Journal of Agricultural Technology

Taxonomical studies on the genus *Botrytis* in Iran

S. Mirzaei, E. Mohammadi Goltapeh[*] and M. Shams-bakhsh

Department of Plant Pathology, Faculty of Agriculture, Tarbiat Modares University, P.O. Box: 14115-336, Tehran, Iran.

Mirzaei, S., Goltapeh, E.M. and Shams-bakhsh, M. (2007). Taxonomical studies on the genus *Botrytis* in Iran. Journal of Agricultural Technology 3(1): 65-76.

A total of 355 isolates were collected from all over the country. They were isolated from apple, arum lily, briar rose, bride wort, broad bean, camellia, canola, carnation, cucumber, egg plant, feijoa, geranium, gerbera, gladiolus, grape, guilder rose, hibiscus, iris, kiwifruit, oleander, onion, orange, pear, pomegranate, primrose, quince, redbud, robinia, rose, rubber plant, sow thistle, spathe flower, strawberry, tomato, violet, wall flower and wheat. To identify their species, morphological characters such as conidiophore length and conidial and sclerotial dimensions were measured. Conidiophore length was (527)662 - 2999 (4334) µm, conidial dimension fell in the range of (4)6-13 (20) × (3) 4-8 (12) µm and sclerotial dimension was (0.36) 1- 11 (20) × (0.36) 1-8 (11) mm. Based on these characteristics, all isolates belonged to morphospecies *Botrytis cinerea*. 47 isolates were selected for molecular studies and using C729 primers, a single 0.7 kb band, specific to *B. cinerea*, was amplified in all of them.

Key words: *Botrytis*, taxonomy, conidiophore, conidia and sclerotia

Introduction

Botrytis cinerea is an important pathogen of stored and transported fruits, vegetables, ornamental crops, and nursery stock (Jarvis, 1977). They occur wherever their host crops are grown, ranging from cool temperate zones to subtropical areas (Jarvis, 1977). Symptoms range from restricted lesions to dry or spreading soft rots, with or without the appearance of conspicuous sporulating colonies (Elad *et al*., 2004). *Botrytis* and its sexual form *Botryotinia* Whetzel comprise 22 species and one hybrid (Hennebert, 1973; Yohalem *et al*., 2003). *Botrytis* classification is largely based on morphological and cultural characteristics. Species of *Botrytis* have been named based on host association (Hennebert, 1973; Jarvis, 1977). Features such as sclerotial size, form and conidium size are useful in delimiting some species, but many species are morphologically similar and growing conditions significantly influence variation (Beever and Weeds, 2004). *B. cinerea* is the commonest species of the genus growing on a wide range of host plants as a parasite or

[*]Corresponding author: E. Mohammadi Goltapeh; e-mail: emgoltapeh@yahoo.com

saprophyte; most other species of the genus have a more restricted host range (Domsch *et al*., 1993). Most restricted host specificity occurs on crolliferous monocotyledons and on members of eudicot families Fabaceae, Geraniaceae, Paeoniaceae and Ranunculaceae (Jarvis, 1977). Considerable effort is invested in protecting the agricultural products against *Botrytis* before and after harvest. The market size for anti-*Botrytis* products have been US$ 15-25 million in recent years (Elad *et al*., 2004).

Despite the importance of this pathogen, there have been few studies in Iran especially regarding its taxonomy. Since awareness of the existing species is essential for effective disease management, the aim of this study was to investigate *Botrytis* species in Iran.

Materials and methods

Fungal isolates

A total of 355 isolates were collected from all over the country. The number of isolates from different hosts and location are listed in table 1. The isolates were purified by single spore isolation and growing mycelium was transferred to PDA slants.

Morphological studies

Morphological characteristics such as conidiophore length, conidial dimension and sclerotial dimension were measured. In order to produce the conidia, *Botrytis* isolates were grown in 9-cm Petri dishes containing PDA for 7 days at 20-22 ^{o}C under light, and to produce the sclerotia, isolates were cultured on PDA and kept in dark condition at 8±1 ^{o}C.

Length and width of 50 conidia from each isolate were measured at ×40 magnification on an Olympus microscope (BH2). Also 30 conidiophore lengths and sclerotial dimensions were measured with each isolate. Cultural characteristics such as colony appearance, conidial shape, colour and shape and distribution of sclerotia over the plates also were examined.

DNA extraction

Monoconidial cultures were grown on malt extract agar (Merk, Germany) for 5-12 days in the dark at 20 ^{o}C. Mycelial mass was harvested by scraping the culture using a sterile scalpel. Mycelium was submerged in liquid nitrogen and ground into a fine powder. Genomic DNA was extracted from

fine mycelial powder according to Moller *et al*. (1992). DNA pellets were dissolved in 50 µl of desterilized water and stored at 4 ^{o}C or -20 ^{o}C.

Table 1. List of isolates collected from different hosts and location.

Locations (province)	Number of isolates	Host(s)	Date(s) of sampling
Ardebil	3	onion	August 2004
Azarbayejan Gharbi	9	grape, pear, quince and wheat	October 2004 & February 2006
Ghazvin	3	grape	September 2004
Ghom	1	strawberry	May 2005
Gilan	57	arum lily, briar rose, bride wort, camellia, canola, cucumber, feijoa, guilder rose, hibiscus, kiwifruit, oleander, pome granate, red bud, robinia, rose, rubber plant, sow thistle and violet	May 2004 & June 2005
Golestan	10	broad bean, canola and strawberry	May 2005 & May 2006
Hamedan	3	peony	June 2006
Khozestan	3	egg plant and rose	May 2005
Kordestan	53	strawberry	May 2005
Lorestan	1	apple	May 2005
Markazi	96	arum lily, carnation, geranium, gerbera, gladiolus, iris, oleander, primrose, rose, spathe flower, violet and wall flower	January 2002, January 2003 & February 2005
Mazandaran	81	kiwifruit	March 2004 & March 2005
Semnan	24	grape	September 2004
Tehran	11	onion, orange, strawberry and tomato	May 2005 & February 2006

PCR amplification

PCR amplification was performed in a 25 µl reaction volume containing 1 µl of template DNA, 2.5 µl PCR buffer (10x), 0.6 µl MgCl2 (50mM), 0.5 µl dNTP (10mM), 1µl of each primer (12.5 pmol/µl) and 0.5 µl Taq (5 u/µ Fermentas). Primers sequences were 5'-AGCTCGAGAGAGATCTCTGA-3' (forward) and 5'-AAGGTGCGTCTTGTAACGTC-3' (reverse). PCR reactions were performed in a thermocycler (eppendorf, Germany). The program applied for amplification was as: 1 cycle of 2 min at 94 ^{o}C, 35 cycles of 30 s at 94 ^{o}C, 30 s at 60 ^{o}C, 1 min at 72 ^{o}C ; 1 cycle of final extension for 10 min at 72 ^{o}C.

The PCR product was separated by electrophoresis on a 1.2% agarose gel in 1x TBE buffer and visualized by staining with ethidium bromide.

Results

Cultural characters

Different kinds of growth pattern were observed on potato dextrose agar, at 20 ^{o}C under light. Tangent colonies or aerial mycelium were produced. They were compact, cottony, warty, powdery, radial or in concentric rings (Fig. 1). Colonies were white, dirty white, grayish white, or hyaline at first but soon becoming light gray, dark gray to dark brown, celadon, soiled or mousy.

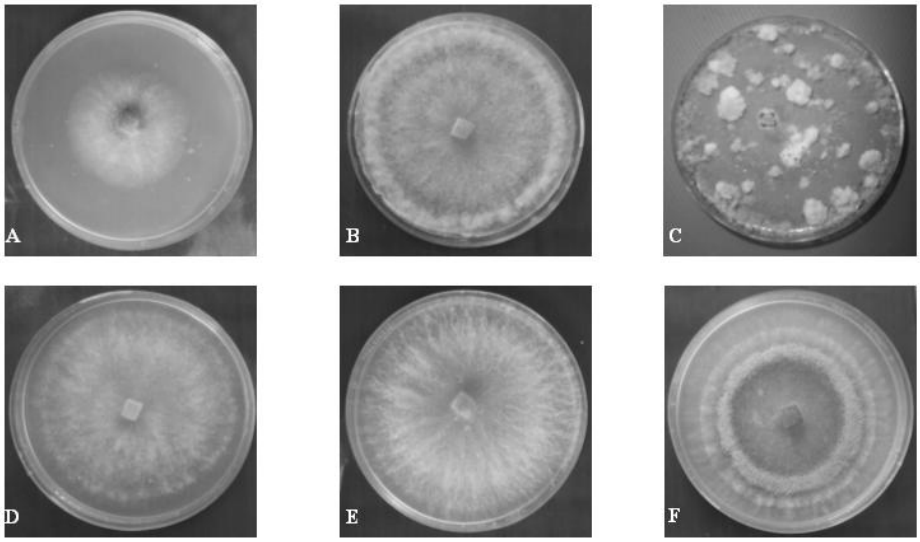

Fig. 1. Growth pattern of *Botrytis cinerea* on PDA. A: compact, B: fluffy (cottony), C: warty, D: powdery, E: appressed (radial) and F: appressed with concentric rings

Conidia usually produced over the surface of the medium, varying in abundance. In some isolates, they were produced all over the plates and in some others in tufts or patches.

Sometimes sporulation began from the old part of the colony and sometimes on the marginal part. In some isolates conidia were produced on concentric rings (Fig. 2).

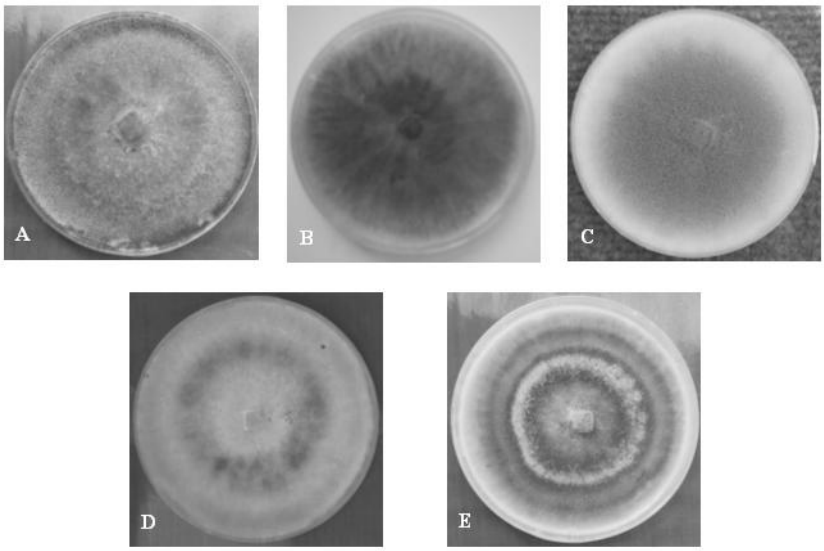

Fig. 2. Sporulation of *Botrytis cinerea* isolates on PDA under light. A: all over the surface, B: in patches, C: from old part of the medium, D: from marginal part of the medium and E: in concentric rings.

Sclerotia also vary in abundance and distribution. In some isolates sclerotia were abundant but rare or absent in others. They were often superficial or deeply imbedded in the agar and adherent to the bottom of the Petri dish. In some isolates both superficial and imbedded sclerotia were produced. They scattered all over the medium in Petri dish and covering the entire surface of the agar. In some isolates sclerotia were produced on concentric rings, formed along the edges of the Petri dish or scattered irregularly (Fig. 3). They were firmly attached to the surface of the medium and were flat or concave on the attachment surface.

Morphological characters

The mycelium was branched, septate, hyaline to brown. Sometimes hyphal swelling here and there was observed. Conidiophore arising directly from the mycelium or from sclerotia. They were more or less straight, septate, branched towards the apex often dichotomously or trichotomously (Fig. 4). They were brown becoming paler near the apex with the ends of the branches often quite colourless. Conidiophore walls becoming deep brown with the age, but there were some cases that conidiophores were narrower and colourless near the base. Also there were some isolates that their conidiophores were

thicker and darker than the hyphae. Average conidiophore length was (527) 662 -2999 (4334) µm. The ultimate conidiophore branches inflated into a swollen conidiogenous cell, the ampulla, that bearing simultaneous conidia on pedicles. The ampulla was clavate, spherical, subspherical, or somehow lobate. After the conidia abscission, a flat rounded scar were left on the ampula (Fig. 4).

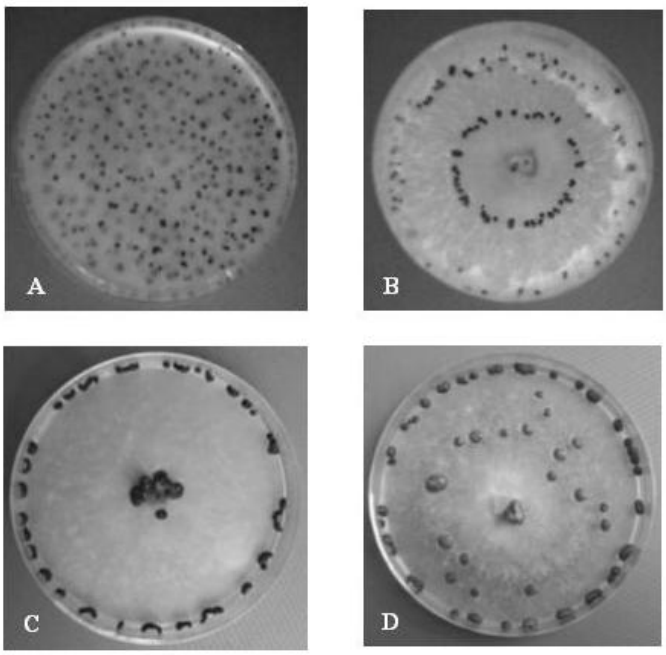

Fig. 3. Distribution and pattern of sclerotia formation on the surface of PDA. A: all over the medium, B: on rings, C: in the edges of the plates and D: irregular form.

Conidia were solitary and attached to the ampulla by fine denticles. They were hyaline or pale brown, but in mass they seemed ashen-gray becoming darker with age. Conidia ovate, ellipsoidal, narrowly ellipsoidal, pyriform or sometimes globose to subglobose or flat in one part. They were smooth, often with a slightly protuberant hilum and usually unicellular but occasionally 1- or 2-septate conidia was observed (Fig. 5). Conidial dimension fell in the range of (4) 6- 13 (20)×(3) 4-8 (12).

Sclerotia were black, shiny black, dark green or white at first becoming black with the age. They were variable in shape and size. They were plano-convexoid, flattened, loaf-shaped, hemispherical, rounded, roughly circular, scaled-like, spongy, pulvinate or irregular in shape, with the surface smooth,

nodolus, or reticulated. Sclerotia discrete or sometimes confluent and in agglomeration. Their size was (0.36) 1-11 (20)×(0.36) 1 -8 (11).

The isolates were grouped according to their morphological characters (conidiophore length, conidial dimension and sclerotial dimension), using MVSP32 software. There were some isolates that did not produce sclerotia, so analysis was carried out twice. One time without these isolates and second time with these isolates and without sclerotial dimensions. In first case isolates grouped into 3 cladograms and second time into 4. In each case there was no relationship between groups and plant hosts or location (data not shown).

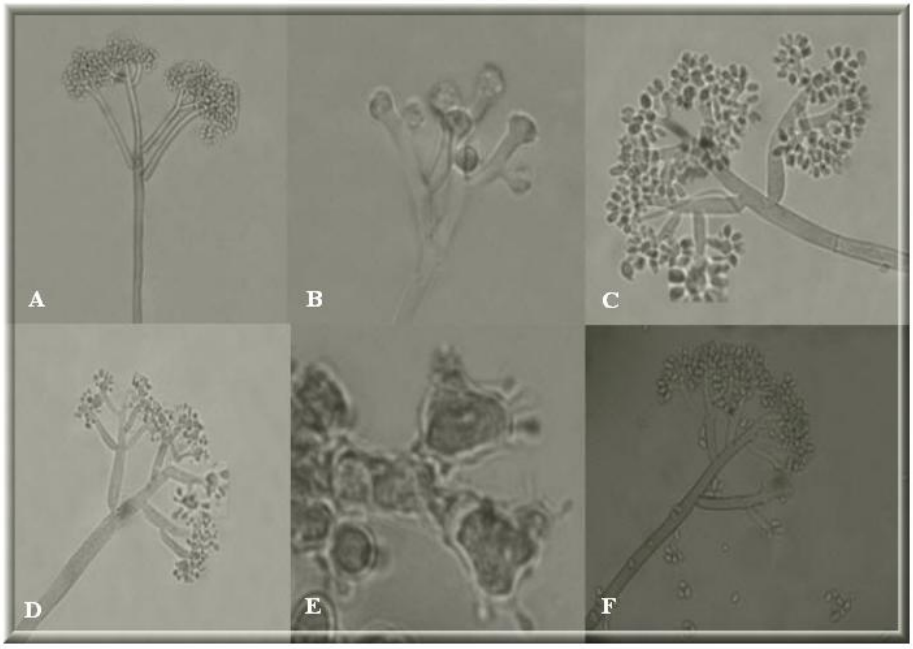

Fig. 4. conidiophore and ampulla in *Botrytis cinerea* isolates. A: whole shape (10x), B: clavate ampula (40x), C: spherical (40x), D: subspherical (20x), E: lobate (cup shape) (100x) and F: scars after conidia abscission (20x).

Molecular characterization

According to morphological characteristics all isolates belonged to *B. cinerea* (Hennebert, 1973; Jarvis, 1977), we decided to confirm this identification by using molecular marker. A set of 48 isolates (table 2) were selected from different clades regarding to their hosts and geographical location. A *B. cinerea* isolate from Netherlands, Bc7, was used as reference.

Rigotti *et al.* (2002) designed primers that were specific to *B. cinerea*. We used these primers in our studies. A single band of 0.7 kb that is specific to *B. cinerea*, was amplified in all 47 isolates and also in the reference isolate. No band was amplified in the negative control (Fig. 6).

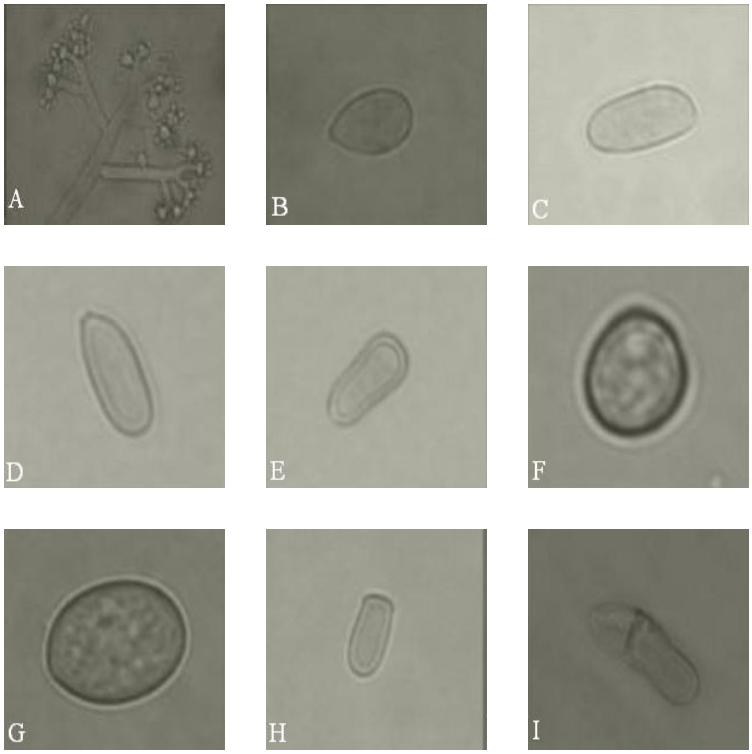

Fig. 5. Conidia formation in *Botrytis cinerea*. A: conidia produced on fine denticle on ampulla (40x), B: ovate (100x), C: ellipsoidal (100x), D: narrowly ellipsoidal (40x), E: pyriform (40x), F: globose (4ox), G: subglobose (100x), H: flat in one part (40x) and I: septate (40x).

Discussion

Botrytis cinerea and other *Botrytis* species are important pathogens of nursery plants, vegetables, ornamental, field and orchard crops and stored and transported agricultural products (Elad *et al*., 2004).

Considering the importance of this pathogen and its significant damage to agricultural products, its management is necessary. The first step in the management of a pathogen is its recognition, and taxonomy is one of the best tools that help us to distinguish pathogens. Despite the importance of *Botrytis*

Table 2. List of selected isolates for molecular characterization.

Isolate code	Host	Location
4	grape	Royan (Semnan)
28	grape	ghazvin
33	grape	Yashil (azarbayejan Gharbi)
54	rose	Mahalat (Markazi)
77	geranium	Mahalat (Markazi)
88	carnation	Mahalat (Markazi)
105	gladiolus	Mahalat (Markazi)
110	gladiolus	Khomeyn (markazi)
111	gerbera	Mahalat (Markazi)
117	primrose	Mahalat (Markazi)
119	oleander	Mahalat (Markazi)
151	kiwifruit	Tonekabon (Mazandaran)
162	kiwifruit	Ramsar (Mazandaran)
188	macronia	Kelachai (Mazandaran)
217	broad bean	Gorgan (Golestan)
225	strawberry	Kordkoy (Golestan)
242	strawberry	Abbas-Abad (Kordestan)
281	rose	Rasht (Gilan)
304	violet	langarud (Gilan)
307	pome granate	Lahijan (Gilan)
312	feijoa	Lahijan (Gilan)
313	sow thistle	Rodsar (Gilan)
316	hibiscus	Lahijan (Gilan)
321	arum lily	Lahijan (Gilan)
322	camellia	Lahijan (Gilan)
324	red bud	Rasht (Gilan)
326	Briar rose	Lahijan (Gilan)
327	Bride wort	Lahijan (Gilan)
328	robinia	Lahijan (Gilan)
329	guilder rose	Lahijan (Gilan)
331	canola	Gorgan (Golestan)
332	kiwifruit	Chayejan (Gilan)
335	cucumber	Rasht (Gilan)
336	quince	Oromiyeh (Azarbayean Gharbi)
339	wheat	Oromiyeh (Azarbayean Gharbi)
340	pear	Oromiyeh (Azarbayean Gharbi)
341	apple	Borojerd (lorestan)
342	egg plant	Ahvaz (khozestan)
344	strawberry	Ghom
345	strawberry	Tehran
350	onion	Tehran
352	rubber plant	Lahijan (Gilan)
353	tomato	Tehran
354	rose	Dezful (khozestan)
356	onion	Tehran
357	orange	Tehran
358	peony	Hamedan

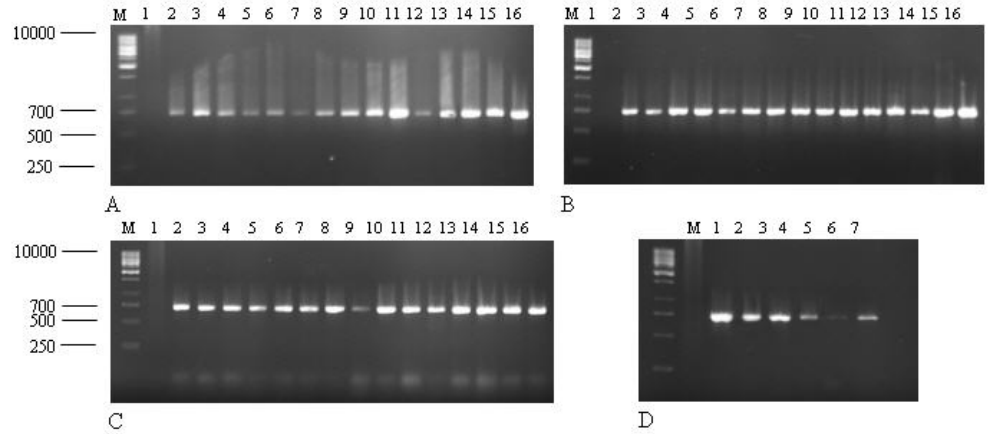

Fig. 6. Polymerase Chain Reaction (PCR) amplification with C729+/- primers on *Botrytis* isolates. M: 1kb ladder (with uppermost band 10000 bp); 1: negative control (no DNA); 2: reference isolate.

A- 3: B4; 4: B28; 5: B33; 6: B54; 7: B77; 8: B88; 9: B105; 10: B110; 11: B111; 12: B117; 13: B119; 14: B151; 15: B162; 16: B188.

B- 3: B217; 4: B225; 5: B242; 6: B281; 7: B304; 8: B307; 9: B312; 10: B313; 11: B316; 12: B321; 13: B322; 14: B324; 15: B326; 16: B327.

C- 3: B328; 4: B329; 5: B331; 6: B332; 7: B335; 8: B336; 9: B339; 10: B340; 11: B341; 12: B342; 13: B344; 14: B345; 15: B350; 16: B352.

D- 3: B353; 4: B354; 5: B356; 6: B357; 7: B358.

grey mould through the world no extensive study has been made in Iran. Therefore a total number of 355 isolate were collected from different hosts and locations in Iran. Their morphological characteristics were examined. Conidiophore length was (527) 662- 2999 (4334) µm, conidial dimensions fell in the range of (4) 6 -13 (20)×(3) 4 -8 (12)µm, and sclerotial dimensions were (0.36)1- 11 (20)×(0.36) 1 -8 (11) µm. According to the key literature all of the isolates belong to *B. cinerea*.

Botrytis taxonomy has traditionally been based on morphological and cultural characteristics coupled with host specificity (Jarvis, 1977, 1980; Hennebert, 1973). Morphological characteristics are influenced by conditions and there is some doubt with their usefulness.

Menzinger (1966a, 1966b) reviewed the taxonomy of *Botrytis* species and showed how cultural conditions could considerably modify taxonomic characters such as dimension and shape of conidia. Venev (cited in Jarvis, 1977) also manipulated conidial size, form and colony characters by altering the temperature and culture medium and found morphological changes to be reversible.

Despite these, morphological characters are so far used in *Botrytis* taxonomy and just in recent years molecular markers have been used in the recognition of *Botrytis* species.

Nielsen *et al*. (2001) 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 were able to distinguish *B. cinerea*, *B. squamosa*, *B. byssoidea* and two groups in *B. aclada* (AI and AII). Staats *et al*. (2005) made use of fragments of three single-copy nuclear DNA (nDNA) genes encoding glyceraldehydes-3-phosphate dehydrogenase (G3PDH), Heat-shock Protein 60 (HSP60) and DNA-dependent RNA polymerase subunit II (RBP2) in *Botrytis* taxonomy. Molecular phylogenetic analysis of the sequences supported the traditional morphological species classification and the hybrid status of *B. allii* (*B. byssoidea* × *B. aclada*) was confirmed. They used ITS regions in their study but the ITS sequence of *B. anthophila* was identical to *Rhizoctonia* sp. ITS sequences, and this sequence was, therefore, excluded from further analysis. Holst-Jensen *et al*. (1998) also analyzed ITS DNA sequences and concluded that the *Botryotinia* teleomorph along with *Botrytis* anamorphs constitute a monophyletic lineage. However variation in the ITS region within *Botrytis* is low and it can't show the relationship among the members of this genus.

There are some species-specific primers (Mathur and Utkhede, 2002; Nielsen *et al*. 2002; Rigotti *et al*. 2002) that had been used in *Botrytis* detection. According to morphological characteristics all of our isolates belonged to *B. cinerea*, we decided to check them by a specific primer. Rigotti *et al*. (2002) designed a primer that was specific to *B. cinerea* and can be used for detection of this species. According to morphological data, hosts and location, we selected 47 isolates and checked them using C729 primers. With all 47 isolates, a single band of 0.7 kb was amplified. These results confirm morphological diagnosis of the isolates.

Although morphological characters can help us in recognition of *Botrytis* species, and Staats *et al*. (2005) showed that molecular studies confirm traditional classification, but this method is time consuming and can be influenced by conditions. So it seems that molecular markers are more useful in delineation of *Botrytis* species. Genetic diversity and pathogenicity tests with the selected isolates will be under taken in our future studies.

Acknowledgements

We thank Dr. M. Staats for his kind provision of the reference isolate, and Dr. J. A. L. van Kan, Dr. D. Yohalem for their valuable comments and suggestions.

References

Beever R.E. and Weeds, P.L. (2004). Taxonomy and genetic variation of *Botrytis* and *Botryotinia*. pp. 29-52 In: Elad, Y., Williamson, B., Tudzynski, P. and Delen, N.(eds). *Botrytis*, Biology, Pathology and Controls. Kluwer Academic Publisher. Netherland.

Domsch, K.H. Gams, W. and Anderson, T. (1993). Compendium of Soil Fungi, New York, Academic Press.

Elad, Y., Williamson, B., Tudzynski, P. and Delen, N. (2004). *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.

Hennebert, G.L. (1973). *Botrytis* and *Botrytis*-like genera. Persoonia, 7: 183-204.

Holst-Jensen, A., Vaage, M. and Schumacher, T. (1998). An approximation to the phylogeny of *Sclerotinia* and related genera. Nordic Journal of Botany 18: 705-719.

Jarvis, W.R. (1977). *Botryotinia and Botrytis* species: Taxonomy, Physiology and Pathogenicity, A guide to the Literature. Monograph No. 15, Canada Department of Agriculture, Ottawa, Canada.

Jarvis, W.R. (1980). Taxonomy. pp. 1-18 In: Coley-Smith, J. R., Verhoeff, K. and Jarvis, W. R. (eds). The Biology of *Botrytis*. Academic Press, London.

Mathur, S. and Utkher, R. (2002). Development of a dot blot technique for rapid identification of *Botrytis cinerea*, the causal organism of grey mould in greenhouse tomatoes. Journal of Horticultural Science and Biotechnology 77: 604-608.

Menzinger, W. (1966a). Zur Variabilitat und Taxonomie von Arten und Formen der Gattung *Botrytis* Mich. I. Untersuchungen zur kulturbedingten Variabilitat morphologischer Eigenschaften von Formen der Gattung *Botrytis*. Aus dem Institut für Pflanzenkrankheiten und Pflanzenschutz der Technischen Hochschule Hannover. Zentralbl Bakteriol Parasitenkd Infektionskr Hyg. 120: 141-178.

Menzinger, W. (1966b). Zur Variabilitat und Taxonomie von Arten und Formen der Gattung *Botrytis* Mich. II. Untersuchungen zur Variabilitat des Kulturtyps unter konstanten Kulturbedingungen. Aus dem Institut für Pflanzenkrankheiten und Pflanzenschutz der Technischen Hochschule Hannover. Zentralbl Bakteriol Parasitenkd Infektionskr Hyg. 120: 179-196.

Möller, E.M., Bahnweg, G., Sandermann, H. and Geiger, H.H. (1992). A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies and infected plant tissues. Nucleic Acids Research 20: 6115-6116.

Neilsen, K. Justesen, A. F., Jensen, D. F. and Yohalem, D. S. (2001). Universally primed polymerase chain reaction alleles and internal transcribed spacer restriction fragment length polymorphisms distinguish two subgroups in *Botrytis aclada* distinct from *Botrytis byssoidea*. Phytopathology 91: 527-533.

Neilsen, K., Yohalem, D.S. and Jensen, D.F. (2002). PCR detection and RFLP differentiation of *Botrytis* species associated with neck rot of onion. Plant Disease 86: 682-686.

Rigotti, S., Gindro, K., Richter, H. and Viret, O. (2002). Characterization of molecular markers for specific and sensitive detection of *Botrytis cinerea* Pers.: Fr. in strawberry (Fragaria ananassa Duch.) using PCR. FEMS Microbiology Letters 209: 169-174.

Staats, M., Baarlen, P.V. and Van Kan, J.A.L. (2005). Molecular phylogeny of the plant pathogenic genus *Botrytis* and the evolution of host specificity. Molecular Biology and Evolution 22: 333-346.

Yohalem, D. S., Neilson, K. and Nicolaisen, M. (2003). Taxonomic and nomenclatural clarification of the onion neck rotting *Botrytis* species. Mycotaxon 85: 175-182.

(Received 18 October 2006; accepted 18 May 2007)