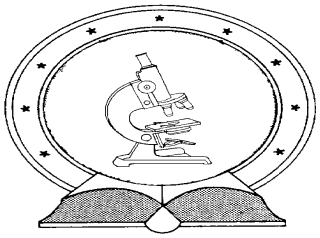

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Genetic diversity of *Botrytis cinerea* and its relevance in the development of fungicide resistance

PhD Thesis

A thesis submitted by:

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Doctorate School of Juhász-Nagy Pál

2013

Ezen értekezést a Debreceni Egyetem Természettudományi Doktori Tanács Juhász-Nagy Pál Doktori Iskola biológia programja keretében készítettem a Debreceni Egyetem természettudományi doktori (PhD) fokozatának elnyerése céljából.
This thesis was prepared in the Juhász-Nagy Pál Doctoral School (program: biology) of the Doctoral Board of Science, University of Debrecen.

Debrecen, 2013.01.14 .

a jelölt aláírása

Tanúsítom, hogy Mojtaba Assadollahi doktorjelölt 2009- 2013 között a fent megnevezett Doktori Iskola biológia programjának keretében irányításommal végezte munkáját. Az értekezésben foglalt eredményekhez a jelölt önálló alkotó tevékenységével meghatározóan hozzájárult. Az értekezés elfogadását javasolom.

Debrecen, 2013.01.14 . *a témavezető aláírása*

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Értekezés a doktori (Ph.D.) fokozat megszerzése érdekében
a biológia tudományágban

Írta: **Mojtaba Assadollahi** okleveles biológus

Készült a Debreceni Egyetem Juhász-Nagy Pál doktori iskolája
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Az értekezés védésének időpontja: 20… . …… … .

To my wife who has always stood by me

Acknowledgments

First of all, I wish to express my sincere gratitude to my supervisors Dr. Karaffa Levente and Dr. Karaffa Erzsébet Mónika for their time, effort and unbounded patience to make my dissertation a success. This work would not have been possible without their guidance, support and encouragement. Throughout my doctoral work they encouraged me to develop my research skills and gave me time whenever I needed without any hesitation. Thank you doesn't seem sufficient but it is said with appreciation and respect to both of them for their support and encouragement.

Besides my supervisors, I warmly thank Dr. Fekete Erzsébet for her valuable advice and unfailing practical support. She has always been kind and helpful to me. I would also like to thank Dr. Michel Flipphi for his precious advice and scientific contributions. My special thanks go to Prof. Dr. Holb Imre and Dr. Kredics László for their detailed review, constructive criticism and excellent advice about my thesis. I am also thankful to Dr. Mahdi Esmaeili who helped me in Data Mining.

During this work I have collaborated with many colleagues from Department of Biochemical Engineering, Institute of Plant Protection and Institute of Food Processing, Quality Assurance and Microbiology for whom I have great regard, and I wish to extend my warmest thanks to all those who have helped me in anyways.

There certainly exist no words that could possibly express the extent of gratitude I owe to my loving wife and her parents. This work is the result of their support, inspiration and understanding. I owe everything to them. At the end, it is a pleasant task to express my thanks to all those who contributed in many ways to the success of this study and made it an unforgettable experience for me.

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Abstract

Botrytis cinerea is a major crop pathogen infesting >220 hosts worldwide. *B. cinerea* has been described as a species complex consistent of two phylogenetic or cryptic "species", group I (also called *B. pseudocinerea*) and group II (*B. cinerea sensu stricto*). The isolates were collected from infected oilseed rape, strawberries and raspberries from various locations in the Nagyréde and Hatvan districts in North-East Hungary during the 2007, 2008 and 2009 growing seasons. Population genetics analysis revealed a high level of diversity within each species and a lack of gene flow between them. The first *B. cinerea* group I strains outside of Western Europe were collected in Hungary in 2008 from strawberry and rape plants. Sympatric *B. cinerea* cryptic species were analyzed using a population genetic approach and phenotypic markers. Statistically significant, but moderate population differentiation was found between the two groups in Hungary. Group I was originally typified by the lack of the transposable elements Boty and Flipper. However, all the Hungarian group I isolates carried the Boty element and one isolate additionally contained Flipper, indicating a much wider genetic variation than previously believed. Phenotypic markers such as fenhexamid resistance or asexual spore size were found unsuitable to differentiate between the cryptic species. Sensitivity of the 157 *B. cinerea* field isolates to a Qo inhibitor (QoI) fungicide azoxystrobin was screened in this study. Resistance has been reported in more than thirty species. In various QoI-resistant monosporic *B. cinerea* isolates from Hungary, a G-to-C point mutation was identified in the mitochondrial gene that encodes the QoI target, cytochrome *b*, resulting in a glycine to alanine substitution at position 143 (G143A). Analysis of Hungarian group I and group II strains further indicated the frequent occurrence of an additional group I-type intron in the *cytb* gene directly downstream of the glycine-143 codon. Mutual presence of

distinct mitochondrial DNAs specifying different *cytb* alleles (heteroplasmy) has also been detected in monosporic strains. Remarkably, a number of group II field isolates were found to be highly resistant to azoxystrobin although they did not appear to carry the G-to-C mutation (G143A) generally associated with fungal QoI-resistance. Moreover, we sampled populations of *B. cinerea* on sympatric strawberry and raspberry cultivars in North-East of Hungary for three years growing seasons. Altogether, 490 of group II *B. cinerea* isolates were analyzed. Standard population genetic data were computed from three different data sets: (i) PCR-RFLP pattern of *ADP-ATP translocase* and *nitrate reductase* genes, (ii) MSB1 minisatellite sequence data, and (ii) fragment size of five microsatellite loci. The structure of the different populations was similar as indicated by Nei's gene diversity and Shannon's information index, and also by haplotype diversity. It should be noted, that a reduced sample size was accompanied by reduced population diversity. The computed index of differentiation (G_{st}), and gene flow indicated differentiation within the sympatric populations. However, the differentiation was also affected by the sample size and the year of isolation. Population genetic parameters were also influenced by the level of polymorphism of the data sets. Data mining and Bayesian approach were implemented in this work. The results support the possibility of sympatric divergence associated with host use in generalist parasites.

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Chapter 1

Introduction

1. 1. *BOTRYTIS*

Botrytis and its sexual form *Botryotinia* Whetzel consist of more than 22 species, and they are classified within the family Sclerotiniaceae Whetzel (Inoperculate Discomycetes). The most important species with respect to economic damage are *B. elliptica*, *B. tulipae*, and *B. gladiolorum*, each potentially causing yield losses up to 60%. *Botrytis* species infecting flower bulb crops are considered specialists with a narrow host range, except *B. cinerea*, which is an opportunistic pathogen. *B. cinerea* infects only flowers and dead or senescing plant parts of bulb flowers. Sexual stage, disease symptoms, and typical host plant species were recognized by Hennebert (1973) and are shown in Table 1. 1. The species of *Botrytis/Botryotinia* are delimited on the basis of morphological and cultural characteristics, although host specificity is also used as discriminatory trait (Hennebert, 1973; Yohalem et al. 2003; Zhang et al. 2010). Morphological characteristics such as the size and form of sclerotia, mycelia and conidia, are useful in delimiting some species but many species are morphologically similar and growing conditions significantly influence variation, moreover, a key to all recognized species is not available (Jarvis, 1977; Beever and Weeds, 2004; Nielsen and Yohalem, 2001). A multiple-gene gene genealogy study recently showed that this genus could be subdivided into two clades, one consisting of *Botrytis* species acting as parasites on both monocots and dicots, and the other containing *Botrytis* species acting as parasites on eudicots only (Staats et al. 2005). Most species have a worldwide distribution or occur wherever their host crops are grown.

Table 1. 1: Species Recognized by Hennebert (1973), Sexual Stage, Disease Symptoms, and Host Specificity

Species	Sexual stage	Common Disease Name	Typical Host	Host-Plant Species	Host-Plant Family
B. cinerea (de Bary) Whetzel	Yes	Gray mould	Fallen leaves, fruits, and flowers	>235 plant species	Polyphagous on eudicotyledons
B. fabae Sardina	No	Chocolate spot	Leaves of bean	*Vicia*, *Pisum* & *Lens* spp. L.,	Fabaceae
B. calthae Hen.	Yes	-	Stem of marsh-marigold	*Caltha palustris*	Ranunculaceae
B. ranunculi Hen.	Yes	-	Buttercup	*Ranunculus* spp. L.	Ranunculaceae
B. ficariarum Hen.	Yes		Buttercup	*Ficaria verna*	Ranunculaceae
B. pelargonii Roed	Yes	-	Leaves of *geranium*	*Pelargonium* spp. L.	Geraniaceae
B. paeoniae Oud.	No	Peony blight	Stems of cultivated peonies	*Paeonia* spp. L.	Paeoniaceae
B. hyacinthi Wes. & Bey.	No	Hyacinth fire	Leaves of hyacinth	*Hyacinthus* spp. L.	Hyacinthaceae
B. tulipae Lind	No	Tulip fire	Leaves, stems & flowers of cultivated tulips	*Tulipa* spp. L.	Liliaceae
B. elliptica (Berk.) Cooke	Yes	Lily fire	Leaves, stems & flowers of cultivated lilies	*Lilium* spp. L.	Liliaceae
B. squamosa Walker	Yes	Onion leaf blight	Leaves of onion	*Allium cepa*	Alliaceae
B. aclada (Fresen.) Yohalem	No	Gray-mould neck rot	Bulbs of onion, garlic, and leek	*Allium* spp. L.	Alliaceae
B. allii [a] (Munn) Yoh.	No	Gray-mould neck rot	Bulbs of onion, garlic, and leek	*Allium* spp. L.	Alliaceae
B. byssoidea Wal.	Yes	Mycelial neck rot	Bulbs of onion, garlic, and leek	*Allium* spp. L.	Alliaceae
B. globosa Raabe	Yes	Neck rot	Wild garlic	*Allium ursinum.*	Alliaceae
B. porri Buchw.	Yes		Bulbs of garlic, leek	*Allium* spp. L.	Alliaceae
B. sphaerosperma Buchw.	Yes	Blight	Three-cornered Leek	*Allium triquetrum*	Alliaceae
B. narcissicola Kleb.	Yes	Smoulder mould	Bulbs of *narcissus*	*Narcissus* spp. L.	Amaryllidaceae
B. polyblastis Dow.	Yes	Narcissus fire	Leaves of *narcissus*	*Narcissus* spp. L.	Amaryllidaceae
B. galanthina Sacc.	No	Blight	Snowdrop	*Galanthus* spp. L.	Amaryllidaceae
B. convoluta Whe. & Dra.	Yes	*Botrytis* rhizome rot	Rhizomes of cultivated iris	*Iris* spp. L.	Iridaceae
B. croci Coo. & Mas.	No	Crocus blight	Leaves of cultivated crocus	*Crocus* spp. L.	Iridaceae
B. gladiolorum Timm.	Yes	Gladiolus blight	Stems of cultivated gladiolus	*Gladiolus* spp. L.	Iridaceae

[a] Hybrid species according to Yohalem, Nielsen, and Nicolaisen 2003.

Based on Staats et al. (2005).

Recently, DNA-based molecular techniques have been widely used to identify fungi, including *Botrytis* species. Nielsen and Yohalem (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*. Staats et al. (2005) made use of fragments of three single-copy nuclear DNA (nDNA) genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH), HSP60 encoding heat-shock protein 60 and DNA-dependent RNA polymerase subunit II (RBP2), which have been successfully used for *Botrytis* taxonomy. There are some species-specific primers that had been used in *Botrytis* detection (Mathur and Utkhede, 2002; Nielsen et al. 2002). Rigotti et al. (2002) and Mirzaei et al. (2008) designed a primer that was specific to *B. cinerea* and can be used for detection of this species.

Morphological traits, together with the application of molecular tools, such as DNA sequence data of multiple protein-coding genes and phylogenetic analysis, can be used to unambiguously identify *Botrytis* species and open the way to a better understanding of the genetic diversity within the genus.

Most of the knowledge on mechanisms that generate genetic variability in *Botrytis* is based on studies in *B. cinerea* till now. This well-studied species causes serious economical damage on many eudicot plant species, including most vegetable and fruit crops, flowers, woody ornamentals and greenhouse-grown crops.

1. 2. *BOTRYTIS CINEREA*

Botrytis cinerea is characterized by abundant hyaline conida (asexual spores) borne on grey, branching tree-like conidiophores. The fungus also produces highly resistant sclerotia as survival structures in older cultures. It overwinters as sclerotia or intact mycelia, both of which germinate in spring to produce conidiophores. The conidia are dispersed by wind and rain-water and cause new infections. Conidia of *B. cinerea* are considered to be the main produced and dispersed inoculum (Holz et al. 2004). The fungus is usually referred to by its anamorph (asexual form) name, because the sexual phase is rarely observed. The sexual cycle involves the spermatization of sclerotia, leading to the production of apothecia. The apothecia form of *Botryotinia fuckeliana* is the teleomorph stage of *Botrytis cinerea* (Fig. 1. 1) (Beever and Weeds, 2004; Williamson et al. 2007).

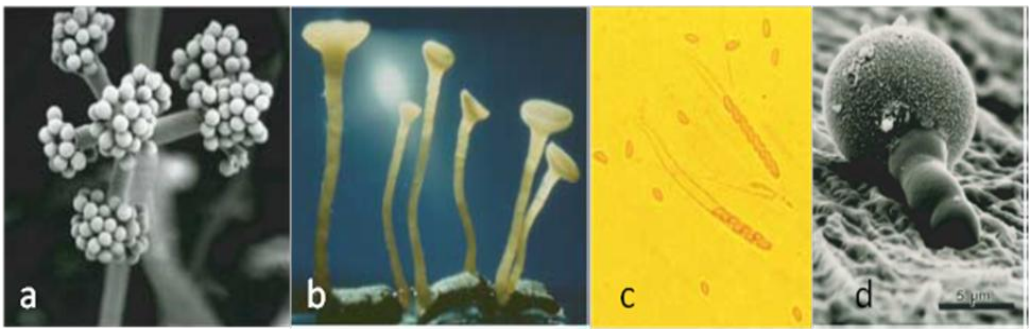

Fig. 1. 1: (a) *B. cinerea* conidiophore with mature conidia. (b) Apothecia of *Botryotinia fuckeliana*, approximately 10 weeks after spermatization. (c) Two asci each containing eight ascospores, surrounded by ascospores released from damaged asci. (d) Conidium germinating in absence of water droplet on abaxial surface of rose petal. Source: Williamson et al. (2007).

Under laboratory conditions, *B. cinerea* sclerotia continue to sporulate for about 12 weeks after the production of the first crop of conidia (Nair and Nadtotchei, 1987). The species name *Botrytis cinerea* is derived from the Latin for "grapes like ashes"; although poetic, the "grapes" refers to the

bunching of the fungal spores on their conidiophores, and "ashes" just refers to the greyish colour of the spores *en masse* (Liddell and Scott, 1940). The life cycle of *B. cinerea* is shown in Figure 1. 2.

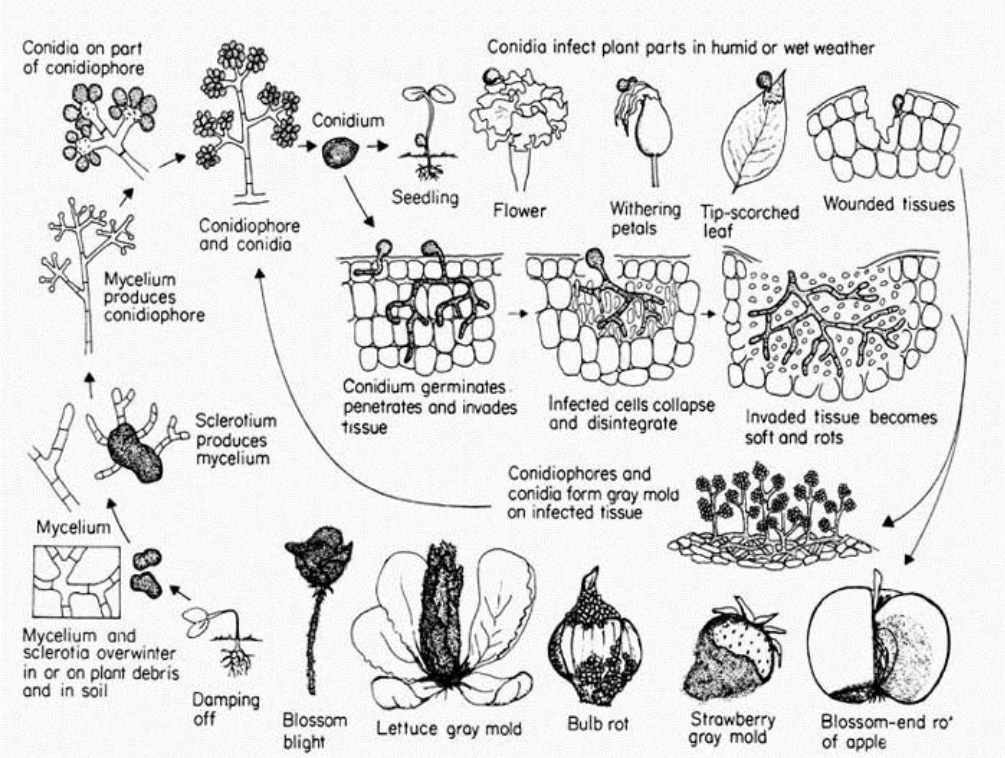

Fig. 1. 2: Life cycle of *B. cinerea*. Source: Agrios (1997).

B. cinerea is a parasitic fungus that affects many plant species, although its most notable hosts may be wine grapes. In viticulture, it is commonly known as *botrytis bunch rot*; in horticulture, it is usually called *grey mould* or *gray mold*. The fungus gives rise to two different kinds of infections on grapes. The first, grey rot, is the result of consistently wet or humid conditions, and typically results in the loss of the affected bunches. The second, noble rot, occurs when drier conditions follow wetter, and can result in distinctive

sweet dessert wines, such as Aszú of Hungary, Sauternes of France and the Trockenbeerenauslese of Germany (Elad et al. 2004). In the *Botrytis* infection known as "noble rot", the fungus removes water from the grapes, leaving behind a higher percent of solids, such as sugars, fruit acids and minerals. This results in a more intense, concentrated final product. The wine is often said to have an aroma of honeysuckle and a bitter finish on the palate (Ribéreau-Gayon et al. 1980). *Botrytis* complicates winemaking by making fermentation more complex. *Botrytis bunch rot* is another condition of grapes caused by *B. cinerea* that causes great losses for the wine industry. It is always present on the fruit set, however, it requires usually a wound to start a bunch rot infection. Wounds can come from insects, wind, accidental damage, etc.

B. cinerea affects many other plants. It is economically important on soft fruits such as strawberries, raspberry and bulb crops. *Botrytis fruit rot* is one of the most important diseases of strawberry worldwide. This disease causes severe pre-harvest and post-harvest losses, primarily due to infection that is initiated in the field during flowering and remains quiescent until fruit ripens. Although the disease can be important pre-harvest, it is most important when developing during shipment or storage. After harvest, this disease is the major limiting factor in cold-chain marketing of strawberries (Droby and Lichter, 2007). Disease cycle of gray mold on strawberry is shown in Figure 1. 3.

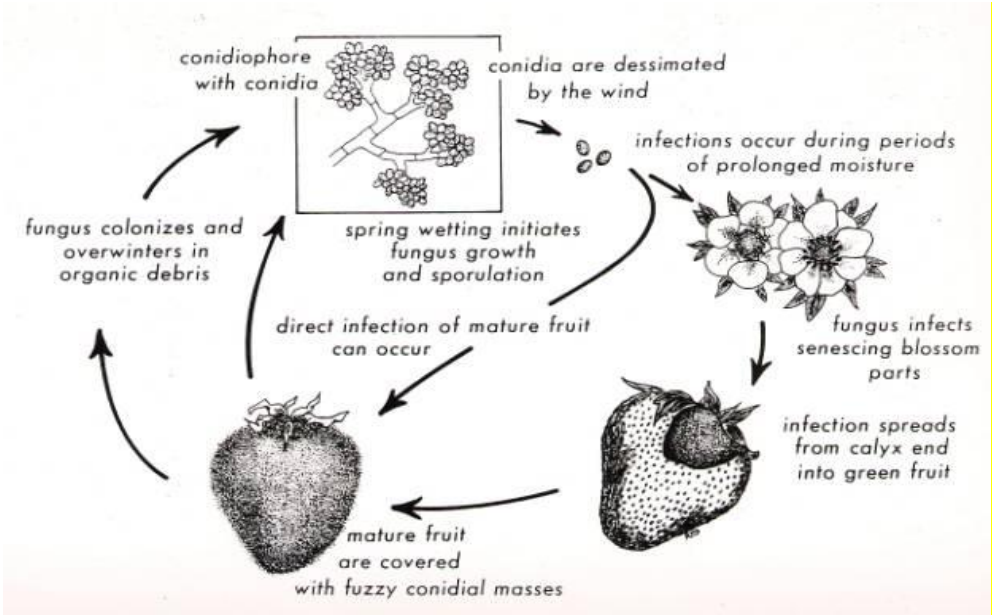

Fig. 1. 3: Infection cycle of gray mold on strawberry. Source: Ellis (2008).

1. 2. 1. Fungicide resistance

B. cinerea is a ubiquitous fungus causing pre- and post-harvest diseases in more than 230 plants, such as economically important crops, including vegetables (e.g. tomato, cucumber, and lettuce), fruits (e.g. grapevine, strawberry, and kiwifruit), bulbs (e.g. onion) and ornamental (e.g. rose) plants. It can attack many organs including leaves, stems and fruits as a nectrotroph, often with heavy losses after harvest. It is also a saprophyte on senescent and dead plant material. *B. cinerea* infects fruit (e.g. apple, grapevine, strawberry) primarily through wounds such as bruises, punctures, or insect damage created near or at harvest and during postharvest handling. It can also cause latent infections of floral parts of the fruit during the early growing season leading to calyx-end gray mold during storage (Lennox and Spotts, 2004). *B. cinerea* is difficult to control because it has a variety of modes of attack, diverse hosts as inoculum sources, and it can survive as

mycelia and/or conidia or for extended periods as sclerotia in crop debris (Williamson et al. 2007). For these reasons the use of any single control measure is unlikely to succeed that is why a more detailed understanding of the host–pathogen interaction, the microenvironment in which the fungus operates and its microbial competitors on the host is needed. The current cost of bringing a new fungicide or biological control agent to market is so high that only major crops attract sufficient interest by agribusiness (Williamson et al. 2007).

Chemical control is one of main approaches to reduce the incidence of grey mould diseases on major crops. The most common interventions consist of spraying aerial parts of plants with fungicides. The applied doses (Table 1. 2) vary from 2000-3000 g/ha (e.g. maneb, thiram, dichlofluanid) to 400-500 g/ha (e.g. carbendazim, fludioxonil, pyrimethanil). The number of treatments during a season ranges from one or two, to more than twenty. Treatments of seeds or bulbs, as well as fungicide applications after the harvest of fruits, are also used (Gullino and Kuijpers, 1994; Leroux, 2004). The chemicals used for control of *B. cinerea*, can be achieved by several families of fungicides. According to Rosslenbroich and Stuebler (2000) five categories of fungicides are recognized, namely (i) those affecting respiration, (ii) microtubule assembly, (iii) osmoregulation, (iv) sterol biosynthesis inhibitors and (v) those whose toxicity is reversed by amino acids.

In the group of fungicides affecting fungal respiration, multi-site toxicants (e.g. dichlofluanid, thiram) are considered the oldest ones. Newer ones are uncouplers (e.g. fluazinam), inhibitors of mitochondrial complex II (e.g. boscalid) or complex III (e.g. strobilurins). Among anti-microtubule botryticides, negative-cross resistance can occur between benzimidazoles (e.g. carbendazim) and phenylcarbamates (e.g. diethofencarb), a

phenomenon determined by a mutation in the gene encoding *β-tubulin*. Aromatic hydrocarbon fungicides (e.g. dicloran), dicarboximides (e.g. iprodione, procymidone, vinclozolin) and phenylpyrroles (e.g. fludioxonil) affect the fungal content of polyols and resistance to these various compounds can be associated with mutations in a protein histidine kinase, probably involved in osmoregulation. However, dicarboximide-resistant field strains of *B. cinerea* are sensitive to phenylpyrroles. Anilinopyrimidines (e.g. cyprodinil, mepanipyrim, pyrimethanil) inhibit methionine biosynthesis but their primary target site remains unknown. Among sterol biosynthesis inhibitors those inhibiting 14α- demethylase (DMIs) which are widely used against many fungal diseases are of limited interest against *Botrytis* spp., though the hydroxyanilide fenhexamid, which inhibits the 3-keto reductase involved in sterol C4-demethylations, is a strong botryticide (Leroux, 2004).

The chemical control of *Botrytis* diseases is impeded because of the development of resistance to many fungicides and also the negative public perception regarding the safety of pesticides. As a consequence, in many countries, the regulatory authorities have restricted the use of new and established pesticides (Gullino and Kuijpers, 1994).

Table 1. 2: Intrinsic toxicity of fungicides towards *Botrytis cinerea* and application rates

Fungicide		EC50 value (mg/L)			Typical Application rate (g/ha)
Common name	**Selected trade names**	**Conidia germination**	**Germtube elongation**	**Mycelium growth**	
Dichlofluanid	Elvaron, Euparen	0.20	0.05	3.00	2000
Folpet	Folpan	3.00	0.40	10.00	1500
Thiram	Pomarsol, Thiram	~ 1.00	0.10	10.00	2000
Fluazinam	Frownicide, Shirlan	0.10	0.04	0.08	750
Azoxystrobin	Amistar, Heritage, Quadris Top	> 10.00	2.50	10.00	200
Boscalid	Endura	0.50	0.10	0.40	600
Carbendazim	Bavistin, Derosal	> 10.00	0.04	0.03	500
Diethofencarb[a]	Sumico[b]	> 10.00	0.08	0.04	500
Iprodione	Kidan, Rovral	2.00	0.80	0.15	750
Procymidone	Sumiclex, Sumilex	2.50	0.80	0.15	750
Fludioxonil	Geoxe, Switch[c]	0.06	0.015	0.004	500
Cyprodinil	Switch[c], Unix	~ 0.10	0.008	0.01	375
Pyrimethanil	Scala	~ 0.30	0.05	0.08	800
Prochloraz	Octave, Sportak	> 10.00	0.03	0.10	250
Tebuconazole	Folicur, Horizon	> 10.00	0.20	0.30	250
Fenhexamid	Teldor	> 10.00	0.05	0.01	750

[a]The EC50 values were recorded by testing benzimidazole-resistant strains (BenR1).
[b]Mixture of diethofencarb and carbendazim.
[c]Mixture of fludioxonil and cyprodinil.

Based on Elad (2004).

- **Fungicides affecting respiration**

The organic molecules such as sugars, fats and proteins provide energy for the survival of living systems. In fungi, like other eukaryotes, the final steps of this catabolic process take place in mitochondria and lead to the synthesis of the high energy intermediate ATP. In aerobic prokaryotes and in the mitochondrion of eukaryotes, cytochrome *b* is a component of respiratory chain complex III also known as the *bc*1 complex or ubiquinol-cytochrome *c* reductase (Fig. 1. 4). Cytochrome *b* is encoded by the cytochrome *b* (*cyt b*) gene located in the mitochondrial genome. Fungicides inhibiting mitochondrial respiration by binding to cytochrome *b* were first introduced to the market in 1996. Quinol oxydation inhibitors (QoIs) are probably the most successful class of agricultural fungicides. The discovery of QoI fungicides was inspired by a group of natural fungicidal derivatives of *β*-methoxyacrylic acid, such as strobilurin A, produced by the basidiomycetes *Strobilurus, Mycena,* and *Oudemansiella* (Kraiczy et al. 1996). Strobilurin A, the first QoI molecule, was obtained from liquid cultures of *Strobilurus tenacellus* in 1977 (Anke et al. 1977). The strobilurins (QoIs) quickly became one of the most important agricultural fungicides, accounting for over 20% of the global fungicide market within the first ten years of their commercial offering. The fungicidal activity of QoIs relies on their ability to inhibit mitochondrial respiration by binding at the so-called Qo site (the outer quinol-oxidation site) of the cytochrome *bc*1 enzyme complex (complex III) (Fig. 1. 4). This inhibition blocks electron transfer between cytochrome *b* and cytochrome *c*1, which, in turn, leads to an energy deficiency in fungal cells by halting the production of ATP. QoI fungicides usually have low toxicity towards birds, mammals (including humans), and bees at limited doses and are thus considered low risk, but they do show

dose-dependent toxicity towards aquatic organisms (Fernández-Ortuño et al. 2008).

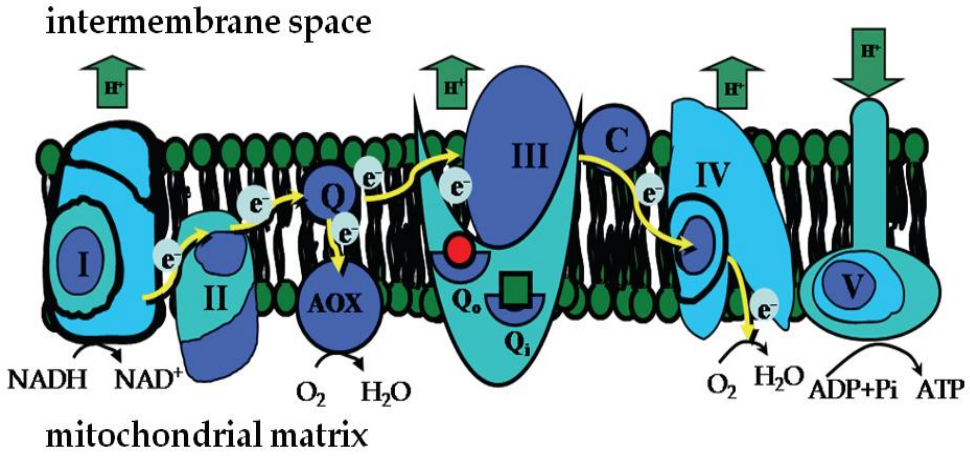

Fig. 1. 4: Schematic representation in mitochondria. I, II, III and IV are the different complexes of the transfer chain. V is the ATP synthase complex. Q is the ubiquinone pool and C is the peripheral protein cytochrome *c*. The yellow arrows inside the membrane indicate the direction of electron flow. The Qo and Qi binding sites of the cytochrome *bc1* enzyme complex (complex III) are delineated by a red circle and a green square representing Qo- and Qi-inhibitor molecules, respectively. In some fungi, inhibitors of the respiratory pathway induce the synthesis of alternative oxidase (AOX), an enzyme that diverts electrons at the ubiquinone pool (Q), but generates much less energy. Sorce: Fernández-Ortuño et al. (2008).

Based on structural similarities, eight chemical classes (Table 1. 3) of Qo inhibitors can be distinguished (Balba, 2007). They share a common mode of action that involves binding to the Qo site of the cytochrome *bc*1 complex and are generally cross-resistant when tested in different assays such as in-planta tests, spore germination, mycelial growth, cell-free enzyme tests, and on artificial mutants, although their spectra and intrinsic levels of biological activity are quite different (Fernández-Ortuño et al. 2008). Presently, there are ten QoI-containing active ingredients commercially available for agricultural use, including azoxystrobin, the world's most commonly used fungicide (Balba, 2007) (Fig. 1. 5).

Table 1. 3: The QoI fungicides

Classes	Fungicides	Company	Announced	First sales
I. Methoxyacrylates	Azoxystrobin	Syngenta	1992	1996
	Picoxystrobin	Syngenta	2000	2002
	Enestrobin	SRICI[a]	In progress	In progress
II. Methoxycarbamates	Pyraclostrobin	BASF	2000	2002
III. Oximinoacetates	Kresoxim-methyl	BASF	1992	1996
	Trifloxystrobin	Bayer	1998	1999
IV. Oximinoacetamides	Metominostrobin	Shionogi	1993	1999
	Dimoxystrobin	BASF	2005	2006
	Orysastrobin	BASF	In progress	In progress
V. Oxazalidinediones	Famoxadone	Dupont	1996	1997
VI. Dihydro-dioxazines	Fluoxastrobin	Bayer	2004	2005
VII. Imidazolinones	Fenamidone	Bayer	1998	2001
VIII. Benzyl-carbamates	Pyribencarb	K-I Chemical	In progress	In progress

[a]Shenyang Research Institute of Chemical Industry. Source: Fernández-Ortuño et al. (2008).

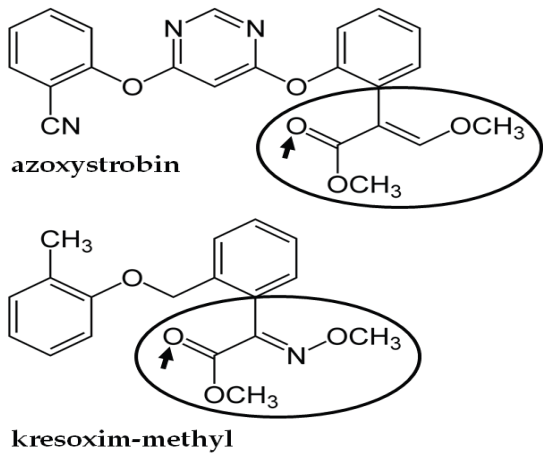

Fig. 1. 5: Chemical structure of azoxystrobin. The toxophores are highlighted with a circle and the carbonyl oxygen moiety responsible for binding is indicated by the arrow. Source: Fernández-Ortuño et al. (2008).

The development of resistance is a concern with the use of QoI fungicides. Following the commercial introduction of QoIs in 1996, field resistance to QoI fungicides has been observed in Europe, Asia, and North American in more than 22 pathogens. Resistance to QoIs have been detected in different plant pathogens, like *Alternaria alternata* (Ma et al. 2003), *Colletotrichum graminicola* (Avila-Adame and Köller, 2003), *Pyrenophora teres* (Sierotzki et al. 2007), *Corynespora cassiicola* and *Mycovellosiella nattrassii* (Ishii et al. 2007), *Pseudoperonospora cubensis* (Zhang et al. 2008) and *Venturia inaequalis* (Lesniak et al. 2011).

- **Mechanism of resistance to QoI fungicides**

Studies on the molecular mechanisms of QoI resistance showed that it primarily arises from a target-site-based mechanism that involves mutations in the mitochondrial cytochrome *b* gene (Gisi et al. 2002, Kuck and Gisi, 2007). Three important amino acid substitutions (glycine to alanine at position 143 (G143A), glycine to arginine at position 137 (G137R) and phenylalanine to leucine at position 129 (F129L)), have been detected in the cytochrome *b* of several phytopathogenic fungi that are resistant to QoIs. Isolates carrying F129L or G137R express moderate resistance, which is usually overcome by the recommended field levels of QoIs. By contrast, isolates with G143A express high resistance (Fig. 1. 6), which is always associated with the failure of QoIs to control disease (Gisi et al. 2002; Kim et al. 2003).

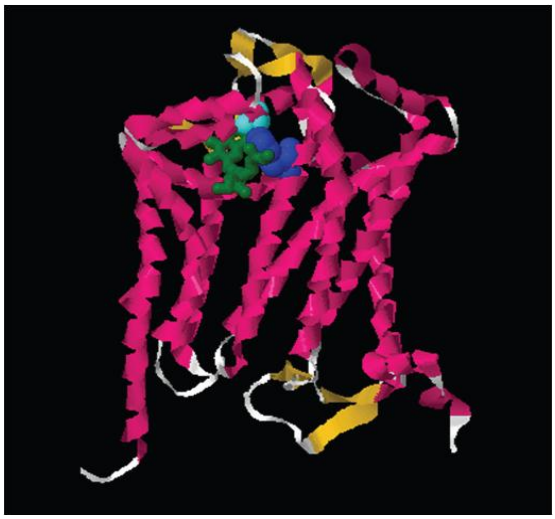

Fig. 1. 6: Cytochrome *b* and QoI resistance. Molecular model of the *cyt b* protein of *S. cerevisiae*. A QoI fungicide molecule bound to the Qo site of the cytochrome *bc1* complex is depicted in green. The amino acids substitutions G143A and F129L that are responsible for QoI resistance are depicted in cyan and blue, respectively. Note how these amino acids interact with the fungicide molecule. Source: Fernández-Ortuño et al. (2010).

The second mechanism of resistance to QoI fungicides is mediated by the induction of an alternative, cyanide-resistant respiration that is sustained by alternative oxidase (AOX) (Wood & Hollomon, 2003). In this rescue mechanism, mitochondrial electron transfer is diverted by circumventing the inhibitory site of QoI in the cytochrome *bc*1 complex (Fig. 1. 4). However, the energy provided by alternative respiration only seems to counteract QoI effects *in vitro* but not *in planta* but several reports using alternative-oxidase-deficient mutants and specific inhibitors of this enzyme have revealed that alternative respiration also limits QoI effectiveness in planta, especially once the infection has been established (Olaya and Köller, 1999; Avila-Adame and Köller, 2003; Miguez et al. 2004). Under field conditions, alternative respiration appears to have a limited impact on the protective activities of QoI fungicides for two main reasons. First, this pathway provides low levels of ATP that represent only 40% of the normal efficiency for energy conservation. This is due to the fact that complexes III and IV of the mitochondrial electron transport system are bypassed, and AOX lacks proton pumping activity. Consequently, processes that demand large amounts of

energy and are critical steps for successful colonization of the plant, such as spore germination and host-penetration, are not supported. Second, plant antioxidants, such as flavones, are released during infection and interfere with the induction of alternative respiration by quenching reactive oxygen species that are necessary to induce the *AOX* gene and are generated by QoIs (Wood & Hollomon, 2003).

1. 2. 2. Genetic variation

The morphological characteristic based on appearance, the biological based on interbreeding, and the phylogenetic based on common descent are three species concepts which have been emphasized in fungi, although plant pathogens host specificity is also emphasized (Harrington and Rizzo, 1999; Taylor et al. 2000). Morphological traits such as the characteristics of conidia, mycelia and sclerotia are useful in delimiting some species. However, many species are morphologically similar. Furthermore, a key to all recognized species is not available. Rather than on morphological traits, species may be identified by phylogenetic analyses of variable nucleic acid sequences. Morphological characters, together with DNA sequence data of multiple protein-coding genes can be used to unambiguously identify *Botrytis* species and open the way to a better understanding of the genetic diversity within the genus and species (Staats et al. 2005). Most of the knowledge on mechanisms that generate genetic variability in *Botrytis* is based on studies in *B. cinerea*. A recurrent theme of variation in *B. cinerea*, indeed perhaps the main reason for its reputation as an unusually variable fungus, is the existence of morphologically distinct cultural types (Jarvis, 1977; Lorbeer, 1980). Typically, such morphotypes are essentially stable when subcultured using mass inoculum, but subcultures from single conidia

often differ from the parents and each other. It is obvious that mycelia, sclerotia and conidia have different abilities for survival and dispersal, and the relative roles of these structures will vary greatly depending on ecosystem and season (Beever and Weeds, 2004).

- **Somatic compatibility**

Vegetative compatibility provides an opportunity for population sub-structuring. Strains that are capable of forming viable heterokaryons with each other are referred to as vegetatively compatible and are members of the same vegetative compatibility group (VCG; Leslie, 1993). Members of the same VCG can undergo hyphal fusion, with the potential for transferring nuclear and cytoplasmic elements. VCGs have been very useful for identifying clones of fungi that are largely asexual, and VCGs often correlate with pathogenicity and other traits (Korolev et al. 2000, 2008). However, heterokaryosis has been shown to account for variability. Evidence for the existence of heterokaryons was initially obtained by the observation of distinct morphotypes in macroconidial subcultures from the same parent (Lorbeer, 1980). Beever and Parkes (2003) showed the existence of multiple VCGs in *B. cinerea* with all six field strains examined being in different groups. Subsequent studies have shown that a large number of VCGs exist within *B. cinerea* populations (59 VCGs have been recognized among 82 field strains of the fungus in New Zealand).

Variations in ploidy levels and chromosomal polymorphism among strains of *B. cinerea* are reported and they were suggested to be haploid, diploid, or even triploid (Büttner et al. 1994; Vallejo et al. 1996). Heteroploidy may arise from the failure of mitosis or fusion of nuclei, coupled with loss or gain of chromosomes. Shirane et al. (1988), using an elegant method to release chromosomes from the tips of germlings, provided clear evidence for the

presence in mitotic metaphase of 16 chromosomes, and 16 chromosomes have also been reported in developing asci (Faretra and Grindle, 1992).

- **Transposable elements**

B. cinerea possesses a variety of extrachromosomal genetic elements including the chromosomes of mitochondria, viruses, plasmids and transposable elements (Rosewich and Kistler, 2000). Transposable elements (TEs) are DNA sequences in prokaryotic and eukaryotic organisms and are a common cause of spontaneous genetic changes that can have a wide range of effects on the biology of the host and on its evolution (Smith and Corces, 1991; McDonald, 1993). There are two main classes of TEs (Kidwell and Lisch, 2001): class I, retroelements, are TEs that transpose by RNA-mediated reverse transcription, and class II, DNA elements, are elements that transpose directly from DNA to DNA (Levis et al. 1997a).

B. cinerea strains have at least two different transposable elements, named *Boty* and *Flipper* (Diolez et al. 1995; Levis et al. 1997a). *Flipper* is present in up to 20 copies per genome, and was confirmed to be mobile by the detection of its insertion into nitrate reductase during spontaneous mutant selection (Levis et al. 1997b). *Boty* is characterized by a long terminal repeat (LTR), present in multiple copies in different regions of the genome (Diolez et al. 1995; Giraud et al. 1999). Initially dot blot methods were used to detect these elements, PCR methods also have been developed (Muñoz et al. 2002). First two types of strain have been defined, based on the presence or absence of two transposable elements, *boty* and *flipper* (Diolez et al. 1995; Giraud et al. 1999; Levis et al. 1997a): transposa strains carry both *boty* and *flipper*, whereas both these elements are absent from vacuma strains. *Boty* and *Flipper* have therefore frequently been used to characterise *B. cinerea* populations worldwide. Recently, molecular studies of different nuclear

genes have suggested that *B. cinerea* populations are grouped into two different clades in the various gene phylogenies, group I and group II, which were therefore proposed to be phylogenetic species (Albertini et al. 2002; Fournier et al. 2003, 2005). Described group I isolates are exclusively from the *vacuma* transposon type, while group II can feature *transposa*, *flipper* (containing only Flipper), *boty* (containing only Boty) or *vacuma* genotype (Fournier et al. 2005; Ma and Michailides, 2005; Munoz et al. 2002; Albertini et al. 2002; Ben Ahmed and Hamada, 2005; Milicevic et al. 2006; Isenegger et al. 2008; Váczy et al. 2008; Rajaguru and Shaw, 2010; Esterio et al. 2011).

- **Population studies using molecular markers**

The development of molecular techniques has revolutionized and energized fungal population genetics by providing numerous readily available genetic markers (Bridge et al. 1998). Multilocus techniques such as RAPDs and AFLPs are convenient and allow ready scoring of numerous polymorphic loci, but are limited by features including difficulties of reproducibility and the assumption that co-migrating bands are identical. Single-locus techniques such as RFLPs are highly reproducible and allow greater precision for estimating genetic parameters, but are more labor intensive (McDonald, 1997). Microsatellite markers, which offer numerous polymorphisms coupled with high reproducibility and convenience, have been developed for *B. cinerea* (Fournier et al. 2002).

Several studies have shown, however, that *B. cinerea* was likely to form a species complex, with restricted gene flow between different cryptic genetic groups (Giraud et al. 1997; Albertini et al. 2002; Fournier et al. 2003, 2005). First, Giraud et al. (1997) studied genetic diversity of *B. cinerea* in France using a range of markers including the presence or absence of transposable

elements *Boty* and *Flipper*, and restriction fragment length polymorphisms (RFLPs) of PCR amplified DNA regions. This study revealed that the *B. cinerea* population is genetically very diverse, with no indication of widespread clonal lineages and a significant role for recombination. Furthermore, *B. cinerea* showed to be composed of two subgroups, *transposa* and *vacuma* that are genetically isolated and occur in sympatry on the same host plants and in the same region. Giraud et al. (1999) in another study has shown that there is a significant genetic differentiation among isolates collected from different host plants in France and that the level of fungicide resistance differs significantly in *transposa* and in *vacuma*. Genetic diversity of *B. cinerea* was also studied in Spain by Alfonso et al. (2000). This study showed that the population as a whole was highly heterogeneous, with little differentiation of the subpopulations between different greenhouses or regions; nor was significant differentiation detected when isolates from other countries were included in the analysis. Muñoz et al. (2002) studied genetic characterization of *B. cinerea* populations between grapevine and tomato in Chile and indicated that *B. cinerea* population is genetically very diverse with no indication of widespread clonal lineages, even in relation to fungicide resistance that might have imposed a genetic bottleneck on some populations. Ma & Michailides (2005) studied the genetic structure of *B. cinerea* populations in California on different hosts using transposable elements and microsatellite primed (MP)-PCR and found no differentiation between *B. cinerea* populations from different hosts.

Fournier and Giraud (2008) reported a significant genetic structure within *B. cinerea* group II according to two natural host plants, grapevine and bramble, using microsatellite markers. They showed that the fungal populations on the two hosts were significantly differentiated, indicating restricted gene flow, even in sympatry. However, Rajaguru and Shaw (2010) showed genetic

differentiation between hosts and locations in populations of latent *Botrytis cinerea* in southern England. Muñoz et al. (2010) studied the genetic diversity of a local population of *B. cinerea* isolates obtained from grapevine in Argentina using transposable elements and PCR-based RFLP molecular markers and showed a high degree of genetic diversity and a significant difference in allele frequency between the local and French populations. Karchani-Balma et al. (2008) studied the genetic structure of *B. cinerea* populations in Tunisia on different hosts and different regions using microsatellite markers and could not detect any significant effect of plant on pathogen population differentiation.

DNA polymorphism and vegetative incompatibility studies revealed that the genetic diversity is lower in group I than group II (Fournier et al. 2005). In addition, the two groups present differences in morphology, phenotypic characteristics and host range, e.g., asexual spores from group I isolates are reported to be significantly larger than those from group II strains (Fournier et al. 2005). While group I isolates have been mainly found in spring on grapevine, group II isolates have been collected both in spring and in fall (Martinez et al. 2005). Group I appears to have a narrower host range than group II. It is likely that the various differences observed between *vacuma* and *transposa* isolates correlate with the divergent evolution of group I and group II and/or to the variable proportion of *vacuma* and *transposa* isolates of the two cryptic species (Fournier et al. 2005). These cryptic species have also been shown to coincide with resistance to the fungicide fenhexamid, and synonymously known as resistant (Group I) and sensitive (Group II) (Albertini et al. 2002). At the present, *B. cinerea* is considered a species complex consistent of two phylogenetic or cryptic "species", group I (also called *B. pseudocinerea*) and group II (*B. cinerea sensu stricto*). Albeit extremely difficult to be distinguished by classical taxonomic criteria, they

can be identified easily using certain molecular markers (Fournier et al. 2003; Walker et al. 2011).

1. 2. 3. Pathogenesis of *B. cinerea*

Pathogenicity refers to the ability of an organism to cause disease. This ability represents a genetic component of the pathogen and the overt damage done to the host is a property of the host-pathogen interactions. Virulence, a term often used interchangeably with pathogenicity, refers to the ability of the pathogen to multiply within the host and the degree of pathology caused by the organism. *Botrytis* spp. possesses virulence factors that enable the pathogen to complete the consecutive stages of its necrotrophic lifecycle. These virulence factors include phytotoxic metabolites and proteins, as well as extracellular enzymes that decompose plant material or inactivate plant defence mechanisms (Staats et al. 2007). Most of the information about pathogenicity in the genus of *Botrytis* comes from research on *B. cinerea*. It can enter the host via stomata and other natural openings (Clark and Lorbeer, 1976; Fourie and Holz, 1995). The interaction of this fungus with plants begins with conidial attachment to the host surface and continues with conidial germination. The fungus grows through polarized tip extension, resulting in the formation of a tubular cell (hyphae), which is subsequently compartmentalized by the formation of perforated septa. Then, penetration structures are formed, which colonize the plant tissue (Aguayo et al. 2011). *B. cinerea* produces a range of cell wall-degrading enzymes, toxins and other low-molecular-weight compounds such as oxalic acid. Williamson et al. (2007) suggested that the pathogen triggers the host to induce programmed cell death as an attack strategy.

The goal of the thesis

The aims of this thesis were to provide a better understanding of Hungarian *B. cinerea* populations firstly, by recognizing and distinguishing group I and group II *B. cinerea* isolates in Hungary using several genetic markers and comparing their suitability for assessing the population structure and diversity of this widespread plant pathogenic fungus. Secondly, molecular mechanisms of QoI fungicide resistance and genetic basis of this resistance were investigated in Hungarian *Botrytis cinerea*. Furthermore, pathogenicity of group I and group II of *B. cinerea* were examined. Thirdly, we investigated the genetic structure of *B. cinerea* group II sympatric populations, infecting two cultivated perennial hosts (strawberry and raspberry) in the open field in Hungary. We employed three different molecular methods (RFLP analysis microsatellites and a minisatellite) to study the population genetics.

Chapter 2

Materials and Methods

2. 1. ISOLATES AND STRAIN STORAGE

Monosporic isolates of *B. cinerea* were collected from infected oilseed rape, strawberries and raspberries on the 17 hectares of plantations of the Benedek Gyümölcs farm in Nagyréde and Hatvan districts in the North-East of Hungary in 2007, 2008 and 2009. All sampling from Nagyréde was done in an area of 2 by 2 km. "Autumn Bliss" raspberries occupied 2 hectares in the center of the area and were planted in 2003. The "Honeoye" strawberry fields were so close to each other that they could be considered one continuous field comprising some hectares. The remaining hectares of the farm were planted with wheat, and involved in the rotation of strawberry; Strawberries are typically maintained for three years. Hence, one third of the strawberry plants were the same throughout our study. Each year, the entire area was screened for grey mould at multiple occasions during the flowering and fruiting season (April 18 to October), and all encountered were collected. The large majority of grey mould samples (94%) were collected from fruits. In 2007, the strawberry plants were treated twice with the anti-botrytis fungicide thiophanate-methyl (Topsin-M) on the 13th of May and the 20th May, and once with fenhexamid (Teldor) on the 1st of June. Raspberry needed no fungicide treatment in 2007. Next year, strawberry was treated with Topsin-M on the 9th of May and with azoxystrobin (Quadris) on the 18th of May. In 2008, raspberry was treated with mankoceb (INDOFIL M-45) on the 23rd of May. In the third and final year of this study (2009), strawberry was treated twice with Topsin-M (19th and 26th of April), while raspberry was treated twice with Topsin-M (19th of May, 5th of June) and once with Teldor (28th of June).

All *B. cinerea* isolates were collected by single-spore isolation. Sporulation was induced by incubating *B. cinerea* colonies at 25 °C. Single-spore isolates

were prepared from each strain and maintained on potato dextrose agar (PDA, Scharlau, Spain). Conidial suspensions were stored in 50% glycerol at –80°C. Numbering of strains was performed according to the chronology of collection, irrespective to the local provenance.

2. 2. FUNGICIDE SENSITIVITY ASSAYS

Sensitivity of *B. cinerea* to azoxystrobin was determined by measuring radial mycelial growth on plates. Quadris (Syngenta, UK), a product that contains 250 g/L azoxystrobin, was added to PDA medium after sterilization to give final concentrations of 0, 1 and 100 mg azoxystrobin/L. To inhibit the alternative respiratory pathway (Wood and Hollomon, 2003), 100 mg/L salicylhydroxamic acid (SHAM) (Sigma–Aldrich Kft, Hungary) was also added. A 10 mm mycelial plug was taken from the edge of a 3-day-old colony and placed on the center of PDA plates containing SHAM and azoxystrobin. Three parallel replicates of each concentration were prepared for each isolate. Plates were incubated at 25 ºC for 3 days in the dark and subsequently, the diameter of the colonies was measured. Isolates that exhibited considerable colonial growth (colony diameter > 50 % of that on the fungicide-free control medium) on PDA plus 100 mg/L azoxystrobin were designated as highly resistant (HR) isolates. Isolates that grew on PDA plus 1 mg/L azoxystrobin (colony diameter > 50 % of that on control medium) but were unable to grow on PDA plus 100 mg/L azoxystrobin, were designated as lowly resistant (LR) isolates, while those that suffered severe growth inhibition in the presence of the fungicide (colony diameter on 1 mg/L < 50 % of that on control medium) were designated as azoxystrobin-sensitive (S) isolates.

- **DNA extraction**

DNA was extracted from aerial mycelium of *B. cinerea* strains grown on PDA medium for 10 days at 20 °C. Magnalyser (Roche, Osterode, Germany) was used for the disruption of fungal cells, and DNA was isolated with the Plant II DNA Purification Kit (Macherey- Nagel GmbH and Co. KG, Germany). The yield and integrity of the DNA were checked by agarose gel electrophoresis. The genomic DNA was used as a template for the subsequent PCR.

- **PCR amplification and analysis of *cyt b* gene**

Based on the sequence of *cyt b* gene from *B. cinerea*, the pair of gene-specific PCR primers cytb-BcF and cytb-BcR was used as previously described (Jiang et al. 2009) to amplify a *cyt b* fragment from all *B. cinerea* isolates. This *cyt b* fragment contains the codon corresponding to glycine residue 143, mutation of which is known to affect azoxystrobin sensitivity in many fungal species. PCR was programmed as follows: initial denaturation for 5 min at 95 ºC; 5 cycles of denaturation for 1 min at 95 ºC; annealing for 1 min at 50 ºC; and elongation for 1 min 30 sec at 72 ºC; 30 cycles of denaturation for 1 min at 90 ºC; annealing for 1 min at 50 ºC; and elongation for 1min 30 sec at 72 ºC; and finally, a post elongation for 15 min at 72 ºC. Amplifications were done in a final volume of 50 µL containing 0.4 µM of each primer, 100 ng of fungal DNA and 25 µL of Green Master Mix (Promega). PCR products were examined by electrophoresis in a 1.5% agarose gel in 1 X Tris–acetate-EDTA (TAE) buffer and stained with ethidium bromide. For a selected number of stains, this PCR fragment was purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega Corp., Madison, WI) and subsequently sequenced over both strands at

MWG-Biotech AG (Ebersberg, Germany). Sequences were manually edited and deposited at GenBank (accession nos. JQ362461 – JQ362470).

- **Allele-specific PCR of the mutated *cyt b* gene**

To detect the G-to-C point mutation in codon 143 of the *cyt b* gene, PCR primers BcAR-F and BcAR-R were used as described by Jiang et al. (2009). The primer BcAR-F was designed so that it could only amplify genomic DNA carrying the G-to-C point mutation in the middle of codon 143. The primer BcAR-R was located within the last intron of the *cyt b* gene. The primer pair was expected to generate a ~ 260-bp fragment from mtDNA of HR isolates of *B. cinerea*.

PCR was programmed as follows: initial denaturation 5 min at 95 ºC; 5 cycles of denaturation for 1 min at 95 ºC; annealing for 1 min at 55 ºC; and elongation for 30 sec at 72 ºC; 30 cycles of denaturation for 1 min at 90 ºC; annealing for 1 min at 55 ºC; and elongation for 30 sec at 72 ºC; and finally, a post elongation for 15 min at 72 ºC. Amplifications were done in a final volume of 50 µL containing 0.4 µM of each primer, 100 ng of fungal DNA, 25 µL of Green Master Mix (Promega). PCR products were analyzed in a 1.5% agarose gel in TAE buffer and stained with ethidium bromide.

- **Diagnosis of QoI resistance by PCR-RFLP**

PCR-RFLP was used to confirm fungicide-resistant isolates lacking the additional intron between codons 143 and 144. A PCR-RFLP protocol with the restriction enzyme *Ita*I was previously developed to identify resistance to strobilurin (Sierotzki et al. 2000; Furuya et al. 2009). *B. cinerea* DNA was amplified using the primer set cytb-BcF/cytb-BcR, purified and digested

with *Sat*I (*Fnu*4HI) (Fermentas, Biocenter Ltd., Szeged, Hungary), an isoschizomer of *Ita*I. The G-to-C point mutation resulting in the G143A substitution creates a *Sat*I restriction site (GC|NGC). Digests were run on a 1.5% agarose gel with TAE buffer and stained with ethidium bromide.

- **Induced resistance toward azoxystrobin**

To induce resistance of *B. cinerea* strains toward azoxystrobin, 5 isolates of *B. cinerea* were used, in which 3 strains (9028, 9043 and 9058) were sensitive and gave positive PCR reaction for detecting point mutation G143A responsible for resistance and do not contain large intron in *cyt b* gene. Strain 9022 was used as a positive control and 9001 as a negative control (Table 2. 1).

Table 2. 1: Characteristics of *B. cinerea* strains used in induced azoxystrobin resistance test.

Strain number		**PCR results**	
	Sensitivity toward azoxystrobin[a]	G143A[b]	Presence of large intron
9028	S	+	**no**
9043	S	+	**no**
9058	S	+	**no**
9022 (+)	HR	+	**no**
9001 (-)	S	-	**no**

[a]S: sensitive, HR: Highly resistant
[b] +: Presence of G143A point mutation detected with specific primers and digestion with restriction endonuclease

To determine sensitivity of *B. cinerea* to azoxystrobin, a 10-mm mycelial plug was taken from the edge of a 3-day-old colony and placed on the center of PDA plates amended with SHAM and 0.5 ml azoxystrobin. Two replicates of each concentration were used for each isolate. After the plates were incubated at 25 ºC for 5 days in the dark place, the mycelial growth was

recorded. A mycelial plug (10 mm in diameter) was taken from the edge of this colony and placed on the center of PDA plates amended with SHAM and 5 ml of azoxystrobin and after 5 days, another mycelial plug was taken from the edge of this colony and placed onto the center of PDA plates amended with SHAM and 50 ml of azoxystrobin (Fig 2. 1).

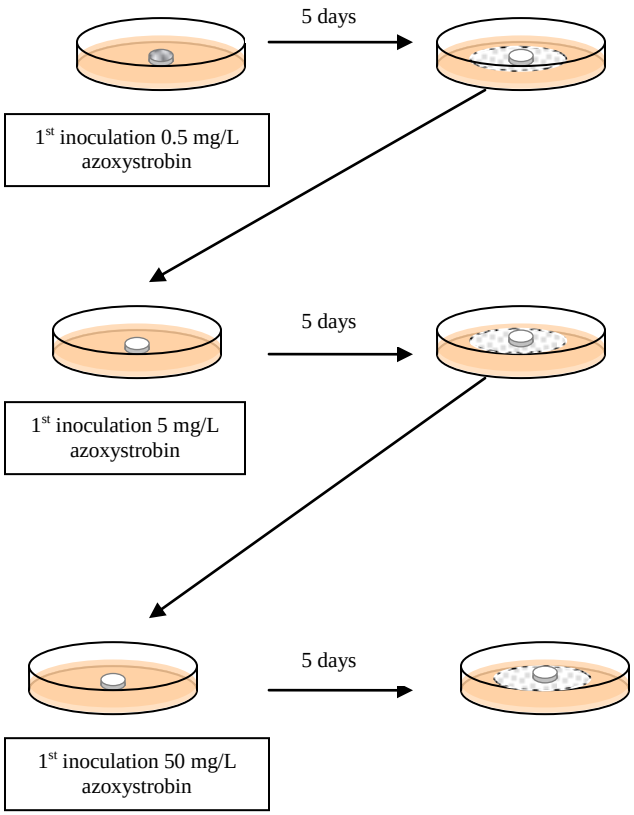

Fig. 2. 1: Inoculation of *B.cinerea* in three concentrations

Based on the sequence of the *cyt b* gene from *B. cinerea*, the pair of PCR primers cytb-BcF + cytb-BcR was developed earlier by (Jiang et al. 2009) to amplify a *cyt b* fragment. This *cyt b* fragment contains the codon position 143, which is known to affect azoxystrobin sensitivity in many fungal species. This fragment was digested by FastDigest® *Fnu4*HI (*sat*I). Digestion only occurs in the presence of G143A mutation. The products were analyzed in a 1.5% agarose gel in TAE buffer.

- **Fenhexamid resistant tests**

Fungicides used in the study were the commercial formulations of fenhexamid (Teldor 500 SC, Bayer CropScience, Hungary). The fungicides were dissolved in sterilized distilled water, and stock solutions were prepared. Autoclaved PDA was cooled to 50 °C and amended with aqueous fungicide solutions at discriminatory doses of 0, 2, and 5 mg/1 fenhexamid by adding appropriate volumes of the fungicide stock solutions into the medium while it was still liquid. For determining pathogen sensitivity to the fungicide, a minimal medium was used containing 10 g glucose, 1.5 g K_2HPO_4, 2 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 5 g $MgSO_4 \cdot 7H_2O$, 2 g yeast extract (Scharlau), and 12.5 g agar (Scharlau) per liter (Leroux et al. 1999). Control medium was not amended with fungicides. Tests for each isolate were replicated three times per concentration. Mycelial plugs were removed with the aid of a cork borer (10 mm in diameter), from the colony margins of actively growing 3-day-old colonies on PDA and placed upside down on the centers of 9 cm diameter plastic petri dishes containing the fungicide-amended or unamended media. Cultures were incubated at 20 °C in the dark for 3 days. Then, the mean colony diameter was measured and expressed as percentage of the mean diameter of the untreated control. Three phenotypes were distinguished: sensitive strains were resistant to estimated effective fenhexamid concentrations lower than 2 mg/1; lowly resistant isolates were resistant to estimated effective fenhexamid concentrations between 2 and 5 mg/1; and resistant isolates survived estimated effective fenhexamid concentrations higher than 5 mg/1.

2. 3. MOLECULAR METHODS

- ***Bc-hch* amplification and digestion**

PCR-RFLP of the *Bc-hch* gene (the *B. cinerea* homolog of the *Neurospora crassa het-c* vegetative incompatibility locus) was used to identify group I and group II isolates as it was described by Fournier et al. (2003). Two primers, 262 (5'- AAGCCCTTCGATGTCTTGGA-3') and 520L (5'- ACGGATTCCGAACTAAGTAA-3') were used to amplify the *Bc-hch* gene. These primers amplified a 1171 bp fragment between position 701 and 1871 of the *Bc-hch* gene. The amplification was conducted in a final volume of 50 µl containing 0.4 µM of each primer, 100 ng of fungal DNA and 25 µl of Green Master Mix (Promega). Reactions were performed in a Primus (MWG-Biotech, Germany) thermal cycler programmed as follows: 1 cycle of 2 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 51 °C, 1 min and 15 s at 72 °C. A final extension of 10 min was carried out at 72 °C. Amplified products were purified with Wizard® SV Gel and PCR Clean-Up System (Promega) and finally eluted with 40 µl nuclease free water. Digestion was carried out on 10 µl of the purified PCR product using 2 µl of FastDigest® *Hha*I restriction enzyme (Fermentas). Incubation was performed for 2 h at 37 °C. Fragments were separated on a native 1% agarose gel by electrophoresis and visualized by ethidium bromide under UV illumination.

- ***β*-tubulin amplification and sequencing**

A portion of *tub1* was amplified and sequenced with primers 155 (5'- CAACCTTCAAAATGCGTGAG-3') and 1174 (5'- AGATGGGTTGCTGAGCTTCA-3') (Fournier et al. 2005). Amplification conditions were the same as described for the *Bc-hch* gene except for the 55

°C annealing temperature. Purified PCR products were sequenced at MWG-Biotech AG, Ebersberg, Germany. Sequences were edited manually and deposited in GenBank (accession nos. HQ890436–HQ890461).

- **Transposon detection**

The presence of transposons was detected with dot-blot method (Váczy et al. 2008). Genomic DNA (200 ng) was spotted onto positively charged nylon membrane (Roche), denaturated in 0.5 M NaOH and 1.5 M NaCl for 20 min, and neutralized in 0.5 M Tris–HCl (pH 7.5) and 1.5 M NaCl for 20 min. The membrane was equilibrated in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 5 min, baked at 120 °C for 30 min and prehybridized in DIG Easy Hyb (Roche) for 30 min.

Hybridizations were performed overnight at 42 °C in the same solution after addition of Boty (648 bp) or Flipper (1250 bp) probe. Primers used were LTR98 (5'-AGCCTGTAGAATCACCAACG- 3') and LTR728 (5'-CGGTATTTCTGGTTGGCA-3') for Boty and F300 (5'-GCACAAAACCTACAGAAGA-3') and F1500 (5'-ATTCGTTTCTTGGACTGTA-3') for Flipper (Levis et al. 1997a; Munoz et al. 2002). The amplification protocol consisted of an initial denaturation step (3 min, 95 °C) followed by five cycles (denaturation: 1 min, 95 °C, annealing: 1 min, 60 °C, elongation: 1 min 72 °C), and 30 slightly different subsequent cycles (denaturation: 1 min, 90 °C, annealing: 1 min, 60 °C, elongation: 1 min, 72 °C. Reaction was completed with a final 15 min elongation step at 72 °C. Probes were DIG-labeled with the PCR DIG Probe Synthesis Kit (Roche) following the manufacturer's protocol.

- **Microsatellite amplification**

Microsatellite analysis was performed as previously described by Váczy et al. (2008). Five of the nine microsatellites described by Fournier et al. (2002) were analyzed. Amplification protocols and the primers used for amplification of Bc2, Bc3, Bc6, Bc7 and Bc10 microsatellites were identical to those described by Fournier et al. (2002). Fragment analysis was performed by MWG-Biotech AG, Ebersberg, Germany. The 5' ends of the forward primers were labeled fluorescently (Bc2: FAM, Bc3: NED, Bc6: NED, Bc7: FAM, Bc10: VIC) as suggested by the analyzer's manufacturer.

- **Minisatellite amplification**

Amplification was done in a total volume of 50 µl containing 50 ng fungal DNA, 0.4 µM of a single primer, and 25 µl GoTaq® Green Master Mix. The PCR used 30 cycles of 1 min at 95 ºC, 1 min at 60 ºC, and 2 min at 72 ºC. The reactions were performed in a Peltier Thermal Cycler 200 (MJ Research). A fragment of the intron of the ATPase gene *atp1* containing the minisatellite MSB1 with primers MSB1fw (5-AAGTTGCTGGTTCCTTGA-3) and MSB1rev (5- GTTGCAACCGGCGTAGAT-3) was amplified. Purified PCR products were subjected to automatic sequencing at MWG-Biotech AG, Ebersberg, Germany. The results were analyzed by DnaSP (Librado, and Rozas, 2009).

- **RFLPs and Amplification Length Polymorphism**

The primers used in PCR of the nitrate reductase- and ADP-ATP translocase genes were as described by Giraud et al. (1997). Amplification reactions

were performed in a total volume of 50 µ1 containing 50 ng fungal DNA, 0.4 µM of each primer, and 25 µl GoTaq® Green Master Mix. Cycling conditions were 30 cycles of 1 min at 95ºC, 1 min at 52 ºC, and 2 min at 72°C for amplification of the nitrate reductase gene. For the ADP-ATP translocase gene, the PCR conditions were the same as described above, with the except that the anneal temperature was 51 ºC, and the elongation time, 1 min 30 s. The ADP/ATP translocase amplifications were digested with *Eco*RI, while those of the nitrate reductase gene were cut either with *R*saI or with *Hinc*II. The DNA fragments were then separated by electrophoresis in 1.5% native agarose gels and 1 X Tris/Acetate/EDTA buffer.

2. 4. DATA ANALYSIS

- **Sequence analysis**

DNA sequences were aligned first with Clustal X 1.81 (Thompson et al. 1997) and then visually adjusted with Genedoc 2.6 (Nicholas et al. 1997). Microsatellite data analysis was performed as previously described in Fekete et al. (2012). The software Popgene Version 1.31 (Yeh and Boyle, 1999) provided online by the University of Alberta, CA was used for data analysis. Allele frequencies, effective number of alleles and gene diversity were determined in the total sample and in both cryptic species. Nei's coefficient of gene variation (G_{ST}) was calculated which is equal to the weighted average of F_{ST} for all multiple alleles (Nei, 1973). Gene flow (N_m) was estimated using the equation $N_m = 0.05\ (1 - G_{ST})/G_{ST}$ (McDermott and McDonald, 1993). Nei's (1972) genetic identity and genetic distance also were calculated with this programme. A second programme, called Multilocus 1.3b (Agapow and Burt, 2003) published online by Multi Locus Sequence Typing was used to calculate the genotypic diversity in relation to

the number of loci employed. This analysis provides information on whether the inclusion of additional markers is needed to capture the breadth of the genotypic diversity present in the two cryptic species. The number of different multilocus genotypes (MLG) and the standardized version of the index of association rD (Agapow and Burt, 2001; Giraud et al. 2006) were also calculated using Multilocus 1.3b. The rD is independent of the number of loci considered, and varies between 0 (complete panmixia) and 1 (no recombination). The null hypothesis of complete panmixia (rD = 0) was tested with the procedure implemented in the software, by comparing the observed dataset to 100 randomized datasets in which infinite recombination has been imposed by randomly shuffling the alleles among individuals, independently for each locus. Population differentiation was tested by comparing allele frequencies among the cryptic species using Weir and Cockerham's θ (F_{ST}) value (Weir and Cockerham, 1984) beyond G_{ST}. The θ-value was estimated under the null hypothesis of non-differentiation among subpopulations, when $\theta = 0$. Statistical analysis was done by comparing the calculated θ-values to datasets in which isolates have been randomized across populations 10,000 times using the programme Multilocus 1.3b. The null hypothesis of lack of genetic differentiation between the two cryptic species was tested using the χ^2 test (Nei, 1987) implemented in Popgene software.

- **Popgene32**

Computation of the fixation index (F_{ST}) as a measure of population differentiation was done with POPGENE32 version 1.31 (provided online by the University of Alberta, CA) and by calculating G_{ST}, which is equal to the weighted average of F_{ST} for all multiple alleles (Nei, 1973). The gene

diversity (Hs) at each locus in each population was calculated by the Nei's method (1973): $Hs = 1 - \sum pi^2$, in which *pi* is the frequency of the i^{th} allele. The mean gene diversity was the mean of Hs over loci in each population.

- **Bayesian inference**

The existence of a population structure in the total sample was investigated using the Bayesian approach implemented in Structure version 2.3 (Pritchard et al. 2000 and 2010; Falush et al. 2003). This clustering algorithm assumes a model in which there are K populations (where K may be unknown), each of which is characterized by a set of allele frequencies at each locus. Individuals in the sample are assigned probabilistically to these K populations, or jointly to two or more populations if their genotypes indicate that they are admixed, without consideration of their region or host of origin.

Loci are assumed at Hardy–Weinberg equilibrium and linkage equilibrium within the K populations, but this approach proved to be robust to deviations from these assumptions (Falush et al. 2003). We used the model with admixture, recommended when little is known about the existence of admixture (Falush et al. 2003), and assumed uniform priors for the vector of proportion qi of the individual i's genome in each cluster. The scores of individuals in the clusters (i.e. the posterior estimates of the qi) correspond to the probability of ancestry in any one of them. We varied K from 1 to 20, each with three independent simulations to check the consistency of the results. Each simulation consisted in 500 000 Markov Chain Monte Carlo (MCMC) iterations preceded by a burn-in period of 500 000 iterations (the burn-in period is the first set of iterations of the MCMC that is dependent on the configuration at the start – the iterations are not incorporated in the final

calculation of the posterior probability). Using the distribution of maximum likelihoods, we calculated the posterior probability for each K to determine the most probable structure. Following Evanno et al. (2005), The best estimation of *K* was that associated with highest LnP(D) or log $P(X/K)$ of STRUCTURE runs expressed as a modal value or magnitude of ΔK. This approach is less ambiguous than using $P(K/X)$ alone and reduces the risk of overestimating *K* (Evanno et al. 2005).

- **Data Mining**

Nowadays, with the rapid development of technology, tremendous amounts of data have been collected and knowledge discovery process is essential. The field of knowledge discovery in databases has evolved in the recent past to address the problem of automatic analysis and interpretation of larger and larger amounts of data. In one point of view, this process is including three phases: preprocessing phase, data mining methods and post processing phase. In real world usually datasets are noisy, incomplete, and inconsistent. For this reason, preprocessing step of data analysis is one of the important steps which can include data cleaning, data integration, data transformation and data reduction (Han et al. 2011). Data mining is an essential process where intelligent methods are applied in order to discover interesting patterns from large datasets. In general view we can classify data mining tasks into two categories: descriptive and predictive.

Despite of the existence of a lot of data mining algorithms, decision trees and rules induction are among the most popular methods. A decision tree is a tree data structure, where each node denotes a test on a feature value, each branch represents an outcome of the test and tree leaves represent classes or class

distribution. The decision trees classification and rules induction methods are the most useful methods in data mining (Maimon and Rokach, 2005). The decision trees are also most common used way for obtaining attributes importance (Esmaeili and Fazekas, 2009).

However, it is clear that the manual analysis of huge amounts of data is impossible. For this reason, it is important to develop tools that can analyze such data to extract interesting knowledge that can be helpful in the decision making process. The Rapidminer package provides implementations of variety of learning algorithms and original data could be preprocessed. Then feed data into a method and analyze results. This package includes methods for all the standard data mining problems: classification, clustering, association rule mining. Rapidminer also includes many data visualization facilities and data preprocessing tools. Three different algorithms are applied (J48, BFTree, LADTree) and their performance compared in order to choose important features in dataset. The discovery of relationships among attributes is useful in decision making. Many data mining packages specialize in one data mining function, such as classification, or just one approach for a data mining function, such as decision tree classification. Weka and Rapidminer provide a broad spectrum of data mining functions and explore multiple knowledge discovery techniques. Weka, developed at the University of Waikato in New Zealand, is open-source data mining software in Java. It contains a collection of algorithms for data mining tasks, including data preprocessing, association mining, classification, regression, clustering, and visualization. The RapidMiner project was started in 2001 at the Artificial Intelligence Unit of the Dortmund University of Technology. It provides several data mining functions, including data cleaning, classification, prediction, clustering, and statistical analysis packages, along with visualization tools. Both Weka and Rapidminer are free softwares. We utilize

decision tree and rule induction algorithms in these package and these algorithms are called with a couple of parameters. The algorithm used in this thesis is C4.5 that is called J48 in Rapidminer application. The terms C4.5 and J48 are used interchangeably throughout this thesis.

The software Rapidminer version 5.1 isvailable at http://rapid-i.com/content/view/26/201/ and Weka is available at http://www.cs.waikato.ac.nz/ml/weka/index_downloading.html.

There are many data mining tasks, as previously mentioned, classification is one of them. This kind of learning is analogous to human learning from past experiences to achieve new knowledge. Classification is a form of data analysis that extracts models describing important data classes. For example, we can build a classification model to categorize microsatellite applications as either strawberry or raspberry host. Such analysis can help provide us with a better understanding of the data at large. Many classification methods have been proposed by researchers. A dataset used in the classification algorithm consists of a set of records, which are described by a set of attributes. The dataset also has a special attribute which is called the class. In this thesis, we focus on decision trees and rule induction algorithms. A decision tree can be changed to rules and used to select attributes.

Rules are a good way of representing information or bits of knowledge. A rule-based classifier uses a set of IF-THEN rules for classification. An IF-THEN rule is an expression of the form: IF *condition* THEN *conclusion*. An example is following rule R1:

R1: IF bc7>117 THEN host=strawberry

This rule shows that attribute bc7 plays important role in recognizing the host. A rule can be assessed by its coverage and accuracy. That is, a rule's coverage is the percentage of examples that are covered by the rule (i.e., their attribute values hold true for the rule's antecedent). For a rule's accuracy, we

look at the examples that it covers and see what percentage of them the rule can correctly classify.

A rule-based classifier uses a set of IF-THEN rules for classification. Rules can be extracted from a decision tree. Rules may also be generated directly from training data using sequential covering algorithms. Measures that assess a classifier's predictive ability include accuracy, sensitivity (also known as recall), specificity, and precision. Detailed explanation about classification algorithms is beyond the scope of this thesis and it can be found in data mining books (Han et al. 2011; Tan et al. 2005).

Datasets for analysis may contain a lot of attributes, many of which may be irrelevant to the mining task. Although it may be possible for expert person to pick out some of the useful attributes, this can be a difficult and time-consuming task, especially when the data's behavior is not well known. Attribute subset selection is a method of dimensionality reduction in which irrelevant, weakly relevant or redundant attributes or dimensions are detected and removed. The "best" (and "worst") attributes are typically determined using tests of statistical significance, which assume that the attributes are independent of one another (Han et al. 2011). In this work, we use decision tree and rule induction algorithms to select best attributes or features to describe our dataset.

The attribute selection measure provides a ranking for each attribute describing the given training examples. The attribute having the best score for the measure is chosen as the splitting attribute for the given examples. We select the attribute that best discriminates the given examples according to class (strawberry or raspberry). Many popular attribute evaluation measures, such as the information gain measure, can be used. In this study we employ attribute selection measures such as Gain Ratio and Chi-square. Notice that all measures have some bias.

The use of chi-square tests for detecting relationships between variables. It was pointed out that if the variables are numerical we can use a simple correlation analysis. However the chi-square test is usually the only option if we have nominal or categorical variables. The chi-square test can also be used with numerical variables by converting them into nominal or categorical types.

2. 5. PATHOGENICITY ASSAY

For the competitive infection assay between the group I and group II *B. cinerea* isolates, 3 strains of each group were selected. Single-spore isolates were incubated at 18°C in a humid, dark chamber. Cucumber and paprika seedlings were grown from seeds in a greenhouse for 4 weeks. When the second leaf was emerged, the first leaf was detached. The grape leave was detached from vineyard. To examine the pathogenicity of both groups of *B. cinerea* isolates, mycelial disks (diameter 10mm) from the colony margins of actively growing 3-day-old colonies on PDA were placed on detached first leaves of cucumber, paprika and grape and incubated at 18°C for 3 days in a humid, dark chamber. The spreading lesions of *B. cinerea* infection were then scored.

Chapter 3
Results

3. 1. COMPARISON OF GROUP I AND GROUP II

- **Genotypic differentiation among group I and group II strains**

More than a thousand *B. cinerea* isolates collected between 2007 and 2009 were analyzed to identify any group I isolates. Fungi were isolated from infected oilseed rapes, strawberries and raspberries. The electrophoresis profile of a PCR-amplified *Bc-hch* fragment digested with the *Hha*I endonuclease resulted in two distinct restriction patterns (Fig 3.1). The size of the upper band was found to be either 601 bp for group I or 517 bp for group II strains. The PCR-RFLP profile of the *Bc-hch* locus suggested that only 13 strains belonged to the group I *B. cinerea* cryptic species isolated from oilseed rape and strawberries in April, May and early June in 2008 (Fig. 3. 1 and Table 3. 1). These comprised only 14% of all the strains isolated from the same year in 2008. Group II strains were isolated only from strawberry and raspberry hosts.

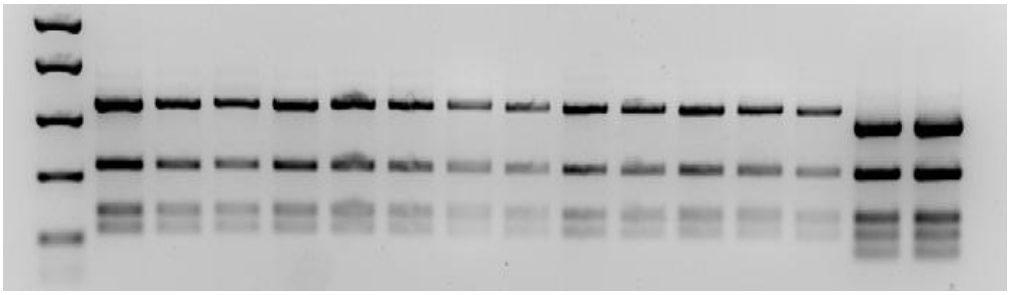

Fig. 3. 1: Analysis of the fragment sizes produced by *Hha*I, digestion of a *Bc-hch* gene amplification from thirteen *B. cinerea* group I strains. Samples from left: molecular marker, group I 8001–8008, 8029–8032, 8047, and 8049, 8061 strains. Molecular marker standards from top in base pairs: 2000, 1000, 500, 250, 100.

Table 3. 1: *Botrytis cinerea* group I and group II strains.

Strain	number of isolates	Isolation date	Host	*β-tubulin* accession numbers	*β-tubulin* sequence[a]	*Bc-hch* + *Hha*I[b]
group I						
8001 - 8008	8	24 April 2008	rape	HQ890436-43	**1**	**1**
8029 - 8032	4	18 May 2008	strawberry	HQ890444-47	**1**	**1**
8047	1	07 June 2008	strawberry	HQ890448	**1**	**1**
group II						
8009 - 8022	14	18 May 2008	raspberry	HQ890449-51	**2**	**2**
8023 – 8028 8033 - 8046	20	18 May 2008	strawberry	HQ890452-58	**2**	**2**
8048 - 8092	45	07 June 2008	strawberry	HQ890459-61	**2**	**2**

[a]1: group I sequencing result, 2: group II sequencing result
[b]1: group I RFLP pattern, 2: group II RFLP pattern

The parsimony analysis of the *β-tubulin* sequences using PAUP* 4.0 clearly separates group I from group II. This clustering was supported by a bootstrap score of 100 (Fig. 3. 2). The *β-tubulin* sequences of thirteen Hungarian and nine French isolates (taken from GenBank) belonging to group I clustered into a monophyletic clade supported by a bootstrap value of 99. All the other Hungarian and French isolates clustered into a second clade, including the *B. fabae* isolate. The *β-tubulin* sequence analyses supported the results of *Bc-hch* PCR-RFLP results, identifying the same 13 isolates as belonging to the group I cryptic species (Table 3. 1).

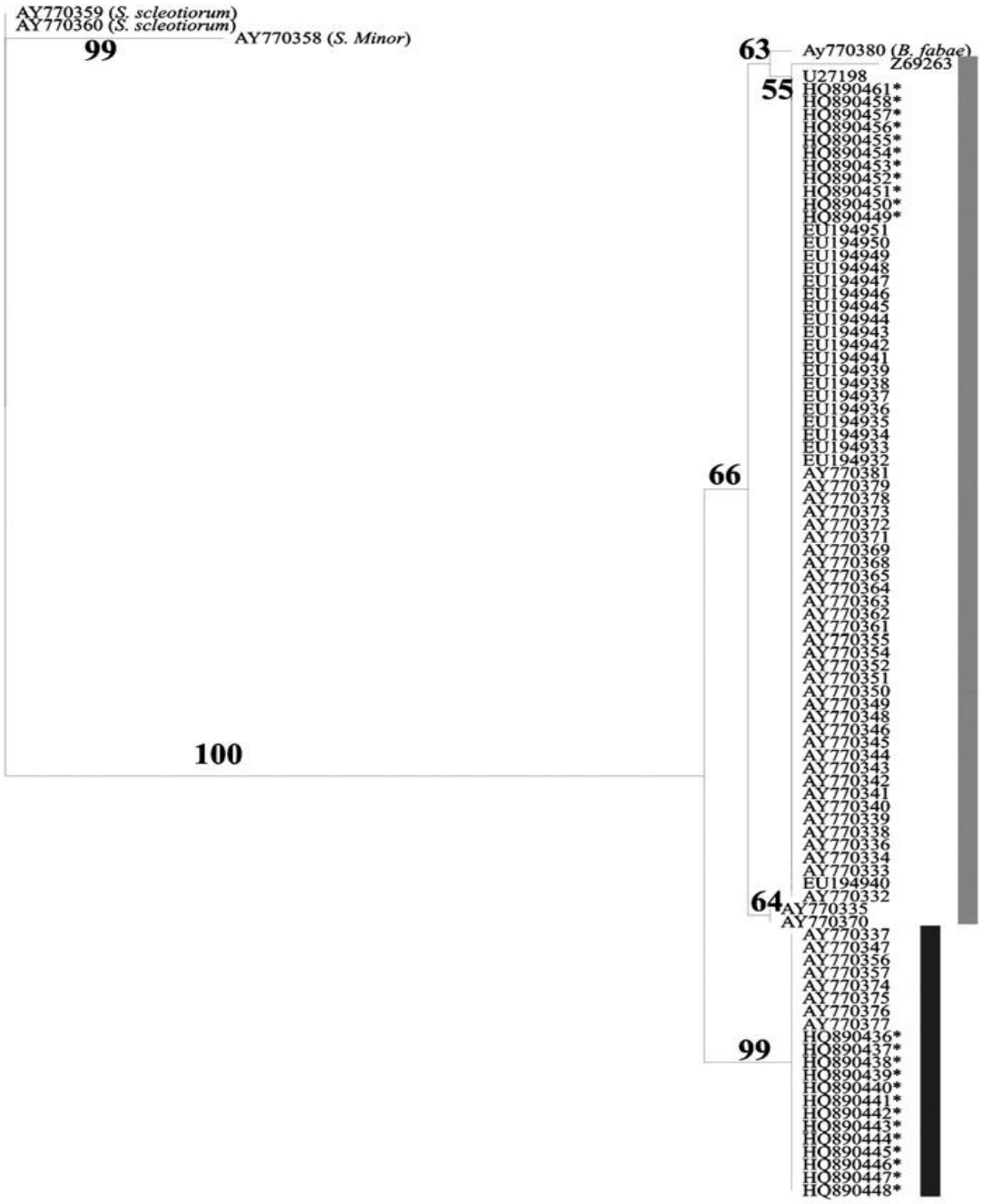

Fig. 3. 2: Parsimony phylogenetic tree based on *β-tubulin* gene. Bootstrap values more than 50% (N = 500 replicates) are indicated on the corresponding nodes. Stars indicate Hungarian *Botrytis cinerea* isolates from 2008. Grey bar and black bars indicate group II and group I clades, respectively.

- **Transposon genotypes in group I and group II**

Botrytis cinerea group I earlier has been described to exclusively feature the *vacuma* genotype, i.e., harboring neither Boty nor Flipper transposable elements. On the other hand, Group II is characterized by a complex transposon spectrum representing four possible genotypes: (i): *vacuma*; (ii): *transposa*, harboring both Boty and Flipper elements; (iii): *flipper*, containing only Flipper; and (iv): *boty*, containing only Boty. To determine whether these characteristics apply to our Hungarian isolates, we tested for the presence of the two transposons in 92 strains, including all 13 group I isolates identified above and a further 79 group II isolates, by means of dot blot hybridization. Intriguingly, transposable elements could be detected in the entire group I collection and in all but one of the group II isolates (Table 3. 2.) The large majority (92%) of group I isolates harbored only the Boty transposon, while *transposa* (62%) and *boty* (34 %) were the most frequent genotypes encountered among the group II isolates. Few group II isolates (2 strains) carried only the Flipper element (2.5%). We could not detect any transposon-specific hybridization in one of the group II isolates (1.26%).

Table: 3. 2: Transposon content and transposon types of group I and group II *B. cinerea* isolates

Strain	Transposon content (number of isolates)		Transposon type (number of isolates)			
	Boty	Flipper	*transposa*	*vacuma*	*flipper*	*boty*
group I	13	1	1	0	0	12
group II	76	51	49	1	2	27

- **Molecular population genetics approaches**

All 92 *B. cinerea* isolates were completely genotyped for five microsatellites (Table 3. 3). The MULTILOCUS program was used to test for the discriminatory power of the five microsatellites. The genotypic diversity topped at 0.711 for a randomly chosen, single locus and increased to 0.913 and 0.927 for four or all five loci, respectively. These results indicate that the set of microsatellite markers used was sufficient to correctly estimate existing diversity. All examined microsatellite loci were polymorphic. The five microsatellite markers exhibited between six and eight alleles, with an average of 7.0 alleles per marker in the examined *B. cinerea* isolates (Table 3. 3). The thirteen isolates of *B. cinerea* group I did not carry any typical allele for any of the five microsatellite loci (with respect to all the group II samples collected between 2007 and 2009) and no indistinguishable microsatellite haplotypes were found between group I and II isolates. The number of alleles and the genetic diversity appeared lower for all five microsatellites in group I, but it should be noted that we analyzed far more group II isolates (Table 3. 3).

Table 3. 3: Characteristics of five examined microsatellite loci from *Botrytis cinerea* group I and group II isolates.

		Size range (bp)[b] and number of alleles (between brackets)					**Gene diversity[c]**				
	n[a]	Bc2	Bc3	Bc6	Bc7	Bc10	Bc2	Bc3	Bc6	Bc7	Bc10
group I	13	166 (1)	121-217 (2)	82-119 (2)	118-124 (2)	161-183 (3)	0.00	0.4734	0.1528	0.320	0.4615
group II	79	138-172 (6)	209-221 (6)	82-149 (6)	110-130 (6)	161-188 (8)	0.624	0.63	0.58	0.663	0.6406
Total	92	138-172 (6)	209-221 (6)	82-149 (6)	110-130 (6)	161-188 (8)	0.630	0.6628	0.667	0.654	0.6973

[a] n: sample size
[b] bp: base pairs. There was no amplification in some cases for Bc2, Bc6 and Bc7 both in group I and in group II strains.
[c] Nei's (1973) gene diversity. Absence of a microsatellite was handled as missing value.

Consistent with previous studies (Fournier and Giraud, 2008; Karchani-Balma et al. 2008; Rajaguru and Shaw, 2010), the level of genetic diversity – indicated by the number of alleles, the effective number of alleles and Nei's gene diversity index – was higher for group II isolates than for group I strains (Table 3. 4). Within the group II population of 79 isolates, 32 distinct multilocus genotypes (MLG) were recovered, displaying a genotypic diversity of 0.909. The group I population from the same area exhibited a lower level of diversity: 5 MLG could be distinguished among 13 strains, with a genotypic diversity of 0.756. The standardized version of the index of association (rD) computed on a subset of clone-corrected data, on the total sample (containing both group I and II results) was significantly different from zero (rD = 0.100, P < 0.01, Table 3. 4), indicating genetic differentiation between populations.

Table 3. 4: Genetic diversity indexes within *B. cinerea* in Hungary calculated from microsatellite data

	n[a]	**n_a[b]**	**n_e[c]**	**h[d]**	**No. of distinct MLG[e]**	**Max. no. of repeated MLG[f]**	**Genotypic diversity**	**r_D[e]**
group I	13	2.0 (0.7071)	1.4814 (0.3994)	0.2815 (0.2040)	5	6	0.756	0.238*
group II	79	6.40 (0.8944)	2.6977 (0.2153)	0.6274 (0.0308)	32	21	0.909	0.08*
Total	92	6.40 (0.8944)	2. 9742 (0.2170)	0.6624 (0.0241)	35	21	0.927	0.1005*

[a] n: sample size,
[b] n_a: observed number of alleles,
[c] n_e: effective number of alleles
[d] h: Nei's gene diversity,
[e] No. of distinct MLG: number of distinct multilocus genotypes
[f] Max. no. of repeated MLG: maximum number of repeated multilocus genotypes
[e] r_D: standardized index of association (clone corrected); standard deviation between brackets,
*p<0.01

The estimate of linkage disequilibrium (Table 3. 4) was lower in group II (rD = 0.08, P < 0.01) than in group I (rD = 0.238, P < 0.01). This suggests a sexual reproductive mode mainly with a low but significant level of clonality for both. We should like to note however, that the sample size for group I was rather small. Statistically significant, but moderate population differentiation was found between the two sympatric *B. cinerea* cryptic species based on Weir and Cockerham's (1984) population differentiation statistics θ (θ = 0.1106, P = 0.007) of clone corrected data. Similarly, both the calculated Nei's coefficient of gene variation (G_{ST} = 0.1305) and the estimated gene flow (N_m = 3.3315) indicated moderate differentiation and gene flow between the two sympatric populations. Nei's genetic identity (0.3837) and genetic distance (0.9579) indexes also suggested moderate identity between the two sampled populations. Results for Nei's χ^2 test were significant only for Bc6, Bc7 and Bc10 microsatellite loci (Table 3. 5). However, for loci Bc2 and Bc3, the results of χ^2 test were not significant. Overall, these data indicated that the null hypothesis – no genetic differentiation between group I and group II – could not be completely rejected.

Table 3. 5: Tests of the null hypothesis of no genetic differentiation between groups I and II

	χ^{2a}	P	df
Bc2	9.43	0.398	9
Bc3	7.18	0.517	8
Bc6	30.84	$<10^{-3}$	8
B7	30.04	$<10^{-4}$	7
Bc10	23.41	0.002	8

[a] χ^2: chi-square test for homogeneity (Nei, 1987),
[b] P: probability,
[c] df: degree of freedom

3. 2. FUNGICIDE RESISTANCE IN *BOTRYTIS CINEREA*

A total of 157 Hungarian *B. cinerea* isolates were tested for QoI fungicide resistance on media containing azoxystrobin (Table 3. 6). Twenty eight (18 %) isolates were classified as highly resistant (HR) (Fig. 3. 3) and forty eight (30 %) exhibited a low resistance (LR) (Fig. 3. 4), while the majority of the isolates, eighty one (52 %) were sensitive (S) to azoxystrobin (Fig. 3. 5) when the plates were incubated at 25 ºC for two days.

Table 3. 6: Origin, host and azoxystrobin resistance of *Botrytis cinerea* isolates

N[a]	**Origin**	**Host**	**Sensitivity to azoxystrobin[b]**
Group I			
6	Hatvan	Rape	S
1	Hatvan	Rape	LR
5	Nagyréde	Strawberry	S
Group II			
34	Nagyréde	Raspberry	LR
4	Nagyréde	Raspberry	HR
19	Nagyréde	Raspberry	S
51	Nagyréde	Strawberry	S
13	Nagyréde	Strawberry	LR
24	Nagyréde	Strawberry	HR

[a]N: Number of isolates
[b]S: sensitive; LR: lowly resistant, HR: highly resistant

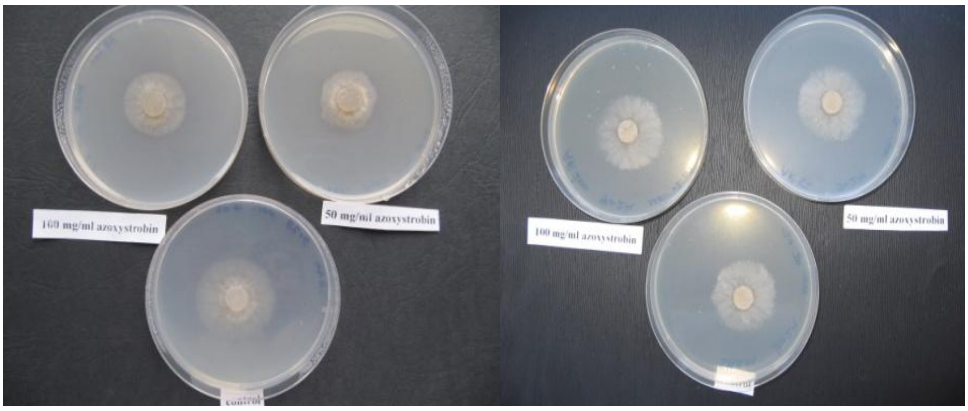

Fig.3. 3: Highly resistant isolates (HR) growing at three concentrations

Fig.3. 4: Lowly resistant isolates (LR).

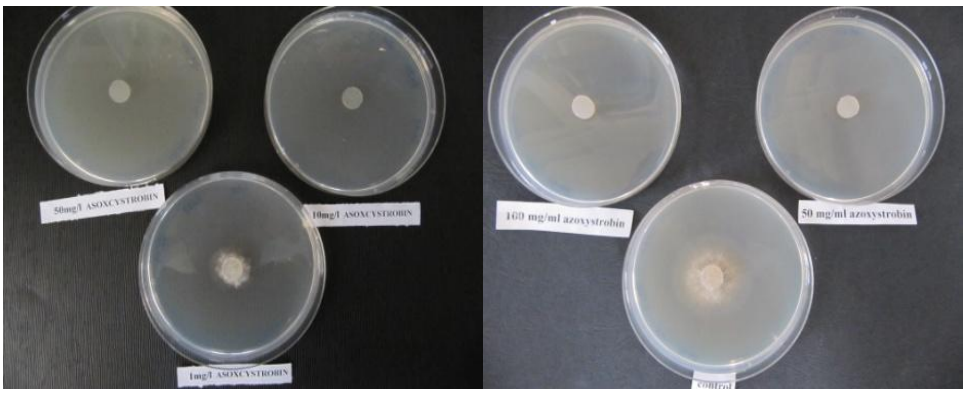

Fig.3. 5: Azoxystrobin-sensitive (S) isolates. They did not grow in the presence of the fungicide

High azoxystrobin resistance was exclusively encountered amongst group II strains, while group I isolates, with one exception, were all classified as sensitive to the QoI.

- **Sequence variation in the partial *cyt b* gene**

A major sequence variation in the *cyt b* gene was observed among the Hungarian *B. cinerea* isolates. Grasso et al. (2006) described a 1204-nt long, group I intron splitting exon 3 between codons 143 and 144, which featured alternatively in the *cyt b* gene of a number of *B. cinerea* variants (see Fig. 3. 6).

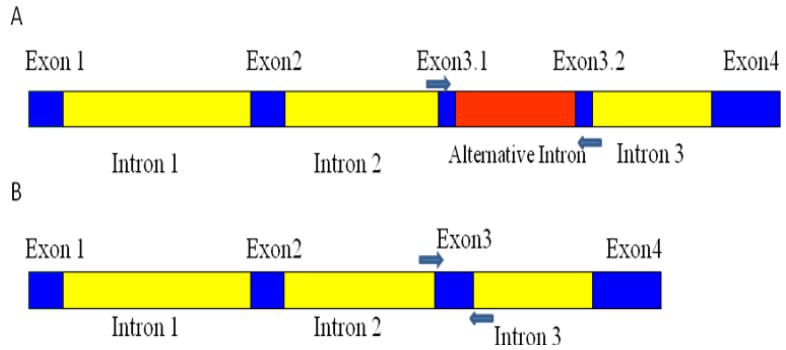

Fig. 3.6: Schematic representation of the cytochrome *b* (*cyt b*) gene, with (A) and without (B) the alternative group-I intron. Black boxes indicate exons, white boxes indicate introns, while the hatched box signifies the alternative intron separating the codons for Gly143 and Ala144. Arrows indicate the approximate position of cytb-BcF and cytb-BcR primers used for amplification. Introns, exons and primers are not drawn to scale.

The primer pair cytb-BcF/cytb-BcR amplified two different PCR fragments (Fig. 3. 7 and Supplementary Figure 1). The larger ~ 1750-bp fragment, indicated the presence of the alternative intron and was amplified in forty six (29 %) isolates. The smaller ~ 560-bp fragment, without the alternative intron, was generated in 106 (68 %) isolates. Interestingly, five (3 %) isolates gave rise to both PCR fragments (Fig. 3. 7, slot 8 from the left) indicating the occurrence of *cyt b* heteroplasmy in the Hungarian *B. cinerea* population.

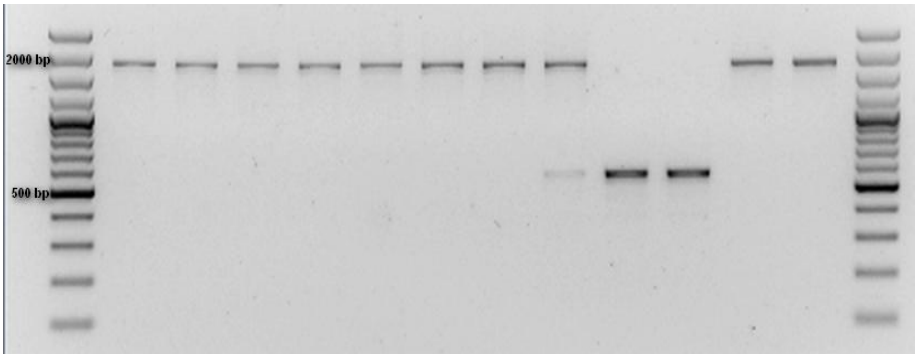

Fig. 3. 7: PCR fragments (the larger ~ 1750-bp and smaller ~ 560-bp) with the primer pair cytb-BcF/cytb-BcR. First and last lane: molecular marker (O'GeneRulerTM 100bp plus DNA Ladder, Fermentas).

Analysis of the sequences of the *cyt b* PCR products amplified with cytb-BcF/cytb-BcR from six S strains and four HR strains (Fig. 3. 8) showed that the wild-type GGT codon of the glycine at position 143 was present in S isolates where a GCT codon occurred in the HR isolates. The latter strains thus feature the glycine to alanine substitution at position 143 (G143A) of cytochrome *b*, associated with QoI resistance in *Alternaria* spp. (Ma et al. 2003), *Erysiphe graminis* (Sierotzki et al. 2000) and *Pyricularia grisea* (Gisi et al. 2002; Kim et al. 2003).

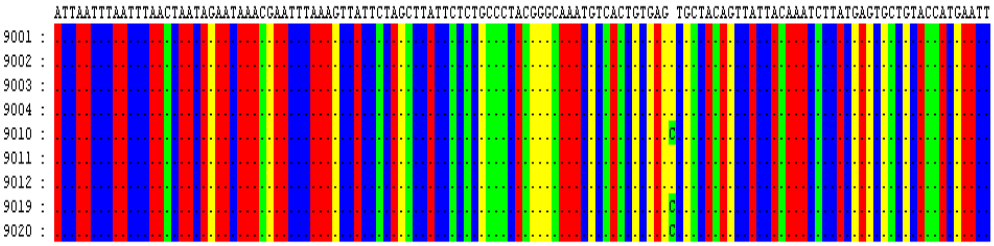

Fig. 3. 8: Partial *cyt b* sequences in Hungarian *B. cinerea* isolates. Strains number 9010, 9019 and 9020 specify a C instead a G in the middle of codon 143, located in exon 3. The sequence at the top is the consensus sequence (with its conceptual translation given in three-letter code). Everywhere else, dots indicate nucleotides identical to consensus sequence.

- **Allele-specific PCR for detecting G143A mutation**

Based on the point mutation G143A, a pair of allele-specific primers BcAR-F + BcAR-R were developed for the detection of *B. cinerea* isolates having this mutation. A specificity test showed that the primer pair amplified a 260 bp fragment only from the HR isolates, but not from any other S and LR isolates of *B. cinerea* (Fig. 3. 9 and Supplementary Figure 2), which indicated that the primer pair was specific to the HR isolates having the point mutation.

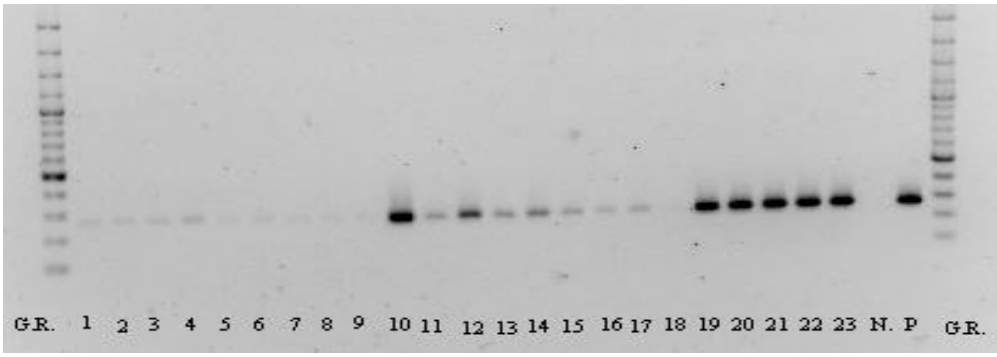

Fig. 3. 9: Allele-specific PCR with the primer pair BcAR-F + BcAR-R for detecting of azoxystrobin-resistant isolates of *B. cinerea*. First and last lanes: molecular marker (O'GeneRulerTM 100bp plus DNA Ladder, Fermentas), numbers indicate strain numbers (1-23: 9001 – 9023)

PCR-RFLP analysis was carried out by digesting the cytb-BcF/cytb-BcR amplification products with *Sat*I restriction enzyme to confirm the results from (G143A) allele-specific PCR. Figure 3. 10 and Supplementary Figure 3 show the results for a selection of strains that do not specify the alternative intron. None of the *cyt b* PCR fragments generated from group I strains and azoxystrobin-sensitive (S) group II isolates were digested following enzyme treatment, whereas the ~ 560-bp *cyt b* fragments from eighteen HR group II strains were digested. The same strains were also identified with (G143A) allele-specific PCR.

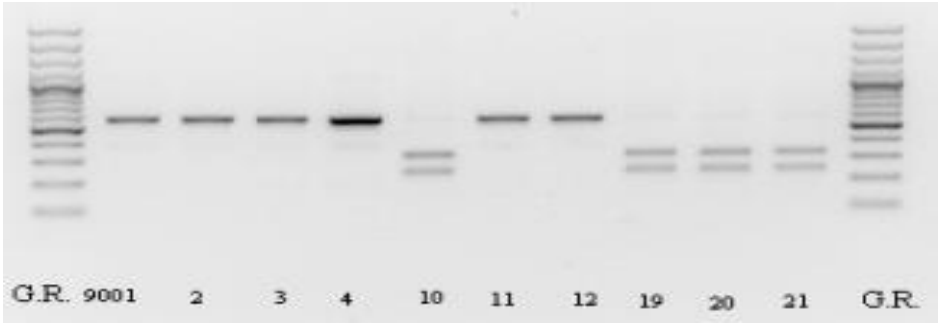

Fig. 3. 10: PCR-RFLP of the small *cyt b* amplification fragment (~ 560 bp) digested with *Sat*I endonuclease. First and last lanes: molecular marker (O'GeneRulerTM 100bp plus DNA Ladder, Fermentas). Numbers indicate strain numbers (2-21: 9002 – 9021). Appearance of the two bands of lower molecular mass results from *Sat*I digestion of the fragment of higher molecular mass, evidencing the G-to-C mutation that causes the QoI-resistant phenotype.

- **Induced resistance of *B. cinerea* strains toward azoxystrobin**

The primer pair cytb-BcF + cytb-BcR amplified 564 bp fragments from all of the *B. cinerea* isolates. All isolates were purified and digested with *Fnu4*HI enzyme (Fig 3. 11).

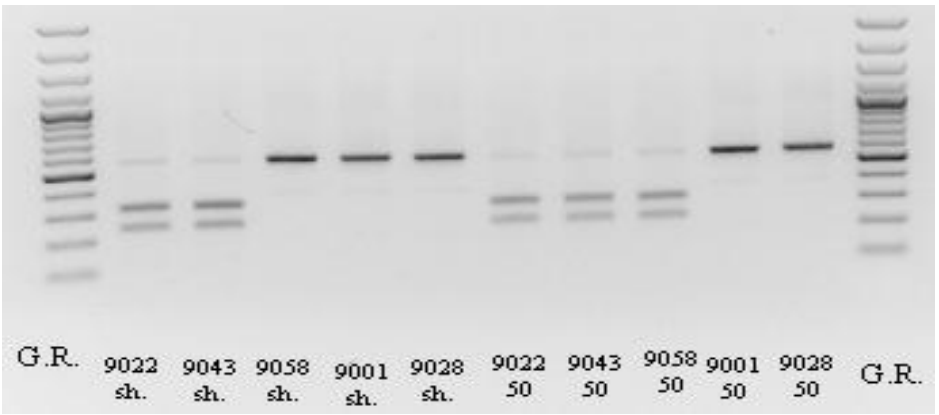

Fig. 3. 11: Induced resistance in *cyt b* gene that was PCR-amplified with primers cytb-BcF and cytb-BcR. PCR-RFLP of the small *cyt b* amplification fragment (~ 560 bp) digested with *Sat*I endonuclease. First and last lanes: molecular marker (O'GeneRulerTM 100bp plus DNA Ladder, Fermentas), numbers indicate strain numbers (with SHAM (st.) and with 50 ml azoxystrobin).

Interestingly the results demonstrated that the strain 9058 grown in SHAM was not digested and didn't mutate, but digested and had mutation in 50 ml azoxystrobin (Fig. 3. 11).

- **Fenhexamid resistance**

To determine fungicide resistance profiles in group I and group II, sensitivity to fenhexamid was studied in all 13 isolates belonging to group I and 74 group II strains. In accordance with *in vitro* responses of these field strains of *B. cinerea*, three phenotypes could be distinguished: four isolates were catgorised as sensitive strains which were resistant to estimated effective fenhexamid concentrations lower than 2 mg L^{-1}; seventy four isolates were catgorised as lowly resistant isolates which were resistant to estimated effective fenhexamid concentrations between 2 and 5 mg L^{-1}; and four isolates were catgorised as resistant isolates that survived estimated effective fenhexamid concentrations higher than 5 mg L^{-1}. High resistance could be detected only among group I strains, while lowly resistant and sensitive isolates could be identified in both groups. A majority of group II isolates proved to be sensitive to fenhexamid.

3. 3. PATHOGENICITY OF GROUP I AND GROUP II

Pathogenicity of group I and group II of *B. cinerea* on detached cucumber, paprika and grape leaves were studied. Three isolates of group I (8001, 8005 and 8047) and three isolates of group II (8020, 8025 and 8061) were incubated on potato dextrose agar and their mycelial disks (10 mm in diameter) were placed on the detached first leaves of cucumber and grape and incubated at 18°C for 3 days in a humid, dark chamber (Fig 3. 12).

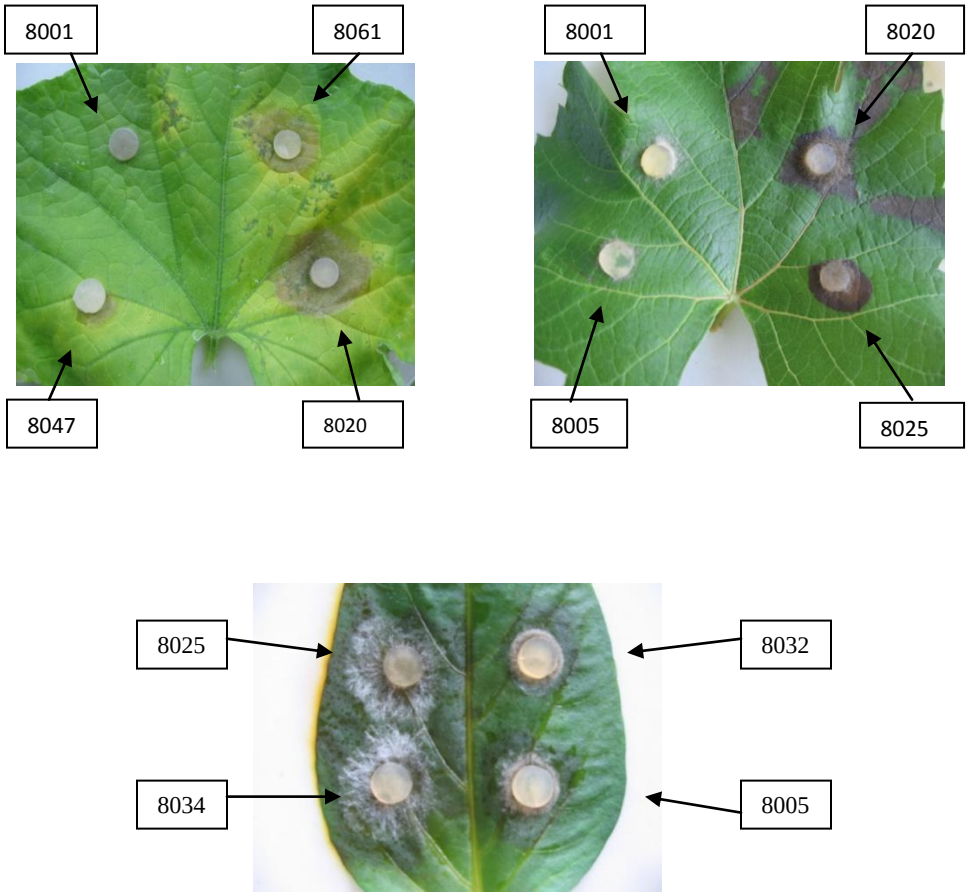

Fig. 3. 12: Pathogenicity of group I and group II of *Botrytis cinerea* on detached cucumber, paprika and grape leaves. The isolates of group I (8001, 8005, 8032 and 8047) and group II (8020, 8025, 8034 and 8061) isolates were incubated on potato dextrose agar plates at 18°C for 3 days. Their mycelial disks (10 mm in diameter) were placed on the detached first leaves of cucumber and grape and incubated at 18°C for 3 days in a humid, dark chamber.

The results showed that the pathogenicity of group II is stronger than pathogenicity of group I. On the other hand, group I and group II are different in competitive ability in terms of pathogenicity.

3. 4. COMPARISON OF *BOTRYTIS CINEREA* POPULATIONS ISOLATED FROM TWO CULTIVATED HOST PLANTS

- **PCR-RFLP markers**

We sampled populations of *B. cinerea* on sympatric strawberry and raspberry cultivars for three years. Altogether, 490 of group II *B. cinerea* isolates were analyzed. Isolates were first analyzed with RFLPs by amplifying two coding sequences (the genes encoding a nitrate reductase and an ADP/ATP translocase) and PCR amplicons were then digested with restriction enzymes. Two different alleles were identifiable for both genes with PCR-RFLP analysis (Fig 3. 13). For the first marker (ADP/ATP translocase), 368 strains (75% of the total) could be digested with *Eco*RI, while the remaining 25% of the isolates could not. For nitrate reductase, literature suggested to use two other restriction enzymes, *Rsa*I and *Hinc*II (Giraud et al. 1997, Muñoz et al. 2002). The amplicons from 464 (95%) of the strains were digested with *Rsa*I in five fragments (774bp, 503bp, 457bp, 404bp, 303bp) while 26 strains (5%) also gave five fragments but with, in most cases, slightly different sizes (785bp, 515bp, 465bp, 409bp, 222bp) (Fig 3. 13). Upon digestion with *Hinc*II, the nitrate reductase amplification of 79 (16%) of the strains was digested in three fragments (1431 bp, 1090bp, 442bp) while for 411 strains (84%) three fragments of different size were observed (1379 bp, 1117 bp, 451bp). The sizes of the RFLP-fragments of both genes found in all distinct types of Hungarian isolates were essentially identical to those detected in French and Chilean *B. cinerea* isolates (Giraud et al. 1997, Muñoz et al. 2002).

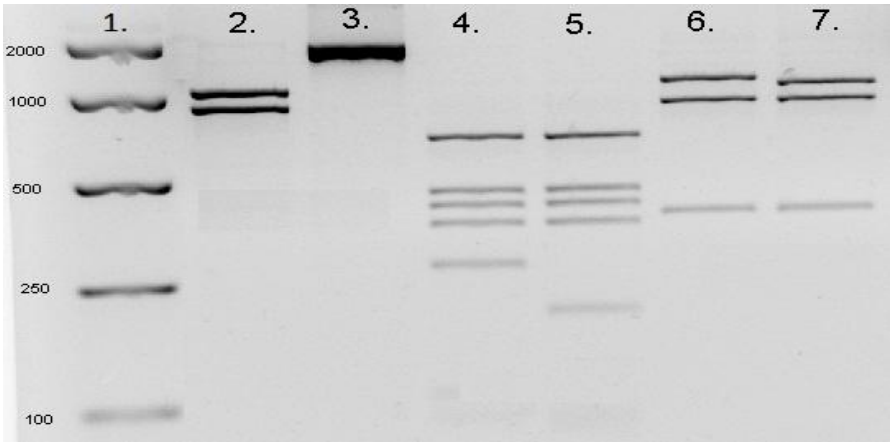

Figure 3. 13: ADP/ATP translocase and nitrate reductase gene alleles detected by PCR-RFLP. At the left, molecular weight markers (Easy Ladder I, BioRad); The numbers give marker length in bp. Fragments from ADP/ATP translocase alleles 1 and 2 upon *Eco*RI digestion, lanes 2 and 3, respectively; *Rsa*I-fragments from nitrate reductase alleles 1 and 2, lanes 4 and 5; *Hinc*II-fragments from nitrate reductase alleles 1 and 2, lanes 6 and 7.

The software POPGENE32 version 1.31 was used to compute observed number of alleles (n_a), effective number of alleles (n_e), Nei's gene diversity (h), Shannon's information index (I), index of differentiation (G_{st}) and gene flow (N_m) in the total samples and in each populations (Table 3. 7 and 3. 8).

Table 3. 7: Analysis of PCR-RFLP results with POPGENE

host	**n**	**n_a**	**n_e**	**h**
strawberry 2007	81	2.00 (0.00)	1.45 (0.44)	0.26 (0.23)
raspberry 2007	28	2.00 (1.41)	1.30 (0.10)	0.22 (0.06)
strawberry 2008	117	1.67 (0.58)	1.41 (0.39)	0.25 (0.22)
raspberry 2008	126	1.67 (0.00)	1.30 (0.28)	0.20 (0.18)
strawberry 2009	84	1.17 (0.58)	1.29 (0.25)	0.20 (0.17)
raspberry 2009	54	1.67 (0.58)	1.15 (0.17)	0.12 (0.13)
Total	490	2.00 (0.00)	1.36 (0.24)	0.25 (0.14)

n: sample size
n_a: Observed number of alleles,
n_e: effective number of alleles,
h: Nei's gene diversity, standard deviation between brackets

Table 3. 8: Comparison of *B. cinerea* populations from two different hosts: strawberries and raspberries. Computed from PCR-RFLP results with POPGENE

Year	2007	2008	2009
Nei's genetic identity	0.7742	0.9957	0.9925
G_{st}	0.290	0.008	0.022
N_m	1.225	61.758	21.87

G_{st}: index of differentiation,
N_m: gene flow

The results showed that the population structure of sympatric *B. cinerea* populations was similar, and all loci were polymorph. Nei's gene diversity and Shannon index indicated similar gene diversity for all populations, except for isolates from raspberry collected in 2009. Populations showed similar structures with PCR-RFLP results. G_{st} and N_m values indicate great differentiation and restricted gene flow between the two sympatric populations in 2007 only, when fungi could be collected only in June from strawberries, while mainly in August from raspberries (Fig. 3. 15).

- **Microsatellite analysis**

Microsatellite analysis at five SSR loci produced 167 haplotypes among the 469 isolates screened (Table 3. 9).

Table 3. 9: Characteristics of five amplified microsatellite loci of Hungarian *B. cinerea* isolates

Locus	Repeat motif	Number of alleles	Allele size range (bp)
Bc2	$(AC)_{20}AT(AC)_4$	20	138-198
Bc3	$(GA)_{10}$	12	209-231
Bc5	$(AT)_{12}$	11	82-149
Bc7	$(TA)_9$	13	108-130
Bc10	$(AC)_{13}$	14	161-190

The number of alleles (n_a), effective number of alleles (n_e), Nei's gene diversity (h), index of differentiation (G_{st}) and gene flow (N_m) were computed by POPGENE32 version 1.31 (Table 3. 10 and 3. 11).

Table 3. 10: Analysis of *B. cinerea* microsatellite results with POPGENE

host	n	n_a	n_e	h
strawberry 2007	78	7.40 (0.89)	3.76 (0.21)	0.73 (0.01)
raspberry 2007	28	3.80 (1.48)	1.69 (0.36)	0.38 (0.15)
strawberry 2008	117	8.20 (0.83)	4.31 (1.12)	0.75 (0.07)
raspberry 2008	122	10.80 (3.11)	5.40 (1.23)	0.81 (0.05)
strawberry 2009	76	8.80 (2.49)	4.21 (1.65)	0.74 (0.07)
raspberry 2009	48	7.00 (1.87)	3.59 (1.12)	0.69 (0.13)
Total	469	14.00 (3.53)	5.89 (1.22)	0.82 (0.03)

[a] n: sample size
n_a: Observed number of alleles,
n_e: effective number of alleles,
h: Nei's gene diversity, (standard deviation between brackets)

Similarly to PCR-RFLP results, all loci were polymorph, and population structure of sympatric *B. cinerea* from the two different hosts showed similarity except for fungi isolated from raspberries in 2007 with the smallest sample number. Nei's gene diversity and Shannon index indicated similar gene diversity for all populations, except again for the 2007 raspberry isolates. Populations showed similar structures with microsatellite analysis, except for one population with the smallest number of isolates.

Table 3. 11: Comparison of *B. cinerea* populations from two different hosts: strawberries and raspberries. Computed from microsatellite results with POPGENE

Year	2007	2008	2009
Nei's genetic identity	0.4483	0.6323	0.8637
G_{st}	0.1889	0.0497	0.0272
N_m	2.1473	9.5621	17.8773

G_{st}: index of differentiation
N_m: gene flow

The result of microsatellite data demonstrated that the genetic diversity of *B. cinerea* from raspberry was lower in 2007 and also G_{st} and N_m values indicate great differentiation and restricted gene flow between the two sympatric populations in 2007 only.

- **Minisatellite analysis**

The fragment of the intron of the ATPase gene *atp1* containing the minisatellite was amplified and sequenced. Thirty-seven haplotypes among the 490 isolates could be detected. One or two alleles were dominant (occurred in more than 50 % of the isolates) in all examined population. The results were analyzed by DnaSP (Librado and Rozas, 2009) available from http://www.ub.edu/dnasp/ (Table 3. 12).

Table 3. 12: Analysis of *B. cinerea* MSB1 sequencing results with DnaSP.

year	host	n	h	H_d	K	Gst
2007	strawberry	79	11	0.806	29.089	
	raspberry	28	5	0.384	19.928	0.2531
2008	strawberry	131	15	0.842	63.268	
	raspberry	112	11	0.768	32.346	0.1730
2009	strawberry	83	13	0.801	40.998	
	raspberry	54	8	0.638	16.919	0.1198
Total/Mean		487	37	0.855	46.027	

[a] n: sample size
h: number of haplotypes,
H_d: haplotype diversity (Nei's 1987)
K: average nucleotide diversity
G_{st}: index of differentiation

The genetic diversity of *B. cinerea* from raspberry was lower in populations with fewer samples. G_{st} values indicate moderate (2009) to great (2007) differentiation of the sympatric populations.

According to three different datasets (PCR-RFLP pattern of *ADP-ATP translocase* and *nitrate reductase* genes, MSB1 minisatellite sequence data,

and fragment size of five microsatellite loci) the structure of the different populations was similar as indicated by Nei's gene diversity and Shannon's information index, and also by haplotype diversity. It should be noted, that a reduced sample size was accompanied by reduced population diversity. The computed index of differentiation (G_{st}) and gene flow indicated differentiation within the sympatric populations. However, the differentiation was also affected by the sample size and the year of isolation. Population genetic parameters were also influenced by the level of polymorphism of the datasets.

- **Transposon elements**

The presence or absence of *Flipper* was checked by PCR for some strains. All 490 strains were then scored for the presence of two transposable elements (*Boty* and *Flipper*) by dot blots. Based on the presence and absence of *Boty* and *Flipper* transposon amplicons, all four possible types of *B. cinerea* transposons were distinguished.

- **Bayesian inference of population structure**

With structure analysis, we performed Bayesian clustering using the 469 group II (*B. cinerea sensu stricto*) isolates. To confirm the genetic differentiation of the populations on the two host plants, we performed Bayesian assignment analyses for 5 loci of microsatellite data. The highest magnitude of ΔK was found with $K = 2$ and therefore two genetic clusters were detected for all data (Fig. 3. 14). Similar results were achieved when samples from each year were analysed separately (results not shown).

At $K = 2$, the structure bar plot for isolates collected in the same year indicates nice separation of isolates from strawberries and raspberries in collection years 2007 and 2008 (Fig. 3. 15).

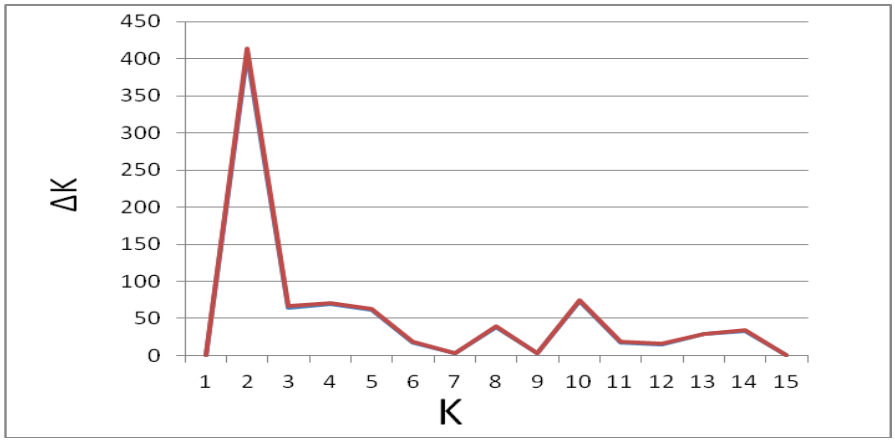

Fig. 3. 14: The magnitude of Δ*K* at each level of *K* (1-15) used to determine the most probable number of genetic clusters (*K* = 2) with *B. cinerea* haplotypes from Hungarian population determined by structure analysis.

In 2007, all the samples from strawberries were collected at the same sampling time (6th July), while all but four fungal samples were collected one month later (6th August). Next year the better conditions (longer humid periods with rains) resulted in more expressed infection, and more strains could be collected from both hosts between middle of June and first part of October in 2008. Beyond overlapping sampling times, *B. cinerea* isolates could be collected only from strawberries in June. The majority of those samples we indicated belong to 2nd (red or dark gray) cluster. On the other hand, fungi could be isolated only from raspberries in August and September. The majority of those isolates were put to the other cluster (green or light grey). In the third year, fungi were collected in June from both hosts, and the majority of them were put to the 2nd (red or dark grey) population irrespective of their hosts, the population has dramatically changed in July following a fungicide treatment of raspberries. Similar population shift was deleted in the case of the pathogen population from strawberry at the beginning of the vegetation period (April-June).

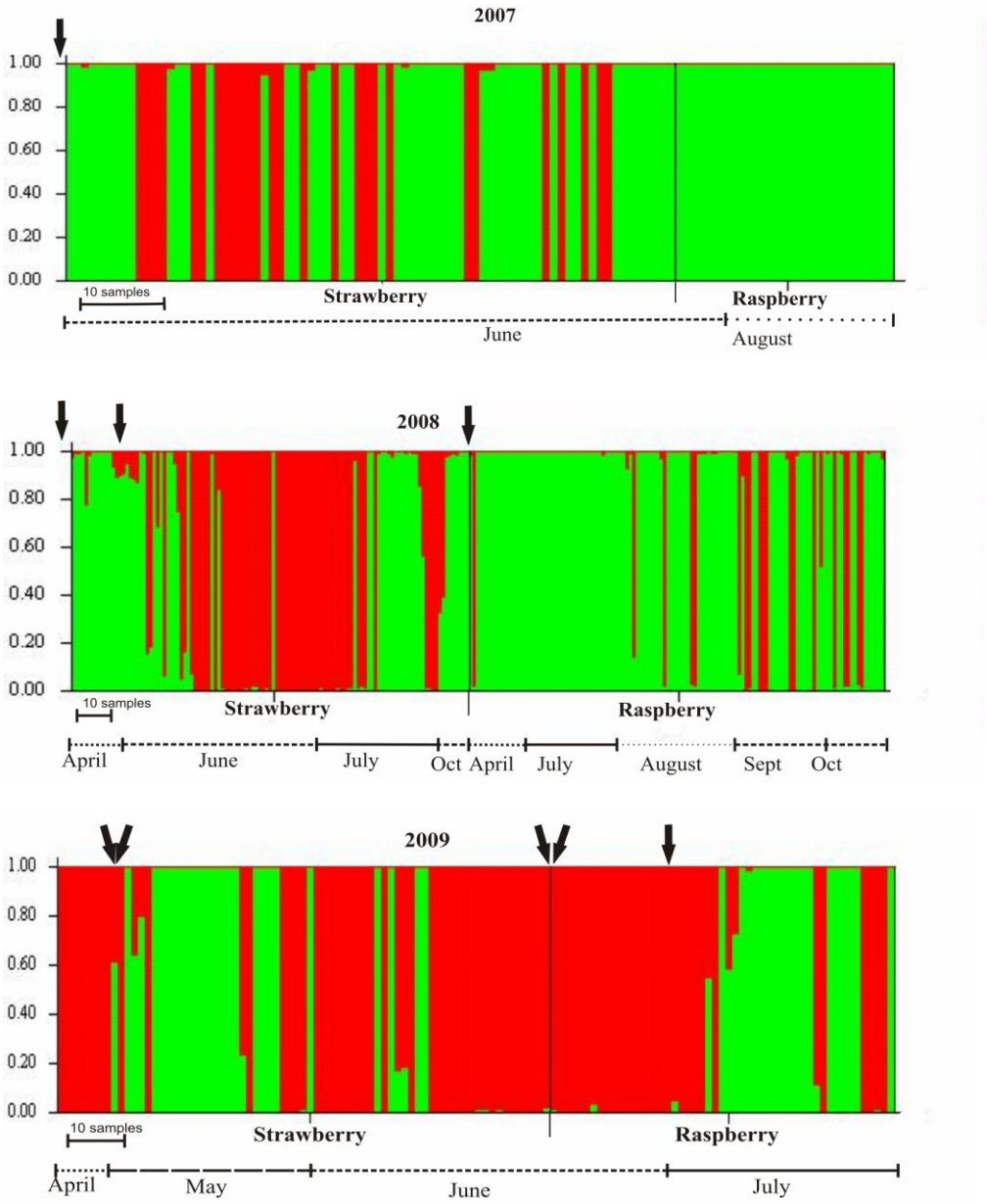

Fig. 3. 15: Bayesian assignment of individuals into two clusters. Structure bar-plot that reveals the assignment of haplotypes at $K = 2$ clusters *B. cinerea* from strawberry and raspberry. 2007, 2008, 2009 indicate collection years. Arrows indicate fungicide treatment on cultivars. Timescale below bar-plots indicates collection time of fungal isolates.

- **Data Mining Results**

Attributes were paraphrased (Table 3. 13) and grouped in four datasets (Table 3. 14). Differentiations between attributes of two hosts (Strawberry and Raspberry) were analyzed by different algorithms (Rule Induction, J48, and Conjunctive Rule) and two tests (Gain Ratio and Chi-square) using the Rapidminer software package. Rule Induction learns a pruned set of rules with respect to the information gained. We set sample ratio from 0.8 to 0.9, step by 0.5. J48 is the most common way to generate decision tree.

Table 3. 13: Characteristics of attributes and their descriptive statistics

Attributes	**Type** [a]	**Range**
Host	Binominal	Strawberry and Raspberry
Microsatellite (Bc2, Bc3, Bc6, Bc7 and Bc10)	Integer	$0 \leq$ Bc2 ≤ 198 [b] $0 \leq$ Bc3 ≤ 231 $0 \leq$ Bc6 ≤ 149 $0 \leq$ Bc7 ≤ 130 $0 \leq$ Bc10 ≤ 190
Minisatellite	Integer	$1 \leq$ Minisatellite ≤ 25 [b]
Flipper	Boolean	0 and 1
Boty	Boolean	0 and 1
Fenhexamid resistance	Binomial	LR and S
*Hinc*II (Nitrate reductase + *Hinc*II)	Binominal	A and B
*Rsa*I (Nitrate reductase + *Rsa*I)	Binominal	A and B
*Eco*RI (ADP-ATP translocase + *Eco*RI)	Binominal	A and B

a: Binominal, Integer, and boolean are statistical variable types
b: Allele size range (bp)

Table 3. 14: Dataset description for test

Name	Attributes	Number of attribute[a]
Dataset 1	strain, host, year, Bc2, Bc3, Bc6, Bc7, Bc10, Flipper, Boty, *Eco*RI, *Hinc*II, *Rsa*I	13
Dataset 2	Bc2, Bc3, Bc6, Bc7, Bc10	5
Dataset 3	Bc2, Bc3, Bc6, Bc7, Bc10, Fungicide	6
Dataset 4	Bc2, Bc3, Bc6, Bc7, Bc10, Minisatelitte	6

[a] Original number of features

This clarifier generates a pruned or unpruned C4.5 decision tree (Table 3. 15). Conjunctive Rule implements a single conjunctive rule learner that can predict for numeric and nominal class labels (Table 3. 16). As we stated above, using these algorithms we gain importance attributes among original features. In table 3. 15, the first column defines classification algorithm, the second column consists of selected attributes that implemented on two dataset (Dataset 1 and Dataset 4) and last column specifies accuracy. Table 3. 16, describes datasets with one rule and obtains examples covered by class label (Strawberry and Raspberry).

Table 3. 15: Algorithms Results over Dataset 1 and Dataset 4

Classifiers Model	Attributes importance respectively[a]		Accuracy[b]	
	Dataset 1	Dataset 4	Dataset 1	Dataset 4
Rule Induction 1	Bc10, Bc7, Bc3, Bc2, Bc6, *Eco*RI, Boty, Filipper	Bc7, Bc10, Bc3, Bc6, Bc2, minisattelite	%91	%88
Rule Induction 2	Bc10, Bc7, Bc3, Bc2, Bc6, *Eco*RI, *Hinc*II, Boty	Bc7, Bc10, Bc3, Bc6, Bc2	%90	%81
Decision Tree	Bc7	Bc7	%68	%61

[a] set of ordered attributes by model
[b] a criterion for evaluation of model

Table 3. 16: Performance of Single Rule Induction over Datasets

Dataset	Rule	Covered by the Rule[a]		Not Covered by the Rule	
		Strawberry	Raspberry	Strawberry	Raspberry
Dataset 2	If (117 < Bc7 ≤ 129) and (Bc10 ≤ 176) then Host = Strawberry	0.890909	0.109091	0.416309	0.583691
Dataset 3	If (Bc7 > 117) then Host = Strawberry	0.867257	0.132743	0.304762	0.695238
Dataset 4	If (Bc7 > 117) then Host = Strawberry	0.820313	0.179688	0.258929	0.741071

[a] percentage of rules covered by model

Gain Ratio and Chi-square tests were used to test the attributes important in ordering between two hosts (Table 3. 17). With other words, after using three classification algorithms (C4.5 and Rule Induction and Conjunctive Rule), we used two most popular criteria to choose importance attributes (Gain Ratio and Chi-square). We did this to compare current results with previous ones.

Table 3. 17: Attribute importance based on Gain Ratio and Chi-square criteria

Dataset	method	Attribute importance respectively
Dataset 1	Gain Ratio	Bc7, Bc6, Bc2, Bc10, Bc3, *Eco*RI, date, filipper, HincII, boty, RsaI
	Chi-square	Bc2, Bc7, Bc6, Bc10, Bc3, date, *Eco*RI, filipper, HincII, boty, RsaI
Dataset 2	Gain Ratio	Bc7, Bc6, Bc2, Bc10, Bc3
	Chi-square	Bc2, Bc7, Bc6, Bc10, Bc3
Dataset 3	Gain Ratio	Bc7, Bc2, Bc6, Bc3, Bc10, fungicide
	Chi-square	Bc2, Bc7, Bc6, Bc10, Bc3, fungicide
Dataset 4	Gain Ratio	Bc7, minisatelitte, Bc6, Bc2, Bc10, Bc3
	Chi-square	Minisatelitte, Bc7, Bc2, Bc10, Bc6, Bc3

[a] attributes selection by 2 criteria: Gain Ratio, Chi-square.

By using Rule Induction and Decision tree models, the results showed that the best markers to demonstrate genetic differentiation in populations of *B.cinerea* on two different sympatric host plants, strawberry and raspberry, are microsatellites, especially Bc7 and Bc10 (table 3. 15). Also the table 3. 16 shows by using Conjunctive Rule model in datasets 2, 3 and 4: if Bc7 is between 117 and 129, the host plant is strawberry. Using Gain Ratio and Chi-square models (Table 3. 17) showed that the microsatellites are the most important markers to differentiate these populations.

These results indicate that the set of microsatellite markers used here was sufficient to correctly estimate the existing diversity.

Chapter 4
Discussion

Sympatric *B. cinerea* cryptic species were analyzed using a population genetic approach and phenotypic markers. The results have not confirmed a complete genetic differentiation of the two cryptic species (originally proposed by multiple gene sequence data analysis, genealogical concordance of the phylogenetic species recognition – GCPSR; Fournier et al. 2005), but point instead either towards a recent divergence or to the existence of genetic exchange between *B. cinerea* group I and group II cryptic species. Before the discovery of appropriate genetic markers, *B. cinerea* was regarded as one, variable and polyphagous species exhibiting a great genetic diversity (Van der Vlugt-Bergmans et al. 1993; Diolez et al. 1995), and morphological variability (Grindle, 1979; Di Lenna et al. 1981; Lorenz and Eichhorn, 1983; Leone, 1990; Movahedi and Heale, 1990). Several genetic characterizations of *B. cinerea* populations revealed that this plant pathogen was genetically extremely diverse which suggested that there is no unique, large and panmictic population (Giraud et al. 1997, 1999; Albertini et al. 2002; Munoz et al. 2002; Fournier et al. 2003). Initially, two genotypes were described characterizing sympatric sibling species: (1) *transposa* that harbors DNA transposons Boty and Flipper, and (2) transposonless *vacuma* (Diolez et al. 1995; Levis et al. 1997; Giraud et al. 1997). However, Fournier et al. (2005) showed that genetic differentiation determined from multiple gene sequences was not in accordance with either of the previously described transposon genotypes (*transposa* or *vacuma*) and suggested partitioning of *B. cinerea* into group I and group II phylogenetic cryptic species. Group I is also known as '*Botrytis pseudocinerea*' while group II is referred to as '*B. cinerea sensu stricto*' (Fournier et al. 2005). The genetic diversity was lower within group I isolates, as revealed by DNA polymorphisms and vegetative incompatibility tests (Fournier et al. 2005). However, *B. cinerea* group II has been shown to be predominant on infected plants (Fournier et al. 2005; Isenegger et al.

2008; Karchani-Balma et al. 2008; Váczy et al. 2008). Diagnostic molecular markers for the two cryptic species have been developed based on the difference of *Hha*I restriction pattern of PCR-amplified *Bc-hch* gene (Albertini et al. 2002; Fournier et al. 2003). To date, the *vacuma* transposon genotype has been detected within group I while in contrast, all four transposon genotypes (*vacuma, transposa, flipper*-only, and *boty*-only) have been detected in group II (Fournier et al. 2005; Ma and Michailides, 2005; Isenegger et al. 2008; Karchani-Balma et al. 2008; Martinez et al. 2008; Váczy et al. 2008; Munoz et al. 2010; Rajaguru and Shaw, 2010). High resistance to the fungicide fenhexamid appeared to characterize group I isolates (Fournier et al. 2005; Martinez et al. 2005). Here, we describe for the first time the existence of the *B. cinerea* group I ('*B. pseudocinerea*') cryptic species outside of Western Europe, possibly suggesting that it was able to spread beyond geographic barriers such as the Alps and to settle in the Carpathian Basin. In addition, oilseed rape was identified as a new host for *B. cinerea* (group I). GCPSR of *B. cinerea* cryptic species is based on the analysis of four nuclear loci, i.e., *β-tubulin*, *Bc-hch*, CYP51 (eburicol 14a-demethylase) and 63R (a noncoding region containing a microsatellite-like motif and flanking regions with numerous SNPs) (Fournier et al. 2005). The identification of Hungarian group I strains was based on (a) PCR-RFLP of *Bc-hch* gene (Albertini et al. 2002; Fournier et al. 2003) and (b) on sequence analysis of *β-tubulin* gene. Phylogenetic analysis of partial *β-tubulin* sequences put these Hungarian isolates into the same clade with Western-European group I isolates collected earlier. However, the population genetic analysis of the microsatellite fragment length data of the two sympatric *B. cinerea* cryptic species indicated only moderate population differentiation. Both population differentiation statistics (θ, G_{ST}, gene flow) and Nei's genetic identity index suggest moderate differences between the two sampled

populations with a low level of gene flow. Finally, the standardized version of index of association (as computed on the total sample) was significantly different from zero, likewise indicating genetic differentiation between populations. However, the examined *B. cinerea* group I isolates did not carry any group-specific microsatellite alleles or haplotypes, while Nei's χ^2 test results supported the genetic differentiation between the two groups for only three out of the five examined microsatellites. The group I (*B. pseudocinerea*) cryptic species represents between 2.5 and 15% of French isolates (Albertini et al. 2002; Fournier et al. 2003, 2005; Martinez et al. 2005) and was equally low (14%) among the Hungarian isolates collected in 2008 from different host plants from the same area. Group I strains were present at spring, in agreement with other studies (Fournier et al. 2005; Martinez et al. 2005). In agreement with previous studies (Fournier and Giraud, 2008; Karchani-Balma et al. 2008; Rajaguru and Shaw, 2010), group II isolates (obtained predominantly from infected plants) showed higher genetic diversity than group I strains. This finding suggests a higher adaptive potential (e.g. towards new hosts), and in general may indicate that these strains are more successful pathogens. Regarding transposon content, it was initially suggested that sympatric transposa and *vacuma* type *B. cinerea* strains were genetically isolated (Giraud et al. 1997, 1999). This definition was later revised by differentiating between group I *vacuma* isolates (Fournier et al. 2005) and group II isolates, that may feature *transposa*, *flipper*, *boty* or *vacuma* genotype (Fournier et al. 2005; Ma and Michailides, 2005; Munoz et al. 2002; Albertini et al. 2002; Ben Ahmed and Hamada, 2005; Milicevic et al. 2006; Isenegger et al. 2008; Váczy et al. 2008; Rajaguru and Shaw, 2010). Phylogenetic studies (Albertini et al. 2002; Fournier et al. 2003, 2005; Isenegger et al. 2008) could not provide hard evidence for genetic differentiation between transposon genotypes. It must

be mentioned, that transposon detection has many uncertainties in *B. cinerea* depending on the test used. Martinez et al. (2008) showed discrepancies between PCR and dot blot results of detecting Boty and Flipper transposon elements. Moreover, weak (or no) correlations were found between transposon frequency or transposon type and the geographic origin of the isolate (Munoz et al. 2002; Váczy et al. 2008), or the year of isolation (Váczy et al. 2008), while results were also contradictory regarding temporal distribution (Martinez et al. 2005, 2008) and host specificity (Ma and Michailides, 2005). Our results indicated that molecular markers based on transposon content alone has limited value on defining population structures and are not useful in *B. cinerea* cryptic speciation either. Significant differences in transposon content could only be detected on a continental scale (Isenegger et al. 2008). Our fenhexamid resistance studies confirm previous results of Albertini et al. (2002) and Fournier et al. (2005) that fenhexamid-resistant strains could only be found among group I isolates. However, three Hungarian group I strains were found sensitive to the fungicide. Moreover, fenhexamid-resistant group II isolates have been reported recently from Chile (Esterio et al. 2010). Therefore, fenhexamid resistance cannot be used for the identification of group I *B. cinerea* isolates. Unlike Fournier et al. (2005), we could not detect statistically relevant differences in conidial size among the Hungarian group I and II isolates, dismissing the spore morphology as a possible means to discriminate between the cryptic species. Up until today, group I isolates could be detected in Europe only. As this and other study (Walker et al. 2011) pointed out, they comprise a small minority of the *B. cinerea* isolates even there. While the reason for this unknown, we speculate that speciation may have occurred in this area, and the lower diversity indicates a bottleneck effect. Other explanations are that group I strains successfully adapted to noncrop

hosts or have a mainly saprophytic lifestyle, thereby avoiding the attention of plant protection-oriented research programmes.

QoI resistance and related cytochrome *b* gene allelism in Hungarian group I and II *B. cinerea* populations were investigated. As it was expected, PCR-amplified cytochrome *b* gene fragments of many QoI-resistant field isolates showed the mutational change causing the glycine to alanine (Ala) (G143A) substitution and presence of the resistance-conferring G-to-C mutation was thus confirmed in Hungarian group II populations. All isolates, where the G-to-C mutation could be detected with both PCR-RFLP and allele-specific PCR, showed high resistance against azoxystrobin. In six cases, the mutation could only be detected with allele-specific PCR (i.e., not with PCR-RFLP) and these strains were sensitive to azoxystrobin. This would indicate marginal presence of the resistance-conferring, mutated mtDNA and these strains may well develop resistance rapidly when faced with QoIs on the field. PCR fragment length analysis of *cyt b* strongly suggested the presence of the alternative group I intron in a considerable part (> 32 %) of the Hungarian *B. cinerea* field isolate collection. Some group II isolates gave rise to both fragments (~ 1750 bp and ~ 560 bp), which could imply *cyt b* heteroplasmy in monosporic isolates. Grasso et al. (2006) reported that the absence of the alternative intron immediately downstream the GGT codon for glycine 143 is positively correlated to the ability of plant pathogenic fungi to develop QoI resistance. This appears to be the case for the twelve Hungarian group-I isolates tested in this study. Neither of them were highly resistant to azoxystrobin and in concordance, neither of them carried the G-to-C mutation in *cyt b* provoking QoI resistance while all possessed the alternative *cyt b* intron. However, Leroux et al. (2010) described at least one group-I isolate, I3, without the alternative intron (accession FJ217742). On

the contrary, four HR group-II strains were identified in this study that possessed the alternative intron (without detectable heteroplasmy) in the *cyt b* gene. For one of those, amplification with the mutant-(G143A)-specific primer pair BcAR-F/BcAR-R was recorded. Remarkably, a considerable number of group-II strains (nine of twenty eight HR strains) exhibited high azoxystrobin resistance where the resistance-conferring G143A substitution could not be evidenced. We currently investigate these nine field isolates for potential alternative mutations that could have rendered them QoI resistant. *B. cinerea* poses a high-risk to develop resistance against single-target fungicides like QoIs, because of its high genetic variability, short life cycle, abundant inoculum production and ease to disseminate it. *B. cinerea* field isolates with azoxystrobin (a QoI fungicide) resistance have been found in several countries, such as France (Leroux et al. 2010), Germany (Weber and Hahn, 2011), Greece (Samuel et al. 2011), USA (Wedge et al. 2007), China (Jiang et al. 2009) and Japan (Ishii et al. 2009). In the large majority of the cases, high resistance against QoIs is associated with one particular G-to-C point mutation in the cytochrome *b* (*cyt b*) gene. The resulting glycine-to-alanine mutation (G143A) changes the conformation at the azoxystrobin target binding site (Esser et al. 2004). *Cyt b* is a mitochondrial gene and as a consequence; inheritance of fungal QoI resistance is uniparental (in analogy with mammals, via the maternal lineage) rather than Mendelian. Other mechanisms, such as bypassing the electron transfer chain blocked by QoI fungicides via the alternative oxidase pathway can also result in resistance to QoIs (Wood and Hollomon, 2003; Fernández-Ortuño et al. 2008).

B. cinerea was previously considered a generalist fungal plant pathogen. However, recently it was shown that significantly structured populations exist according to the host plant, suggesting sympatric specialization (Karchani-Balma et al. 2008). Differentiation among transposon genotypes

within *B. cinerea* group II has been described and these genotypes were found in sympatry on various hosts irrespective of geography (Giraud et al. 1997, 1999; Muñoz et al. 2002; Martinez et al. 2003, Samuel et al. 2012). On the other hand, Ma and Michailides (2005) studied the genetic structure of *B. cinerea* populations in California on different hosts using transposable elements as markers and found no differentiation between *B. cinerea* populations from different hosts. Phylogenetic studies by Albertini et al. (2002), Isenegger et al. (2008) and Fournier et al. (2003, 2005) provided only weak indications for genetic differentiation between transposon genotypes. Fournier and Giraud (2008) focused on distribution of the *Flipper* element in different host plants and could not detect significant differences, while distribution varied significantly according to geographic location. The detection of all four possible transposon genotypes in Hungarian group II isolates in this work indicated that there is no significant barrier to gene flow of transposons or any relevant difference among isolates from the different host plants under study. Moreover, data-mining analysis did not identify transposon content information as particularly important or even indicative for the differentiation of *B. cinerea* populations. Similar to our present results, weak correlations were found between transposon frequency or type and the *B. cinerea* cryptic species (Fekete et al. 2012), host specificity (Ma and Michailides, 2005), or other observed variation within *B. cinerea* populations (Muñoz et al. 2002; Váczy et al. 2008). Using PCR-RFLP markers, several studies (Giraud et al. 1999; Muñoz et al. 2002) indicated that *B. cinerea* group II populations are structured according to infected host plants, i.e., isolates from the same host exchange genes more frequently with each other than with isolates from another host. In this work, we employed three different data sets (i.e., PCR-RFLP patterns of the *ADP-ATP translocase* and nitrate *reductase genes*, MSB1 minisatellite sequence data,

and fragment size of five microsatellite loci) and conclude that the structure of the different populations under study was similar for all three datasets as indicated by Nei's gene diversity and haplotype diversity (in the case of the minisatellite markers). It should be noted that a reduced sample size (strawberry isolates in 2007) coincided with a reduced population diversity. Population genetic parameters were influenced by the extent of polymorphism within the data sets. Nevertheless, the F statistic results, and gene flow strongly suggested differentiation within the sympatric populations on strawberry and raspberry. Data mining analysis corroborated that there were significant differences between the *B. cinerea* sympatric populations infecting the two hosts. The most informative markers turned out to be the microsatellites, while transposons were not informative and suitable for population differentiation in our study. The Bayesian analysis of the microsatellite data set highlighted the basis of differentiation. Because of the different phenological characteristics of the perennial hosts, the influence of meteorology, and variable sensitivity during the annual life cycle (flowering, fruit maturation) for *B. cinerea* infection, only a restricted number of isolates could be collected at the same time from the two hosts. The importance of spore migration and a subsequent, rapid change in *B. cinerea* populations in tomato greenhouses has been noticed previously (Alfonso et al. 2000; Decognet et al. 2009). The rapid shift in predominance between two population clusters with the progress of the annual phenology and after fungicide application(s) in 2008 and, particulary, in 2009 (Fig. 3. 15) indicated that the same phenomena occurred with open-field hosts on adjacent fields. Sympatric specificity of *B. cinerea* growing on different hosts may be influenced by several parameters, like differences in the phenology of the hosts, as populations of migrating spores encounter host plants in a different phenophase. The sudden change of fungal population

observed following fungicide treatment of infected plants supports the hypothesis that a change of the *B. cinerea* population in the air, in the form of vegetative spores, could result in an abrupt change of *B. cinerea* populations on hosts. However, eventual host preferences of *B cinerea* variants may also play as role.

Summary

We described for the first time the existence of the *B. cinerea* group I ('*B. pseudocinerea*') cryptic species outside of Western Europe, in Hungary, possibly suggesting that it was able to spread beyond geographic barriers such as the Alps and to settle in the Carpathian Basin. In addition, oilseed rape was identified as a new host for *B. cinerea* (group I). Phylogenetic analysis of partial *β-tubulin* sequences put these Hungarian isolates into the same clade with Western-European group I isolates collected earlier. Sympatric *B. cinerea* cryptic species were analyzed using a population genetic approach and phenotypic markers. Population genetics analysis revealed a high level of diversity within each species and a lack of gene flow between them.

Sensitivity of the Hungarian *B. cinerea* field isolates to a Qo inhibitor (QoI) fungicide azoxystrobin was screened in this study. Resistance has been reported in more than 20 % species. In various QoI-resistant monosporic *B. cinerea* isolates from Hungary, a G-to-C point mutation was identified in the mitochondrial gene that encodes the QoI target, cytochrome *b*, resulting in a glycine to alanine substitution at position 143 (G143A). Analysis of Hungarian group I and group II strains further indicated the frequent occurrence of an additional group I-type intron in the *cytb* gene directly

downstream of the glycine-143 codon. Mutual presence of distinct mitochondrial DNAs specifying different *cytb* alleles (heteroplasmy) has also been detected in monosporic strains.

We analyzed populations of group II *B. cinerea* on sympatric strawberry and raspberry cultivars in North-East of Hungary for three years growing seasons. Standard population genetic data were computed from three different data sets: (i) PCR-RFLP pattern of *ADP-ATP translocase* and *nitrate reductase* genes, (ii) MSB1 minisatellite sequence data, and (ii) fragment size of five microsatellite loci. The structures of the different populations were similar as indicated by Nei's gene diversity and haplotype diversity. The F statistics (F_{st}, G_{st}), and the gene flow indicated ongoing differentiation within sympatric populations. The population genetic parameters were influenced by polymorphisms within the three data sets as assessed using Bayesian algorithms. Data mining analysis pointed towards the five microsatellite loci as the most defining markers to study differentiation in all isolates. The results suggest the occurrence of host-specific, sympatric divergence of generalist phytoparasites in perennial hosts.

New scientific results:

I. We described for the first time the existence of the *B. cinerea* group I ('*B. pseudocinerea*') cryptic species outside of Western Europe, in Hungary, possibly suggesting that it was able to spread beyond geographic barriers such as the Alps and to settle in the Carpathian Basin.

II. Oilseed rape was identified as a new host for *B. cinerea* (group I).

III. Sympatric *B. cinerea* cryptic species were analyzed using a population genetic approach and phenotypic markers. Population genetics analysis revealed a high level of diversity within each species and a lack of gene flow between them.

IV. Analysis of Hungarian group I and group II strains indicated the frequent occurrence of an additional group I-type intron in the *cytb* gene directly downstream of the glycine-143 codon.

V. Mutual presence of distinct mitochondrial DNAs specifying different *cytb* alleles (heteroplasmy) has also been detected in Hungarian monosporic strains.

VI. The occurrence of host-specific, sympatric divergence of group II *B. cinerea* populations in perennial hosts (strawberry and raspberry) were proved with the molecular population biology analysis of three different data sets: (i) PCR-RFLP pattern of *ADP-ATP translocase* and *nitrate reductase* genes, (ii) MSB1 minisatellite sequence data, and (ii) fragment size of five microsatellite loci.

VII. Data mining analysis pointed the five microsatellite loci as the most defining markers to study differentiation of *B. cinerea* group II isolates from different hots (strawberry and raspberry).

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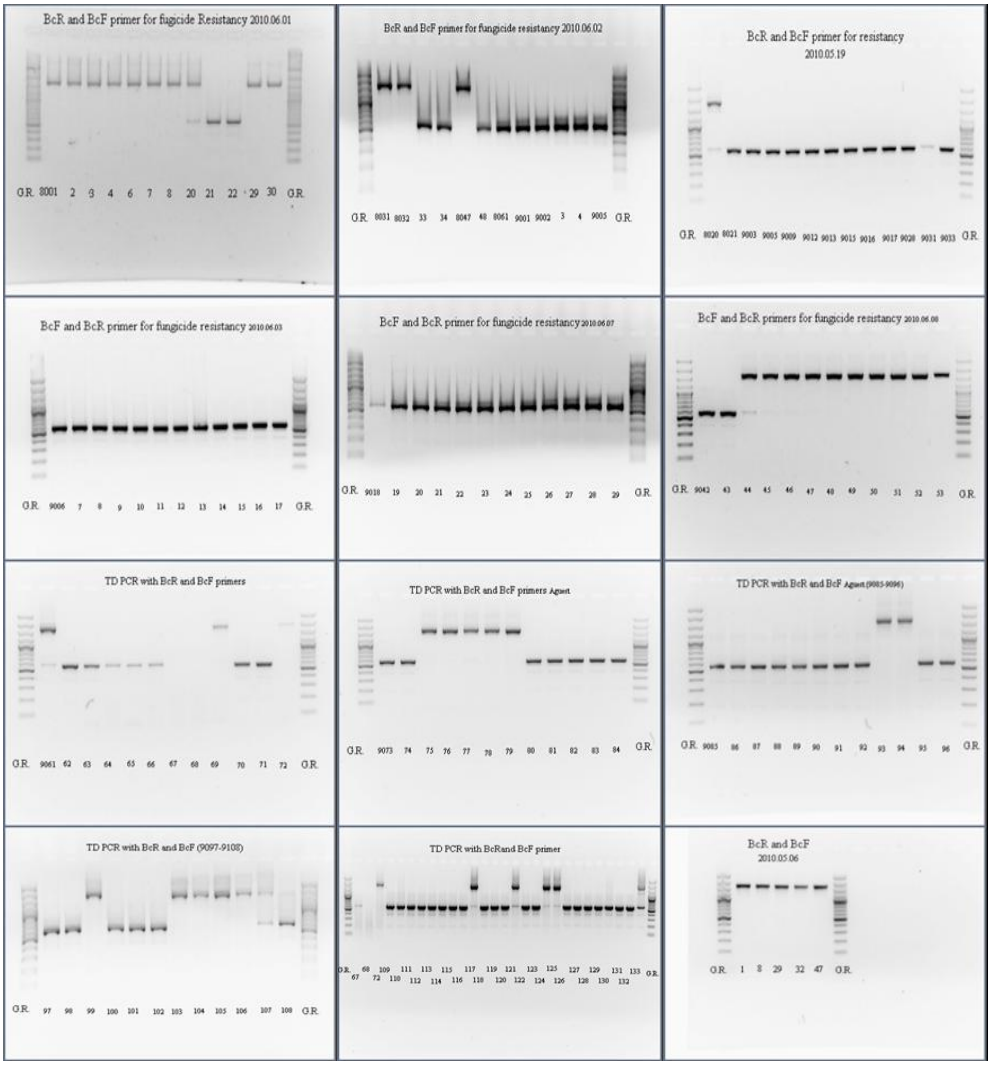
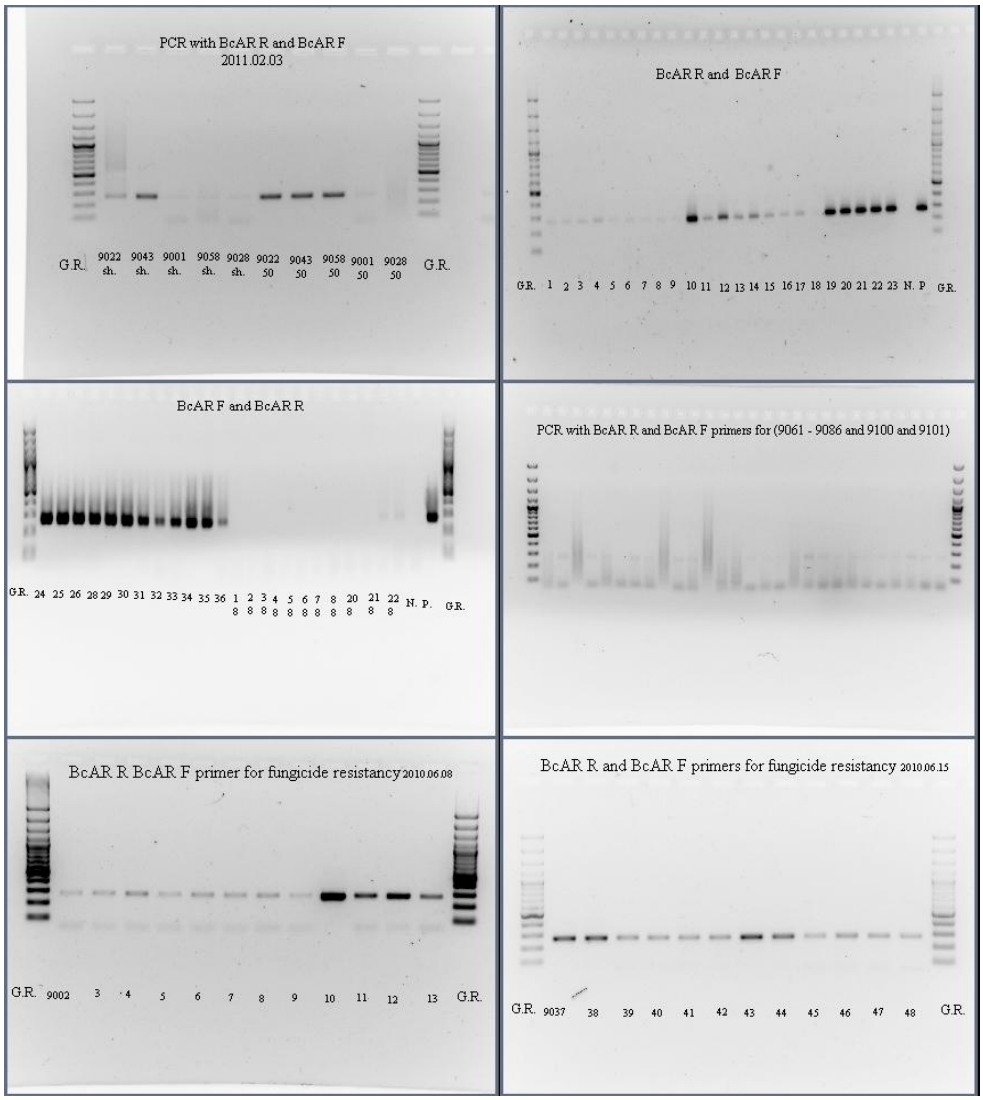
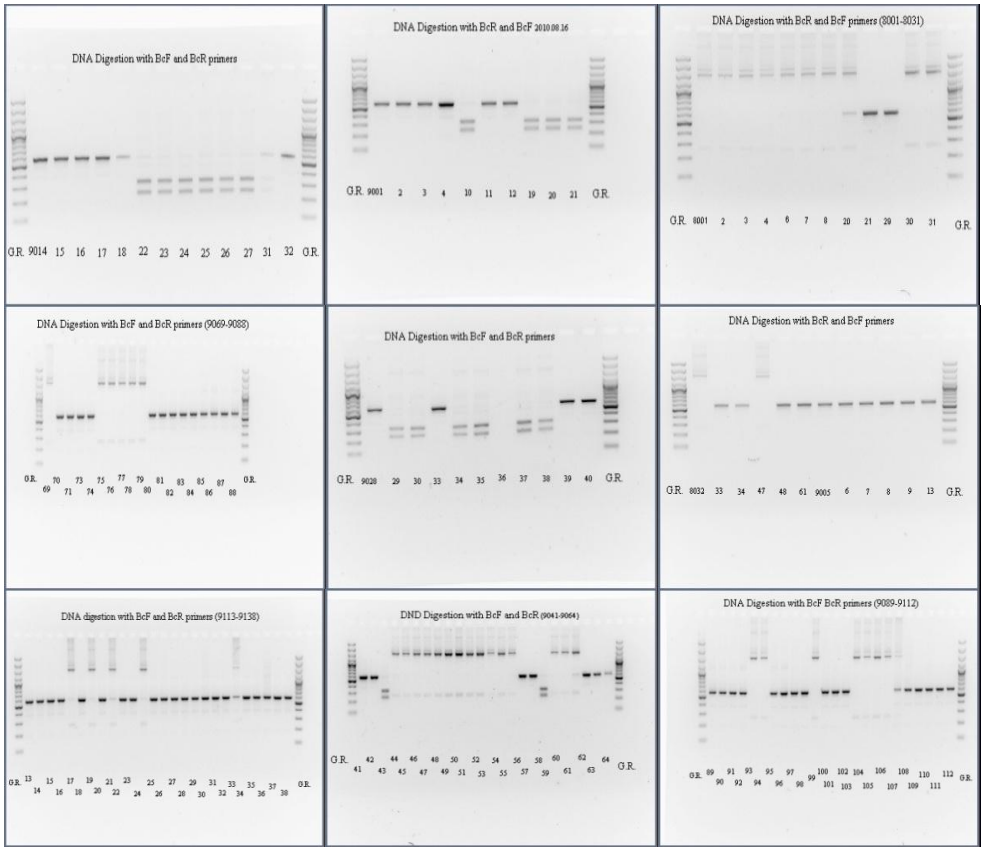
Supplementary Chapter



Supplementary Figure 1: Cytochrome *b* (*cyt b*) gene size differentiation in the Hungarian *B.cinerea* field isolate collection. For each of the 157 monosporic strains, part of the *cyt b* gene was PCR-amplified with primers cytb-BcF and cytb-BcR. A representative selection of PCR amplifications is shown. First and last lane: molecular marker (O'GeneRulerTM 100bp plus DNA Ladder, Fermentas). The large fragment (~ 1750 bp) indicates the presence of the alternative intron between the codons 143 and 144. GR: nucleotide ladder (indicate main fragment sizes, or list them), numbers indicate strain numbers.



Supplementary Figure 2: Allele-specific PCR with the primer pair BcAR-F + BcAR-R for the detection of azoxystrobin-resistant isolates of *B. cinerea*. First and last lane: molecular marker (O'GeneRulerTM 100bp plus DNA Ladder, Fermentas).



Supplementary Figure 3: PCR-RFLP of the small *cyt b* amplification fragment (~ 560 bp) digested with *Sat*I endonuclease. First and last lane: molecular marker (O'GeneRulerTM 100bp plus DNA Ladder, Fermentas). Appearance of the two bands of lower molecular mass results from *Sat*I digestion of the fragment of higher molecular mass, evidencing the G-to-C mutation that causes the QoI-resistant phenotype. PCR-RFLP analysis of strains that carry the alternative intron was not performed, as in those strains the G-to-C mutation does not result in a novel *Sat*I (GC|NGC) restriction site.

International, peer-rewieved publications related to the thesis / A doktori értekezés témájához kapcsolódó nemzetközi, referált közlemények

Asadollahi, M., Fekete, E., Karaffa, L., Flippi, M., Árnyasi, M., Esmaeili, M., Váczy, K., Sándor, E. (2013): Comparison of *Botrytis cinerea* populations isolated from two open-field cultivated host plants. *Microbiological Research*, (in press) http://dx.doi.org/10.1016/j.micres.2012.12.008. **IF: 2.308**

Asadollahi, M., Szojka, A., Fekete, E., Karaffa, L., Takács, F., Flippi, M., Sándor, E. (2013): Quinol oxidation inhibitor (QoI) fungicide resistance and cytochrome *b* diversity in the Hungarian *Botrytis cinerea* population. *Journal of Agricultural Science and Technology* Vol. 15 (2): (in press). **IF: 0.436**

Fekete, É., Fekete, E., Irinyi, L., Karaffa, L., Árnyasi, M., **Asadollahi, M.,** Sándor, E. (2012): Genetic diversity of a *Botrytis cinerea* cryptic species complex in Hungary. *Microbiological Research* 167, 283-291. **IF: 2.308**

Other publications related to the thesis / A doktori értekezés témájához kapcsolódó további közlemények

Asadollahi, **M.,** Fekete, É., Fekete, E., Karaffa, L., Irinyi, L., Sándor, E., (2010): Cytochrome *b* diversity of Hungarian *Botrytis cinerea* strains. *Acta Agraria Debreceniensis*, 39, 18-21.

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Poster and oral presentations related to the thesis

M. Asadollahi, É. Fekete, E. Fekete, L. Karaffa, L. Irinyi, E. Sándor (2010): Cytochrome *b* diversity of Hungarian *Botrytis cinerea* strains. *Power of Microbes in Industry and Environment*, 22-25 Sep. Malinska, Croatia. (Poster Presentation)

É. Fekete, L. Irinyi, **M. Asadollahi**, E. Fekete, L. Karaffa, E. Sándor (2010): Possible genetic information exchange between *Botrytis cinerea* group I and II isolates in Hungary. *Power of Microbes in Industry and Environment*, 22-25 Sep. Malinska, Croatia. (Poster Presentation)

É. Fekete, **M. Asadollahi**, L. Irinyi, E. Fekete, L. Karaffa, E. Sándor (2010): Differences between sympatric *Botrytis cinerea* populations in Hungary. *Power of Microbes in Industry and Environment*, 22-25 Sep. Malinska, Croatia. (Poster Presentation)

M. Asadollahi, É. Fekete, E. Fekete, L. Irinyi, L. Karaffa, M. Árnyasi, A. Szojka, G. Kövics, E. Sándor (2011): Comparision of *Botrytis cinerea* populations isolated from two cultivated host plants. 15th Evolutionary Biology Meeting at Marseilles, September 27 - 30, 2011, Marseilles, France, Abstract Book pp 135. (Poster Presentation)

Fekete É., Fekete E., Irinyi L., Karaffa L., Árnyasi M., **Asadollahi M.**, Kövics Gy.J., Sándor E. (2011): Genetic diversity within the cryptic species of *Botrytis cinerea* causing grey mold on different crops in Hungary. 15th Evolutionary Biology Meeting at Marseilles, September 27 - 30, 2011, Marseilles, France, Abstract Book pp 74-75. (Poster Presentation)

Asadollahi M., Fekete É., Fekete E., Irinyi L., Karaffa L., Árnyasi M., Szojka A., Kövics Gy.J., Sándor E. (2011): Comparison of *Botrytis cinerea* populations isolated from two cultivated host plants. *Botrytis-Sclerotinia* Post-Genome workshop, 15th-17th September 2011, Lyon, France, Abstract Book pp 27. (Poster Presentation)

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Javidnia, K., Miri, M., **Assadollahi, M.**, Gholami, M. and Ghaderi, M. (2009): Screening of selected plants growing in Iran for antimicrobial activity. *Iranian Journal of Science and Technology, Transaction A: Science*, 33 (4), 329-333. **IF: 0.01**

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