Morphological and Molecular Identification of *Botrytis Cinerea* Causal Agent of Gray Mold in Rose Greenhouses in Centeral Regions of Iran

P.Khazaeli[1]*; H. Zamanizadeh[1]; B. Morid[2]; H. Bayat[3]

1: Department of Plane Pathology, Science and Research Branch, Islamic Azad University, Tehran, Iran
2: Takestan Branch, Islamic Azad University, Tehran, Iran
3: Research International Station, Mahallat, Iran

ABSTRACT

Botrytis cinerea is an important pathogen that causes diseases in ornamental crops. In present research several greenhouses of roses located in central region of Iran were surveyed to identify the Botrytis cinerea. A total of 80 isolates were collected from rose greenhouses in central region of Iran. Morphological identification was based on characters such as conidiophore and conidial length. According to the results conidiophores length was (587) 657-3123 (3896) µm, conidial dimension were in the range of (4)8–13(16)×(2)4–7(10) µm. Based on these characteristics, all isolates belonged to morphospecies Botrytis cinerea. Using specific primer pairs C729 and PCR molecular method, a DNA fragment of 700bp was amplified to the gene isolates for 30 representative isolates identified by morphological assessment indicating the presence of Botrytis cinerea rose samples.

Keywords: *Botrytis Cinerea*; Gray mold; Morphological and Molecular identification.

* **Corresponding Author Email:** parisa_khazaeli82@yahoo.com

INTRODUCTION

Botrytis cinerea Pers.: Fr. is a common and economically important pathogen of numerous greenhouses-grown ornamental crops (Daughtery & Petrson, 1995)Ornamental plants grown in greenhouses are become the valuable agricultural production in Iran, the ornamental crop grown in greenhouses alone had an estimated commercial value of 2 million dollars in the year 2006. One of the most important ornamental crops that cultivated in Iran are roses, with a total production of approximately 270 million root stocks in a year. The gray mold which is caused by *Botrytis cinerea* is one of the most important diseases of roses, that create highly damage in the rose greenhouses every year quantitatively and qualitatively. Botrytis and its sexual form Botryotinia Whetzel comprise 22 species and one hybrid (Hennebert, 1973; Yohalem *et al.,* 2003). Botrytis genus classification is largely based on morphological and cultural characteristics (Hennebert, 1973). Characteristics such as sclerotia size, form and conidium size are useful in delimiting some species, but many species are morphologically similar and growing conditions significantly influence variation (Beever & Weeds, 2004). B. cinerea is a common species and inhabited on a wide range of host plants as a parasite or saprophyte (Domsch & Anderson, 1993). Considerable effort is invested in protecting the agricultural products against *Botrytis cinerea* before and after harvest. The market size for anti-Botrytis products has 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. The aim of this study was to detection of *Botrytis cinerea* in several greenhouses of roses located in the centeral region of Iran.

MATHERIALS AND METHODS

Fungal isolates

A total of 80 isolates were collected from rose greenhouses in center of Iran. The number of 30 isolates that used in this study, from different location is listed in table 1.

All isolates were purified by single spore: isolates were grown on Potato dextrose agar (PDA) at 25°c. Mycelium was allowed to grow out and sporulate, and conidia were transferred to a microfuge tube containing 100 µl of sterile water. An aliquot of this suspension was spread onto a 6 cm diameter water agar plate, for the isolation of single spore derived isolates, and were incubated at 20°c for 24 h, and sporulation induced after 1d with black light for 1 h. After purification all isolates were stored on PDA slopes at 4°c.

Table 1: List of isolates collected from rose greenhouses in central region of Iran

Locations	Number of isolates	Date(s) of sampling
Mahallat	52	March.May/2009
Tehran	7	April /2009
Varamin	18	May/ 2009
Yazd	3	June/2009

Morphological studies

Morphological characteristics such as conidiophores length, conidial dimension were measured. In order to produce the conidia, *Botrytis cinerea* isolates were grown in 9-cm Petri dishes containing PDA for 7 days at 20-22^{0}c under light. Length and width of 30 conidia from each isolate were measured at × 40 magnification on a Leica DNLB. Cultural characteristics such as colony appearance, conidial shape, color and shape also were examined.

DNA extraction

B.cinerea isolates were grown in 100-ml Potato dextrose broth, at 21 under alternate light (12h/12h) without agitation. The incubation time varied from 5 to 10 days, until the fungi colonized the surface of the medium. Mycelia mat was harvested by filtration. Mycelia were grounded in liquid nitrogen using a mortar and pestle.

The procedure for extracting DNA was modified from the CTAB method (Rigotti *et al*., 2002). DNA pellets were dissolved in 50 µl of distilled water and stored at -20°c.

PCR amplification

PCR amplification was performed in a 25 µl reaction volume containing 2 µl of template DNA, 2.5 µl PCR buffer (10x), 0.75 µl MgCl2 (50mM), 0.75 µl dNTP (10mM), 1µl of each primer and 0.2 µl Taq (5 u/µ Fermentas). Primers sequences were 5'-AGCTCGAGAGAGATCTCTGA-3'(C729+) and 5'- CTGCAATGTTCTGCGTGGAA-3'(C729-). 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 45 s at 94 ^{o}c, 50 s at 50 ^{o}c, 50 s at 72 ^{o}c ; 1 cycle of final extension for 5 min at 72 ^{o}c. The PCR product was separated by electrophoresis on a 1.2 percent agarose gel in 1x TBE buffer and visualized by staining with ethidium bromide.

RESULTS AND DISCUSSION

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 cottony, powdery, compact or radial pattern (Fig. 1). Colonies were white, dirty white or grayish white in color, or hyaline at first but soon becoming light gray, dark gray to dark brown.

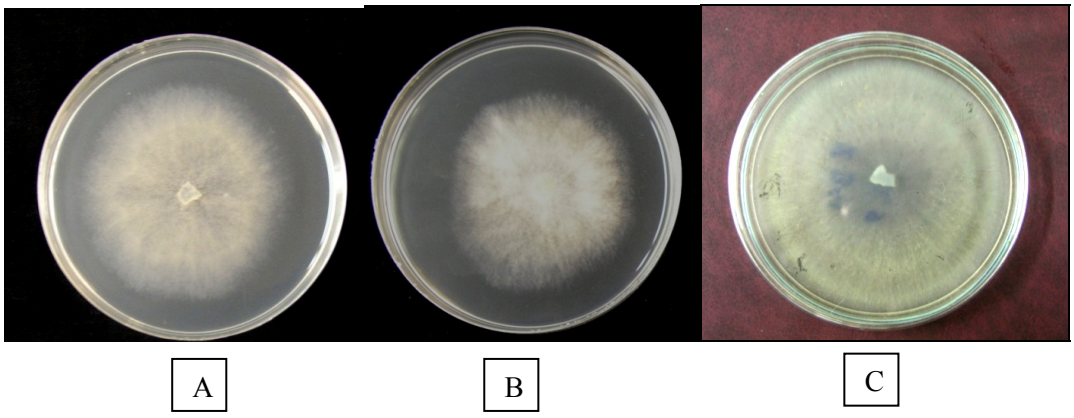

Fig. 1: Growth pattern of *Botrytis cinerea* on PDA. A: powdery, B: compact, C: radial

Morphological characters

The mycelium was branched, septate, hyaline to brown. Conidiophores arise directly from the mycelia or from sclerotia. They were more or less straight, septate, branched at the apex often dichotomously or trichotomously. Conidiophores and conidium were grape shape and conidia Average conidiophore length was (587)657 - 3123 (3896) µm. Each conidial were one cell with egg-shape hyaline. Conidial dimension fell in the range of (4)8–13(16)× (2)4–7(10) µm [length (min) average range (max)× width (min) average range (max)].

Molecular characterization

According to morphological characteristics all isolates belonged to B. cinerea (Hennebert, 1973). we decided to confirm this identification by PCR molecular method. A set of 30 isolates (Table 2) were selected from different rose greenhouses in different regions. We used primers that Rigotti *et al*., (2002) designed specifically for B. cinerea detection by Rigatti *et al.,*(2002). A single band of 0.7 kb that is specific to B. cinerea, was amplified in all 30 isolates and also in the reference isolate. No band was amplified in the negative control (Fig.2).

Table 2: List of selected isolates

Isolate code	location
Bc1-12	Mahallat (March/2009)
Bc18-27	Mahallat (May/2009)
Bc13-17	Varamin
Bc28-Bc29	Tehran
Bc30	Yazd

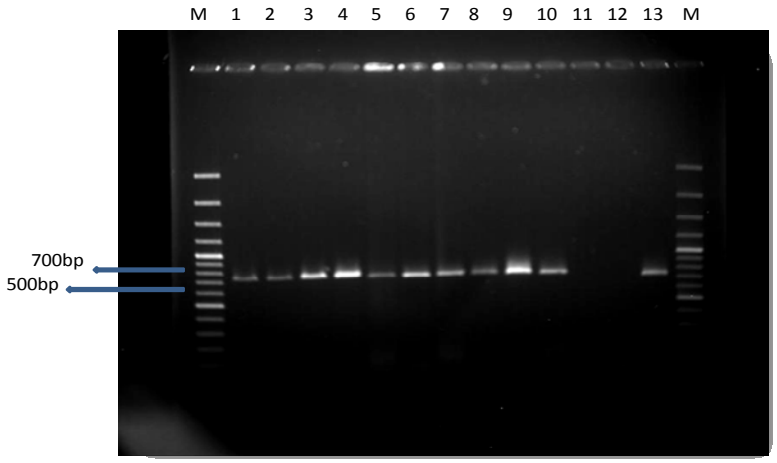

Fig. 2: Polymerase Chain Reaction (PCR) amplification with C729+/- primers on *Botrytis cinerea*

isolates. M: 1kb ladder (with uppermost band 10000 bp); 1:Bc36, 2: Bc12,3:Bc22,4:Bc1, 5:Bc6, 6:Bc45, 7:Bc75, 8:Bc:33,9:Bc62, 10:Bc51, 11: negative control (no DNA);12*:Botrytis alli,*13: positive control

DISCUSSION

Botrytis cinerea Pers. is a phytopathogenic fungus which causes grey mould on over 230 hosts (Vallejo *et al*., 2002). Considering the importance of this pathogen and its significant damage to agricultural products, its control management is necessary. The first step in the management of a pathogen is its identification. Monitoring the infected regions is one of the best tools that help us to identify pathogens.

Therefore a total number of 80 isolate were collected from several greenhouses of roses located in Markazi and Tehran province in center of Iran. According to the key literature all of the isolates identified as B. cinerea.

Botrytis cinerea identification has traditionally been based on morphological and cultural characteristics coupled with host specificity (Jarvis, 1977). Morphological characteristics are influenced by conditions and there is some doubt with their usefulness. Menzinger (1966) reviewed the taxonomy Botrytis species and showed how cultural conditions could considerably modify taxonomic characters such as dimension and shape of conidia and also it is not good for identify the Botrytis species. Venev 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 cinerea* identification and just in recent years molecular markers have been used in the recognition of Botrytis species. There are some species-specific primers that had been used in *Botrytis cinerea* detection (Rigotti *et al*., 2002). 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 and is one of the important one that used to detection of Botrytis cinerea. According to morphological data and location, we selected 30 isolates and checked those using C729 primers. With all 30 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 cinerea, 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 detection of *Botrytis cinerea* species. The results of this study showed the monitoring of widespread of *Botrytis cinerea* in rose producing greenhouses in central region of Iran. By molecular method and using species specific primer we can identify isolates of *Botrytis cinerea* carefully and rapidly.

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