (No. 5 and 2) respectively which isolated from El- Gharbeia caused highly incidence of fruit rot of cucumber followed by isolate (No.7) from E Beheira Governorate. Fungal isolates were identified as *Botrytis cinerea* according to cultural, morphological and molecular characterizations based on sequencing of internal transcribed space (ITS). Some nucleotides sequences were registries of fungi in Gene Bank under accession number of MF996362, MF996363, MF996364, MF996365, MF996366, MF996367 and MF996368.

Key words: Molecular characterization, *Botrytis cinerea*, fruit blight, cucumber

Introduction

Cucumber plants (*Cucumis sativus* L.) is an important economic vegetable and one of the most popular members of the Cucurbitaceae family (Lower and Edwards, 1986). Most cucumber fruits production in plastic greenhouses conditions are attacking by various foliar and soil borne fungal diseases, *i.e*., *Fusarium oxysporum*, *Sphaerotheca fuliginea, Alternaria alternata, Cladosporium tenuissimum, F. solani, Aspergillus* spp., *Curvularia* sp. and *Bipolaris* sp. (Farrag *et al*., 2007; Al-Sadi *et al*., 2011; Sani *et al*., 2015; Ziedan and Saad , 2016). Grey mold (*Botrytis* blight) caused by *Botrytis cinerea* Pers. (teleomorph: *Botryotinia fuckeliana*) is an airborne plant pathogen and wide spread in greenhouses worldwide (Williamson *et al*., 2007), and considered a very common and serious disease to cucumber in greenhouses due to suitable weather conditions, where high humidity is prevalent (Davidson *et al.,* 2004; Elad *et al.,* 2004 and 2016). It has a global distribution and wide host range of more than 200 plant species worldwide (Hahn *et al.,* 2014). Under favourable conditions grey mould symptom of *Botrytis* infection on cucumber fruits develop quickly in a greenhouse as aggressive grey rot on cucumber and other fruits causing problem to growth (Blancard *et al.,* 2005; An and Ma 2005-2006; Soliman, *et al.,* 2015 and Elad *et al.*, 2016). Molecular biology has offered a number of insights into the detection and enumeration of fungal pathogens and information on identifying unknown species from their DNA sequences. rRNA genes are attractive and a rapid assay and accurate identification of fungal pathogens can be important for initiating treatment in the earliest stages of infection and for guiding antifungal therapy (Khot *et al.*, 2009). The most molecular protocols used in the identification of fungal species are based on the variability of the ribosomal genes 5.8S, 18S and 26S rRNA (Cai *et al*., 1996; James *et al*., 1996; Kurtzman, 1992; Khot *et al.,* 2009 and Schoch *et al*., 2012). The interest in ribosomal genes for species identification comes from the concerted fashion in which they evolve showing a low intraspecific polymorphism and a high interspecific variability (Li, 1997). Previous results have demonstrated

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that the complex ITS regions (non-coding and variable) and the 5.8S rDNA gene (coding and conserved) are useful in measuring close fungus phylogenetic relationships, since they exhibit far greater interspecific differences than the 18S and 26S rDNA genes (Cai *et al*., 1996; James *et al.*, 1996 and Kurtzman 1992). The internal transcribed spacer (ITS) has been used innumerous systematic studies at genus and species levels of a wide array of plant taxa (Sang *et al.*, 1995 and Alice and Campbell, 1999). ITS-1 and ITS-2 are two internal spacers which locate between genes encoding the 18, 5.8 and 28S nuclear ribosomal RNA (nrRNA) subunits. In addition, the 5.8s nrRNA are referred as nrDNA ITS region (Baldwin, 1992). This investigation was initiated to survey and epidemiology of grey mould of cucumber fruits in growing greenhouse cultivation. Also, identification of pathogenic fungi causing grey mould by molecular methods based on sequencing of internal transcribed space (ITS1) and 5.8S rDNA regions.

Materials and Methods

Incidence and severity of fruit blight on cucumber

Survey of fruit blight of cucumber was performed in different famous cultivations within greenhouse of Ministry of Agriculture Station in Dokki, El-Giza Governorate, Sakha Station in Kafr El-Shiehk Governorate, Salah El-Deen village, Badr Distract in El-Beheira Governorate, Kafr El-Abida village, and El-Mehalla El-Kobra District in El- Gharbeia Governorate, Egypt. Diseases incidence and their severity were determined 1, 2 and 3 months after cultivation on cucumber fruits using the following formula:

$$\text{Disease incidence} = \frac{\text{No. of infected plants}}{\text{Total No. of plant assessed}} \times 100$$

Disease severity was assessment as a percentage of rotten tissue of cucumber fruits using linear scale from 0 to 4 (Zoeir *et al*., 2017) as follows:

0 = healthy fruits

1=1-25% soften rot of fruit

2=26-50% soften rot of fruit

3= 51-75% soften rot of fruit

4= 76- 100% soften rot of fruit

2.2. Isolation and identification of fungi.

Samples of diseased tissue of rotten fruits were washed thoroughly with tap water several times cut into small pieces then rinsed with 5% Clorox (Sodium hypochlorite) for 3 minutes and cultured on potato dextrose agar (PDA) medium. Five specimens of rot tissue will rotten tissue were placed on (PDA) medium in each Petri dish. Plates incubated at 25°C for five days according (Zoeir *et al.*, 2017). Different fungal colonies morphological identified after purification using single spore isolation technique according to (Ellis 1971; Jarvis 1977 and Barnett and Hunter, 1998). Frequency of isolated fungi was recorded using the following formula:

$$\text{Frequ. occurrence \%} = \frac{\text{No. of colonies of the genera or Spp.}}{\text{No. of colonies of the genera or Spp.}} \times 100$$

Pathogenicity test of fungi on cucumber fruits

The isolate fungi were tested for their ability to induce rot in healthy cucumber fruits. Fruits of cucumber (Cv. Golden) of greenhouse cultivation were washed with tap water several time and sterilized in sodium hypochlorite (1%) for 1 minute then dried under sterilized conditions. Ten fruits were sprayed with conidial spore suspension of 10^4/ml^{-1} obtained from 20 days old PDA cultures of each fungal isolate tested. Ten healthy cucumber fruits sprayed with sterilized water were served as a control according to (Zoeir *et al.,* 2017). Cucumber fruits were incubated at 25°C for 20 days. After symptoms appeared reisolaton were made from infected tissues using the technique described previously and the morphological and cultural characteristics of the pathogen were compared with the original fungus. Percentage of rotten fruits and disease severity were determined by each fungal isolates as mentioned before.

Molecular identification of *Botrytis* strains

Isolation and extraction of DNA.

DNA was extracted from 25 mg of the harvested culture mycelia of *Botrytis* spp.*,* which was frozen in liquid N2 and ground in a mortar, according to the protocol recommended for the DNA tissue purification mini kit (Qiagene) according to Abd Murad *et al.* (2016). The genomic DNA was checked by agarose gel electrophoresis and the concentrations of the purified total genomic DNA were determined with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and stored at -20°C for further use.

Molecular characterization using ITS primer

Molecular identification of *Botrytis* spp. cultures was carried out based on conserved ribosomal internal transcribed spacer (ITS) region, between the small nuclear 18S rDNA and large nuclear 28S rDNA, including 5.8S rDNA using universal primer pairs ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS 5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') Geiser (2003). Amplification was performed on a Thermal Cycler (Bio-Rad T100) with 25 µl reaction mixtures containing 2.5 µl

of 10X buffer (10 mM Tris– HCl, pH8.8); 2.5 mM MgCl2; 2 mM each of dNTP; 25 pmol ml-1 primer (each of ITS-1, ITS-4 and ITS-5); 1U of Taq DNA Polymerase; 60–100 ng genomic DNA. The amplification cycle consists of an initial denaturation at 95°C for 2 min followed by 40 cycles at 94°C for 30 s, 50°C for 1 min, and 72°C for 2 min and a final extension at 72°C for 10 min. Amplified PCR products were separated on an agarose gel (2% w/v) in 1X TAE buffer at 75V for 150 min and photographed under Gel Doc™ XR+ Gel Documentation System Gene ruler ladder 1oopb (Ferments) was used as a size standard. They were then eluted by MinElute PCR purification kit (Qiagen). DNA from All isolates was subjected to automated DNA sequencing and subsequently used for sequencing.

ITS data analysis

The ITS nucleotide sequences for each isolate were then compared to those in the public domain databases NCBI (National Center for Biotechnology information; www.ncbi.nih.gov)using Basic Local Alignment Search Tool for Nucleotide Sequences (BLASTN). Alignment of ITS DNA sequences was done using Clustal_W program (Lenc *et al.* 2008 and Abd Murad *et al.*, 2016). Phylogenetic tree was created using CLC Sequence Viewer Version 6.3 based on UPGMA (un weighted pair group method for arithmetic analysis). The confidence of the branching was estimated by bootstrap analysis.

Statistical analysis

The obtained data were statistically analyzed according to (Snedecor and Cochran, 1980). Means were compared by using LSD test at 0.05 level

Results

Fruit blight syndromes of cucumber

Typical *Botrytis* rot symptoms of cucumber fruits were observed in different locations in this study *i.e.*, yellowing of fruits from one end followed by browning and rotting gray mold as shown in fig. (1) . Data in table (1) and fig. (1) showed that the rate of infection of young cucumber fruits, progress increased by increasing growth age of cucumber plants under protective plastic greenhouse. On the other hand, no observation of cucumber fruit rot incidence at Kafr El-Sheikh Governorate.

Fungal isolation and morphological identification

Seven *Botrytis* fungal colonies were isolated from El Beheira and El Gharbeia Governorates on PDA at 25°C were initially white and turned grey to dark grey after 3 days. Numerous hard, small, irregular and blackish sclerotia were observed on well- developed colonies (fig. 2).

Pathogenicity test of fungal isolates

All the fungi isolates were found to be pathogenic on cucumber fruits as shown in table (2). Rotten symptoms obtained were similar to those observed previously on diseased fruits .The mould seen were the same as those of the isolated fungi of fresh fruits which were subject to spoilage. Isolated fungi, *Botrytis cinerea* (No. 5 and 2) from El-Gharbeia were highly pathogenic leading to rapid disintegration of treated fruits in 3-5 days followed by isolates (No. 1 and 7) of El-Beheira and El-Gharbeia, respectively. On the other hand, isolates (No. 3 and 4) which isolated from El-Gharbeia were moderately pathogenic

Molecular identification of fungal isolates

In this study, phenotypic classification is often complicated expertise, time consuming, and misleading due to the various characters involved**.** Fungal diagnostics

Table 1: *Botrytis* blight incidence of cucumber fruits under natural infestation in protective plastic houses during three months.

Location	*Botrytis* fruit blight disease of cucumber plants (month) after cultivation					
	Dec. 2016		Jan 2017		Feb. 2017	
	%	D.S	%	D.S	%	D.S
El- Giza	0.0 c	0.0 c	0.25 b	2.0 a	0.5 b	3.0 a
Kafr El- Sheickh	0.0 c	0.0 c	0.0 c	0.0 c	0.0 c	0.0 c
El- Beheira	0.0c	0.0 c	0.0 c	0.0 c	2.0 b	3.0 a
El -Gharbeia	1.0 a	1.0 b	25.0 a	1.1b	35.0 a	2.0 b

-Value followed by the same letter are not significantly different at P≤ 0.05 according to Duncan's multiple range.

Table 2: Pathogenicity test of different isolates of *Botrytis* spp. obtained from blight cucumber fruits under greenhouse conditions.

Location	No.	Isolate		Fruit blight incidence	
		Accession	Name	Infection %	D. severity
El- Gharbeia	1	MF996362	*Botrytis cinerea*	80.0b	0.8 d
	2	MF996363	*Botrytis fuckeliana*	100.0 a	3.7 a
	3	MF996364	*Botrytis cinerea*	20.0 d	1.0 d
	4	MF996365	*Botrytis cinerea*	20.0 d	1.0 d
	5	MF996366	*Botrytis cinerea*	100.0 a	4.0 a
El- Bebeira	6	MF996367	*Botrytis eucalypti*	60.0 c	2.1 b
	7	MF996368	*Botrytis fuckeliana*	80.0 b	1.5 c
-----		--------	Control	0.00 e	0.0 e

-Value followed by the same letter are not significantly different at P≤ 0.05 according to Duncan's multiple range.

Fig. 1: Fruit blight of cucumber at El Gharbeia Governorate showing yellowish, brownish and soften gray mold of ucumber fruits

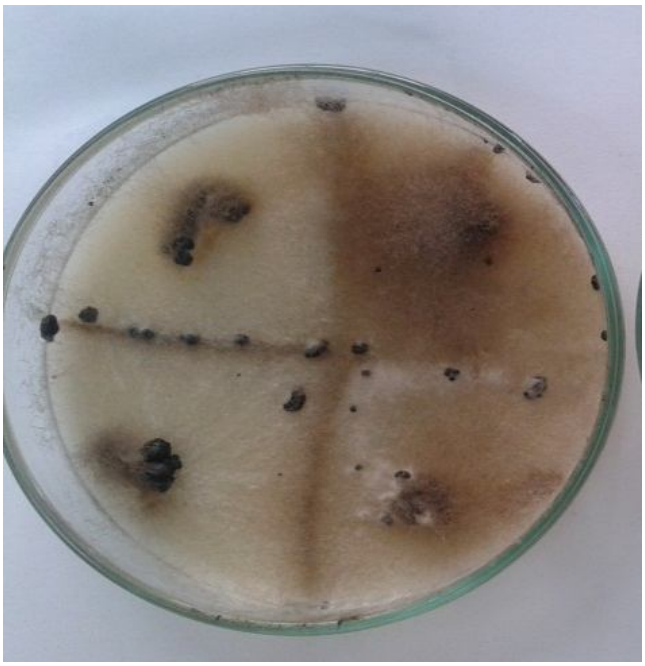

Fig. 2: Culture of *B. cinerea* isolates from El-Gharbeia Governorate.

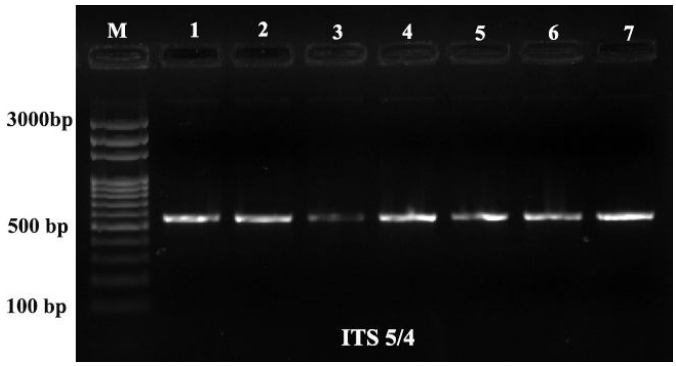

Fig 3: Agarose gel showing Amplification of conserved ribosomal regions of *Botrytis* isolates using the primers ITS-4 and ITS-5. Lane M: 100bp DNA ladder, Lane 1-7 *Botrytis* isolates .

using molecular techniques can be used to confirm cultural and morphological characterization and classification**.** ITS region I between the 18S rDNA and the 28S rDNA is flanked by ITS-5 and ITS-4 also between ITS-1 and ITS-4 and the region II between the 18 S rDNA is flanked by ITS-5 and ITS-2**.** In subsequent experiments, primers ITS-4 and ITS-5 were used with the *Botrytis* species. DNA from all isolates was amplified with PCR primers. The amplified DNA was cleaned with a MinElute PCR purification kit (Qiagen) by standard procedures. DNA from All isolates was subjected to automated DNA sequencing and subsequently used for sequencing. PCR amplification of *Botrytis* with ITS 5/4 yielded an estimated 600-bp product. (Fig. 3). PCR amplification of DNA samples from *Botrytis* using the universal primer pairs designed from the ITS region of rDNA can also offer useful and reliable information. The method helped to investigate the polymorphism between *Borytis* spp. used to identify a specific ITS region. This ITS region which has a size of 280 to 600 bp can also be easily amplified by universal primers that can complement with the rRNA genes Hence, the PCR protocols specified in this study can be used as a reference for proper amplification of the required product size and quality. A sequence of nucleotides was done for all isolates and recorded in gene bank under accession No. illustrated in

Fig. 4: Phylogenetic tree of *Botrytis* species based on maximum likelihood (ML) analysis of DNA sequence data of the ITS region.

table (2). Phylogenetic analysis tree of all *Botryti*s isolates which collected in this study were aligned using the online version of MAFFT (https://mafft.cbrc.jp/alignment/ server) with the interactive refinement method (FFT-NS-i) setting. The alignments were further edited manually in MEGA v. 6.0 and deposited in Tree BASE (http://treebase.org). The Phylogenetic tree (cladogram) based on the ITS sequences showed in fig. (4). These results indicated that all the seven isolates of *Botryti*s strains had three main group. On the other hand, the main group divided into four distinct groups (clusters) from G1 to G3. The first group (G1) includes 4 strains. The two *Botrytis cinerea* strain 1 and 3 local isolates had one sub-group with very close distance between them, about 90% similarity, also strain 1and 3 were identified as *Botrytis fuckeliana*. The second group (G2) includes two strains in one *Botrytis*. sp (strain 4&5) with very close distance between them. The last group (G3) included one strain of *Botrytis eucalypti* (strain 6). In addition, the four strains in group one, *Botrytis cinerea* and *Botrytis fuckeliana* were closely genetic relation.

Discussion

Survey of fruit blight (grey mould disease) on fruits of cucumber plants cultivations during growing winter season 2016- 2017 in protective plastic houses of some Governorates in Egypt revealed, the epidemiological presence of the disease. Typical *Botrytis* rot symptoms on cucumber fruits were observed in three locations in this study *i.e*., yellowing of fruits flower end followed by browning and finally, rotting gray mould. This observation likely observed on cucumber fruits by (Yunis *et al*., 1991; Blancard *et al*., 2005 and Al-Sadi *et al*., 2011). In this study, the rate of infection of young cucumber fruits was increased by increasing growth age of cucumber plants under protective plastic greenhouse. On the other hand, no observation of cucumber fruit rot incidence at Kafr El-Sheikh Governorate. These findings may be appearing to agree with results which demonstrated that, the prevalence of favourable environmental conditions such as temperature and relatively high humidity prevailed during the period from December to February and apparently favoured the pathogenesis leading to substantial development of the disease. The present results were agreed with the reports emphasising the major role of *B. cinerea* as a causal agent of fruit rot on cucumber (Elad 1996; Davidson *et al*., 2004 and Blancard *et al*., 2005). Pathogenicity tests provided evidence for fruit rot symptoms of cucumber to be caused by all isolates of *Botryti*s were found to be pathogenic on all tested fruits. Isolated fungi, *Botrytis cinerea* isolates (No. 5 and 2) which isolated from El-Gharbeia Governorate were highly pathogenic leading to rapid disintegration of treated fruits in 3-5 days followed by isolates (No. 1 and 7) which isolated from El-Beheira and El-Gharbeia respectively, *Botrytis cinerea* has long been known to be the main organism causing fruit rot of cucumber (Blancard *et al*. , 2005; An and Ma 2005-2006 and Al-Sadi *et al*., 2011). In the present study, we conducted molecular phylogenetic analyses of seven *Botrytis* species isolates. Morphological characters, together with nucleotide sequencing variation of DNA information, can be used to identify *Botrytis*

fungal isolates species; for instance, in a diagnostic key of *Botrytis* species. Such a key may be a potentially powerful tool for diagnostics of this important group of plant pathogens. (O'Donnell *et al.,* 1998). DNA from all isolates was amplified with the internal transcribed spacer (ITS) primers under study and was subjected to automated DNA sequencing. In addition, the (ITS) regions were sequenced to study the diversity of the seven isolates of Botrytis. These sequences were compared with those of Botrytis species available in the GenBank database (www.ncbi.nlm.nih.gov) these similar to data obtained by (Mokrini *et al*., 2017 and Ibrahim *et al*., 2014) and confirmed again the identity of the specie by using the sequence alignment program CLUSTAL (Higgins and Sharp, 1989). PCR amplification of DNA samples from all *Botrytis* strains using the universal primer pairs designed from the ITS region of rDNA can also offer useful and reliable information. The ITS region which has a size from 280 to 600 bp can also be easily amplified by universal primers that can complement with the rRNA genes as previously mentioned (White *et al.*, 1990). Moreover, the high copy number of rRNA genes from the ITS region makes easy to amplify from a small, diluted or highly degraded DNA samples (Gardes *et al.,* 1991; Gardes and Bruns 1991 and Alwakeel 2013). The Phylogenetic tree (cladogram) based on the ITS sequences indicated that all the seven isolates of *Botrytis* strains had three main group with closely genetic relation as previously shown by Humbert and Le Berre (2001) which suggested that the sequence homogeneity could be due to frequent transfers of genetic material between strains. PCR amplification and sequencing of internal transcribed space regions (ITSI-5.8S and ITS2-28S) and several new nucleotide sequence was registration in Gene Bank with new accession number. This work will be helpful to study the pathogenesis, rapid detection and molecular evolutionary of the *Botrytis cinerea* fungal strains.

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