JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Nov. 2013. Vol. **7**(Spl. Edn.), p. 79-91

Molecular Identification of Four *Botrytis* Species Three of Them Associated with Neck Rot and Blasting Diseases of Onion with Special Reference of Two, *aclada* and *byssoidae*

Elsayed E. Hafez[1], Emad el-Dein H. Wasfy[2], Ahmed H.M. Ramadan[3] and M. A. Abdel-Gayed[3]

[1]Plant Protection and Biomolecular Diagnosis Department, Arid Land Research Institute, City for Scientific Research and Technology Applications, Egypt.
[2]Plant Pathology Department, Faculty of Agriculture, Alexandria University, Egypt.
[3]Onion & Garlic Diseases Research Department, Plant Pathology Research Institute, Agriculture Research Center, Giza, Egypt.

(Received: 15 August 2013; accepted: 20 October 2013)

Botrytis aclada **and other** ***Botrytis*** **spp. can cause neck rot, a storage disease and blasting diseases on onions.** ***Botrytis byssoidea*** **is investigated and recorded for the first time in Egypt on onion plants associated with seeds and bulb neck rot. Specific primers (BA2f/BA1r) were designed based on genome of** ***Botrytis*** **spp. associated with neck rot and blasting of onions. The partial sequences of the amplified PCR products and the constructed phylogenetic tree distinguished the examined Botrytis isolates into four groups:** ***Botrytis aclada*** **types AI and AII (*B. allii*); *B. byssoidea*; and *B. cinerea*. Results revealed that the major dominant species associated with onion neck rot and blasting was type AI (*B. aclada*). Whenever, *Botrytis byssoidea* was not investigated and recorded before on onion plants associated with seeds and bulb neck rot in Egypt. Specificity of the designed primers confirm that only *Botrytis spp.* can amplify a product of approximately 413 bp but no amplified products were obtained with the other tested genera and species isolated from infected and/or healthy tissues of onion bulbs**

Key words: Neck Rot, Blasting Diseases of Onion, *Botrytis* spp. and Molecular Identification by PCR.

The onion (*Allium cepa* L.) crop is regarded as one of the most important economic crops in Egypt as well as in many countries of the world. *Botrytis* species are important pathogens of nursery plants, vegetables, ornamental, field and orchard crops and stored and transported agricultural products (Elad *et al*., 2004). *Botrytis* diseases are considered one of the major diseases of onion, which need special vigilance in seed and bulb crops. *Botrytis* and its teleomorph *Botryotinia* Whetzel involve 27 species and 1 hybrid species (Hennebert, 1973; Wang *et al.*, 1996; Yohalem *et al.*, 2003; O'Gorman *et al.*, 2008; Zhang *et al.*, 2010). *Botrytis* classification and identification are largely based on morphological and cultural characteristics of macroconidia, conidiophores, and sclerotia. Species of *Botrytis* have been named based on host association (Hennebert, 1973; Jarvis, 1977). At least, seven *Botrytis* spp. (Common Names of Plant Diseases, APS website) have been associated with diseases of onion crop as recorded by Hennebert (1963) and Ramsey and Lorbeer (1986 a and b). Four of these species have known teleomorphs in the genus *Botryotinia*, family Sclerotiniaceae, order Helotiales, and class Discomycetes (Jarvis, 1977). Three species are considered the primary causal agents of neck rot

* To whom all correspondence should be addressed. Home: +203 4595100; Work Tel.: +203 4593418 Mob.: +2010 6019801; Fax: +203 4593423.

of onion, namely *B. aclada*, *B. allii*, and *B. byssoidea* (Kritzman *et al.*, 1978). *B. aclada* was first described by Fresenius (1850) as a pathogen of onion. *B. allii* was described as the causal pathogen of onion neck rot by Munn (1917). Walker (1925) described *B. byssoidea* as the causal pathogen of mycelial neck rot of onion. *Botrytis squamosa* was first described as the cause of small sclerotial neck rot of onion (Walker, 1925b). Later the fungus *B. squamosa* was shown to be the cause of a leaf spot and blight disease of onion that for many years was described as 'blast', but is now referred to as Botrytis leaf blight (Hancock and Lorbeer, 1963; Lorbeer, 1992). Neck rot species of *Botrytis* are found in all areas of the world where onions are produced, but the greatest losses have been reported in temperate regions, where *B. allii* and *B. aclada* appear to be the predominant onion neck rot pathogens as cited by Ellerbrock and Lorbeer, (1977); Maude and Presly (1977); Crowe *et al.*, (1995); Nielsen *et al., (*2001) and du Toit *et al.*, (2002). In anticipation of the recent taxonomic and nomenclatural clarification by Yohalem *et al.*, (2003) of the primary onion neck rot species of *Botrytis*, *B. aclada* (Fresen.) Yohalem was considered by many to be synonymous with *B. allii* (Munn) Yohalem, while *B. byssoidea* J.C. Walker was regarded by some as nonspecific with *B. aclada* (Lacy and Lorbeer, 1995). The lack of distinction among these species was due, in part, to limitations at differentiating these species using classical morphological and cultural methods (Presly, 1985). Nevertheless, Owen *et al.*, (1950) confirmed that *B. byssoidea* and *B. aclada* are valid species.

Two subgroups within *B. aclada* (AI and AII) have been distinguished based on chromosome number and conidial dimensions (Hennebert, 1973; Shirane *et al.*, 1989). The development of molecular techniques has revolutionized and energized fungal population genetics by providing numerous readily available genetic markers (Bridge *et al.*, 1998). Hennebert (1973) suggested that *B. aclada* and *B. allii* are synonymous; but Yohalem *et al*., (2003) showed that they are distinct species. According to cumulative data they suggested that *B. byssoidea*, *B. aclada* and *B. allii* are genetically distinct and that isolates of *B. aclada* and *B. byssoidea* are the ancestors of the polyploid *B. allii*. Recent molecular and taxonomic studies have indicated that *B. allii*, *B. aclada*, and *B. byssoidea* are each valid species associated with a neck rot disease of onion (Nielsen *et al.*, 2001 and 2002; Nielsen and Yohalem, 2001; Yohalem *et al.*, 2003). The results of molecular studies conducted by Nielsen *et al.*, (2001), which focused on *B. aclada* and *B. byssoidea*, suggest there are two genetically distinct populations within the *B. aclada* group and that the populations are distinct species. They used universal-primed polymerase chain reaction (UP-PCR) fingerprinting coupled with restriction analysis of ITS DNA regions for onion neck-rotting species of *Botrytis*. They reported that *B. aclada* and *B. allii* produce conidia which are smaller than those produced by *B. byssoidea*, and that the conidia produced by *B. aclada* are slightly smaller than those produced by *B. allii*. Nielsen *et al.*, (2002) explained that the *Botrytis* specific primer pair BA2f/BA1r amplified all neck rot– associated *Botrytis* isolates were used. A restriction digest of the amplification product with *Apo*I separated the *Botrytis* isolates into five groups: *B. aclada* types AI and AII; *B. byssoidea*; *B. squamosa*; and *B. cinerea*. DNA was not amplified using the BA2f/BA1r primers from any other species tested. Among the other species tested were isolates of *Sclerotium cepivorum*, causal agent of white rot in onions; *Penicillium* spp., representing many common saprophytes and onion pathogens associated with blue mold of onions; *Fusarium* spp., which cause damping- off and basal rot in onions; *Cladosporium* sp., which causes leaf blotch on onions; and *Sclerotinia sclerotiorum*, causing rot of onions, as well as other non-neck rot-associated *Botrytis* species demons-trating the specificity of the primers to *Botrytis* onion neck rot–associated pathogens.

Yohalem *et al.*, (2003) have proposed that the name *B. aclada* Fresenius be applied to the small-spored subgroup and that the name *B. allii* Munn be applied to the larger-spored subgroup within the formerly recognized *B. aclada* species as recognized by Hennebert (1973). In addition, since molecular sequence data indicated that two different cloned fragments from *B. allii* are identical with those of *B. aclada* and *B. byssoidea*, Yohalem *et al.*, (2003) have suggested that during the synchronized infection of onion by *B. aclada* and *B. byssoidea* a hybridization event involving

hyphal anastomosis between the two species occurred. This event resulted in the origin of *B. allii* which has 32 mitotic chromosomes and other distinct molecular characteristics. *B. aclada* and *B. byssoidea* each have 16 mitotic chromosomes. More recently, polymerase chain reaction (PCR) alleles and internal transcribed spacer restriction fragment length polym-orphisms (ITS-RFLPs) have been used to demonstrate that isolates in subgroups AI and AII are distinct from *B. byssoidea* (Nielsen *et al.,* 2001; Nielsen and Yohalem, 2001; Nielsen *et al.,* 2002).

MATERIAL AND METHODS

Fungal isolates

Fungal isolates (Table 1) were isolated from natural diseased bulbs of onion, showing typically symptoms of neck rot disease and likewise in onion seed crops, blighted scapes, flowers and umbels showing typically symptoms of blasting disease as illustrated in Figure 1 and harvested onion seeds were collected during 2004–2005 winter season, from eighteen localities of eleven Governorates in Egypt. On the other hand, natural diseased strawberry fruits and broad bean leaves showing typically symptoms of gray mold and chocolate spot diseases, respectively, were obtained from two localities of two Governorates. Isolation trails showed the occurrence of twenty isolates of the causal pathogens. Obtained isolates were maintained on potato dextrose agar (PDA). For long-term preservation, isolates were either stored in a 15% glycerol suspension at –80°C, or they were grown on PDA in sealed tubes and stored at 4°C**.**

Culture morphology, linear growth and spore measurements

Morphological characteristics of twenty *Botrytis* spp. isolates such as color, type of mycelium and rate of linear growth were studied. Disk (5 mm in diameter) of vegetative culture edge of each isolate was inoculated at the center of Petri-dish (90 mm diameter) containing sterile Czapek's Dox medium (2.0 gm $Na\ NO_3$, 1.0 gm K_2HPO_4, 0.5 gm KCl, 0.5 gm $Mg\ SO_4$-$7H_2O$, 0.01 gm $Fe\ SO_4$-$7H_2O$, and 30 gm $C_6H_{12}O_6$ up to 1000 ml with d.d. H_2O and 20 gm agar were added and autoclaved) and three dishes were used as replicates for each treatment. Dishes were incubated at 20 ºC. Linear growth was estimated, two diameters were measured at right angles across on growth surface back after 7 days of inoculation. Afterwards, dishes were kept under light for another week for colony appearance and complete sporulation. Types of mycelia, also, were recorded and photographed. Conidia of the aforementioned cultures were harvested by adding 40 ml d.d. H_2O for each dish and using spatula for collecting spores. Number of conidia per 1 ml of 40 ml was calculated using hemicytometer slide. Length and width of 200 conidia from each isolate were measured after 21 days of inoculation and incubation at 20°C by ×40 magnifications using an Olympus microscope (BH2). The volumes of conidia were computed according to Shirane *et al.*, (1989) using the following formula: Conidial volume (μm^3) = $L.W^2\ \pi/6$, in which L = conidial length (µm) and W = conidial width (µm). Conidial shape was examined and photographed by ×40 magnifications. The obtained fungal isolates of causal pathogens from different hosts (onion, strawberry, and broad bean) were identified based on morphological, microscopical and cultural charact-eristics of mycelia, conidiospores, conidiophores as reported by Abdel-Gayed (2004), du Toit *et al.,* (2004) and Chilvers *et al*., (2006). Twenty obtained isolates of *Botrytis* spp. were cultivated on 100 ml of sterile PD liquid media in Erlenmeyer flasks for 10-15 days in the dark at 20˚C. After incubation period, mycelia of each *Botrytis* isolate individually, were harvested, filtrated and stored at -20˚C until used.

The genomic DNA was isolated according to protocol of Möller *et al.*, (1992). DNA pellet was re-suspended in 100 µl of TE buffer (consisted of 10 mM Tris-HCl [pH 8.0], and 1mM di-Na EDTA [pH 8.0]) and finally stored at -20°C until use. DNA concentration was estimated and adjusted to 100 ng μl^{-1} and visualized on 1% (w/v) agarose gel for DNA quality. Based on these sequences [GeneBank accession numbers: AJ2914 76 - AJ291487(Table 2)], two sequence characterized amplified region (SCAR) primers (BA2f/BA1r) were designed using the program Primer Select in the program package DNASTAR (Windows 32 Primer Select 4.00, 1993-1999 DNASTAR Inc.) those amplify a 413-bp PCR product in the *Botrytis* groups associated with onion diseases.

Specific-PCR amplification and conditions

PCR amplification was performed in a total

reaction volume of 25 µl. The following compounds were added, with final concentrations indic-ated: 1µl (50 pmol) of each oligonucle-otide primer (Table 2), 0.3 µl *Taq* DNA polymerase (5 unit μl^{-1}) (Promega, Germany), 2.5 µl PCR buffer, 1 µl 10 mM $MgCl_2$, 1 µl 2.0 mM dNTP (dATP, dGTP, dCTP, and dTTP), 1 µl of fungal DNA (approximately, 50 ng) as template and 17.2 µl sterilized distilled water. A GeneAmp PCR System 9700 (Perkin Elmer, Norwalk, CT) thermo-cycler device was used with the following program: 94 °C for 5 min, as initial denaturation cycle and 35 cycles consisting of denaturation at 94 °C for 30 sec, annealing of primer at 60°C for 30 sec and extension for 1 min at 72 °C finally addition of 3‘ terminal at 72 °C for 10 min.

PCR products electrophoresis and visualization

2% agarose gel (prepared by dissolving 1g agarose (Sigma) powder in 50 ml of 1X TBE buffer) was used to separate amplified products in 1X TBE running buffer (10X stock solution was prepared from: 108 g Tris base (Amresco, USA), 115 g Boric acid (Amresco, USA), and40 ml of 0.5M disodium EDTA, final volume fill up to 1000 ml with d.d. H_2O, and pH adjusts to 8.0). Five µl of the PCR amplification products were mixed with 3 µl of 6X gel loading buffer (38% Sucrose, 0.1% Bromophenol blue, and 67mM di-Na EDTA). 1.5 kbp DNA ladder New England Biolabs) with size marker, ranged from 100 to 1500 bp was used as molecular size standard. Electrophoresis was performed at 80V, the gel was stained with ethidium bromide solution (0.5µg/ml) for 10 min, and visualized on an ultraviolet dual intensity transilluminator (WVP, USA), and photographed using gel documentation system (WVP, USA). Amplification of fungal isolates with the *Botrytis* specific primers pair BA2f/BA1r was performed at least twice. QIAquick PCR purification kit (Qiagen, Germany) was used to purify the amplified products of BA2f/BA1r specific primer of 20 fungal isolates. Five volumes of the buffer PS were mixed with one volume of the PCR product and the mixture was loaded onto the PCR purification column placed into 2 cm^3 collection tube. The column was centrifuged at 9447 *g* for 1 min. After which it was placed into a new collection tube in which 750 µl of PE buffer were pipetted. A centrifugation step at 9447 *g* for 10 min was conducted twice to ensure getting rid of ethanol. The amplified fragment was eluted from the column using 50µl sterile distilled water with a centrifugation step at 5590 *g* for 1 min. The clean PCR product was subjected cycle sequencing in both directions using specific primer. The sequencing was done with ABI PRISM Dye Terminator Cycle sequencing method (Perkin Elmer) that used four different colored fluorescence-dyes to label four ddNTPs terminators Sequencing reactions were setup according to instructions manual. Briefly, a total of 20 µl reaction mixture consisting of 8 µl terminator ready reaction mix, 50 ng of PCR product and 3.2 pmole of primer. The cycle of sequencing was performed on the GeneAmp PCR system 9600 (Perkin Elmer) with the following parameters: 25 cycles at 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min.

Alignment and phylogenetic analysis

The nucleotide sequences were edited and carried out using CLUSTAL/W(1.82) http:// www2.ebi .ac.uk/clustalw (Thompson *et al*., 1994). Bootstrap neighbor-joining tree was generated using MEGA version 3.1 (Kumar *et al*., 2004) from CLUSTAL/W alignments. Comparisons with sequences in the GeneBank database were achieved in BLASTN searches at the National Center for Biotechnology Information (NCBI) site (http://www.ncbi. nlm.nih.gov). The GeneBank access-ion numbers of partial sequences of tested *Botrytis* isolates are listed in Table (3).

Specificity of the primer

Isolated genomic DNA from *Fusarium oxysporum f.sp.cepae* and *F. solani* {basal rot}, *Sclerotium cepivorum* {white rot}, *Alternaria porri* {purple blotch}, *Penicillium* spp. {blue rot}, *Aspergillus niger* {black rot}, *Phoma terrestris* {pink root}, and *Cladosporium* sp. {leaf blotch}, *Trichoderma harzianum* as bioc-ontrol agent and onion bulb tissues cv. Giza 20 was used to check the specificity of the primer. PCR reaction, conditions, products electrophoresis and visualization were done as described above.

Statistical Analysis

The data obtained were statistically analyzed using the Statistical Analysis System (SAS). Data of all experiments were subjected to statistical analysis using analysis of variance. Means were compared using L.S.D. test as described by (Snedcor and Cochran, 1967).

RESULTS

The obtained fungal isolates of causal pathogens from different hosts (onion, strawberry, and broad bean) were identified as *Botrytis* spp. based on morphological, microscopical and cultural characteristics of mycelia, conidiophores and conidiospores as illustrated in Figure (2) and also based on their pathogenicity tests. At the 7th day of incubation, linear growth of all tested isolates was approximated to others and to fill surface of Petri-dishes 90 mm in diameter except one isolate from Shbein El-koum its linear growth was 62.5 mm in diameter. Type and color of mycelia were illustrated and demonstrated in in Figure (2) and Table (3). Measurements of conidia showed in Table (3) and Figure (2) indicated that isolates from Dommitta (*B. allii*) and Nagh-Hammady (*B. aclada*) were the biggest ones in their dimensions whereat, recorded conidial volume 782.31 and 865.28 µm, respectively. Whereas, isolate from Maghagha (*B. aclada*) recorded the smallest dimensions in all tested isolates (conidial volume 147.46 µm). The conidial volumes of other isolates were in between two recorded isolates. Isolate from Noubaria (*B. cinerea*) scored the largest one in conidial volume (1097.85 µm). Isolates obtained from onion plant were identified as following (15 isolates as *Botrytis aclada*, 1 isolate as *B. allii* and 2 isolates as *B. byssoidea*), but isolates obtained from strawberry and broad bean plants were identified as *B. cinerea*. This identification was confirmed by molecular studies as coming next.

PCR analysis and partial sequences determination of PCR product amplified by BA2f/ BA1r specific primer

Specific oligonucleotide primer (BA2f / BA1r) to *Botrytis* spp. designed by Nielsen *et al.*,

Table 1. Isolates of *Botrytis* spp. isolated from different hosts, collected from divers' localities during 2004-2005 winter season and used in this study

Isolate(s) from different host(s)	No.	Governorate	Locality	Origin of * diseased part
Onion	1	Beni-Suef	Sids	Bulb neck
	2	Beni-Suef	Somosta	Flowers
	3	Beni-Suef	Fashn	Bulb neck
	4	Beni-Suef	Beni-Suef	Bulb neck
	5	Fayoum	Ellahoun	Bulb neck
	6	Minia	Maghagha	Bulb neck
	7	Minia	Mallawi	Bulb neck
	8	Minia	Minia	Bulb neck
	9	Assiut	Manfalout	Flowers
	10	Sohag	Tama	Seed-stalk
	11	Sohag	Shandaweill	Seeds
	12	Kena	Nagh-Hammady	Flowers
	13	Kalubiya	Kalub	Bulb neck
	14	Gharbiya	Gmmeiza	Bulb neck
	15	Gharbiya	Tannta	Bulb neck
	16	Dommitta	Dommitta	Bulb neck
	17	Dakahliya	Miniet-elnasr	Bulb neck
	18	Menofiya	Shbein El-koum	Bulb neck
Strawberry	19	Esmailliya	Fayed	Fruits
Broad bean	20	Behaira	Noubaria	Leaves

Table 2. Nucleotide sequences of primer used to *Botrytis* amplification

Primer name	Oligonucleotide sequence, 5‘ to 3‘	Product	Reference
BA2f BA1r	GTGGGGGTAGGATGAGATGATG TGAGTGCTGGCGGAAACAAA	H 413 bp.	Nielsen *et al.*, (2002)

(2002) was used in molecular studies to identify *Botrytis* spp. associated with onion diseases. Genomic DNA isolated from twenty isolates of *Botrytis* spp. (18 isolates from onion, Sids, Somosta, Fashn ,Beni-Suef, Ellahoun, Maghagha, Mallawi, Minia, Manfalout, Tama, Shandaweill, Nagh-Hammady, Kalub, Gmmeiza, Tannta, Dommitta, Miniet-elnasr, and Shbein El-koum and 1 isolate from strawberry, Fayed and finally 1 isolate from broad bean, Noubaria) were examined in PCR procedure with the *Botrytis* spp. specific primer.

The obtained results in figure (3) showed that specific primer (BA2f/BA1r) can amplify PCR product (approximately 413 bp) in a single sharp band for all *Botrytis* spp. isolates from different hosts. PCR products (≈ 413 bp) of all twenty isolates of *Botrytis* spp. were subjected to sequencing using ABI PRISM Dye Terminator Cycle sequencing method (Perkin Elmer) that used four different colored fluorescence-dyes to label four ddNTPs terminators. The resulted partial sequences were obtained for twenty isolates of *Botrytis* spp. Search in databases to identify the fungus was accomplished in BLAST search at the National Center for Biotechnology Information site (http://www.ncbi.nlm.nih.gov). The search disclosed that these sequences were identical to the four obtained *Botrytis* spp.

Alignment and phylogenetic analysis

Alignment of partial sequences of *Botrytis* spp. isolates investigated in this study with the RAPD fragments L45-550 partial sequences of *Botrytis* spp. collected from the GeneBank was carried out using CLUSTAL W (1.82) (http://www2.ebi.ac.uk/clustalw; Thompson *et al*., 1994) from which MEGA version 3.1 (Kumar *et al*., 2004) was used to generate the Bootstrap neighbor-joining tree. Results of the sequences alignment confirmed that identification of all twenty tested isolates of *Botrytis* spp. used in this study. Fifteen isolates isolated from infected onion parts and obtained from Sids, Somosta, Fashn , Beni-Suef,

Table 3. Morphological characteristics of 18 *Botrytis* onion isolates and, moreover, one isolate from each strawberry and broad bean on Czapek's Dox medium after inoculation and incubation at 20 °C

Isolate(s) from different host(s)		*Botrytis* spp.	Type of mycelium	Shape of conidium of inoculation	Linear growth[(1)] (mm.) after 7 days	Conidial volume (μm^3) [(2)]
Onion	Sids	*B. aclada*	Compact	Wanded	90.0±0.00 a	627.99±36.33 c
	Somosta	*B. aclada*	Compact	Spindle	84.5±3.97 cd	493.87±34.87 ef
	Fashn	*B. aclada*	Softy	Ovate	79.0±6.06 e	513.56±35.82 def
	Beni-Suef	*B. aclada*	Cottony	Ovate	88.0±1.41 ab	398.09±39.49 g
	Ellahoun	*B. aclada*	Powdery	Spindle	90.0±0.00 a	375.54±40.49 hi
	Maghagha	*B. aclada*	Fluffy	Ovate	90.0±0.00 a	147.46±19.41 k
	Mallawi	*B. aclada*	Watery	Spindle	82.8±1.53 d	310.72±16.19 ij
	Minia	*B. aclada*	Fluffy	Ovate	90.0±0.00 a	354.36±38.50 hi
	Manfalout	*B. aclada*	Light warty	Ovate	89.7±0.76 a	509.26±44.16 def
	Tama	*B. aclada*	Powdery	Ovate	85.7±2.25 bcd	308.57±36.84 ij
	Shandaweill	*B. byssoidea*	Heavy cottony	Ovate	89.5±0.50 a	563.16±46.41 cde
	Nagh-Hammady	*B. aclada*	Downy	Narrowly elongated	89.3±1.15 a	782.31±87.74 b
	Kalub	*B. aclada*	Softy	Ovate	89.3±0.58 a	436.11±44.70 fgh
	Gmmeiza	*B. byssoidea*	Warty	Spindle	89.7±0.58 a	641.16±34.34 c
	Tannta	*B. aclada*	Downy	elongated	89.0±1.73 ab	366.61±30.01 hi
	Dommitta	*B. allii*	Watery	Bacillary	88.3±1.76 ab	865.28±75.79 b
	Miniet-elnasr	*B. aclada*	Compact	Ovate	87.3±0.76 abc	241.8±30.46 j
	Shbein El-koum	*B. aclada*	Downy	Spherical	62.5±3.99 f	480.66±45.35 efg
Straw-berry	Fayed	*B. cinerea*	Cottony	Large Spherical	90.0±0.00 a	587.05±53.60 cd
Broad bean	Noubaria	*B. cinerea*	Heavy	Large global cottony	90.0±0.00 a	1097.85±114.15 a

Ellahoun, Maghagha, Mallawi, Minia, Manfalout, Tama, Nagh-Hammady, Kalub, Tannta, Miniet-elnasr, and Shbein El-koum were identified as *Botrytis aclada*, and encoded to Zeyad-1,Zeyad-2, Zeyad-3, Zeyad-4, Wasfy-5, Sohaila-6, Sohaila-7, Sohaila-8, Hafez-9, Hafez-10, Gayed-12, Gayed-13, Gayed-15, Gayed-17, and Gayed-18, respectively. Two isolates isolated from infected onion parts and obtained from Shandaweill, and Gmmeiza were identified as *Botrytis byssoidea*, and encoded to Nouma-11, and Nouma-14, respectively. One isolate isolated also from infected onion parts and obtained from Dommitta was identified as *Botrytis allii*, and encoded to Muhammad-16. One isolate isolated from infected strawberry fruits and obtained from Fayed was identified as *Botrytis cinerea*, and encoded to Hema-19. Finaly, one isolate isolated from infected broad bean leaves and obtained from Noubaria was identified as *Botrytis cinerea*, and encoded to Hema-20.

Figure (4) presented the phylogenetic tree of *Botrytis* spp. isolates (Zeyad-1, Zeyad-2, Zeyad-3, Zeyad-4, Wasfy-5, Sohaila-6, Sohaila-7, Sohaila-8, Hafez-9, Hafez-10, Nouma-11, Gayed-12, Gayed-13, Nouma-14, Gayed-15, Muhammad-16, Gayed-17, Gayed-18, Hema-19, and Hema-20) currently used in this study and *Botrytis* spp. strains obtained from the GeneBank, and demonstrated the similarity matrix and linkage distance among different *Botrytis* spp. isolates. The phylogenetic tree revealed that four main clusters exist. Cluster 1 divided into 2 sub-clusters: Sub-cluster I included *B. allii* isolated from Dommitta (Muhammad-16; HM597769), whereas sub-cluster 2 included *B. byssoid*ea isolated from Shandaweill (Nouma-11; HM597764). Cluster II included *B. cinerea* (*Botryotinia fuckeliana* - XM001551951) obtained from the GeneBank. Cluster III included *B. allii* (AJ716056) obtained from the GeneBank. Cluster IV divided into 2 sub-clusters: Sub-cluster 1 divided into 2 groups: Group 1 included *B. byssoid*ea isolated from Gmmeiza (Nouma-14; HM597767), whereas Group 2 included *B. byssoid*ea strain BB6 (AJ291483) obtained from the GeneBank. Sub-cluster 2 divided into 2 groups: Group 1 included 3 isolates of *B. aclada* isolated from Maghagha (Sohaila-6; HM597759), Beni-Suef (Zeyad-4; HM597757), and Minia (Sohaila-8; HM597761) and 1 isolate of *B. cinerea* isolated from Fayed (Hema-19; HM597772). Group 2 included 2 sub-groups:

Table 4. The accession numbers of partial sequences of *Botrytis* isolates used in this study in the GeneBank

Isolate(s) from different host(s)	No.	Isolate code	Locality	*Botrytis* spp.	Accession number
Onion	1	*Zeyad-1*	Sids	*B. aclada*	HM597754
	2	*Zeyad-2*	Somosta	*B. aclada*	HM597755
	3	*Zeyad-3*	Fashn	*B. aclada*	HM597756
	4	*Zeyad-4*	Beni-Suef	*B. aclada*	HM597757
	5	*Wasfy-5*	Ellahoun	*B. aclada*	HM597758
	6	*Sohaila-6*	Maghagha	*B. aclada*	HM597759
	7	*Sohaila-7*	Mallawi	*B. aclada*	HM597760
	8	*Sohaila-8*	Minia	*B. aclada*	HM597761
	9	*Hafez-9*	Manfalout	*B. aclada*	HM597762
	10	*Hafez-10*	Tama	*B. aclada*	HM597763
	11	*Nouma-11*	Shandaweill	*B. byssoidea*	HM597764
	12	*Gayed-12*	Nagh-Hammady	*B. aclada*	HM597765
	13	*Gayed-13*	Kalub	*B. aclada*	HM597766
	14	*Nouma-14*	Gmmeiza	*B. byssoidea*	HM597767
	15	*Gayed-15*	Tannta	*B. aclada*	HM597768
	16	*Muhammad-16*	Dommitta	*B. allii*	HM597769
	17	*Gayed-17*	Miniet-elnasr	*B. aclada*	HM597770
	18	*Gayed-18*	Shbein El-koum	*B. aclada*	HM597771
Strawberry	19	*Hema-19*	Fayed	*B. cinerea*	HM597772
Broad bean	20	*Hema-20*	Noubaria	*B. cinerea*	HM597773

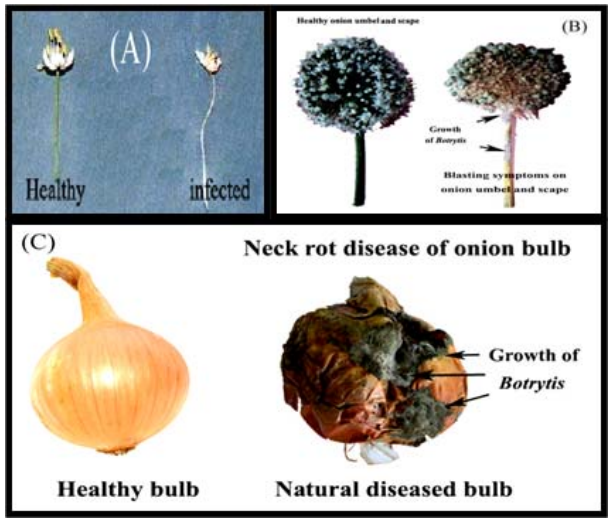

Fig. 1. (A)Symptoms of blasting on onion flowers, (B) Blasting symptoms on onion umbel and scape, (C) Symptoms and signs of neck rot disease on onion bulb

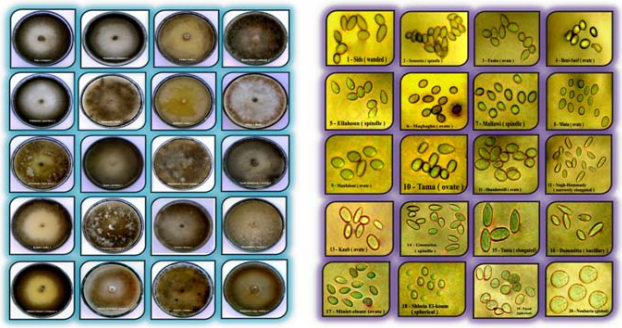

Fig. 2. Cultural and conidial morphology of 18 *Botrytis* onion isolates and, moreover, one isolate from each strawberry and broad bean on PDA medium after 21 days of inoculation and incubation at 20 °C. (Isolates from left to right, gradually, Sids, Somosta, Fashn, Beni-Suef, Ellahoun, Maghagha, Mallawi, Minia, Manfalout, Tama, Shandaweill, Nagh-Hammady, Kalub, Gmmeiza, Tannta, Dommitta, Miniet-elnasr, Shbein El-koum, Fayed and Noubaria)

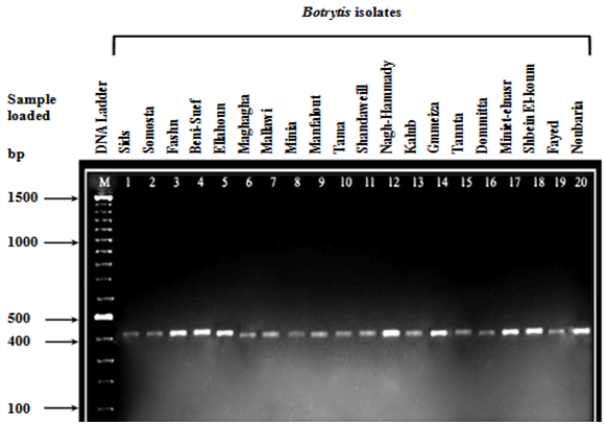

Fig. 3. Electrophoretic pattern of PCR product (≈ 413 bp) amplified by specific primers BA2f/BA1r for *Botrytis* spp. The lane marked M at the far left side of the gel is loaded with a 100 bp DNA ladder with uppermost band 1500 bp. Samples from 1 to 20 are the twenty *Botrytis* isolates from different hosts and localities

sub-group 1 included 2 isolates of *B. aclada* isolated from Manfalout (Hafez-9; HM597762) and Tannta (Gayed-15; HM597768), whereas sub-group 2 included 13 isolates, via. ten isolates of *B. aclada* isolated from Tama (Hafez-10; HM597763), Shbein El-Koum (Gayed-18; HM597771), Miniet-elnasr (Gayed-17; HM597770), Kalub (Gayed-13; HM597766), Nagh-Hammady (Gayed-12; HM597765), Sids (Zeyad-1; HM597754), Somosta (Zeyad-2; HM597755), Fashn (Zeyad-3; HM597756), Mallawi (Sohaila-7; HM597760), and Ellahoun (Wasfy-5; HM597758) and 2 isolates of *B. aclada* obtained from the GeneBank (strain BA8; AJ291477 and strain SAL005; AJ291487) and finally 1 isolate of *B. cinerea* isolated from Noubaria (Hema-20; HM597773).

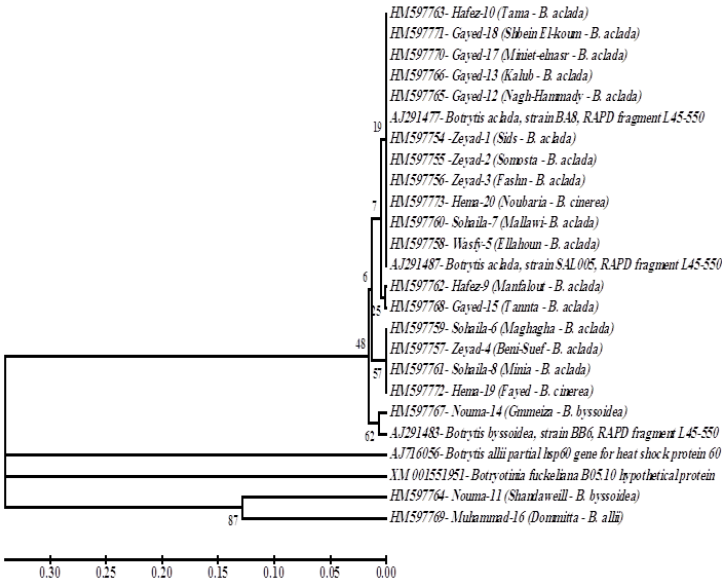

Fig. 4. Phylogenetic tree and the similarity matrix of *Botrytis* spp. isolates from the alignment of PCR product (≈ 413 bp) amplified by specific primer BA2f/BA1r for aforementioned different *Botrytis* spp. and related *Botrytis* spp. isolates accessioned in GenBank. Digital discriminations inserted into the tree represent degree of similarities and the base scale indicates linkage distance

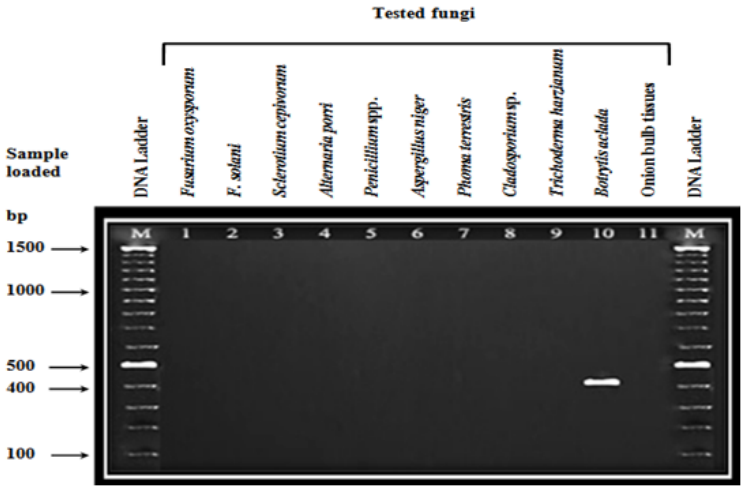

Fig. 5. Electrophoretic pattern of PCR product amplificated by specific primer BA2f/BA1r indicated specificity of the primer. M: 100-bp DNA ladder (left and right site) with uppermost band 1500 bp, lanes 1 to 9: *other tested genera*, 10: *Botrytis aclada* (Nagh-Hammady isolate) and 11: healthy onion bulb tissues cv.Giza 20

Specificity of the primer

Genomic DNA isolated from different 10 genera and species of fungi associated with onion diseases, via. one isolate of *B. aclada* (Nagh-Hammady, Gayed-12; HM597765) causing onion neck rot disease as well as one isolate of each : *Fusarium oxysporum f.sp.cepae* {basal rot}, *F. solani* {basal rot}, *Sclerotium cepivorum* {white rot}, *Alternaria porri* {purple blotch}, *Penicillium* spp. {blue rot}, *Aspergillus niger* {black rot}, *Phoma terrestris* {pink root}, *Cladosporium* sp. {leaf blotch}], *Trichoderma harzianum* as biocontrol agent and healthy onion bulb tissues, cv. Giza 20 as host of these fungi were used as template DNA in PCR reaction with the *Botrytis* specific primer (BA2f/BA1r).Electrophoretic pattern illustrated in Figure (5) indicated that the only isolate of *B. aclada* (Nagh-Hammady) can amplify a product of approximately 413 bp with the primer and gave a specific band. On the other hand, no amplified products were obtained with the other tested genera and species or with healthy tissues of onion bulbs.

DISCUSSION

The onion (*Allium cepa* L.) crop is considered one of the most important economic and exported crops in Egypt and many of other countries in the world. It is the third top cultivated area and agricultural resulting of vegetable crops after tomato and potato. Onion is invaded by several fungal, bacterial, and viral pathogens in the field and some of them spoil the bulbs during storage, transporting and marketing and subsequently, they decrease both yield and quality.

Botrytis diseases are considered one of the most important fungal diseases of onions which decrease greatly yield, and can devastate the bulb yield during post harvest period and also seed crop in the field. Extensive effort is invested for protecting the agricultural production against *Botrytis* pre and post harvest. The intensity of anti-*Botrytis* measures taken by farmers continued unabated throughout the last 20 years but our understanding of the processes that govern *Botrytis* life cycles, pathogenicity and epidemiology have become inclusive. Over the last 125 years, *Botrytis* spp. have been investigated by an increasing number of specialists in diverse fields including chemistry, biochemistry, molecular and cell biology, genetics, morphology and histology, taxonomy, host-parasite interaction, ecology and epidemiology (Jarvis 1977; Coley-Smith *et al.,* 1980; Verhoeff *et al.,* 1992).

In this study, natural diseased bulbs of onion, showing typical symptoms of neck rot disease develop only after bulbs harvest and placed in storage, and also in onion seed crops, blighted scapes (seed stalks), flowers and umbels showing typical symptoms of blasting disease develop in the field, and harvested onion seeds were collected during 2004–2005 winter season, from eighteen localities of eleven Governorates in Egypt (Beni-Suef, Fayoum, Minia, Assiut, Sohag, Kena, Kalubiya, Gharbiya, Dommitta, Dakahliya, and Menofiya). On the other hand, natural diseased strawberry fruits and broad bean leaves showing typical symptoms of gray mold and chocolate spot diseases, respectively, were obtained from two localities of Esmailliya, and Behaira Governorates, respectively. Isolation trails showed the occurrence of twenty isolates of the causal pathogens.

The obtained data showed that, eighteen isolates of *Botrytis* spp . (4 from Beni-Suef; 1 from Fayoum; 3 from Minia; 1 from Assiut; 2 from Sohag; 1 from Kena; 1 from Kalubiya; 2 from Gharbiya; 1 from Dommitta; 1 from Dakahliya and 1 from Menofiya) were isolated from different parts, bulbs, scapes, flowers, umbels and seeds of onion plant showing clearly symptoms of neck rot or blasting diseases, and also 2 isolates of *Botrytis* spp. (1 from Esmailliya and 1 from Behaira) were isolated from strawberry fruits and broad bean leaves showing natural symptoms of gray mold and chocolate spot diseases, correspond-dingly. All obtained fungal isolates of causal pathogens from different hosts, onion, strawberry, and broad bean were identified as *Botrytis* spp. based on morphological, microscopical and cultural characteristics of mycelia, conidiophores and conidiospores and also based on their pathogenicity tests. Isolates obtained from onion plant were subsequently, identified 15 isolates as *Botrytis aclada*, 1 isolate as *B. allii* and 2 isolates as *B. byssoidea*, but isolates obtained from strawberry and broad bean plants were identified as *B. cinerea*. Different studies indicated that, *Botrytis* identification and classification were largely based on morphological and cultural

characteristics of conidia, conidiophores, and sclerotia. Species of *Botrytis* have been named on bases of host association (Hennebert 1973; Jarvis 1977). Features such as sclerotial size and form and conidium size are useful in delimiting some species, but many species are morphologically similar and growing conditions significantly influence these characters (Beever and Weeds 2004). *B. cinerea* is the most commonly isolated species of the form genus, growing on a wide range of host plants as a parasite or saprophyte; most other species of the genus have a more restricted host range (Domsch *et al.,* 1993). At least, seven *Botrytis* spp. (Common Names of Plant Diseases, APS website) have been associated with diseases of onion crop as recorded by Hennebert (1963) and Ramsey and Lorbeer (1986). Four of these species have known teleomorphs in the genus *Botryotinia*, family Sclerotiniaceae, order Helotiales, and class Discomycetes (Jarvis 1977). Three species are considered the primary causal agents of neck rot of onion, namely *B. aclada*, *B. allii*, and *B. byssoidea* (Kritzman *et al.,* 1978). *B. aclada* was first described by Fresenius (1850) as a pathogen of onion. *B. allii* was described as the causal pathogen of onion neck rot by Munn (1917). Walker (1925b) described *B. byssoidea* as the causal pathogen of mycelial neck rot of onion. *Botrytis squamosa* was first described as the cause of small sclerotial neck rot of onion (Walker 1925b). Later the fungus *B. squamosa* was shown to be the cause of a leaf spot and blight disease of onion that for many years was described as 'blast', but is now referred to as Botrytis leaf blight (Hancock and Lorbeer 1963; Lorbeer 1992). Although *B. squamosa* and *B. tulipae* have also been associated with neck rot, these species are not typically primary causes of neck rot (Netzer and Dishon 1966; Ramsey and Lorbeer 1986). Although primarily a pathogen of garlic and leek, *B. porri* has been isolated from naturally-infected onion seed crops and onion seeds as reported by du Toit *et al.,* (2002) and du Toit *et al.,* (2004).

Identification of our obtained *Botrytis* isolates was analogous to previous studies. Hennebert (1963) synonymized *B. aclada* and *B. allii* as *B. allii* and then later accepted *B. aclada* as the valid name for the species (Hennebert 1973). He also accepted *B. byssoidea* as a valid species. Two species of *Botrytis* pathogenic to onion which have been studied relatively intensively in recent decades are *B. squamosa*, the causal agent of Botrytis leaf blight (Lorbeer 1992 and 1997), and *B. allii* (*B. aclada)*, the causal agent of Botrytis neck rot (grey mould) of onion (Lacy and Lorbeer 1995). *B. cinerea* is also pathogenic on onion, causing Botrytis brown stain (Clark and Lorbeer 1973b) and superficial leaf flecks (tiny leaf spots), when artificially inoculated on to onion leaves under controlled environmental conditions (Hancock and Lorbeer 1963). Neck rot species of *Botrytis* are found in all areas of the world where onions are produced involved Egypt, but the greatest losses have been reported in temperate regions, where *B. allii* and *B. aclada* appear to be the predominant onion neck rot pathogens as cited by Ellerbrock and Lorbeer, (1977); Maude and Presly (1977); Crowe *et al.,* (1995); Nielsen *et al., (*2001) and du Toit *et al.,* (2002). In expectancy of the recent taxonomic and nomenclatural clarification by Yohalem *et al.,* (2003) of the primary onion neck rot species of *Botrytis*, i. e. *B. aclada* (Fresen.) Yohalem was considered by many to be synonymous with *B. allii* (Munn) Yohalem, while *B. byssoidea* J.C. Walker was regarded by some as conspecific with *B. aclada* (Lacy and Lorbeer 1995). The lack of distinction among these species was due, in part, to limitations at differentiating these species using classical morphological and cultural methods (Presly 1985 a and b). Nevertheless, Owen *et al.,* (1950a) confirmed that *B. byssoidea* and *B. aclada* are valid species. Two subgroups within *B. aclada* (AI and AII) have been distinguished based on chromosome number and conidial dimensions (Hennebert 1973; Shirane *et al.,* 1989).

During the present study, *Botrytis* identification was confirmed by molecular studies using specific oligonucleotide primer (BA2f/BA1r) to *Botrytis* spp. designed by Nielsen *et al.,* (2002) to detect and identify *Botrytis* spp. associated with onion diseases. Based on these sequences (GeneBank accession numbers: AJ291476 - AJ291487), two sequence characterized amplified region (SCAR) primers (BA2f/BA1r) were designed using the program Primer Select in the program package DNASTAR (Windows 32 Primer Select 4.00, 1993-1999 DNASTAR Inc.) that amplify a 413-bp PCR product in the *Botrytis* groups associated with onion diseases. Genomic DNA isolated from

twenty isolates of *Botrytis* spp. (18 isolates from onion, 1 isolate from strawberry, and finally 1 isolate from broad bean) were examined in PCR procedure with the *Botrytis* spp. specific primer. The obtained results showed that specific primer (BA2f/BA1r) can amplify PCR product (approximately 413 bp) in a single sharp band for all *Botrytis* spp. isolates from different hosts. PCR products (≈ 413 bp) of all twenty isolates of *Botrytis* spp. were subjected to sequencing. Alignment of partial sequences of *Botrytis* spp. isolates investigated in this study with the RAPD fragments L45-550 partial sequences of *Botrytis* spp. collected from the GeneBank was carried out using CLUSTAL W (1.82) (http://www2.ebi.ac.uk/clustalw; Thompson *et al*., 1994) from which MEGA version 3.1 (Kumar *et al*., 2004) was used to generate the Bootstrap neighbor-joining tree.

The obtained results from alignment confirmed the identification of all twenty tested isolates of *Botrytis* spp. used in this study. Eighteen isolates isolated from infected onion parts were identified as following: Fifteen isolates as *Botrytis aclada*, two isolates as *Botrytis byssoidea*, and finally one isolate as *Botrytis allii*. One isolate isolated from infected strawberry fruits was identified as *Botrytis cinerea*, and one isolate isolated from infected broad bean leaves was identified as *Botrytis cinerea*. The phylogenetic tree revealed that four main clusters exist. In our study, *Botrytis byssoidea* is investigated and recorded for the first time in Egypt on onion plants associated with seeds and bulb neck rot. Specificity of the primer indicated that the only isolate of *B. aclada* (Nagh-Hammady) can amplify a product of approximately 413 bp with the primer and gave a specific band. On the other hand, no amplified products were obtained with the other tested genera and species isolated from onion or healthy tissues of onion bulbs. A similar result was obtained by Nielsen *et al.*, (2002) who demonstrated that, all isolates of other fungi, i.e. *Ulocladium atrum; U. consortiale; Clonostachys rosea; Trichoderma harzianum; Penicillium* sp. and *Cladosporium* spp were amplified with ITS primers but not with the BA2f/BA1r primer pair.

REFERENCES

1. Abd El-Gayed, M.A., Studies on blasting disease of onion seed-head in Egypt. M.Sc. Thesis, Fac. Agric., Sues Canal Univ, 2004.
2. Bridge, P.; Couteaudier, Y. and Clarkson, J., Molecular variability of fungal pathogens. CAB International, Wallingford, UK, 1998.
3. Chilvers, M.I. and du Toit, L.J., Detection and identification of *Botrytis* species associated with neck rot, scape blight, and umbel blight of onion. Online. Plant Health Progress doi: 10.1094/PHP-2006-1127-01-DG, 2006.
4. Crowe, F.J.; Mohan, S.K. and Schwartz, H.F., Other *Botrytis* diseases. Pages 19-20 in: Compendium of Onion and Garlic Diseases. H. F. Schwartz and S. K. Mohan, eds. American Phytopathological Society, St Paul, MN, 1995.
5. du Toit, L.J.; Derie, M.L. and Pelter, G.Q., *Botrytis* species associated with onion seed crops in Washington State. Plant Dis. 2004; **88**: 1061-1068.
6. du Toit, L.J.; Derie, M.L.; Hsiang, T. and Pelter, G.Q., *Botrytis porri* in onion seed crops and onion seed. *Plant Dis*. 2002; **86**:1178.
7. Elad, Y., Williamson, B., Tudzynski, P., and Delen, N., *Botrytis* spp. and diseases they cause in agricultural systems- an introduction. pp. 1-8 In: Elad, Y., Williamson, B., Tudzynski, P. and Delen, N. (eds). *Botrytis*: Biology, Pathology and Control. Kluwer Academic Publisher. Netherland, 2004.
8. Ellerbrock, L.A. and Lorbeer, J.W., Etiology and control of onion flower blight. Phytopathology 1977; **67**:155-159.
9. Hancock, J.G. and Lorbeer, J.W., Pathogenesis of *Botrytis cinerea, B. squamosa* and *B. allii* on onion leaves. Phytopathology 1963; **53**: 669-673.
10. Hennebert, G.L., *Botrytis* and *Botrytis*-like genera. *Persoonia* 1973; **7**:183–204.
11. Hennebert, G.L. and Groves, J.W., Three new species of *Botryotinia* on Ranunculaceae. *Can. J. Bot*. 1963; **41**: 341-371.
12. Jarvis, W.R., *Botrytinia* and *Botrytis* Species: Taxonomy, Physiology, and Pathogenicity, A guide to the Literature. Monograph No. 15, Canada Department of Agriculture, Ottawa, Canada, 1977.
13. Kritzman, G. and Netzer, D., A selective medium for isolation and identification of *Botrytis* spp. from soil and onion seed. *Phytoparasitica* 1978; **6**: 3-7.
14. Kumar, S.; Tamura, K. and Nei, M., MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* 2004; **5**(2): 150-163.
15. Lacy, M.L. and Lorbeer, J.W., *Botrytis* neck rot. In: Schwartz H and Mohan S (eds) Compendium of Onion and Garlic Diseases. (pp. 18-19)

American Phytopathological Society Press, St. Paul, MN, USA, 1995.

16. Lorbeer, J.W., Botrytis leaf blight of onion. In: Chaube H, Kumar J, Mukhopadhyay A and Singh U (eds) Plant Diseases of International Importance. volume II. Diseases of vegetables and oil seed crops. (pp. 186-211) Prentice-Hall, Inc., Englewood Cliffs, New Jersey, USA, 1992.
17. Maude, R.B. and Presly, A.H., Neck rot (*Botrytis allii*) of bulb onions, I. Seed-borne infection and its relationship to the disease in the onion crop. *Ann. Appl. Biol*. 1977; **86**:163-180.
18. Möller, E.M.; Bahnweg, G.; Sandermann, H. and Geiger, H.H., A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. *Nucleic Acids Res.* 1992; **20**: 6115-6116.
19. Munn, M., Neck rot disease of onions. New York (Geneva) Agricultural Experimental Station *Bulletin* 1917; **437**: 363-455.
20. Nielsen, K. and Yohalem, D.S., Origin of a polyploid *Botrytis* pathogen through interspecific hybridization between *Botrytis aclada* and *B. byssoidea*. *Mycologia* 2001; **93**:1064-1071.
21. Nielsen, K.; Justesen, A.F.; Funck Jensen, D. and Yohalem, D.S., Universally primed polymerase chain reaction alleles and internal transcribed spacer restriction fragment length polymorphisms distinguish two subgroups in *Botrytis aclada* distinct from *B. byssoidea*. Phytopathology 2001; **91**: 527-533.
22. Nielsen, K.; Yohalem, D.S. and Jensen, D.F., PCR detection and RFLP differentiation of *Botrytis* species associated with neck rot of onion. Plant Dis. 86:682-686.
23. O'Gorman, D.T.; Sholberg, P.L.; Stokes, S.C. and Ginns, J. 2008. DNA sequence analysis of herbarium specimens facilitates the revival of *Botrytis mali*, a postharvest pathogen of apple. Mycologia 2002; **100**: 227–235.
24. Owen, J.H.; Walker, J.C. and Stahmann, M.A., Variability in onion neck-rot fungi. Phytopathology 1950; **40**:749-768.
25. Presly, A.H., Studies of *Botrytis* spp. occurring on onions (*Allium cepa*) and leeks (*Allium porrum*). *Plant Pathol*. 1985; **34**:422-427.
26. Ramsey, G.R. and Lorbeer, J.W., Pathogenicity of *Botrytis* species on onion umbels and scapes under controlled conditions. *Phytopathology* 1986a; **76**: 604-612.
27. Ramsey, G.R. and Lorbeer, J.W., The role of temperature and free moisture in onion flower blight. *Phytopathology* 1986b; **76**: 612-616.
28. Shirane, N.; Masuko, M. and Hayashi, Y., Light microscopic observation of nuclei and mitotic chromosomes of *Botrytis* species. Phytopathology 1989; **79**: 728-730.
29. Snedecor, G.W. and Cochran, W.G., Statistical methods, The Iowa State Univ. Press Ames, Iowa, U.S.A., 1967; 593 pp.
30. Thompson, J.D.; Higgins, D.G. and Gibson, T.J., CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research* 1994; **22**(22): 4673-4680.
31. Walker, J.C., Two undescribed species of *Botrytis* associated with the neck rot diseases of onion bulbs. *Phytopathology* 1925; **15**: 708-713.
32. Wang, X.Y.; Zhang, L.X. and Zhang, Z.Y., A new species of *Botrytis* and five known *Botrytis* species in China. *Acta Phytopathol Sin* 1996; **26**: 79-85.
33. Yohalem, D.S.; Nielsen, K. and Nicholaisen, M., Taxonomic and nomenclatural clarification of the onion neck rotting *Botrytis* species. Mycotaxon 2003; **85**:175-182.
34. Zhang, J.; Zhang , L.; Li, G.Q.; Yang, L.; Jiang, D.H.; Zhuang, W.Y. and Huang, H.C., *Botrytis sinoallii*: a new species of the grey mould pathogen on *Allium* crops in China. Mycoscience. DOI: 10.1007/s10267-010-0057-4, 2010.