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Faculty of Agricultural Science

Department of Food Improvement and Protection

Study of Italian isolates of *Alternaria* spp.: molecular and morphological characterization and pathogenesis on apple tree

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1. Summary

In 1999, a symptomatic disease was reported among a number of apple tree orchards (Golden cultivars) in North Italy (Trentino Alto Adige region) (Marshall et al., 2004, and Marshall et al., 2006). By 2002 the disease was found in an area 10 times larger than the initial infection sites and had started attacking the Gala variety of apple, broadening to Veneto, Piedmont (Cuneo and Alessandria) (Ortalda, 2006) and some areas of Emilia Romagna (Ferrara and Ravenna with spots present only on leaf) (Antonacci and Montuschi, 2006). Originally, the disease was attributed to a lack of microelements or a nutritional disequilibrium. In 2003, the Experimental Centre of Laimburg (BZ) demonstrated the pathogenicity of *Alternaria* isolates on healthy apple foliar tissues and proposed a pathotype of *Alternaria alternata* as the causal agent of the disease (Gobber et al., 2004 and Marshall et al., 2006). The severe damages inflicted on orchards been the impetus to more deeply investigate the diversity and spatial distribution of *Alternaria* spp. isolated from these diseased tissues and discover the relationship of the Italian pathogen to the *Alternaria alternata mali* pathotype, causal agent of the Apple Blotch disease.

It was decided to carry out a morphological and molecular characterization of the isolates, evaluating their pathogenicity and, subsequently, combining the collected data to more fully characterize the strains. The strain collection (174 isolates) was constructed by collecting material (received from extension service personnel) between June and August of 2007, 2008, and 2009. All the isolates were sampled from symptomatic tissues (leaves and fruits) from apple orchards in different geographical area in the North of Italy. All the colonies isolated were tested for pathogenicity. Preliminary bioassays were performed on detached plant materials (fruit and leaf wounded and unwounded), belonging to the Golden cultivar, with two different kind of inoculation (conidial suspension and conidial filtrate). Symptoms were monitored daily, and a value of pathogenicity score (P.S.) was assigned on

the basis of the diameter of the necrotic area that developed. On the basis of the bioassays, the number of isolates to undergo further molecular analysis was restricted to a representative set of single spore strains (44 strains).

Morphological characteristics of the colony and sporulation pattern were determined according to previous systematic work on small-spored *Alternaria* spp. (Pryor and Michailides, 2002 and Hong et al., 2006). Reference strains (*Alternaria alternata*, *Alternaria tenuissima*, *Alternaria arborescens* and four Japanese strains of *Alternaria alternata mali* pathotype), used in the study were kindly provided by Prof. Barry Pryor, who allowed open access to his own fungal collection.

Molecular characterization was performed combining and comparing different data sets obtained from two distinct molecular approaches: 1) investigation of specific loci and 2) fingerprinting based on diverse randomly selected polymorphic sites of the genome. For the specific locus investigations, it was chosen to sequence the EndoPG partial gene and three anonymous regions (OPA1-3, OPA2-1 and OPA10-2). These markers proved to be a powerful tool in the latter systematic works on small-spored *Alternaria* spp. It has been reported in the literature that small-spored *Alternaria* taxonomy is complicated due to the inability to resolve evolutionary relationships among the taxa because of the lack of variability in the markers commonly used in fungal systematics, making this choice of loci appropriate. The three data sets together provided the necessary variation to establish the phylogenetic relationships among the Italian isolates of *Alternaria* spp. On Italian strains these markers showed a variable number of informative sites (ranging from 7 for EndoPg to 85 for OPA1-3) and the parsimony analysis produced different tree topologies that were all concordant to define *A. arborescens* as a monophyletic clade.

Fingerprinting analysis (nine ISSR primers and eight AFLP primers combination) led to the same result: a monophyletic *A. arborescens* clade and one clade containing both *A. tenuissima* and the *A. alternata* strains. This first attempt to characterize Italian *Alternaria*

species recovered from apple produced concordant results with what was already described in a similar phylogenetic study on pistachio (Pryor and Michailides, 2002), on walnut and hazelnut (Hong et al., 2006), apple (Serdani et al., 2002) and citrus (Peever et al., 2004). Together with these studies, this research demonstrates that the three morphological groups are widely distributed and occupy similar ecological niches. Furthermore, this research suggests that these *Alternaria* species exhibit a similar infection pattern despite the taxonomic and pathogenic differences.

The molecular characterization of the pathogens is a fundamental step to understanding the disease that is spreading in the apple orchards of the north Italy. At the beginning the causal agent was considered as *Alteraria alternata* (Marshall and Bertagnoll, 2006). Their preliminary studies proposed a pathogenic system related to the synthesis of toxins. Experimental data from our bioassays suggest an analogous hypothesis, considering that symptoms could be induced after inoculating plant material with solely the filtrate from pathogenic strains. Moreover, some positive PCR reactions using AM-toxin gene specific primers, designed for identification of apple infecting *Alternaria* pathovar, led to a hypothesis that a host specific toxin (toxins) could be involved. It remains an intriguing challenge to discover whether or not if the agent of the “Italian disease” is the same of the one previously typified as *Alternaria mali*, casual agent of the apple blotch disease.

2. Introduction

2.1 The genus *Alternaria* – Historical overview

The *Alternaria* genus is spread worldwide and able to colonize many different habitats, as a parasite on living plant tissue or a saprophyte on organic substrata. It is not always easy to distinguish between the two different lifestyles due to the intermediate position of some specimens that are able to shift from the saprophytic to parasitic strategy when faced with a weak host (Rotem, 1994). The genus *Alternaria* was originally described by Nees in 1817 with *A. tenuis* as the type and only member of the genus. In 1917 Elliott discussed the generic traits of *Alternaria* stressing the form of conidia: obclavate, pointed, and often beaked. Then in 1945, Neergaard published his treatise, *Alternaria*, which was helpful in delineating the main characteristics of the genus but was limited to an accepted 16 species, two varieties, and a few *formae speciales* known in Denmark. Despite his inclusion of names not always accepted by other taxonomists, the Neergaard treatise was useful in assistance and in identification because it remarked the differences among isolates of the same species.

Later, Joly (1964) focused on 28 species, including several *Ulocladium* taxa within his *Alternaria* concept. Ellis (1971, 1976) published precise descriptions and drawings of 44 *Alternaria* taxa focusing, principally, on macro sporulating pathogens. Furthermore, the author performed one of the first descriptions concerning the conidia shape and their morphological characteristics. This was an important milestone even if the key was too simplified, did not give any information regarding media, and did not include beaks in measuring the conidia dimensions.

Recently, Simmons, with his taxonomic essays enclosed in *Alternaria Themes and Variations* (1981-2003), intended to cover the entire genus with detailed descriptions, drawings, and culture/isolation media and procedures. Exploiting the diversity in spore

shape, size, and branching among the *Alternaria* spp., the author organized the genus into species-groups by referring to a representative species. Most important groups under the pathogenic profile are: the *Alternaria alternata* group, the *Alternaria tenuissima* group, the *Alternaria infectoria* group, the *Alternaria arborescens* group, the *Alternaria brassicicola* group, the *Alternaria porri* group and the *Alternaria radicina* group (Simmons 1992, Simmons & Roberts 1993, Simmons 1995 and Simmons 1999). Even with the precise descriptions of the differences among various species, these characters remain difficult to exploit by plant pathologists in routine identifications.

Because of the absence of an identified sexual stage for the vast majority of *Alternaria* species, this genus was classified into the division of mitosporic fungi, or the phylum Fungi imperfecti. Latter taxonomic studies have recognized some *Alternaria* as the anamorphic form of the genus *Lewia*, and the current classification reported on Dictionary of Fungi 10th edition (Kirk et al., 2008) is:

Phylum: Ascomycota

Class: Dothiideomycetes

Subclass: Pleosporomycetes

Order: Pleosporales

Family: Pleosporaceae

Genus: *Alternaria* (anamorph.) / *Lewia* (telomorph.)

2.2 The genus *Alternaria* – Morphological overview

The key taxonomic feature of the genus *Alternaria* is the production of multicellular, dark-colored (melanized) conidia with longitudinal as well as transverse septa (phaeodictyospores). These conidia are broadest near the base and gradually taper to an elongated beak, providing a club-like appearance (Thomma, 2003). They are produced in

single or branched chains on short, erect conidiophores. *Alternaria* forms conidia that arise as protrusions of the protoplast through pores in the conidiophore cell wall.

Classification based on conidial characteristics is complicated by the existence of other fungal genera, such as *Stemphylium* and *Ulocladium*, which produce phaeodictyosporic conidia that resemble those of *Alternaria*. Based on the characteristics defined by Simmons (1995), *Stemphylium* and *Alternaria* species are discriminated by the appearance of the conidiophores apex, and *Ulocladium* and *Alternaria* species by the appearance of the basal end of immature conidia (Thomma, 2003). A misclassification can occur due to environmental conditions which affected the development and morphological traits of the fungus, resulting in a single species being accidentally divided into several (Rotem, 1994). This phenotypic variation does not justify assigning *A. alternata*-like specimens to other species. However, it is this morphological variation that has made the classification of this genus really difficult and the creation of different synonymies for the same species. As a result, there is no generally accepted classification of *Alternaria*, and the binomials used differ in various descriptions. For example the agent of potato early blight is identified as *A. porri* (Ell.) Neerg. f. sp. *solani* E. & M. according to Neergaard (1945), *A. dauci* f. sp. *solani* (E. & M.) Neerg. according to Joly (1964) and *A. solani* Sorauer in the M.B. Ellis classification (1971).

To characterize a species some morphological traits are more important than others. For instance, the character of the beak distinguishes between clearly different species but it is not sufficient to discriminate between very closely related ones (Rotem, 1994). The same concept can be applied to other traits such as length of conidiophores, the number of transverse septa, and dimension of the spore body, including the beak. It has to be considered that differences in dimension can arise because of a number of factors. The influence of the growing media composition, the incubation temperature, and the age of the colony have been reported to have the most effect on the morphology of the conidia (Tisdale

and Wadkins, 1931, Neergaard, 1945, Hartill, 1968, Lucas, 1971, Mishaghi et al., 1977 and Rotem, 1994). As a result, taxonomists prefer referring to dimensions using ranges rather than means so an overlap among the species is observed.

2.3 Morphological themes and variations: *A. alternata*, *A. tenuissima*, *A. arborescens* and *A. alternata mali* patotype (*Alternaria mali*)

In the following paragraphs are presented the descriptions drawn by Simmons (1999) concerning the species groups encountered in this study. Simmons, in thirty years of monographic approach to *Alternaria* taxonomy, has maintained a closely defined view of morphology so as not to increase the chaos that already existed in *Alternaria* spp. systematic. The isolates used for the descriptions and the illustrations were selected by Simmons (1992, 1995) among his own isolate collection (Fig.1). He chose isolates that appeared morphologically identical and culturally stable under controlled conditions.

Alternaria alternata

The sporulation pattern encloses a single suberect conidiophore and an apical cluster of branching chains of small conidia separated by short secondary conidiophores. The primary conidiophore is comparatively short, 40-70 x 3-4 μm . It can remain single or branched, becoming 1-3 branches or geniculate, with corresponding numbers of primary conidiophores. Single chains of conidia in the branching head, ignoring short lateral branches, have ca. 15-20 conidia. The first conidia in a chain usually remain long and elliptical as they mature; conidia produced in the lateral chain are elliptical or ovoid. Initial elliptical conidia are 25-40 x 5-9 μm with 4-7 transverse septa and a few or no longisepta.

Alternaria tenuissima

The basic pattern is moderate length to long chains of conidia: 5-10 and more conidia, most of which are long and narrow. The initial chain usually remains simple or may produce lateral chains. In older cultures, the main conidiophores may count about 12-15

units with some lateral branches. Initial conidia in a chain often have a narrow-tapered upper half. Conidia in the distal part of a chain usually are ovoid or ellipsoid without a narrow taper in the upper half; each conidium produces a short apical secondary conidiophore of 1-2 cells. The narrowest conidia have only transverse septa while the wider ones present both transept (5-8) and few longitudinal or oblique septa. Conidial size ranges for conidia having only transepta are in the range ca. 32-45 x 11-13 μm and can reach 40-60 x 16-18 μm in obclavate multiseptate conidia.

Alternaria arborescens

Long well defined primary conidiophores characteristically bear a few terminal and subterminal branches. Each conidiophore branch bears a branching chain of conidia. Primary conidiophores are ca 200-300 μm long. Mature conidia are typically 12-42 x 7-11 μm , non-beaked, ellipsoid or short-ovoid, with greatly punctulate to verrucose ornamentation, and with 1-4 transepta and few longisepta or oblique septa. Moreover, conidia come in a branching aggregate with 50-125 conidia.

Alternaria mali or *Alternaria alternata mali* pathotype

Roberts (1924) initially described an *Alternaria* which was isolated from apple fruit and able to cause rot of ripe apple as well as necrosis spots on leaves. Whether or not the species identified by Roberts is related to the disease now generally known as *Alternaria* blotch of apple, it can be demonstrated that his *A. mali* is a fungus morphologically different than the one that figures prominently in HST-related studies. Furthermore, *A. mali* isolates present different and definable patterns and conidial morphologies. They can resemble the *A. tenuissima* and the *A. arborescens* sporulation patterns with some variations in conidia dimensions

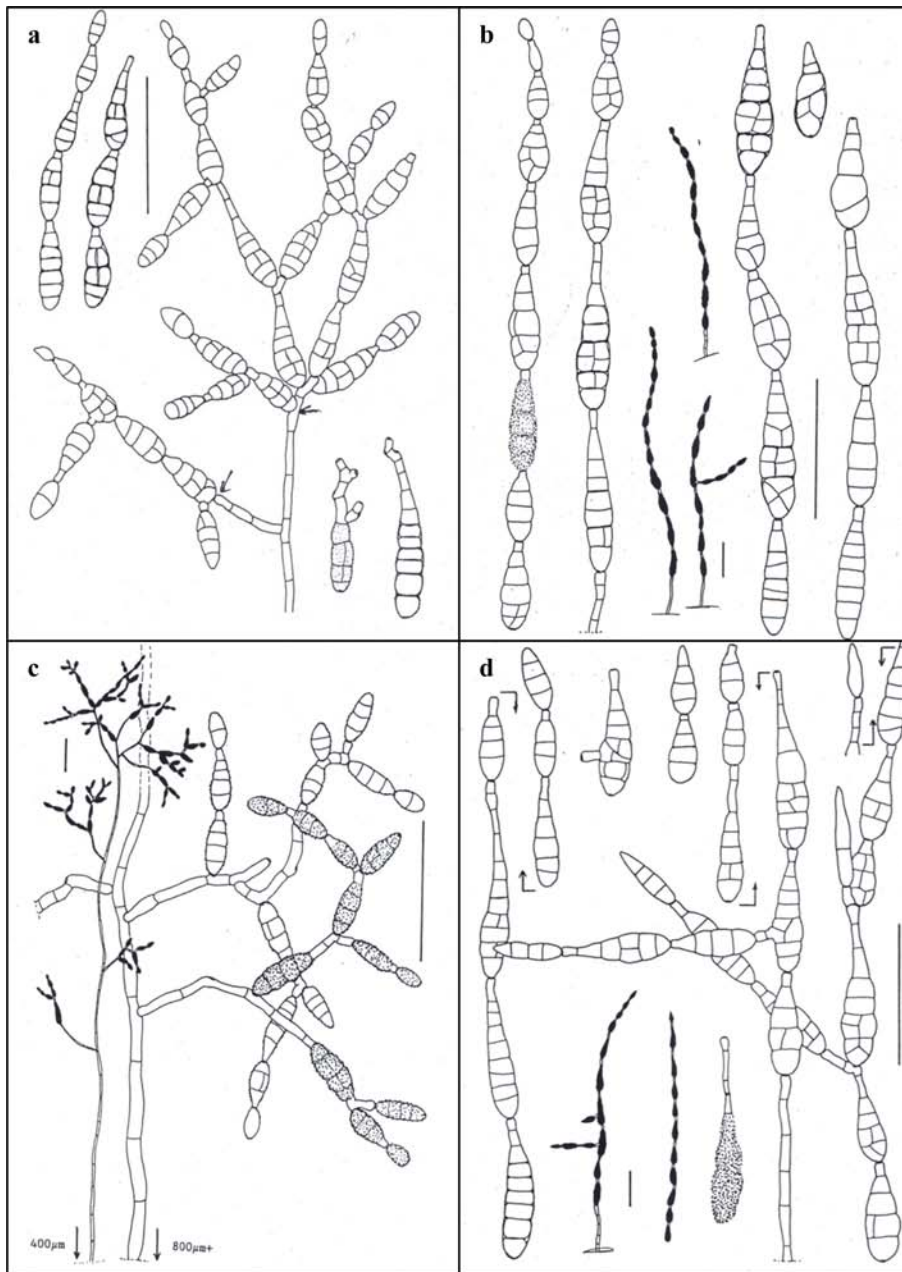


Fig. 1. Simmons's drawings extracted from *Alternaria themes and variations* (Mycotaxon 1999). a) *Alternaria alternata* EGS 34-016, b) *Alternaria tenuissima* EGS 34-015; c) *Alternaria arborescens* EGS 39-128; d) *Alternaria mali* EGS 38-029.

2.4 Factors influencing the sporulation process of *Alternaria* spp.

Light

Witsch and Wagner (1955) and Laech (1967) demonstrated that different species of fungi can be divided in two groups: the diurnal sporulators (such as *Alternaria* spp.) and the constant temperature sporulators (such as *Fusarium* spp.). Here is reported the photomorphogenesis concerning only the first group. The process occurs in two phases: the

first one, inductive phase, leads to the formation of the conidiophores while the second one, terminal phase, leads to the conidia production. The light requirements for these two stage are different: the inductive phase is stimulated by near ultraviolet wave lengths (310-400 nm), while the terminal phase proceeds better in the darkness and it is usually inhibited by light. Other researchers considered also an intermediate phase in which conidiophores are triggered to form conidia. The need for induction by light was recorded also *in vivo* (Rotem, 1989).

Temperature

The temperature range for the sporulation is less wide than the one required by the fungus for vegetative growth. The minimum temperature is around 5 °C and the maximum temperature is enclosed between 30°C and 35°C. The optimum range for different species varies from 15 °C to 30°C. The effect of such conditions are reported both in the study done by Douglas (1972), where an optimum of 25 °C was found for the sporulation of *A. solani* in light and 20 °C in a dark/light photoperiod, and in the Leach's work (1967) in which it is stressed how high temperatures enable the production of conidiophores in *A. dauci* whereas lower temperatures are favorable to spore formation.

Humidity

In general, humidity is a difficult parameter to study *in vitro*. This factor is usually characterized by a high value in agar petri dishes and too difficult to be standardized and controlled. Nevertheless, it's possible to refer to some field studies that point out how high values of dew and/or rain increases the spore production (Kare and Nema, 1981 and Everts and Lay, 1990).

Culture media

Apparently, all *Alternaria* species enclose isolates that differ in the number of conidia produced and the requirements for the induction in culture: some isolates maintain their sporulating potential for years while others loose it after a brief time. Incubation in darkness

on Potato Dextrose Agar (PDA), at optimum growth temperatures, has proved meaningful to differentiate among isolates of *A. alternata*, *A. arborescens* and *A. tenuissima* (Pryor and Michailides, 2002). The use of rich media, such as DRYES (dichloran Rose Bengal yeast sucrose agar; Frisvad, 1983), promotes the vegetative growth of small spored catenulate *Alternaria* spp. with concomitant reduction of sporulation. Moreover, colonies composed primarily by vegetative hyphae reveal much more variation in color and texture. On the other hand typifying the isolates according their sporulation habit requires nutritionally weak media and lighted growth.

Pryor and Michailides (2002) established precise culture parameters for medium, light intensity, and photoperiod within narrow limits avoiding external environmental factors as light and temperature fluctuations. Authors also stressed the importance of the uniform composition of the medium creating a preference for the usage of the weak PDA (0.5% commercial PDA) instead the Potato Carrot Agar (PCA) homemade produced.

Melanin production

Melanins are dark, brown to black, high molecular weight pigments and are produced by diverse organisms from animals to plants to microorganisms. They are formed by the oxidative polymerization of phenolic or indolic compounds. As polymerization exploits different pathways, melanins are molecules with various structures carrying aromatic rings and available hydroxyl groups (Bell and Wheeler, 1986). *Alternaria* melanin production seems to be derived from a monomeric precursor, 1,8-dihydroxynaphthalene (DHN) which is synthesized through the pentaketide pathway (Kimura and Tsuge, 1993) (Fig. 2).

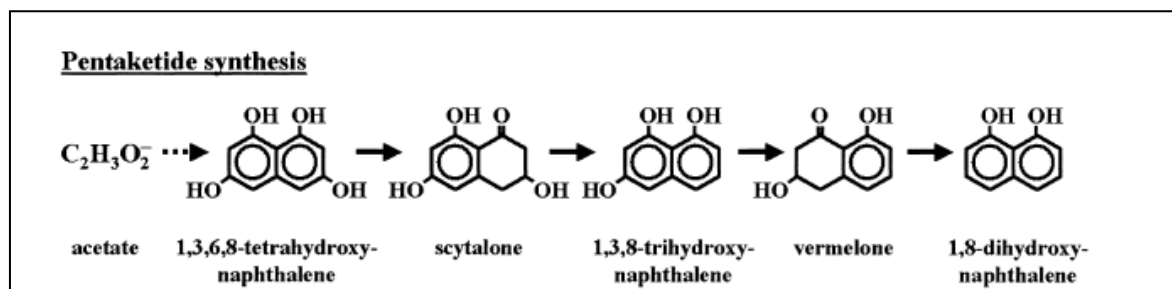


Fig. 2. Pentaketide pathway leading to the formation of DHN from acetate in *Alternaria alternata* and other fungi. Through the condensation and cyclization, 1,3,6,8-tetrahydroxynaphthalene is formed from acetate. Through alternating reduction and dehydration 1,8-dihydroxynaphthalene (DHN) is formed which can be directly formed into melanin through oxidative polymerization.

Melanins have an important role both in conidia formation and in protecting fungi against environmental stress such as UV-radiation, extreme temperatures, and compounds released by microbial antagonists, thus adding longevity and survival (Rehnschorn and Free, 1996 and Kawamura et al., 1999). *In vitro* experiments showed a loss of pathogenicity in melanin-deficient isolates, it has been demonstrated that the melanization of appressoria can contribute to fungal virulence (Howard and Valent, 1996). However, it has also been reported that mutants of *Alternaria* naturally produce unmelanized appressoria and still exhibit their pathogenicity, demonstrating that melanin is not required for virulence (Tanabe et al., 1990). So the influence of melanin on the capability of produce disease among fungi is variable, explaining the diversity in timing, localization and function of melanin synthesis.

2.5 Infection process

Alternaria spp. are usually foliar pathogens causing slow destruction of host tissues through the reduction of photosynthetic potential (Thomma, 2003). The status of the leaf determines the success of infection. For instance, density of stomata, more frequent on the lower surface of the leaf, thickness of cuticle (Bock, 1964), and wettability of the surface (Conn and Tewari, 1989) are the characteristics that mainly affect this process

The histology of sporulation has been investigated in *A. alternata* and in *A. brassicicola*. Dormant spores of *A. brassicicola* have really thick and heavy melanized walls

with plugged septal pores. The germ tube arises from the inner layers of the cell wall. During the germination phase, a multiplication of ribosomes and mitochondria is recorded. Soon cytoplasm shrinks and the vacuoles occupy most of the cell (Campbell, 1970).

Generally, conidia of *Alternaria* do not require extrinsic nutrients even if the presence of such compounds may facilitate the germination. The leachates are composed of amino acids, organic acids, growth substances, vitamins, alkaloids, phenolic substances, and carbohydrates, all of which are externally excreted from the plant tissues. Under favorable conditions most *Alternaria* species germinate in about 1-3 hours, but penetration of host tissue by germ tubes may be markedly delayed. Some species, apparently less virulent, achieve penetration long after the germ tubes emerge and spreads on the leaf surface. The ability to penetrate stomata and wounds has been reported for most *Alternaria* spp. In this system, penetration seems to be accompanied by chemical degradation of cells (Allen et al., 1983). In contrast, active penetration through intact tissues have been rarely observed (Hatzipapas et al., 2002).

The fungus resides usually in the center of the lesion, which is surrounded by an uninvaded chlorotic halo, a symptom that is commonly observed in the infection process of necrotrophic fungi. Fungal metabolites, as toxins, are the determinants for the formation of this area (Tewari, 1983 and Agarwal et al., 1997). Members of *Alternaria* usually cause quiescent infections in which the fungus enters the tissue where it remains dormant until changed conditions favor the infection. Typically, weakened tissues, either due to stress, senescence, or wounding, are more susceptible to *Alternaria* infection than healthy ones. *Alternaria* spp. usually do not affect water and nutrient transport throughout the plant, because they do not specifically target roots and vessels (Rotem, 1994).

Alternaria spp. can survive as mycelium or spores on decaying plant debris for long period of time, or as a latent infection in seeds (Rotem, 1994). If seed-borne, the fungus can

develop symptoms on seedlings once the seed has germinated. In other cases, once spores are produced they are spread mainly by wind onto the surface where infection can occur.

Despite the taxonomic and pathogenic differences, the infection pathways follow similar steps among the species (Fig. 3). Dormant spores, as previously reported, have shown strongly melanized cell walls and, under favorable conditions, produce one or more germ tubes. Subsequently, the germ tubes penetrate stomata, cuticle, and wounds with or without formation of appressoria. In less virulent species wounds and stomata are targeted, while more virulent species can also penetrate directly (Rotem, 1994). Enzymatic processes in *Alternaria* infections are essentially similar to those in other diseases. The cuticle, which consists of a combination of cutin and waxes, comprises the first line of defense to be overcome by directly penetrating fungal pathogens. For *A. brassicola*, the differential expression of cuticle degrading genes was monitored between saprophytic and pathogenic stages of the fungus (Yao and Koller, 1995). Furthermore, it was found that the different cutynolytic enzymes are sequentially induced upon landing on, and penetration of, the cabbage leaf (Fan and Koller, 1998).

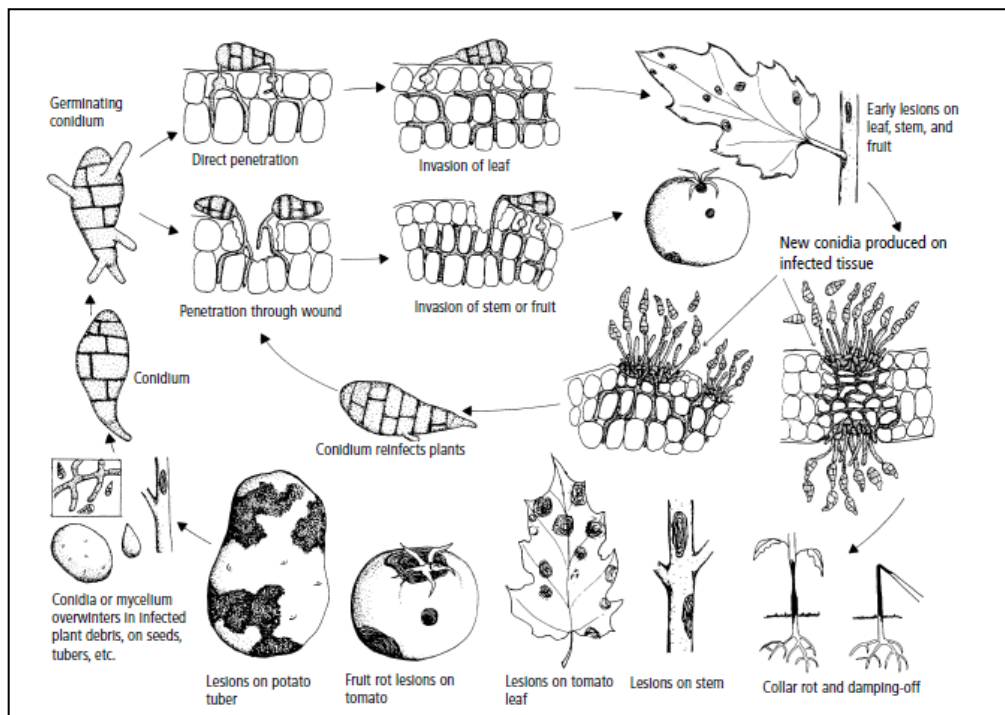


Fig.3. Development and symptoms of disease caused by *Alternaria*. (source Agrios, G.N. 1997 Plant pathology, 4th edition. San Diego: Accademic Press).

Cutinases, constitutively produced, are expressed during the initial contact of the fungus with the cuticle. These cutinases are inducible by cutin monomers, implying a switch between the parasitic and saprophytic stage. In addition to cutinases, lipases might also contribute to establishment of infection (Berto et al., 1997).

About one third of the total cell components in dicotyledonous plants are pectic polysaccharides. These compounds can be easily hydrolyzed by galacturonidases of the fungal enzymatic set. *A. citri* was found to be dependent on the endopolygalacturonase activity to begin the infection process. However, the tangerine pathotype of *A. alternata* did not show this dependency, possibly because this particular pathogen largely depends on toxin production for colonization of its host (Isshiki et al., 2001).

For a specific *A. alternata* endoglucanase, it was demonstrated that its production is triggered by a pathogen-induced pH increase on the host (Eshel et al., 2002b). Correlation-studies between enzymes production and symptoms development suggest that endoglucanases are involved in *A. alternata* pathogenicity (Eshel et al., 2000, 2002 a,b).

2.6 Toxin biosynthesis

Most phytotoxins employed by fungi are chemically diverse secondary metabolites: low molecular weight components that are not required for normal growth or reproduction. Often these toxins are produced as families of related compounds. Based on selectivity, phytotoxins can be divided into two categories: non-host-specific toxins and host-specific toxins. In general, non-host-specific toxins have relatively mild phytotoxic effects, affect a broad spectrum of plant species and are thought to be an additional factor of disease alongside, for instance, penetration mechanisms and enzymatic processes. Although they generally act as virulence factors and intensify disease symptom severity, they are not absolutely required for establishing disease since they are also toxic to plant species outside the host range of the pathogen (Thomma, 2003)

In *Alternaria*, many non-host-specific toxins have been identified, although the precise action of only a few has been studied in detail. Brefeldin A (dehydro-) curvularin, tenuazonic acid, tentoxin and zinniol are examples of toxins that are produced by several *Alternaria* species. The phytotoxic activity is applied through various ways. Brefeldin A causes disassembly of the Golgi complex and acts as an inhibitor of secretion, while curvularin is an inhibitor of cell division through its disturbance of the microtubule assembly, tenuazonic acid inhibits protein synthesis and zinniol affects membrane permeabilization (Meronuck et al., 1972, Robeson and Strobel, 1981, Thuleau et al., 1988 and Fujiwara et al., 1988). Tentoxin is produced by *A. alternata* and acts as a photophosphorylation inhibitor through specific binding to chloroplast ATP synthase, causing the inhibition of ATP hydrolysis and ATP synthesis (Steele et al., 1978). Because these toxins often target basic cellular processes, they are often powerful mycotoxin.

Host-specific toxins are involved in the development of a few, destructive diseases. They generally display severe effects on a rather narrow species-range that serves as host to the fungus and they are indispensable for disease. The mechanisms of host-selective

pathogenesis through the participation of HSTs is well understood (Yoder, 1980; Nishimura, 1980, Nishimura et al., 1982, Nishimura and Khomoto, 1983 and Scheffer and Livingston, 1984) and about 20 HSTs have been documented (Walton, 1996), of which at least seven are from *A. alternata* pathotypes (Otani et al., 1995, Markham and Hille, 2001 and Wolpert et al., 2002) (Fig. 4). It was proposed that these variants should be considered pathotypes of *A. alternata* (Nishimura and Kohmoto, 1983), a hypothesis that is supported by molecular analysis (Kusaba and Tsuge, 1994, 1995, 1997). The toxins that are produced by these pathotypes are chemically diverse, ranging from low molecular weight secondary metabolites to peptides. Akamatsu and his collaborators (1997, 1999), studying pathogenic (host-specific toxin-producing) and non-pathogenic (non-host-specific toxin-producing) *A. alternata* species, demonstrated that the pathogenicity was strictly related to the presence of small extra chromosomes. In fungal species, extra chromosomes, so-called supernumerary chromosomes, can be found in certain subsets of individuals. These are not essential therefore they are not required for normal growth, but they can carry extra traits. If supernumerary chromosomes confer an adaptive advantage to the individual in some habitats, they are referred to as conditionally dispensable chromosomes (Hatta et al., 2002).

This hypothesis is supported by the observation that homologues of toxin biosynthesis genes have not been found in strains from other pathotypes or non-pathogenic *A. alternata* isolates, making it unlikely that mutations or internal genetic rearrangements account for the toxin-producing ability (Tanaka et al., 1999, Masunaka et al., 2000 and Hatta et al., 2002).

There is no advantage for the clustering of genes during gene transmission from one generation to the next (vertical gene transfer), because the entire genome is transferred as a unit. However, during horizontal gene transfer, a relatively small contiguous DNA sequence is generally transferred. Since the total set of biosynthesis genes should be transmitted from one species to the other in order to provide the recipient with a complete biosynthesis

pathway, this chance is higher if genes are clustered. An originally saprophytic fungus like *Alternaria* could have acquired pathogenic capacity in this way (Walton, 2000), an hypothesis which is supported by the finding that single pathotype populations do not form monophyletic groups (Kusaba and Tsuge, 1994, 1995, 1997). The occurrence of horizontal gene transfer is well accepted for prokaryotes and even for fungal mitochondrial genes, but experimental evidence for the horizontal gene transfer of fungal nuclear genes is scarce. However, the difference between patterns of repeated DNA sequences on certain supernumerary chromosomes and the rest of the chromosomes in the same genome suggest that both types have a different evolutionary history (Covert, 1998). Furthermore, it was demonstrated for the fungus *Colletotrichum gloeosporioides* that a small chromosome could be transferred between two genetically isolated strains, thus demonstrating that horizontal gene transfer can occur (He et al., 1998).

A. alternata apple pathotype (previously described) is the causal agent of Alternaria blotch of apple on a strict number of susceptible apple cultivars through the production of a host-specific AM-toxin (Kohomoto et al., 1974). Moreover, studies have recognized that these *Alternaria* pathogens are pathogenic variants within a single variable species, *A. alternata* (Kusaba and Tsuge, 1994; Kusaba and Tsuge, 1995). The production of the cyclic AM-toxin peptide by the apple pathogen is determined by such a chromosome (Johnson et al., 2000a and Johnson et al., 2001). The AK- and AF-toxin biosynthesis genes and their homologues, identified in the Japanese pear and strawberry pathotypes of *A. alternata*, respectively, generally clustered together on a small chromosome (Tanaka et al., 1999, Tanaka and Tsuge, 2000 and Hatta et al., 2002). Physical clustering is a phenomenon that is commonly found for genes involved in the production of secondary metabolites in fungi (Keller and Hohn, 1997). AK- and AF-toxin are related to ACT-toxin, produced by the tangerine pathotype of *A. alternata*, and carry an epoxydecaatrienoicester backbone (Fig. 4). Interestingly, the AF- and ACT producing strains were also found to be pathogenic on pear

lines that are sensitive to AK-toxin, but not vice versa (Maekawa et al., 1984 and Kohmoto et al., 1993). Four genes involved in the biosynthesis of AK-toxin have been cloned from the Japanese pear pathotype genome of *A. alternata*, and homologues of three of these genes have also been identified in the strawberry and tangerine pathotypes. This suggests that these homologues are involved in the production of common moieties between these toxins (Tanaka et al., 1999, Masunaka et al., 2000; Tanaka and Tsuge, 2000 and Hatta et al., 2002). Together with the existence of such homologous toxin biosynthesis genes between pathotypes, the physical clustering of toxin genes on a single chromosome suggests that these genes are acquired through horizontal gene transfer (Tanaka et al., 1999; Walton, 2000 and Akagi et al., 2009).

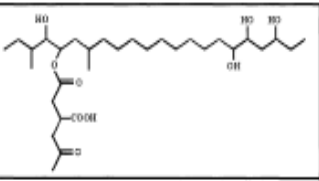
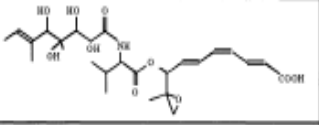
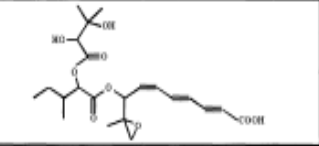
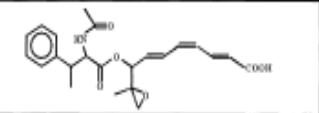
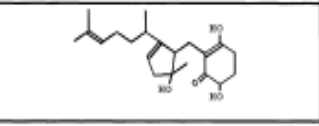
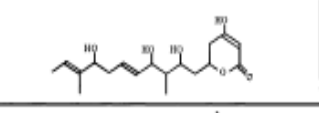
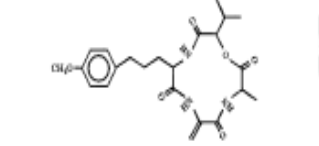
<i>Alternaria alternata</i> f.sp. <i>lycopersici</i> (tomato pathotype)	AAI-toxin (Bottini and Gilchrist, 1981)	
<i>Alternaria alternata</i> f.sp. <i>citri tangerine</i> (tangerine pathotype)	ACT-toxin (Kohmoto et al., 1993)	
<i>Alternaria alternata</i> f.sp. <i>fragariae</i> (strawberry pathotype)	AF-toxin (Nakatsuka et al., 1986)	
<i>Alternaria alternata</i> f.sp. <i>kikuchiana</i> (Japanese pear pathotype)	AK-toxin (Nakashima et al., 1985)	
<i>Alternaria alternata</i> f.sp. <i>citri tangerine</i> (tangerine pathotype)	ACTG-toxin (Kohmoto et al., 1993)	
<i>Alternaria alternata</i> f.sp. <i>citri jambhiri</i> (rough lemon pathotype)	ACR-toxin (Gardner et al., 1985)	
<i>Alternaria alternata</i> f.sp. <i>mali</i> (apple pathotype)	AM-toxin (Okuno et al., 1974)	

Fig. 4. Some chemical structures of *Alternaria* HS-Toxin. Most of these toxins are produced as families of related compounds, of which only the major compound is shown.

Mode of action of host-specific toxins (HST)

The precise mode of action of the most part of HST remain unknown. Interestingly, the site of action of different *Alternaria* toxins varies but they all trigger host cell death. AF- (*A. alternata*, with the strawberry pathotype), ACT- and ACTG- (*Alternaria alternata* tangerine pathotype) toxins act at the plasma membrane and cause permeabilization (Otani et al., 1995). AM-toxin (*Alternaria alternata mali* pathotype) not only affects the plasma membrane (Park et al., 1977), but also acts on chloroplasts, while ACR (*Alternaria alternata* rough lemon pathotype) and AT-toxins (*A. longipes*) were found to affect mitochondria (Otani et al., 1995). ACR-toxin induces swelling and other morphological modifications of mitochondria, and increases NADH oxidation, which is followed by plasma membrane disorders leading to electrolyte leakage and necrosis (Akimitsu et al., 1989). AAL-toxin mechanism of action is the one better understood. AAL-toxin is an aminopentol ester, an analogue of the sphingosine precursor sphinganine, which is produced by the tomato pathogen *A. alternata* f.sp. *lycopersici*. *A. alternata* mutants affected in AAL-toxin production also lose their pathogenicity, demonstrating the requirement of this toxin for pathogenicity (Akamatsu et al., 1997). The application of AAL-toxin leads to an accumulation of sphingoid base precursors, a depletion of complex sphingolipids (ceramide) and subsequently to the cell death of sensitive tomato species (Abbas et al., 1994; Wang et al., 1996a and Wang et al., 1996b). Moreover, host specific toxins are known to suppress the induction of host resistance (Hayami et al., 1982 and Walton, 1996) and in certain cases susceptible cells can be protected from the effects of HSTs with inhibitors to protein and RNA synthesis (Walton and Panaccione, 1993), suggesting that the active transcription and translation by plant cells are required for toxicity.

AM-toxin is a four member depsipeptide containing four catalytic domains responsible for the activation of each residue of AM-toxin and cause *Alternaria* blotch

(Okuno et al., 1974 and Ueno et al., 1977). Alternariolide (AM-toxin I) is the compound most abundant and cytotoxic of the three produced (Johnson et al., 2000a).

For ACR-toxin, specificity seems to be determined by differential post-transcriptional processing of a mitochondrial gene. This gene is present in the mitochondrial DNA of toxin-sensitive as well as resistant species, but the transcript of the gene is shorter in resistant than in sensitive mitochondria. In the end, an oligomeric protein is produced in toxin-sensitive mitochondria whereas the transcript is not translated in resistant mitochondria. Although the gene is likely to encode a mitochondrial membrane protein, no function has been assigned yet (Yamagishi et al., 2006). HSTs can be considered molecular determinants of pathogenesis (Kohomoto and Otani, 1991) and the study of their biosynthesis can improve the knowledge of the molecular basis of pathogenicity as well as provide new elements to how new pathogenic race of fungi evolves.

2.7 *Alternaria* diseases

Diseases caused by *Alternaria* species are very common and worldwide in their occurrence. Important host plants include a variety of crops such as apple (Bulajic et al., 1996 and Sawamura, 1990), pear (Sawamura, 1990), pistachio (Pryor and Michailides, 2002), hazelnut, walnut (Hong et al., 2006) broccoli (Huang and Levy, 1994), cauliflower (Schimmer, 1953), potato (Moore and Thomas, 1942, Moore, 1943, B.P. Singh et al., 1987 and Waals Vander et al., 2004), tomato (Grogan et al., 1975, Malathrakis, 1983, Choi et al., 1989 and Kishore et al., 2007), carrots (Strider, 1963) cabbage (Humpherson-Jones, 1989 and Bansal et al., 1990).

Alternaria attacks the aerial parts of its hosts, symptoms of *Alternaria* infections usually start as a small circular, dark spot. As the disease progresses the necrosis may increase to 1 cm or more in diameter, be characterized by a gray to black color and can also be surrounded by a red halo. Due to variability of the environmental conditions, the

pathogens have a no constant growth rate, thus spots develop in a target pattern of concentric rings. Where host leaves are large enough to allow unrestricted symptom development, the necrotic lesions are diagnostic as there are few pathogens that cause this type of symptoms. Dark, sunken lesions are usually expression of *Alternaria* infections on roots, tubers, stems and fruit.

Alternaria Leaf blotch

Certain species of *Alternaria* are currently enclosed in the European and Mediterranean Plant Protection Organization list. EPPO is responsible for European cooperation in plant health. Its main aims are to protect plants, to avoid the introduction of dangerous pathogens applying international agreements and to promote safe and effective control methods. Therefore the Organization draws and updates the list of the organisms considered threatening under the phytosanitary point of view.

Alternaria alternata mali pathotype is reported among the quarantine pests and has not been recorded in European countries since to 2006, while it has been a serious problem in Japan since 1970 (Sawamura, 1972) in the United States since the late 1980s (Filajdic and Sutton, 1991). Although leaf blotch severity may vary from year to year, there are strong indications that it has spread to new areas in North Carolina (Sawamura, 1990), Virginia, and West Virginia, and could become a problem in more northern areas of the mid-Atlantic region (Farr et al., 1989). Disease severity is aggravated by severe mite infestation. Maintaining good mite management is an important factor in preventing severe disease development. The situation is more complex in Australia where, in addition to *A. mali*, a number of other species of *Alternaria* seem to cause leaf blotch and fruit spot symptoms. In Europe the disease was reported in Yugoslavia in 1996 (Bulacijc et al., 1996) in Turkey in 2006 (Ozgonen and Karaca, 2006). In 2007 the disease was recorded also in the eastern part of Iran (Soleimani and Eesmailzadeh, 2007).

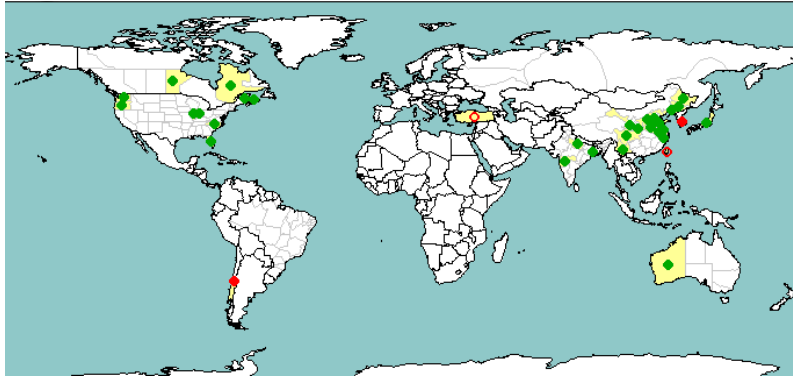


Fig 5. Distribution of *A. alternata mali* pathovar according to the EPPO (data 2006). ● Present (national record), ○ Present only in some areas (national record), ● Present (subnational record).

The fungus can overwinter as mycelium on dead leaves on the orchard floor, in mechanical injuries in twigs, or in dormant buds. Primary infection takes place about one month after petal fall. The disease advances rapidly in the optimum temperature range (25-30 °C) and wet weather. At optimum temperatures, infection occurs with 5.5 hours of wetting, and lesions can appear in the orchard two days after infection, causing a serious outbreak. The fungus produces a chemical toxin which increases the severity of the disease on susceptible cultivars (i.e. Indo and Golden delicious).

Symptoms first appear on leaves in late spring or early summer as small, round, purplish or blackish spots, gradually enlarging (1.5-5 mm) in diameter, with a brownish purple border (Fig. 6). Lesions may coalesce or undergo secondary enlargement and become irregular and much darker, acquiring a "frog-eye" appearance. When lesions occur on petioles, the leaves turn yellow and defoliation may occur. Severe defoliation leads to premature fruit drop. Frogeye leaf spot usually appears earlier in the season and is associated with nearby dead wood or fruit mummies



Fig. 6. Alternaria leaf blotch symptoms on apple fruit and leaf (Golden Delicious cultivar).

Italian situation

In 1999, a symptomatic disease was reported that was similar to apple Blotch caused by *A. mali* among a number of apple trees orchards (Golden cultivars) in the North Italy (Trentino Alto Adige region) (Marshall et al., 2004, and Marshall and Bertagnoll., 2006). By 2002 the disease was found in an area 10 times larger than the initial infection sites and had started attacking the Gala apple variety, broadening to the Veneto, the Piedmont (Cuneo and Alessandria) (Ortalda, 2006) regions and some areas of Emilia Romagna (Ferrara and Ravenna with spots present only on leaf) (Antonacci and Montuschi, 2006). The incidence of the disease was more severe in the upper part of the apple tree and between rows. It has been reported that serious damages have occurred in most rainy years.

Originally, it was confused with a lack of microelements or a nutritional disequilibrium due to an excess of zinc. Usually, symptoms (Fig.7) appear at the end of May on the leaves as small circular brown spots of 2–5 mm diameter, increasing in number and coalescing during the following months. On fruits, spots (1-3 mm) come out, often centered on the lenticels and surrounded by a reddish halo; commonly the spots don't develop extended rot and damages are particularly visible during the harvest time and the post-harvest storage.

In 2003, the Experimental Centre of Laimburg (BZ) demonstrated the pathogenicity of *Alternaria* isolates on healthy foliar tissues in accordance to Koch's postulates. They proposed a pathotype of *Alternaria alternata* as the causal agent of the symptoms described above (Gobber et al., 2004 and Marshall and Bertagnoll, 2006) and found that the suspected isolates do not differ in life cycle and growth habits to those previously described.



Fig.7. Symptoms on fruit and leaf caused by *Alternaria* spp. in Golden apple orchard in the Northern Italy (Trentino Alto Adige).

2.8 The genus *Alternaria* – Molecular characterization overview

The morphological groups, defined on the basis of spore characteristics and sporulation patterns, have been supported by a large number of works. For example, molecular phylogenies have discriminated between the *A. alternata* and the *A. porri* species groups, morphologically distinguished by large (more than 100 μm long) and small (ranged 20-50 μm) conidia size respectively. To date, phylogenetic studies have clearly demonstrated a distinction between large and small-spored *Alternaria* species (Pryor and Bigelow, 2003, Hong et al., 2005 and Peever et al., 2004). However it is among the small spored taxa of *Alternaria* spp. that there is still to debate.

Molecular techniques are a powerful discriminating tool, especially where differentiating these fungi can be difficult for those not familiar with specific morphological

characteristics that separate this species (Pryor and Michailides, 2002). The advancement of these techniques has allowed the examination of taxonomic relationships among small spored catenulate *Alternaria* spp.

DNA-DNA reassociation kinetics pointed out a high similarity among several host specific *Alternaria* spp., which suggests these species are intraspecific taxa (Kuninaga and Yokusawa, 1987). Restriction fragments length polymorphism (RFLP), utilizing the λ phage clone Alt 1 as a hybridization probe, has been used to discriminate among small spored *Alternaria* isolates, segregating the fungi recovered from pistachio into three distinct groups (Ardhya et al., 2001) of *Alternaria alternata* strains. Sequence analysis of nuclear rDNA internal transcribed spacer (ITS) region has revealed a variation among small spored *Alternaria* spp. but failed to resolve these taxa as phylogenetically different from *Alternaria alternata* (Kusaba and Tsuge, 1994 and Kusaba and Tsuge, 1995). RAPD (random amplified DNA) fingerprinting has been largely applied in this taxonomic work. These kinds of markers have highlighted different fragment patterns among the small-spored groups such as *A. alternata*, *A. infectoria*, and *A. tenuissima*, suggesting that these taxa constitute well-defined species (Roberts et al., 2000). Random Amplified Polymorphic DNA (RAPD) profiles and sequencing of ITS and IGS (intragenic spacer) regions were successfully exploited also in the molecular characterization of *Alternaria* isolates associated with *Alternaria* late blight of pistachio (Pryor and Michailides, 2002). The isolates of the *Alternaria infectoria* species group resolved phylogenetically distinct groups separate from isolates in the *Alternaria alternata*, *Alternaria arborescens* and *Alternaria tenuissima* groups. Moreover, while the *A. arborescens* group formed a separate clade, the *A. tenuissima* and *A. alternata* group did not resolve into different clades due to the low rate of variability in the ITS sequences.

Kang and affiliates (2003) employed the universal rice primers (URPs) to detect PCR polymorphisms in 25 isolates of six different *Alternaria* species producing host specific

toxins (HST). URP markers can be used to reveal PCR polymorphisms of *Alternaria* isolates at the intra- and inter-species levels. Peever and associated have focused on the phylogenetic work concerning the *Alternaria* species affecting rough lemon and tangerine. Using variable regions of the genome, such as mitochondrial large subunit (mtLSU), β -tubulin, EndoPG (endopolygalacturonase gene) and two anonymous regions (OPA1-3 and OPA2-1), it was possible to distinguish among the isolates investigated as well supported monophyletic lineages (Peever et al., 2004). Another interesting study was performed by Hong et al. (2006) that created a polyphasic classification, using standardized procedures, to evaluate genetic relationships among *Alternaria* taxa recovered from brown apical necrosis of English walnut and grey necrosis of hazelnut. Amplified Fragment Length Polymorphism (AFLP) markers, inter simple sequence repeat (ISSR) markers, and histone gene sequence data were compared. The analysis of the AFLP profiles allowed separation of the *A. arborescens* group from the *A. alternata* and *A. tenuissima* groups, which resolved as a single lineage. ISSR data supported the groupings by AFLP data except for the isolates of the *A. alternata* group, which clustered with the *A. arborescens* group. The H4 gene sequencing also supported the discrimination of the *A. arborescens* group from the *A. alternata* and *A. tenuissima* groups.

Troubles can occur working with protein-coding genes, most commonly employed in fungal systematic, due to the lack of variation among these taxa. As a result, non-coding, anonymous regions of the genome were developed to assess evolutionary relationships among these organisms. Sequence-characterized amplified regions (SCAR) were screened for phylogenetic utility by comparing sequences among reference isolates of small-spored *Alternaria* species (Peever et al., 2004). In the latter work of Andrew et al. (2009), phylogenetic analyses were performed with 150 small-spored *Alternaria* isolates collected from citrus in Florida, pistachio in California, desert plants in Arizona, walnuts in France and Italy and apples in South Africa. No associations were found between host or

geographic associations and phylogenetic lineage, indicating that these characters are not useful for cladistic classification of small-spored *Alternaria*. In addition, the *A. alternata* and *A. tenuissima* isolates did not separate in distinct clades, and no strict congruence was found between the morphological and phylogenetic characters. On the contrary, the *A. arborescens* group was found belong to a discrete clade in all of the datasets. Should *A. arborescens* be considered a phylogenetic species or an emerging evolutionary lineage within the phylogenetically defined *alternata* species-group?

2.9. Molecular markers in fungal species investigation

In several areas of research, the precise and unequivocal identification, discrimination and characterization of fungal species, populations, isolates and pathotypes are of prime importance. However, this is difficult task if the characterization is led solely with morphological and biochemical criteria. Molecular markers have become part of a repertoire of tools needed to assess the amount of genetic variation in populations. An outstanding advantage of the molecular approach is the immense amount of potential data they provide (Avisé, 1994). Furthermore, rates of evolution of different parts of the genome are extremely variable, allowing molecular data to be applicable at any taxonomic level. Both dominantly (e.g. AFLP, RAPD, and ISSR) and codominantly inherited markers (e.g. allozymes and microsatellites) have been used to study population genetics and life history traits in many species. Among these, polymerase chain reaction (PCR)-derived markers obtained with non-species specific primers have become exceedingly popular since they do not request sequence information for the target species.

The first and so far most commonly used method in this group is RAPD (Random Amplified Polymorphic DNA), which was introduced in 1990 (Weir et al., 1998, Roberts et al., 2000, Pryor and Gilbertson, 2000 and Pryor and Michailides, 2002). A few years later, the relatively similar ISSR (Intersimple Sequence Repeats) (Hong et al., 2006 and Park et al.,

2008) and the somewhat more technically demanding AFLP (Amplified Fragment Length Polymorphism) (Vos et al., 1995) were introduced. In spite of the obvious advantages of these methods related to the efficient and quick PCR amplification of polymorphic DNA fragments starting from small amounts of template, however, they share some limitations in the interpretation of the multi-band profiles produced: heterozygotes cannot be detected because of their dominant nature; homology of comigrating bands cannot be assigned certainly; from a technical point of view, competitive priming (Halldén et al., 1996), and the occurrence of artefactual bands produced by nested primer annealing or interactions within and between DNA strands during PCR (Rabouam et al., 1999) still remain potential problems. The difficulty of achieving robust profiles, particularly in RAPDs, may make the reliability of these markers somehow questionable, but the reproducibility of RAPD analysis can be enhanced through improved laboratory techniques and band scoring procedures (Hansen et al., 1998) while AFLP and ISSR are less affected by the problem of reliability than RAPD (Zietkiewicz et al., 1994 and Vos et al., 1995) because longer primers and higher annealing temperatures are employed. In data compilations, estimates of genetic variation obtained with different types of dominant markers (AFLP, RAPD, ISSR) proved to be quite similar in magnitude, both for within and among populations (Meng and Chen, 2001).

Amplified Fragment Length Polymorphism (AFLP)

Amplified Fragment Length Polymorphism, polymerase chain reaction (AFLP-PCR), is a relatively fast, cheap, easy, and reliable method to generate hundreds of informative genetic markers (Vos et al., 1995 e 1997). The main disadvantage of AFLP-PCR is the difficulty in identifying homologous markers (alleles), rendering this method less useful for studies that require precise assignment of allelic states, such as heterozygosity analyses. However, because of the rapidity and ease with which reliable, high-resolution markers can be generated, AFLPs are emerging as a powerful addition to the molecular toolkit for genetics and evolutionary studies.

The main advantage of AFLP technique is its capacity of analyzing simultaneously many DNA regions distributed randomly throughout the genome. To achieve high reliability of the screen, genomic DNA is prepared in an ingenious, but technically straightforward, way that combines the strengths of two methods, the replicability of restriction fragment analysis and the power of the PCR (Vos et al., 1995, 1997).

AFLP markers can be generated from DNAs of any organism, and no initial investment in sequence analysis is required. Small amounts of DNA (~500 ng) are digested with a combination of one rare cutter and frequent cutter enzymes. Then, the adaptors, designed on the base of restriction site blunt ends, are ligated so do not reconstitute the restriction site. This step is fundamental because it creates a multitude of fragments with known sequences to the ends.

To reduce the amount of fragments generated and consequently obtain a subset of these fragments, primers are extended into the unknown part, usually adding one to three arbitrarily chosen bases beyond the restriction site. Two subsequent PCRs are performed (preamplification and selective PCR) amplification: the first is performed with no extension or a single-bp extension, followed by a more selective primer with up to a 3-bp extension. The primer extension of one, two or three bases reduces the number of amplified fragments by factors of 4, 16 and 64, respectively.

Generally, the profiles are separated using polyacrylamide gel electrophoresis.

AFLP markers have proved useful for assessing genetic differences among individuals, populations and independently evolving lineages, such as species. For a wide range of taxa, including fungi, AFLP markers have been used to uncover cryptic genetic variation of strains, or closely related species, that had been impossible to resolve with morphological or other molecular systematic characters (Huys et al., 1996 and Janssen et al., 1997). For example, AFLP methods were shown to be superior to classic systematic methods allowing finer differentiation of microorganisms (Kothera et al., 2003 and Bayon et

al., 2006). For closely related species, AFLP markers have also been used to infer phylogenetic relationships based on measures of genetic distance (Hong et al., 2006 and Gannibal et al., 2007). On the other hand, phylogenetic inferences based on similarities of AFLP profiles become problematic, because of the high variability of AFLP markers reduces similarities between distant taxa to the level of chance. For these reasons, AFLP markers have found the widest application in analyses of genetic variation below the species level, particularly in investigations of population structure and differentiation.

Indeed, AFLP markers have been applied to evaluate gene flow and dispersal, outcrossing, introgression and cases of hybridization. The high resolution of AFLP markers also enables testing for clonal identity between individuals (i.e. absence of recombination), and thus permits inferences about sexual versus asexual modes of reproduction (Majer et al., 1996, Rosendahl and Taylor, 1997 and Majer et al., 1998). AFLP markers have the potential to resolve genetic differences at the level of DNA fingerprints for individual identification and parentage analysis. In the ideal case, a few primer combinations is sufficient to generate an adequate number of polymorphic markers.

However, in principle, any individual can be profiled with unique combinations of AFLP markers, because a large number of AFLP markers presumably include at least some that are hypervariable (Vos et al., 1995).

Inter-Simple Sequence Repeats (ISSR)

One of the variants following the utilization of microsatellites as molecular marker is based on using single primers complementary to part of SSR sequences in the PCR amplification. The successful application of microsatellite-specific oligonucleotides as PCR primers was first described by Meyer et al. (1993), who amplified DNA from different strains of the human fungal pathogen *Cryptococcus neoformans* with the primers (CA)₈, (CT)₈, (CAC)₅, (GTG)₅, (GACA)₄, and (GATA)₄. The technique was subsequently applied to numerous fungi genetics studies: characterization of species (Tymon and Pell,

2005 and Chadha and Gopalakrishna, 2007), assessment of genetic diversity (Meng and Chen, 2001) and molecular systematics (Zhou et al., 2001 and Hong et al., 2006).

Several acronyms were proposed, including single primer amplification reactions (SPAR), inter-simple sequence repeat PCR (ISSR-PCR), and microsatellite-primed PCR (MP-PCR), all referring to semiarbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite. Gupta et al. (1994) used 23 primers complementary to di-, tri-, tetra-, and pentanucleotide repeats to amplify genomic DNA across a panel of eukaryotes. They found that tetranucleotide repeat primers were most efficient in amplifying polymorphic patterns. GC- as well AT-rich primers worked equally well. Primers representing a combination of two tetranucleotide repeats, or compound microsatellites, were also effective. Single base permutations produced different PCR fingerprints.

ISSRs sample a large portion of the genome, because microsatellites are abundant throughout the genome and evolve rapidly; consequently, ISSRs may reveal a high number of polymorphic fragments per primer. Meyer et al. (1993) stressed that these markers combined some advantages of RAPD analysis (i.e., no need for sequence information) and microsatellite analysis (i.e., use of high-stringency annealing conditions, leading to more reproducible banding patterns). According to Wang et al. (2005) the repeatability of ISSR is better than RAPDs because ISSR primers are longer (15 to 20 bp) and hence have higher annealing temperature. Sometimes complex fingerprintings are produced by ISSRs due to incidental annealing of primers within SSRs during PCR amplification. Initial priming in fact may occur in different registers within the microsatellite target region or the average product size may be continuously reduced by internal priming in successive cycles so that the final product is expected to be primed from the extreme 3'-end of each flanking microsatellite.

The more sophisticated anchored ISSR variant developed by Zietkiewicz et al. (1994), also coined anchored microsatellite-primed PCR (AMP-PCR) uses 5'- or 3'-anchored di- or trinucleotide repeats as single PCR primers. The anchor is composed of nonrepeat bases and ensures that the amplification is initiated at the same nucleotide position in each cycle. AMP-PCR has several advantages over unanchored variants of microsatellite-primed PCR. First, primer design ensures annealing of the primer only to the ends of a microsatellite, thus circumventing internal priming and smear formation. Second, the anchor allows only a subset of the targeted inter-repeat regions to be amplified, reducing the overwhelming number of PCR products sometimes produced from interrepeat regions, and to sets of easily resolvable bands. Third, functional 5'-anchors ensure that the targeted microsatellite is part of the product.

Since the primer is a SSR motif the frequency and distribution of the microsatellite repeat motifs influence the generation of bands. In general, primers with (AG), (GA), (CT), (TC), (AC), (CA) repeats show higher polymorphism than primers with other di-, tri- or tetra-nucleotide repeats. (AT) repeats are the most abundant di-nucleotides in plants but the primers based on (AT) would self- anneal and not amplify. Tri and tetra-nucleotides are less frequent and their use in ISSRs is lesser than the di-nucleotides. Usually di-nucleotide repeats, anchored either at 3' or 5' end reveal high polymorphism. The evolutionary rate of change within microsatellites is considerably higher than most other types of DNA, so the likelihood of polymorphism in these sequences is greater. The source of variability in the ISSRs can be attributed to any one of the following reasons or any combination of these. The extent of polymorphism also varies with the nature (3'-anchored, or 5'-anchored) of the primer employed. The primers anchored at 3' end give clearer banding pattern as compared to those anchored at 5' end (Tymon and Pell, 2005 and Chadha et Gopalakrishna, 2007). When 5' anchored primers are used, the amplified products include the microsatellite sequences and therefore variability in number of nucleotides within the sequence would

result in length polymorphisms. ISSR markers are theoretically inherited in a dominant or codominant Mendelian fashion (Gupta et al., 1994), however, they are interpreted as dominant markers similar to RAPD data (Stenglein and Balatti, 2006). Polymorphism may relate to mutations at the priming site that prevent amplification giving a presence/absence pattern while insertion/deletion events within the SSR region or the amplified region would result in the absence of a product or, more rarely, in length polymorphism, depending on the amplifiability of the resulting fragment size.

2.10. Molecular phylogenetics

Cluster analysis and parsimony analysis

The purpose of any phylogenetic analysis is to estimate the evolutionary relationships between a set of homologous taxa, which can be anything from morphological characteristics to molecular sequences (Mount, 2001). The result is a tree composed of “nodes” and “branches”, where the terminal nodes (or “leaves”) correspond to the taxa being studied, the internal nodes represent ancestral sequences, and the branches represent the topological relationship between the nodes (Saitu, 1996).

A great and ever increasing number of methods have been described for inferring phylogeny, they can all be thought as members of two broad classifications: methods that use an algorithm to directly build a tree through a series of defined steps; and methods that define a criterion to be maximized (or minimized), and then use an algorithm to evaluate potential trees based on this criterion (Swofford et al., 1996).

The first class (Unweighted Pair Group Method with Arithmetic Mean, Neighbor Joining, Fitch-Margoliash) works largely by converting the similarity between pairs of samples into evolutionary distance, and then using a defined set of steps to build a tree. Precisely, the UPGMA methods assume a constant rate of evolution, and its original purpose was to construct taxonomic phenograms, which are trees that reflect the phenotypic

similarities between operational taxonomic units (OTUs). The method uses a sequential clustering algorithm, in which local homology between OTUs is identified in order of similarity, and the tree is built in a stepwise manner. The two OTUs that are most similar to each other are first determined and then these are treated as a new single 'composite' OTU. Subsequently from among the new group of OTUs (composite and simple), the pair with the highest similarity is identified and clustered. This continues until only two OTUs are left.

The second group of methods assumes that shared characters in different entities result from a common descent; groups are built on the basis of such shared characters and the simplest explanation for the evolution of characters is taken to be the correct or most parsimonious one. Moreover, multiple trees are generated when different groupings are equally parsimonious. In such cases, a strict consensus tree should be derived that includes only topologies that are not contradicted in any of the initial trees. If the strict consensus tree results phylogenetic uninformative a majority rule consensus tree can be built. This type of consensus tree shows nodes that are consistent in half to all of most parsimonious trees.

Maximum parsimony (or simply parsimony) is based on finding the simplest solution to an observed set of data (Swofford et al., 1996). Essentially, parsimony attempts to build a tree that minimizes the number of evolutionary changes required to explain the observed data. Therefore, the optimality criterion (which in this case is minimized) is total tree length. The length of the tree is defined as the number of character state transformations (mutations to the biologist) required to explain the existence of the nucleotides at all positions in a set of aligned sequences (Swofford et al., 1996). In simplified terms, parsimony algorithms evaluate a given tree by individually looking at each column of nucleotide characters in a set of aligned sequences, summing the length of the tree required to account for each of the characters in that column, and then summing the results for all columns. To evaluate the tree length required for each column, nucleotide characters are inferred for each internal node, and the cost of traveling along each branch is then evaluated (Swofford et al., 1996).

Principal Component Analysis

Principal Components Analysis (PCA) (Jolliffe,1986) is a way of identifying patterns in data, and expressing the data in such a way as to highlight their similarities and differences. Since patterns in data can be hard to find in data of high dimension, where the luxury of graphical representation is not available, PCA is a powerful tool for analyzing data. The other main advantage of PCA is that once you have found these patterns in the data, and the data can be compressed, by reducing the number of dimensions, without much loss of information.

PCA has several applications, the most important of them are:

- reduction of the data set to only three variables (the three most important components), for plotting and clustering purposes
- determination of a correlation between the three most important components and some other underlying variables.

3. Goals

The present work concerns a new disease caused by *Alternaria* spp. that causes severe yield losses in the most important apple growing regions of Italy. To better understand and define the casual agent of this disease, it was decided to evaluate the pathogenicity of the isolates obtained from symptomatic plant tissue and, subsequently, find correlations among the ability to cause disease and morphological traits and molecular characters.

The study has been developed according the following steps:

- pathogenicity bioassays on apple leaf and fruit,
- morphological characterization of a subset of 44 single spore Italian isolates,
- single locus analyses of the subset of 44 single spore Italian isolate,
- multilocus analysis of the subset of 44 single spore Italian isolate.

Further objectives were: 1) to assess the diversity among the *Alternaria* isolates and 2) to delineate the relationship of the unknown *Alternaria* spp. with the pathogen *Alternaria alternata mali* pathotype, causal agent of apple blotch disease.

4. Materials and methods

4.1 Sampling, strains isolation and identification of micromorphological characters

All sampled plant material was received from extension service personnel working with farmers in affected apple orchards. The strain collection was constructed by collecting material between June and August of 2007, 2008, and 2009. All the isolates were sampled from symptomatic tissues (leaves and fruits) collected from apple orchards in different geographical area in the North of Italy (Fig. 8): Verona (8 locations), Padova (2 locations), Trento (6 locations), Bolzano (4 locations), Udine (1 location), Pordenone (1 location) and Ravenna (1 location). The culture collection (Tab. 1a, 1b, 1c) consisted of a total of 174 isolates, 70 of which were collected from leaf lesions and 104 from fruit lesions. The varieties affected mainly belonged to Gala and Golden cultivars, which seem greatly susceptible to the attack of *Alternaria* spp.

Sections were excised from lesions on fruits and leaves using a sterile scalpel blade. Excised portions were then dipped in 1% hypochlorite for 30 seconds, washed with sterile water for 1 minute, and then placed on potato dextrose agar (PDA) (BBL). Agar was prepared according to manufacturer specifications. Plates were incubated at 23°C with a photoperiod 12 hrs light/dark. Durable microscopy slides were prepared from seven days old culture to identify the pathogen. Length, width, beak length, beak width and number of vertical and horizontal septa were recorded. Positive *Alternaria* spp. were transferred to PDA agar slants for storage at 4°C.

Tab. 1a. List of isolates collected during 2007. For each orchard sampled the table reports: the corresponding geographical location, apple cultivars and the number of isolates, identified by an assigned code, obtained from plant tissue (leaf or fruit).

Isolates from leaf	Isolates from fruit	Location	Cultivar
1	11A ¹ , 1A ²	Villafranca (VR)	Golden clone B
2B	2A, 2B, 2C, 2D, 2E	Villafranca (VR)	Gala Brookfield
-	3A, 3D	Belfiore (VR)	Golden Smoothee
-	4	Belfiore (VR)	Gala Brookfield
	5A, 5Test	Albaro Ronco (VR)	Golden Smoothee
6A, 6B, 6D	6A, 6B	Albaro Ronco (VR)	Early Red Gala
7	7A, 7B, 7D, 7E	Belfiore (VR)	Gala Galaxy Selecta
8A, 8B	8A, 8B, 8C, 8D	Belfiore (VR)	Gala Brookfield
9A, 9C	9A, 9C, 9D, 9F 9A ¹ , 9A ² , 9A ³	Belfiore (VR)	Golden Reinders
10A, 10B, 10C	10A	Belfiore (VR)	Golden Reinders
11A, 11B, 11E	11A, 11C, 11E	Albaro Ronco (VR)	Gala Obrogala
12	-	Randon (VR)	Golden clone B
14A, 14B	14	Alfonsine (RA)	Gala
-	14A ¹ , 14A ² , 14A ³ , 14A ⁴	Alfonsine (RA)	Fuji
18	18	Mane S.Rocco (TN)	Golden D.
19	19A, 19C, 19D	Rovereto (TN)	Golden D.
20A, 20B	20A	Bleggio (BZ)	Golden
21	21A, 21B, 21C	Mottarello (TN)	Golden
22A, 22B	22A, 22B, 22C, 22D	Pressano Lavis (TN)	Golden Smoothee
23	23A, 23B, 23C	Trento	Golden D.
24	24A ¹ , 24A ² , 24A ³	Trento	Golden D.
25A, 25B	25A ¹ , 25A ²	Trento	Golden

Tab. 1b. List of isolates collected during 2008. For each orchard sampled the table reports: the corresponding geographical location, apple cultivars and the number of isolates, identified by an assigned code, obtained from plant tissue (leaf or fruit).

Isolates from leaf	Isolates from fruit	Location	Cultivar
26A, 26B, 26C	26A, 26B, 26C, 26D	Belfiore (VR)	Golden
27A, 27B	27A, 27B, 27C	Belfiore (VR)	Gala
28A, 28B, 28C, 28D	28A, 28B, 28C, 28D	Ronco all'Adige (VR)	Golden
29A, 29B, 29C	29A, 29B, 29C, 29D	Vigasio (VR)	Golden
	4BZ	Merano (TN)	Golden
	9BZ	Nalles (TN)	Golden
	10BZ	Nalles (TN)	Golden
	11BZ	Valvenosta (BZ)	Golden
	A138	Vilpiano (BZ)	Golden
	A152	Vilpiano (BZ)	Golden
	B19	Vilpiano (BZ)	Golden
	B50	Terlano (BZ)	Golden
	F09	Bleggio (BZ)	Golden

Tab. 1c. List of isolates collected 2009. For each orchard sampled the table reports: the corresponding geographical location, apple cultivars and the number of isolates, identified by an assigned code, obtained from plant tissue (leaf or fruit).

Isolates from leaf	Isolates from fruit	Location	Cultivar
2B	2A, 2B, 2C, 2D, 2E	Belfiore (VR)	Gala Brookfield
6A, 6B,	6A, 6B	Albaro Ronco (VR)	Early Red Gala
8A, 8B, 8C	8A, 8B	Belfiore (VR)	Gala Brookfield
9A,9B, 9C	9A, 9B,	Belfiore (VR)	Golden Reinders
34A, 34B	34A, 34B	S.. Martino B.a (VR)	golden
35A, 35B	35A, 35B	S. Martino B.a (VR)	golden
36		Piacenza d' Adige (PD)	golden
	37 Test, 37 Biol, 37A, 37B 37C	Spilimbergo (PN)	golden
	37 Test I	Beano (UD)	golden
38A 38B	38A 38B	Palù (VR)	pink
39A	39 A 39B 39 C	Castelbaldo (PD)	pink

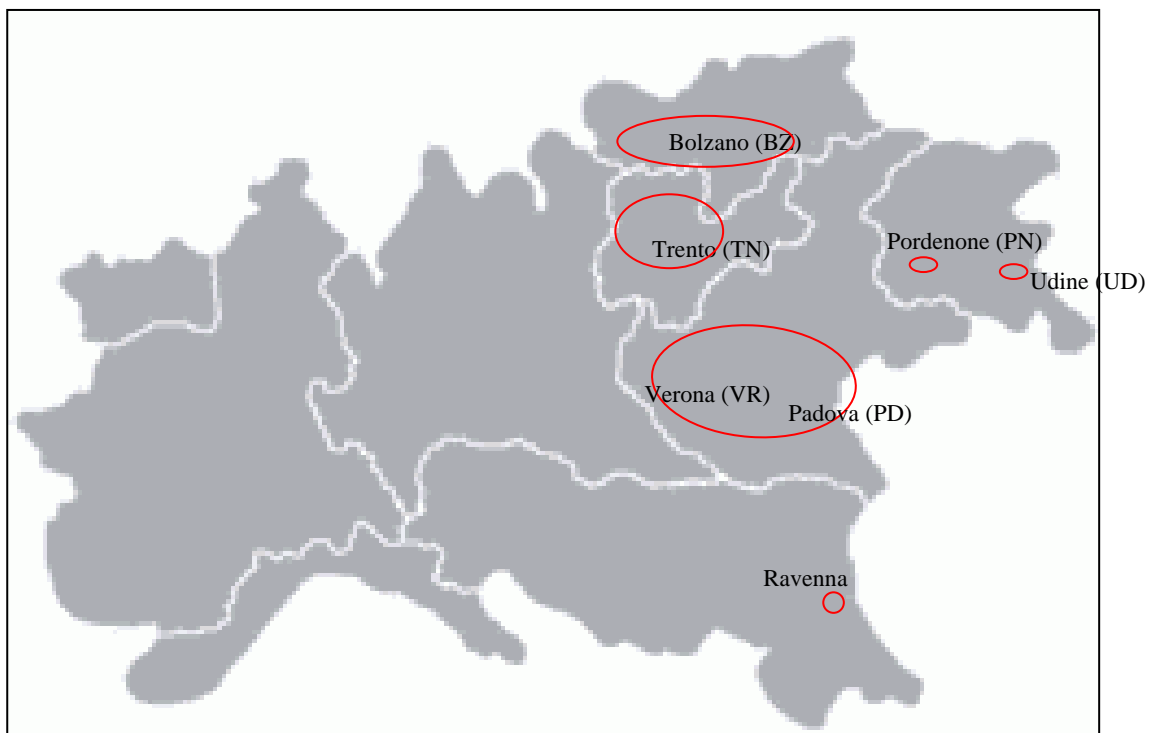


Fig. 8. Map of the geographical areas (ellipses) where symptomatic materials were collected.

4.2 Pathogenicity Bioassays

All the colonies isolated were tested for pathogenicity. Bioassays were performed on detached plant materials (fruit and leaf wounded and unwounded) belonging to the Golden cultivar. Young leaves were collected from both uniform genetic material grown in the greenhouse at the University of Bologna; fruits were from cold storage obtained from organic agriculture.

Tissues were wounded slightly by scratching the lower side of the leaf, or the upper part of the fruit, with a sterile blade. For each isolate six drops (15 μ l for leaves and 30 μ l for fruits) of conidial suspension (10^5 conidia/ml) were inoculated on the tissues (or wounds). The conidial suspension was obtained by flushing 7-days old colonies, grown on PDA as previously described, with sterile water. The spore concentration was adjusted using a hemocytometer. Plant material was placed in plastic trays, covered with plastic bag to maintain the humidity, and incubated in a growth chamber room at 23°C. Symptoms were monitored daily. The experiment was performed twice and independently utilizing three replicates for each isolate.

Isolates were tested for the capability to produce extracellular toxins. One ml of conidial suspension was inoculated in 30 ml Czapek-Dox broth (Difco) enriched with 0.5% Yeast Extract (Difco) and incubated statically for 21 days at 23°C. Then, culture broth was filtered with 0.2 μ m filter (Millipore), and the suspension was applied on wounded and unwounded plant material and incubated as already mentioned. An aliquot of the filtrate was also plated on PDA to check the absence of fungus. Symptoms were monitored daily for 72hrs. A value of pathogenicity score (P.S.) was assigned on the basis of the diameter of the necrotic area that developed (1=no lesions, 2= < 2 mm, 3= 2-4 mm, 4= > 4 mm). As in the previous pathogenicity test, also this experiment was conducted independently two times with three replicates for each isolate per experiment. In order to assess the reliability and the reproducibility of the “Czapek” bioassay, a ring test among three laboratories was carried

out, testing five shared strains on the same plant material. Bioassays on leaf tissues have shown the most reproducibility and informativeness, so it was chosen to carry out only these types of tests to investigate the pathogenic capability of the single spores *Alternaria* isolates. Differences in symptom elicitation were analyzed by Anova and Kruskal-Wallis statistics.

4.3 Single spores cultures

On the basis of the pathogenicity bioassays, the number of isolates to undergo further molecular analysis, and consequently population study, was restricted to a representative set of single spore strains. The fungal spores were distributed on the surface of a 2% water agar plate (9 mm) by depositing 250 µl of conidial suspension on the center of the plate and spreading the drop gently with a sterilized glass rod. After 30 minutes of incubation at 23 °C degree, to increase adherence of conidia onto the agar surface, single conidia were picked up using a needle under a stereomicroscope (20 X magnification) and transferred to PDA agar plates and incubated at the optimal condition for fungus growth. For each isolate, an average of 4-5 single spores colonies were obtained. All the single spore strains were tested for pathogenic capability as previous described. Subsequently, one strain was chosen to represent the uniform “isolate-population”.

4.4 Morphological Characterization

For the subset of single spores strains, morphological characteristics of the colony and sporulation pattern were determined according to previous systematic work on *Alternaria* spp. (Pryor and Michailides, 2002 and Hong et al., 2006). Fungi were grown on PDA plates and incubated at 24°C in darkness for 10 days. After incubation, cultures were examined for colony color, colony margin, colony texture, diameter and the presence of pigments or crystals in the agar medium. Ridgway’s color standards (Ridgway, 1912) and Nobles (Nobles, 1948) descriptions of colony texture were used to classify isolates and

define phenological groups (Hong and Pryor, 2004). The properties were evaluated by naked-eye or were measured.

To characterize isolates by sporulation habit, they were cultured on weak potato dextrose agar (WPDA) (Pryor & Michailides, 2002) and incubated in a fully programmable growth chamber (Conviro ATC 10-3, Conviro Controlled Environments, Pembina, ND) using the following environmental parameters: temperature, constant at 22 C; humidity, constant at 25 % RH; lighting, 60 $\mu\text{moles}/\text{m}^2/\text{s}$ using Sylvania FB031/841/XP 31-watt fluorescent lamps (Osram Sylvania, Danvers, MA) at a 10:14 light/dark cycle. The three dimensional conidial chain formation was investigated with a light microscope at 40 \times magnification. The examination was conducted in two independent steps. First, the morphological systematic key developed by Simmons was used (Simmons, 2007), and then the systematic key developed by Prof B.M. Pryor (Pryor and Gilbertson, 2001 and Pryor and Bigelow, 2003) was used, which assigns different scores are on the basis of each character (chain length, presence of tapering beaked conidia, number of branches and branching structure). Reference strains used in the study were kindly provided by Prof. Barry Pryor, who allows an open access to his own fungal collection. The reference strains were: BMP0269 for *Alternaria alternata*, BMP0304 for *Alternaria tenuissima*, BMP0308 for *Alternaria arborescens* and four Japanese strains of *Alternaria alternata mali* pathotype (M71, IF08984, EGS 38-029 and AKI3).

Morphological data analysis

A matrix was created from the resulting morphological scores (Prof B.M. Pryor's key) and analyzed with PAST software version 1.63 (Hammer et al., 2001), utilizing euclidian distance. Then dendrograms were constructed using an unweighted paired group method of arithmetic means (UPGMA) algorithm based on the euclidean distance. The sporulation characteristics of representative reference cultures (*A. alternata* BMP0269, *A.*

tenuissima BMP0304, *A. arborescens* BMP0308, *A. alternata pathotype mali*: M71, IF08984, EGS 38-029 and AKI3) were included in these analyses for comparative purposes.

4.5 Molecular analysis

DNA extraction

Fungal DNA was extracted from 50 ml of liquid PDA culture prepared dislodging 1 ml of spore suspension (10^4 conidia/ml), obtained from 7 days old PDA plates (single spores colony), with a plastic pipette. Flasks were agitated on a rotary shaker, at 120 rpm for 14 days at 23°C, and mycelia was harvested and lyophilized.

For extraction of fungal total genomic DNA, 200 mg of lyophilized mycelia was ground into a powder in liquid nitrogen. Then 1 ml of lysis buffer (300 mM NaOAc at pH 6, 0.5% SDS, 100 mM EDTA at pH 8.0 and 50 mM Tris HCl at pH 7.5) was immediately added to the powdered mycelia and heated for 1 hour at 65°C in a water bath. After the incubation materials were spin down for two minutes at 10.000 g and transferred in new 1.5 ml tubes. Working in fume hood, equal volume of phenol:chloroform (1:1) was added into samples and mixed well by inversion for ten minutes.

Samples were spun for ten minutes at full speed (14.000 rpm) and the top layer was transferred into a new 1.5 ml tube to which was added an equal volume of chloroform to each sample. The tubes were then mixed by inversion for ten minutes followed by a ten minutes centrifugation at 14.000 rpm. The top layer was pipetted into a new 1.5 ml tubes to which a 2/3rds volume of cold isopropanol was added and mixed by inversion. The DNA clouds were moved into new tubes with sterile tips and washed overnight with 70% ethanol. The following day the ethanol was poured off and the DNAs dried in a laminar flow hood. The nucleic acids were re-suspended in 200 µl of TE 1X buffer pH 8±0. Sample DNA concentration was estimated using a Thermal Scientific ND 1000 Spectrophotometer.

4.6 Single locus analyses

PCR for the EndoPg gene and the Opa1-3, Opa2-1 and Opa10-2 anonymous regions

Polymerase chain reactions were carried out, in a PTC-10 MJ Research Thermal Cycler, for all single spore isolates. The reaction mixture (25 μ l) contained 20ng DNA template, 0.2 μ M of each primers, 0.2mM of PCR nucleotide Mix, 2.5 μ l of 10X Fermentas DreamTaq Buffer (with KCl and (NH₄)₂SO₄ and 20 mM MgCl₂) and 1 unit of Taq Polymerase (Fermentas DreamTaq). The cycling conditions were: initial denaturation step at 94°C for 5 minutes followed by 35 cycles of 94°C for 45 sec, T °C annealing temperature (T °C = 56 for EndoPg primers, T °C = 60 for Opa10-2 primers, T °C = 58 for Opa1-3 and Opa2-1 primers) for 45 sec and 72°C for 45 sec. These cycles were followed by a final extension step at 72°C for 7 min.

The primers utilized were PG2b (5'-GAGAATTCRCARTCRTCYTGRTT-3') and PG3 (5'-TACCATGGTTCTTTCCGA-3') for the segment of EndoPg gene, OPA1-3L (5'-CAGGCCCTTCCAATCCA -3') and OPA1-3R (5'-AGGCCCTTCAAGCTCTCTTC-3') OPA2-1L (5,-TGCCGAGCTGTCAGATAATTG-3') and OPA2-1R (5'-GCCGAGCTGGTGGAGAGAGT-3'), OPA10-2R (5'-GATTTCGCAGCAGGGAAACTA-3') OPA10-2L (5'-TCGCAGTAAGACACATTCTACG-3') for the three anonymous regions.

Sequencing and phylogenetic analysis

Successful PCR amplifications were visualized on 1.2% agarose gel using ethidium bromide. Prior to sequencing, 16 μ l of PCR product were purified using the combination of Shrimp Alkaline Phosphatase and Exonuclease I (1 unit/0.3 unit) in a final reaction volume of 20 μ l. Amplicons were then submitted to University of Arizona Genetics Core at the Arizona Research Laboratories for bidirectional sequencing performed with ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystem) in a ABI PRISM® 3100 automated

sequencer. Automated fluorescent DNA sequencing using a capillary DNA sequencing instrument (ABI PRISM® 310 Genetic Analyzer - Applied Biosystems) is based on the use of a different colored fluorescent dye for each of the four DNA bases. The most popular method for doing this is called the dideoxy method or Sanger method (Sanger et al., 1977).

Resulting sequences were trimmed and edited using MacVector 11.1 software before further analysis. The editing was performed using two combined algorithms: phred which takes chromatogram information from an automated sequencing run and re-evaluates the peaks to produce a "base call", that is usually significantly more accurate than the original call, and phrap which assembles sequences into contigs and creates a consensus sequence with its own set of quality values, based on the quality and strandedness of the overlapping sequences.

Initial sequence alignments for phylogenetic analysis were completed using ClustalW v. 2.0.12 (Thompson et al., 1994) and adjusted by eye using Mesquite software v.2.72 and MacClade Phylogenetic Software (Maddison and Maddison, 1992 and Maddison and Maddison, 2009). A correct multiple sequence alignment is a fundamental step to build a reliable phylogeny. The method applied is known as progressive alignment (Feng and Doolittle, 1987, Higgins and Sharp, 1988). This method builds an alignment up stepwise, starting with the most similar sequences and progressively adding the more dissimilar ones. The cardinal rule of the progressive sequences alignment is "once a gap always a gap"; gaps can only be added or enlarged, never moved or removed.

Phylogenetic trees were calculated following the discrete method of parsimony, where each column of the alignment is examined separately and where a search is conducted to find the dendrogram which best accommodates all of the data. Specifically, a heuristic maximum parsimony trees were constructed for each marker of interest (EndoPG, OPA1-3, OPA2-1, and OPA10-2) using PAUP Phylogenetic Software (version 4.0-beta; Sinauer Associates, Inc., Sunderland, MA; Swofford, 2002). For each search, default

settings were used with the exceptions of 1) setting the sequence addition method to random and 2) setting the number of repetition to 1000. A full heuristic bootstrap search with 1000 random sequence additions and 1000 bootstrap replicates was conducted for each tree to obtain support values. These values were then mapped onto a majority rule consensus tree of the most parsimonious trees.

In addition, two estimates of tree topology were calculated for the most parsimonious trees for each locus, the retention index (RI) and the consistency index (CI). CI is defined as m/s , where m is the minimum possible number of character changes (steps) on any tree, and s is the actual number of steps on the current tree. This index hence varies from one (no homoplasy) and down towards zero (a lot of homoplasy). The ensemble consistency index CI is a similar index summed over all characters. RI is defined as $(g-s)/(g-m)$, where m and s are as for the consistency index, while g is the maximal number of steps for the character on any cladogram. The retention index measures the amount of synapomorphy on the tree, and varies from 0 to 1. After analyzing each locus individually, the loci were concatenated into a larger data set. This concatenated data set was then subjected to a maximum parsimony search as described above. Support values and topology statistics were also recorded as previously described.

Test of neutrality and combinability of dataset

Tajima's D (Tajima 1989), Fu's and Li's D* and F* statistics (Fu and Li, 1993) were estimated with DnaSP (Rozas and Rozas, 1997). Tajima's D and Fu's and Li's D* and F* statistics measure departure from the null hypothesis of neutral evolution where significant values may indicate change in population sizes and/or purifying or balancing selection (Tajima 1989, Fu and Li, 1993). Tree topologies were compared with the parametric Kishino-Hasegawa test and the "winning sites" test, performed with PAUP phylogenetic software version 4.0-beta (Sinauer Associates, Inc., Sunderland, MA; Swofford, 2000). These tests allow us to evaluate if one tree is statistically worse than another. Kishino-

Hasegawa test utilizes differences in the support provided by individual sites for two trees to determine if the overall differences between the trees are significantly greater than expected from random sampling error. The “winning sites’ test sums the number of sites supporting tree A over tree B and vice versa.

4.7 Multi locus analysis

AFLP markers

AFLPs were performed following the method described in Vos et al. (1995), except that the profiles were obtained using PAGE (Polyacrylamide Gel Electrophoresis) and visualized through the silver staining technique (Bassam and Caetano-Allones, 1993). Genomic DNA (250ng) was digested for 2 hours at 37°C with 5U of frequent cutter enzyme (TruI isoschizomer of MseI) and 5 U rare cutter enzyme (EcoRI) in 50 µl of Restriction/Ligase Buffer 5X (50 mM Tris-Cl pH 7.5, 50 mM Mg-Actate, 250 mM K-Acetate). Adaptors (Eco-F: 5'-CTC GTA GAC TGC GTA CC-3', Eco-R: 5'-AAT TGG TAC GCA GTC TAC-3'; Mse-F: 5'-GAC GAT GAG TCC TGA G-3', Mse-R: 5'-TAC TCA GGA CTC AT-3') were ligated, at 37°C for 3 hours, combining 10 µl of ligation solution (5 pmol ADA-EcoRI 5, 50 pmol ADA-TruI, 0.2 mM ATP, 1 U of ligase and 2 µl of R/L buffer 5X) with 40 µl of digestion product. The pre-amplification was carried out adding to 5 µl of 10-times diluted ligation product 2.0 µl Buffer 10X (100 mM Tris-HCl pH 8.3, 500 mM KCl), 2 µl of MgCl₂ 15 mM, 0.16 µl of dNTPs 25mM each, 1U of Taq polymerase and 1.6 µl of non selective primers combination (50 ng/µl each of MseI-0 and EcoRI-0) in a final volume of 20 µl. Cycling conditions were set as a denaturation step of 1minute at 94°C followed by 40 cycles (94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min). Amplicons were checked on a 2% agarose gel as faint smears of DNA. A preamp 1:50 dilution was used to carry out selective PCR. These were performed in a 20 µl reaction volume using different primers combinations of MseI and EcoRI with two selective nucleotides at the 3' end (Tab. 2) with

the same master mix composition. The PCR cycle was edited in the following initial denaturing step at 94°C for 1 min followed by 40 cycles of 94°C for 1 min, 62°C for 30 sec, 72°C for 1 min. Each reaction was performed twice.

Products were subjected to electrophoresis in 4.5% (w/v) acrylamide gel in TBE 1X buffer, by staining with silver nitrate (Bassam and Caetano-Allones, 1993). The size of the DNA fragments was estimated by comparing the DNA bands with a 100bp GeneRuler DNA ladder (Fermentas, Inc). Images were scanned with a HP Scanjet 5590P.

ISSR markers

A set of ISSRs primers were utilized to analyze the genetic diversity within the population of Italian isolates. On Eleven ISSR primers screened, nine (Tab.2) proved to be suitable for this study and gave a polymorphic, reproducible pattern. PCR's were performed on the basis of others fingerprinting works, with a few modifications (Weising et al., 1995, Goldwin, 1997 and Bayraktar et al., 2008). PCR's were carried out in a final volume of 25µl containing 20ng DNA template, 0.7µM of primer, 0.2mM of PCR nucleotide Mix, 2.5 µl of 10X Fermentas DreamTaq Buffer (with KCl and (NH₄)₂SO₄ and 20 mM MgCl₂) and 1 unit of Taq Polymerase (Fermentas DreamTaq). DNA amplification was performed in a thermal cycler MJ Research PTC-100 with an initial denaturing step at 94 C for 5 min, followed by 35 cycles at 94°C for 1 min, 48 C for 75 s, and 72 C for 2 min and a final extension cycle at 72°C for 7 min. The annealing temperature was adapted on the bases of the specific annealing temperature of the primer used (Tab. 2). Each reaction was performed twice.

Products were subjected to electrophoresis in a 1.2% (w/v) agarose gel in TBE 0.5X buffer, and they were visualized by exposure to UV light after staining in ethidium bromide. The sizes of the DNA fragments were estimated by comparing the DNA bands with a 1 kb GeneRuler DNA ladder (Fermentas, Inc). Images were captured using the software LabWorks 4.0, UVP Bioimaging System, Epichemi³ Darkroom.

Tab.2. Sequences of primers utilized for the multi locus analysis.

AFLPs and ISSRs data scoring and statistical analysis

The fingerprinting profiles were analyzed with QuantityOne ver. 4.2.1, Bio-Rad. Only those bands that showed consistent and unambiguous amplification were scored. Smear and weak bands were excluded. Amplified fragments, with the same mobility according to the molecular weight (base pair), were clustered in weight ranges and scored as discrete variables, using 1 to indicate presence and 0 for absence of homologous bands. Since ISSRs and AFLPs are dominant markers, each amplification product was considered to represent the molecular phenotype at a single bi-allelic locus and it was assumed that similarity of fragment size was an indicator of homology (O'Hanlon and Peakall, 2000). The resulting banding profile scores were used to construct two binary qualitative data matrices, which were analyzed according the following statistical methods. Those loci with a low value of Polymorphic Information Content (less than 0.25) were excluded. The formula to determine the Polymorphic Information Content is as follows:

$$PIC = 1 - \sum p_i^2$$

where p represent the frequencies of the allele i for the locus investigated). The discriminatory power of each primers (or primers combination), the ability of a typing system to discriminate between unrelated strains, was measured using Simpson's diversity index (Simpson, 1949) and the raw percentage of polymorphic bands (PPB) produced. Simpson's index values close to 0 indicate that there is little diversity whereas a Simpson's index approaching 1 indicates a high diversity. Parameters were calculated using the software Past v. 1.63 (Hammer et al., 2001).

A cluster analysis of the two binary matrices was performed, utilizing PAST software v 1.63, Dice's similarity coefficients (Dice, 1945) between genotypes were calculated to estimate the diversity among the isolates. These coefficients were used to identify groups

that could be distinguished from neighboring classes and to resolve these classes in a dendrogram. The formula for creating Dice's coefficient is as follows:

$$D = \frac{2a}{2(a + b + c)} \quad (\text{Dice's coefficient})$$

Here, **a** is the number of positions with shared bands between individual *i* and *j*; **b** is the number of position where individual *i* has a band but *j* does not and **c** is the number of position where individual *j* has a band and *i* does not.

In order to assess the molecular diversity within the *Alternaria* single spores strains the Analysis of Molecular Variance, AMOVA, (Excoffier et al., 1992), was performed . The analysis was carried out separately for AFLP and ISSR data sets calculating the partitioning of the genetic variation among the population, defined by the cluster analysis. The estimations of the genetic differentiation (Φ_{PT}) and the Nei's genetic distance (D) (Nei, 1972) were computed using the software GenAlEx version 6.3 (Peakall and Smouse, 2009).

THE MANTEL TEST

In order to estimate the congruence among dendrograms, cophenetic matrices for each marker was computed and compared using the Mantel test. The Mantel Test (Mantel, 1967) was conducted in this study using the program PAST version 1.63 (Hammer et al., 2001). The Mantel's statistic is based on a cross-product term:

$$Z = \sum_{ij} X_{ij} Y_{ij}$$

where \sum_{ij} is the double sum over all *i* and all *j* where $i \neq j$. Because *Z* can take on any value depending on the exact nature of **X** and **Y**, one usually uses a normalized Mantel coefficient, calculated as the correlation between the pair wise elements of **X** and **Y**. Like any product-moment coefficient, it ranges from -1 to 1.

The degree of fit was interpreted subjectively as follows:

0.9 < r Very good fit.

$0.8 < r < 0.9$ Good fit.

$0.7 < r < 0.8$ Poor fit.

$r < 0.7$ Very poor fit.

PRINCIPAL COMPONENT ANALYSIS

A Principal Component Analysis (PCA) (Joliffe, 1986) was performed in order to highlight the resolving power of the ordination. The PCA takes a cloud of data points, and rotates it such that the maximum variability is visible. The first stage in rotating the data cloud is to standardize the data by subtracting the mean and dividing by the standard deviation. The first component extracted in a Principal Component Analysis accounts for a maximal amount of total variance in the observed variables where the “total variance” in the data set is simply the sum of the variances of the observed variables. Because of this, the total variance in a Principal Component Analysis will always be equal to the number of observed variables being analyzed.

Then, under typical conditions, the first component will be correlated with at least some of the observed variables. It may be correlated with many. The second component extracted will have two important characteristics. First, this component will account for a maximal amount of variance in the data set that was not accounted for by the first component. Again under typical conditions, this means that the second component will be correlated with some of the observed variables that did not display strong correlations with component 1.

The second characteristic of the second component is that it will be uncorrelated (or orthogonal) with the first component. Literally, if the correlation between components 1 and 2 is computed, that correlation would be zero. The remaining components that are extracted in the analysis display the same two characteristics: each component accounts for a maximal amount of variance in the observed variables that was not accounted for by the preceding components, and is uncorrelated with all of the preceding components.

A Principal Component Analysis proceeds in this way, with each new component accounting for progressively smaller and smaller amounts of variance (this is why only the first few components are usually retained and interpreted). When the analysis is complete, the resulting components will display varying degrees of correlation with the observed variables, but are completely uncorrelated with one another. The Principal Component Analysis was conducted in this study using the program PAST version 1.63 (Hammer et al., 2001).

4.8 *Alternaria mali* toxin gene specific PCR

The polymerase chain reaction (PCR) has been shown to be a powerful tool to discriminate and identify numerous fungal pathogens which are difficult to differentiate using conventional techniques (Brown et al., 1993; Fulton and Brown, 1997 and Niessen and Vogel, 1998). Recently, Johnson and collaborators (Johnson et al., 2000a) have cloned a gene involved in the biosynthesis of the AM-toxin, AMT (GenBank accession no. AF184074), and demonstrated that it is specific to the apple pathotype of *Alternaria*. Subsequently, they have designed specific primers to specifically detect the *Alternaria alternata* apple pathotype (Johnson et al. 2000b).

A preliminary investigation of the single spores strains was performed utilizing these specific primers to elucidate if the pathogenicity was linked to the presence of AM toxin gene. PCR was conducted with AMT gene specific primers (LinF1, 5'-TATCGC CTGGCCACCTACGC-3' positions 8017–8036; LinR, 5'-TGGCCACGACAACCCACA TA-3' position 8493–8512 within the *AMT* gene Genbank accession no. AF184074). PCRs were carried out following the cited work with some modifications. The reaction mixture (25µl) contained 20ng of genomic DNA template, 1µM of each primers, 0.2mM of PCR nucleotide Mix, 1X of Buffer (with KCl and (NH₄)₂SO₄), 1.2 mM MgCl₂ and 1 unit of Taq Polymerase (Takara). The cycling conditions were: initial denaturation step at 94°C for 5

minutes followed by 35 cycles of 94°C for 45 sec, 65°C of annealing temperature for 45 sec and 72°C for 45 sec. These cycles were followed by a final extension step at 72°C for 7 min. Positive controls to check the DNA quality was performed using GPD primers (GPD1 5'-CAACGGCTTCGGTCGCATTG-3, GPD2 5'-GCCAAGCAGTTGGTTGTGC-3'). Products were visualized as previously described on a 1.2% agarose gel using ethidium bromide and scored as present or absent. PCRs were performed different times changing some parameters that would have affected the appearance of the bands so to exclude the possibility of false positive.

In cases of positive PCR, the amplicons were purified (Sigma Genosys Cleanup kit) and sent to sequence in both direction to BMR Genomics Sequencing Service for bidirectional Sanger sequencing. Then, pherograms were checked and trimmed manually, using the Bioedit software v.7.05. Alignment with the sequence of AM toxin gene, deposited in NCBI genomic Bank, was performed trough Mesquite software v.2.72. To investigate the flanking region of the specific AM-toxin primers, other couples of oligonucleotides have been designed downstream and upstream of the original primer sites. (Tab. 3). PCRs were carried out on the samples that had shown a positive result the first set of specific PCR primers (primer Lin F1 and Lin R). These reactions were carried out using different primers combinations without changing anything other the PCR conditions (with the exception of the annealing temperature) as follows:

AM_ToX F1 (7916-7926) + AM_ToX_R1.3 (8620-8639)

AM_ToX F2 (7938-7959) + AM_ToX_R2.3 (8575-8595)

AM_ToX F3 (7946-7969) + AM_ToX_R1.3 (8620-8639)

AM_ToX F3 (7946-7969)+ AM_ToX_R2.3 (8575-8595)

AM_ToX F1(7916-7926) + LinR (8493–8512)

LniF1(8017–8036) + AM_ToX_R1.3(8620-8639)

Tab.3. Sequences and annealing temperature of the primers designed on the AM-toxin gene.

5. Results

5.1 Strain sampling and isolation

All the obtained colonies were morphologically typified and eventually ascribed to the *Alternaria* genus. Investigations were then performed to test the pathogenic capability of each isolate. The initial step was to identify the colonies belonging to *Alternaria* genus observed under the optical light microscope (40X magnification). This was a preliminary characterization based on the conidia shape. The following is a description of the spore typology observed: I) mature conidia of 10-30 x 5-12 μm , short conical beak or beakless, narrowly ellipsoid to ovoid and elongated on branching chains, juveniles narrowly elliptical, punctate roughened ornamentation especially when old, dull olive in color, 3-7 transepta, 1-5 longisepta; II) mature conidia typically 12-42 x 7-11 μm , non-beaked, ellipsoid or short-ovoid, greatly punctulate to verrucose ornamentation, light tan or brown in coloration, 1-4 transepta, few longisepta or oblique septa; III) mature conidia 30-50 μm x 15-20 μm narrow-ellipsoid taper beaked in the upper part.

5.2 Pathogenic bioassays

All the colonies were subjected to *in vitro* bioassays to evaluate their capability to produce necrotic symptoms. Tests, performed on apple fruit and apple leaf differently treated (wounded and unwounded), highlighted a variable ability of the isolates to produce symptoms related to: the original plant material, the presence or absence of artificial lesions and the different type of inoculation (spore suspension or filtrate) tested in the bioassays. When spore suspension (10^5 conidia/ml) was inoculated, 70% of the isolates were able to produce symptoms on wounded plant material while only 30% were able to induce necrosis on unwounded leaf and 5% on unwounded fruit. A different trend was observed utilizing the filtrate: 45% of the isolates showed pathogenicity on wounded leaves, 30% on wounded

fruits, only 25% on unwounded leaves whereas no symptoms were observed on unwounded fruits.

5.3 Morphological characterization of single spore isolates

A subset of single-spore strains (Tab. 4), both pathogenic and non pathogenic, was considered representative of geographical areas and were subjected to deeper investigation. The comparison with reference isolates distinguished three main species-groups among the pure culture colonies investigated: the *tenuissima*, the *alternata* and the *arborescens* (Fig. 10). Note that the term “species-group” has been adapted from previous usage by Simmons (1992) and when it preceded by the non italicized epithet of representative taxon, it has been adapted as the nomenclatural format for phylogenetically based infragenic groupings of *Alternaria* in general (Pryor and Gilbertson, 2000). Twenty one isolates were ascribed to the *tenuissima* species-group. They were characterized by long principal conidiophores (6-16 conidia), generally not branched and when branching, it was observed that they never exceeded 4 conidia in length. Only four strains were enclosed in the *alternata* group distinguished by primary conidium chains of 6-12 in length with secondary branches (2-6 conidia) growing from terminal, median or basal conidium cells and the tertiary branches were rarely observed. Ten strains were comprised in the *arborescens* group. These were characterized by short conidia chains, primary conidiophores of 2-8 conidia in length, and secondary conidiophores always originating from distal cells (usually 3-5 per conidiophore). Moreover, in this group, tertiary and quaternary conidiophores were observed in a highly geniculate sporulation structure.

Two strains (10FgB, 24FgB) exhibited an intermediate pattern between the *A. alternata* and *A. arborescens* group and were named “open *arborescens*” group. Three isolates (A138, A152 and F09) were registered as “unknown” because spores were too scarce to be able to ascribe to any of the three described groups. Lastly, four isolates were not classified due to failed sporulation, even after repeated attempts to induce sporulation by

changing growth conditions and using different media culture. Concerning the reference strains of *Alternaria alternata mali* pathotype, three of them (AKI3, IF08984, EGS 38029) exhibited sporulation habits typical of the *tenuissima* morpho-species group, while the M71 strain was characterized by an *arborescens* sporulation pattern (Fig. 11)

Morphological descriptions, obtained following both the systematic key elaborated by Simmons (*Alternaria: an Identification manual*, 2008) and the one created by Prof B.M. Pryor, have led to a concordant interpretation. Furthermore, the dataset achieved with Prof B.M. Pryor's key (Pryor and Michailides, 2002) was interpreted with a cluster analysis that clearly shows distinct clusters that coincide with previous classifications (Fig. 9).

Fig. 9. The Cluster Analysis, based on the euclidian distance, calculated on the scores attributed to the different characteristic of the sporulation habits (presence of tapering beaked conidia, length of chain, number of branches and branching structure) following the key of Prof. Pryor. The analysis incorporates also the reference isolates (*A. alternata* BMP0269, *A. arborescens* BMP0308, *A. tenuissima* BMP0304; four reference isolates for *A. alternata mali* pathotype: AKI3, IF08984, EGS 38029 and M71). The non sporulating isolates (11BZ1, 11BZ4, 4Fr and 6Fg) and the isolates classified as "unknown" (A152, A138, F09) were not included.

Tab. 4. Morphological characterization of the strains analyzed in this study. Letters represent affiliation species-group: A= alternata, AR = arborescens, T = tenuissima.

The characterization of the colony morphology led to a similar result, delineating three major morphological groups: tenuissima, alternata and arborescens (Fig.10). Twenty seven colonies that were pale olive gray to olive gray, often with a very thin (1 to 2 mm) white margin. These were characterized by a high growth rate (>7mm), an ability to produce crystals underneath mycelia mat, that was usually from woolly to cottony, and resembled morphological traits of the *A. tenuissima* representative strain . The alternata group (with 4 isolates) produced colonies that were usually green (from lettuce green to olive green), with a smooth margin and a white bordered (2-4 mm), and had a colony textures ranging from felty to woolly. This group produces whitish crystals in the agar medium underneath the mycelial mat. After 10 days of growing, the diameter of these isolates typically exceeded 70 mm. The *Alternaria arborescens* group, with 13 isolate, comprises dark colonies (dark olive gray to olivaceous black 2) with wavy torn border and felty to wolly texture. The production of crystals both on the mycelia and in the agar medium underneath the mycelia mat was observed. A pale orange diffusible pigment was present. After 10 days of growing in darkness, the growth of individuals in this group never exceeded a diameter of 50 mm, as is typical of *A. arborescens*. Concerning the colony morphology, *Alternaria alternata mali* pathotype reference isolates showed different grades of texture and color but all enclosed in the tenuissima species-group (Fig. 11).

Fig.10. Sporulation pattern (20X), conidia shape (40X) and colony morphology of the reference isolates: A) *Alternaria tenuissima* BMP0304, B) *Alternaria alternata* BMP0269, C) *Alternaria arborescens* BMP0308.

Fig. 11. Sporulation pattern (20X), conidia shape (40X) and colony morphology of the *Alternaria alternata mali* pathotype reference isolates: A) EGS38-029, B) AKI3, C) IF08984; D) M71.

5.4 Leaf bioassays with single spores

Concerning the spore suspension bioassays on leaf material, they mirrored a similar trend of the bioassays previously reported. Symptoms (Fig. 12) were induced by 83% of the isolates on wounded tissues and by 35% on unwounded tissues. For each type of inoculation, the mean pathogenicity scores were calculated for each species-groups and according to the ANOVA comparison they did not show statistically significant differences ($P > 0.05$). The ANOVA result was also confirmed by the Kruskal-Wallis test ($p > 0.05$). High variability of pathogenicity score was found within each group highlighting how the capability of produce symptoms is likewise distributed among the three groups. (Fig. 13). Comparison between the two kinds of treatments (wounded/unwounded) were statistically significant according the student's -T statistic ($P < 0.05$).

The inoculation of the filtrate induced symptoms for 42% of tested strains. For this bioassay pathogenicity scores were lower than the ones observed in the spore suspension tests (Fig 13). Moreover, the P.S. referred to each single spore isolate seemed to highlight a specific capability of the strains to produce putative toxins independently of belonging to a morpho-species group. This data was supported by both ANOVA and Krukal-wallis statistics ($P > 0.05$). No correlations were found between geographical area and pathogenic capability exhibited by isolates.



Fig.12. Example of results obtained by pathogenicity bioassays: a) spore suspension, b) filtrate

5. For each strain, sampling location and mean pathogenicity scores, resulting from bioassays on leaf, are reported.

Fig. 13a-b. Mean pathogenicity scores (P.S.) are reported for the three morpho-species group according to the results of the leaf bioassay: (a) Spore suspension (10^5 conidia/ml) test (b) filtrate test. Pathogenicity score was assigned to each isolates (*Alternaria tenuissima* with 29 isolates, *Alternaria arborescens* with 15 isolates and *Alternaria alternata* with 4 isolates) on the base of the diameter of lesions developed following the rating scale: 1=no lesions, 2= < 2 mm, 3= 2-4 mm, 4= > 4 mm. Differences of pathogenicity score (P.S.), resulted from two type of treatment (wounded and unwounded), were significant according a T-student comparison (** $p < 0.05$).

Fig. 14 a-b. For each strains is reported the mean pathogenicity score (P.S.) for the two inoculations performed. Graphs depict a high variability present inside the group. Bars are the standard errors.

Fig. 15 a-b. For each strains is reported the mean pathogenicity score (P.S.) for the two inoculations performed. Graphs depict a high variability present inside the group. Bars are the standard errors

Fig. 16 a-b. For each strains is reported the mean pathogenicity score (P.S.) for the two inoculations performed. Graphs depict a high variability present inside the group. Bars are the standard errors.

5.5 Single locus analyses

Phylogeny and parsimony analysis

PCR experiments carried out on the four loci, to address the relationship among the *Alternaria* spp. strains, successfully amplified the expected products. EndoPg and OpA1-3, OPA2-1 and OPA10-2 primers yielded amplicons averaging 389 bp, 780bp, 520 bp and 620 bp respectively. A majority rules consensus tree (parsimony analysis method) was created from the pool of equally most parsimonious trees per each locus. In all analyses *Alternaria arborescens* was set as an outgroup to highlight the monophyletic origin of the *Alternaria arborescens* species.

The EndoPg sequence alignment showed 7 informative sites and only one most parsimonious tree (tree length = 7) characterized by high scores of consistency index and retention index (C.I.= 1 and R.I.=1). The EndoPg phylogeny (Fig. 17) revealed three main clades: the *A. alternata*-*A. tenuissima* clade (clade 2) was strongly supported by a bootstrap value of 90 while the *A. tenuissima* (clade 1) and the *A. arborescens* (clade 4) clades were characterized by moderate values of bootstrap support (bootstrap = 62 and bootstrap value = 66 respectively). Due to the polarization the *A. arborescens* clade node appears collapsed.

After sequence alignment the OP1-3 region retained 85 informative sites which produced 24 equally parsimonious trees (tree length = 121) with a C.I. = 0.82 and R.I.= 0.97 with. The majority rules consensus tree depicted (Fig. 18) four strongly supported clades. *Alternaria tenuissima* strains were spread among three clades (clade 1, clade 2 and clade 3) while *A. arborescens* strains formed a monophyletic clade (clade 4).

The OPA2-1 sequence alignment highlighted 17 informative characters and yielded 100 equally trees parsimonious (tree length = 12) with C.I. = 0.92 and R.I. = 0.98. The consensus tree resolved two clades: the *A. alternata*-*A. tenuissima*, clade (clade 1) supported

by a moderate bootstrap value (bootstrap value =67), and the *A. arborescens* group (clade 2 and clade 3) (Fig.19).

Despite the high number of informative sites (i.s. = 47) the consensus tree (tree length = 86) defined by the parsimony analysis of OPA10-2 sequences had the lowest tree scores (C.I. = 0.63 and R.I. = 0.95). The resulting tree (Fig. 20) divided the *Alternaria tenuissima* strains in three clades. Clade 1 was supported by a bootstrap value of 93 while clades 2 and 3 showed moderate bootstrap scores (61 and 64 respectively). Moreover, the *A. arborescens* clades (4, 5) appeared polyphyletic even after the polarization in contrast with literature and previous *Alternaria* phylogenetic studies (Pryor and Michailides, 2002 and Andrew et. al., 2009).

Partitioning of the isolates was variable among the four trees, especially concerning the *Alternaria alternata mali* pathotype reference isolates, which were not observed in any particular clustering with other *Alternaria* strains tested. For this reason, tree topologies were compared in a pairwise manner utilizing the “winning sites” test, Tempelton Test and Kishino-Hasegawa test (Tab. 6). Tests showed a significant difference ($p > 0.05$) for nearly all the comparisons computed, the non significant values were due to the low discriminating power of the locus compared with loci that were more informative. Moreover for each locus, the hypothesis of neutral evolution was tested using Tajima (D^*), Fu and Li D^* and F^* statistic tests. According the Fu and Li D^* and F^* statistics the neutrality was accepted for all the loci ($p > 0.05$) while the D^* 's Tajima value for the EndoPG resulted significant ($p < 0.05$) indicating a non random evolution process for this locus. This result was concordant with previous systematic work on *Alternaria* spp. (Andrew et al., 2009).

Tab. 6. Kashino-Hasegawa test and Tempelton-Winning sites tests. For each tree topologies comparison (rows over columns in a pairwise manner), p values are reported.

A parsimony analysis was then conducted for the combined dataset. This dataset was used to build a concatenated tree, combining the four matrices, to optimize the number of informative sites, utilized by the heuristic search. The concatenated tree (tree length = 374) (Fig. 21) was characterized by low tree scores (C.I. = 0.63, R.I = 0.9) but strong statistical support. This support is especially striking for the *A. arborescens* clade (bs = 87) which was separated from the *A. alternaria*-*A. tenuissima* clades (clades1 and calde2, bs= 50 and bs=76 respectively).

Fig. 17. Tree (C.I.=1, R.I.=1) obtained from the parsimony analysis of the EndoPG gene using a heuristic search in the maximum parsimony analysis with 1000 repetition random. Bootstrap values of 1000 replicates are indicated above the branches. ++ indicates strains isolates which produced symptoms for at least two of the three pathogenicity tests. +++ indicates strains that were positive for all the three pathogenicity bioassays.

Fig. 18. Majority rules consensus tree (C.I.=0.82, R.I.=0.97) obtained from the parsimony analysis of the OPA1-3 sequence using a heuristic search in the maximum parsimony analysis with 1000 repetition random. The isolate 36Fg is not present due to failed sequencing . Bootstrap values of 1000 replicates are indicated above and below the branches. ++ indicates the strains which produced symptoms for at least two of the three pathogenicity tests. +++ indicates strains that were positive for all the three pathogenicity bioassays.

Fig. 19. Majority rules consensus tree (C.I.=0.92, R.I.=0.98) obtained from the parsimony analysis of the OPA2-1 sequence using a heuristic search in the maximum parsimony analysis with 1000 repetition random. Bootstrap values of 1000 replicates are indicated above the braches++ indicates strains which produced symptoms for at least two of the three pathogenicity tests. +++ indicates strains that were positive for all the three pathogenicity bioassays.

Fig. 20. Majority rules consensus tree (C.I.=0.63, R.I.=0.95) obtained from the parsimony analysis of the OPA10-2 sequence using a heuristic search in the maximum parsimony analysis with 1000 repetition random. Bootstrap values of 1000 replicates are indicated above the braches++ indicates the isolates which produced symptoms for at least two of the three pathogenicity tests. +++ indicates strains that were positive for all the three pathogenicity bioassays.

Fig. 21. Majority rules consensus tree (C.I.=0.63, R.I.=0.90) obtained from the joint alignment of the combined data sets (EndoPg, OPA1-3, OPA2-1 and OPA10-2) using a heuristic search in the maximum parsimony analysis with 1000 repetition random. Bootstrap values of 1000 replicates are indicated above the braches. ++ indicates the strains which produced symptoms for at least two of the three pathogenicity test. +++ indicates strains that were positive for all the three pathogenicity bioassays.

5.6 Multi locus analysis

AFLPs and ISSRs results

The eight primer combinations utilized in the AFLPs analysis produced a total of 166 polymorphic bands (86%). The size of amplification products ranged between 70 bp to 950 bp (Fig.22). The yield of polymorphic fragment for each couple utilized was between 12 to 34 with an average of 24.75 bands per combination. The Simpson's index (S), calculated to assess the discriminatory power of each primers combination, ranged between 0.85 to 0.95.

A total of 95 bands were scored as polymorphic using the nine ISSRs primers. Each primer generated between 7 to 12 bands (Fig. 23) corresponding to an average of 10,5 bands per primers. The Simpson's index, calculated for each set of data, ranged, from 0.91 to 0.96. Moreover, the polymorphic information content was estimated for each locus and those which had a PIC lower than 0.25 were not included (10 for AFLPs loci and 7 for ISSRs) in the subsequent cluster and principal component analyses.

Fig. 22. DNA banding patterns of the AFLP primer combination EcoAC+MseGA. Each lane corresponds to an isolates. A, T, AR represent the reference isolates BMP0269, BMP0304 and BMP0308 (*A. alternata*, *A. tenuissima* and *A. arborescens* respectively). Electrophoresis was performed on an 4.5 acrylimude gel colored according the silver stain protocol (Bassam, 1993).

Fig. 23. Examples of DNA banding patterns from two ISSR PCR amplifications on the tested isolates. Samples were loaded twice and the belonging species group is reported on the pictures (A=*A. alternata*, T= *A. tenuissima*, AR= *A. arborescens*).

Tab.7. Molecular data for each primer type.

DATA ANALYSIS

The AMOVA (Excoffier et al., 1992), performed on the two sets of data, comparing the genetic diversity among the groups defined by the cluster analysis, showed that molecular variance was higher within the groups than among them (55% vs 45% for the

AFLP dataset and 61% vs 39% for the ISSR data set). Genetic differentiation index (Φ_{PT}) and the Nei's genetic distance (D) were calculated for each pairwise comparison between the groups (tables 8 a-b). For each cluster, the percentage of polymorphic bands, the number of locally common bands and the number of private bands (bands found exclusively in one group) were calculated (Tab.9).

Trees obtained for the two data sets, analyzed with the UPGMA based on Dice's similarity index, are shown in figure 24a and figure 24b. Both trees were able to discriminate the *A. arborescens* group as a well distinct cluster. Furthermore, the AFLP split the remaining strains in two clusters: *A. alternata* – *A. tenuissima* and the *A.tenuissima* cluster.

A high value of correlation, $r= 0.82$, between the two matrices was found (Mantel test with 5000 random permutations. $P >0.05$) showing a concordance of the molecular data obtained with the two different techniques.

The Principal Component Analysis (Fig. 25a , Fig. 25b) was used to describe the variability among the 51 strains of *Alternaria* spp. and to determine if there were hidden factors which influenced the distribution of the samples. PCA of AFLP accounted for 52.11% of the observed variance with the first three components. In contrast, for the ISSR data set the component values were lower and four components were required to explained 51.81% of the total variance. For both markers *A. arborescens* resulted a separated group and no others grouping that correspond to geographical areas or pathogenic capability could be found in this analysis. The relationships among and within the groups obtained with the PCA were concordant with the ones produced by the cluster analysis.

Tab. 8a. Differentiation index (Φ_{PT}) and Nei's genetic distance (D) calculated among the groups defined by AFLP cluster analysis.

*All values are significant with $P < 0.05$, P-values were computed based on a simulation of 999 permutations.

Tab. 8b. Differentiation index (Φ_{PT}) and Nei's genetic distance (D) calculated among the groups defined by ISSR cluster analysis.

*Value is significant with $P < 0.05$, P-value was computed based on a simulation of 999 permutations.

Tab.9a Summary of AFLP band that characterize the different groups defined by the cluster analysis.

Tab.9b. Summary of ISSR bands that characterize the different groups defined by the cluster analysis.

Fig. 24a-b. Cluster analysis, based on Dice's similarity coefficient, of AFLP (a) data set and ISSR data set (b). Thick bars represent the belonging species-group. Δ identifies the alternata isolates; ++ indicate positivity to at least two of the three type of bioassays, +++ positivity to all the bioassays.

Fig. 25a-b Distributions of \square *Alternaria alternata*, + *Alternaria arborescens*, and \times *Alternaria arborescens* strains computed by PCA, based on the correlation matrices of AFLP (a) and ISSR (b). The legend indicates the correspondence code number/strain.

5.7. AMT specific primers PCR

Nine of the 44 single spore isolates, tested with the Johnson's specific primers, produced an ambiguous result. Some of them (Tab. 10, Fig. 26) were characterized by a really faint band of the same molecular weight as those given by the "*A. mali*" reference strains AKI3, IF08984 and M71 (fragments size 500bp), while no amplicon was detected in the rest of isolates or in the reference strain EGS038-29 (*A. mali* strains used by Simmons in morphological characterization of this species, described in Mycotaxon 1999). Amplified products were employed as template in a second PCR to increase the yield. The sequenced retrieved were compared with those available in the online database provided by National Center for Biotechnology Information using the Blast search program. Sequences were affiliated with *A. alternata mali* pathotype (GeneBank accession no. AF184074) at 99% of similarity. Differences present consisted of single nucleotides on the third codon position, and they were silent transitions. The positive strains did not belong exclusively to any

unique species, did not come from a specific geographical location and exhibited different grades of pathogenicity (Tab.10).

Fig. 26. Products obtained from PCR with specific primers for AM-toxin gene. Electrophoresis was performed on 1% agarose gel stained with ethidium bromide.

Tab. 10. Summary of the principal traits of the strains which produced the faint band with the specific Johnson's primers.

*Sp. WL: Spore suspension on wounded leaf, Sp.UL: Spore suspension on unwounded leaf, Fil. WL: filtrate on wounded leaf.

For a preliminary investigation of the possible causes laid behind the weak PCR signal, new primer sets were designed downstream and upstream of the original primer sites. The sequencing, of these new PCR products showed that no differences were present in the original annealing sites of the primers designed by Johnson and collaborators. Consequently the hypothesis of faint band productions due to a mismatching was rejected. Subsequently these sequences were queried against a private on line *Alternaria* genome database (courtesy of Prof. B.M. Pryor and affiliates) using BLAST algorithm to look for significant sequences similarities. No significant matches were identified among any of the sequenced strains, suggesting the gene was not present in the sequences strains (personal communication).

6. Discussion and conclusion

The present study is the first work characterizing the pathogen *Alternaria* spp. on apple trees conducted in Italy. As previously mentioned, the severe damages incurred by orchards in the north of Italy, afflicted by a formerly uncharacterized disease, have pushed scientists to more deeply investigate the diversity and spatial distribution of *Alternaria* spp. isolated from these diseased trees and to link these characteristics to their pathogenic capability. All data are reported in table 11.

Morphological characterization

Within the genus *Alternaria*, species are usually defined based on conidial characteristics. The high variability of the morphological characters, due to intrinsic factors and environmental conditions, led to the definition of several main subgroups and to date there is not any concordant classification (Thomma, 2003) able to combine both morphological characters and molecular traits into unique groups. It is well documented that a morphologically distinct group of small-spored catenulate *Alternaria*, defined broadly as a species complex due to the high variability in these morphological characters from a number of different host association (Roberts et al., 2000, Pryor and Michailides, 2002, Hong et al., 2006 and Andrew et al., 2009).

It was immediately evident after a morphological investigation of the 44 Italian strains that there was a high degree of variability in morphology, even after culturing under standardized growth conditions. The precise definition of the parameters used to investigate the sporulation patterns and the colony morphologies of the isolates gave reproducible results in agreement with the features delineated by previous systematic works, as in Pryor and Michailides's study (2002) on pistachio *Alternaria* strains and in Hong et al.'s study (2006) on hazelnut and walnut *Alternaria* spp. The identification key utilized identifies distinctive conidia and spore apparatus traits used to ascribe each isolate to a defined morpho-species group. The cluster analysis (Euclidian distance similarity) performed on the

morphology key scores assigned to each sporulation characteristic, clearly showed three groups that correlated very well with the representative isolates (previously typified by Prof. Pryor in his work). Hence, according to the sporulation habits, it was possible to achieved distinct taxonomically significant groups. Colony morphology descriptions also produced an analogous division, but the categories were not used in clustering analysis due to the inherently subjective nature of the data. Moreover, there appeared to be no discernable pattern among the isolates, both for sporulation habit and the colony morphology, associated with pathogenesis resulting in pathogenic strains resulted spread among the three groups. Similarly the *Alternaria mali* reference isolates that did not show particular morphologic traits and did not define a unique group. Culture conditions (medium, light intensity and photoperiod) were demonstrated to have a primary role (Pryor and Michailides, 2002 and Hong et al., 2006) in the reproducibility of all morphological traits used to assess the diversity study of the species in this study.

Pathogenicity bioassays

In vitro bioassays were conducted to discern if there were differences in pathogenicity capability among isolates. The use of different plant material (fruit and leaf) differently treated (wounded and unwounded) has immediately highlighted variability in the production of symptoms correlated with both tissue type and treatment. The presence of artificial lesions proved to be significant in lesion development, while unwounded material showed little substantial lesion development (especially on fruit). Tests on leaf tissues seemed to be the most reproducible and reliable with good percentage of symptoms produced for all the bioassays (70% of positive lesion development for spore suspension inoculation on wounded tissues and 30% on unwounded tissues, while for the filtrate the percentage were 45% and 25% respectively). One reason for this could be that *Alternaria* species are usually foliar pathogens that cause relatively slow destruction of host tissues (Rotem, 1994). Rotem also

reported that tissues that are weakened due to stresses, senescence, or wounding are more susceptible to the fungal infection.

Based on the results from these bioassays, single spore isolations of select strains were conducted. The pathogenic investigation of these 44 strains showed that there was no significant correlation of the pathogenicity with either the morphological groupings or the genetics clustering (based on the molecular investigations, see below). Indeed, even if similar results were obtained in previous work (Pryor and Michailides, 2002 and Andrew et al., 2009), the inability to clearly define relationships among morphology, genetic haplotypes and pathogenicity could be due to the unbalanced number of isolates among the three different taxa which could skew the representativeness of the present sample. Future characterizations of this system could benefit from a more even sampling from all the three species-groups.

Molecular characterization

An important feature of this study has been that the molecular characterization was performed combining and comparing different data sets obtained from distinct molecular approach: 1) investigation of specific loci and 2) fingerprinting based on diverse randomly selected polymorphic sites of the genome. However even with the use of this multi-method approach to molecular characterization of the isolates, the results of this study mirrored previous work (Pryor and Michailides, 2002, Serdani et al., 2002, Peever et al. 2004, Hong et al. 2006, Andrew et al., 2009) in its ability to clearly differentiate the species-groups found in this study. As reported in literature small-spored *Alternaria* taxonomy is complicated due to the inability to resolve evolutionary relationships the taxa because of the lack of variability in the markers commonly used in fungi systematic, especially for protein coding genes (Peever et al. 2004, Hong et al., 2006 and Andrew et al., 2009). Sequencing of a portion of the EndoPg gene and the three anonymous OPA regions together provided the necessary variation to establish the phylogenetic relationships among the Italian isolates of

Alternaria spp. On Italian strains these markers showed a variable number of informative sites (ranging from 7 for EndoPg to 85 for OPA1-3) and the parsimony analysis produced different tree topologies. Even so, all four markers were able to distinguish the *A. arborescens* as a phylogenetically distinct clade while not resolving the taxonomic relationship between *A. alternata* and *A. tenuissima* (Serdani et al., 2002, Peever et al., 2004 and Andrew et al., 2009). This work provides further support for a hypothesis which poses that the inability to separate these species-groups reliably is like due to a recent divergence of these taxa, with incomplete lineage sorting, or that these groups, even though recognized as separate species, are still diverging.

Fingerprinting analysis (ISSR and AFLP) led to the same result: a monophyletic *A. arborescens* clade and one clade containing both *A. tenuissima* and the *A. alternata* strains. The estimates of the genetic distance computed supported the evidence that *Alternaria arborescens* is a well defined clade. A Mantel test assessed the strength of the correlation between the AFLP and ISSR data with similar grouping found by the two technique and found to be quite strong. The AMOVA highlighted that the percentage of molecular variance was higher within the groups than among the groups confirming the existence of diverse haplotypes within the same lineage. The high variability within a group was also observed for the single locus analyses and this explains why clades were not always supported by strong bootstrap values.

This first attempt to characterize Italian *Alternaria* species recovered from apple produced concordant results with what was already described in a similar phylogenetic study on pistachio (Pryor and Michaelides, 2002), on walnut and hazelnut (Hong et al., 2006), apple (Serdani et al., 2002) and citrus (Peever et al., 2004). Together with these studies, this research demonstrates that the three morphological groups are widely distributed and occupy similar ecological niches. Furthermore, this research suggest that these *Alternaria* species exhibit a similar infection pattern despite the taxonomic and pathogenic differences.

Pathogenicity correlated to the molecular data

Another aim of this study was to differentiate the pathogenic strain on the basis of molecular profiles. Attainment of this goal would have the most relevant implications concerning the practical management and control of the disease. In fact, the molecular characterization of the pathogen is a fundamental step to understanding the disease that is spreading in the apple orchards of North Italy. At the beginning, the causal agent was considered as *Alternaria alternata* (Marshall and Bertagnoll, 2006). The preliminary studies purposed a pathogenic system related to the synthesis of toxins. Experimental data of our bioassays suggest an analogous hypothesis. In fact, symptoms could be induced after inoculating plant material with solely the filtrate from pathogenic strains. A confirmation could come by investigating the chemical composition of the filtrate, using a different technique as HPLC or mass spectroscopy for the AM-toxin I and other toxic secondary metabolites. The studying of secondary metabolites profile has proved to be an informative method for highlighting differences among small-spored *Alternaria* where the molecular and morphological characterizations have failed (Andersen et al., 2002 and Andersen et al., 2006).

Moreover, some positive PCR reactions using AM-toxin gene specific primers, designed for identification of apple infecting *Alternaria* pathovar, support the hypothesis that a host specific toxin (toxins) could be involved. It remains an intriguing challenge to discover whether or not the agent of the “Italian disease” is the same of the one previously typified as *Alternaria mali*, casual agent of the apple blotch disease.

A number of important conclusions were derived from this research. From this study, it has emerged that the ability to be pathogenic on apple is not an exclusive trait of a specific morpho-group or phylogenetic clade, but this capability is owned by strains independently. Studies on the molecular basis of HS-toxin synthesis in *Alternaria* spp. (Akamatsu et al. 1999) have demonstrated that the genes involved in the AM- toxin host

specific interaction are clustered on a conditionally dispensable chromosome (CDC). It is also accepted that horizontal transmission of these chromosome among closely related fungi can occur (Akagi, 2009). It is possible that these closely related species are sharing this CD chromosome, or have done so in the past. This idea is further supported by the fact that the reference *Alternaria mali* isolates employed in the present study belonged to different species-groups; specifically, the M71 strain showed a typical *arborescens* sporulation pattern and a *tenuissima* colony morphology while the other three strains were comparable to the *A. tenuissima* reference strain. These strains also differed according to the molecular analysis. While it was not the specific goal to examine the distribution of the AMT gene throughout small-spored *Alternaria* spp., its possible presence among several species presents an interesting future research topic. These incongruences also stress a need to further investigate the possibility of horizontal CD-chromosome transfer among these closely related isolates. Different species names such as *Alternaria mali* Roberts and *A. alternata* (Fries.) Keissler have been tied to the Alternaria blotch, but the presence of the host specific toxin seems to be one of the common factors for symptoms development in susceptible apple cultivars. Consequently, in a diagnostics scope, this research points out that it is not possible to strictly define a single species as the causal agent of the Italian Alternaria leaf spot. This work is a helpful starting point for further investigations. Combining these results with the results of other studies examining different aspects of the disease (expression study profile, biochemical characterization, host susceptibility) can strongly define the relationships between the Italian *Alternaria* populations and the *Alternaria alternata mali* pathotype and their capability to produce toxic secondary metabolites (specific or not).

Tab. 11. Summary of the traits obtained for each single spore isolate from the morphological and molecular characterization and from the pathogenicity bioassays.

T= tenuissima, AR= arborescens, AT= alternata-tenuissima, numbers after letters refer to the belonging clade defined by parsimony analysis or cluster analysis. + = positive to the AMT PCR, S. susp. W= spore suspension on wounded leaf, S. susp. Uw= spore suspension on unwounded leaf.

7. References

- Abbas, H.K., Tanaka, T., Duke, S.O., Porter, J.K., Wray, E.M., Hodges, L., Sessions, A.E., Wang, E., Merrill, A.H. Jr, and Riley, R.T. 1994. Fumonisin- and AAL-toxin-induced disruption of sphingolipid metabolism with accumulation of free sphingoid bases. *Plant Physiology* 106:1085–1093.
- Agarwal, A., Garg, G.K., Devi, S., Mishra, D.P., and Singh, U.S., 1997. Ultrastructural changes in *Brassica* leaves caused by *Alternaria brassicae* and destruxin. *Journal of Plant Biochemistry and Biotechnology* 6:25–28.
- Agrios, G.N., 2005. *Plant Pathology* 4th edition, Elsevier Academic Press, San Diego, CA.
- Akagi Y., Akamatsu, H., Otani, H., and Kodama, M. 2009 Horizontal Chromosome Transfer, a Mechanism for the Evolution and Differentiation of a Plant-Pathogenic Fungus. *Eukaryotic Cell* 8(11):1732-1738,
- Akamatsu, H., Itoh, Y., Kodama, M., Otani, H., and Kohmoto, K. 1997. AAL-toxin-deficient mutants of *Alternaria alternata* tomato pathotype by restriction enzyme-mediated integration. *Phytopathology*, 87:967–972.
- Akamatsu, H., Taga, M., Kodama, M., Johnson, R., Otani, H. and Kohmoto, K. 1999. Molecular karyotypes for *Alternaria* plant pathogens known to produce host-specific toxins. *Current Genetics* 35:647–656.
- Akimitsu, K., Kohmoto, K., Otani, H., and Nishimura, S. 1989. Host-specific effects of toxin from the rough lemon pathotype of *Alternaria alternata* on mitochondria. *Plant Physiology* 89:925–931.
- Allen, S.J., Brown, J.F., and Kochman, J.K, 1983. The infection process, sporulation and survival of *Alternaria helianthi* on sunflower. *Annals of Applied Biology* 102: 413-419.
- Andersen, B., Krøger, E., and Roberts, R.G. 2002. Chemical and morphological segregation of *Alternaria arborescens*, *A. infectoria* and *A. tenuissima* species-groups. *Mycological Research*: 106(2):170-182.
- Anderson, B., Kroger, E., and Roberts, R.G. 2002. Chemical and morphological segregation of *Alternaria arborescens*, *A. infectoria* and *A. tenuissima* species-groups. *Mycological Research* 106:170-182.
- Anderson, B., Smedsgaard J., Jørring, I., Skouboe P., and Pedersen, L.H. 2006. Real-time PCR quantification of the AM-toxin gene and HPLC qualification of toxigenic metabolites from *Alternaria* species from apples. *International Journal of Food Mycology* 111:105-111.
- Andrew, M., Peever, T.L., and B.M., Pryor, 2009 An expanded multilocus phylogeny does not resolve morphological species within the small-spored *Alternaria* species complex. *Mycologia*:101(1):95-109.
- Antoniacci L., and Montuschi, C. 2006. Ecco come si riconosce la filloptosi del melo. *Agricoltura*:7/8:78-79.

- Aradhya, M. K., Chan, H. M., and Parfitt, D.E. 2001. Genetic variability in the pistachio late blight fungus, *Alternaria alternata*. *Mycological Research* 105:300-306.
- Avise, J.C., 1994. Molecular markers, natural history and evolution. Chapman and Hall, New York
- Bansal, V.K., G. Seguin-Swartz, G.F.W. Rakow, and Petrie, G.A.. 1990. Reaction of *Brassica* species to infection by *Alternaria brassicae*. *Canadian Journal of Plant Science* 70:1159-1162.
- Bassam, B. J., and Caetano-Allones, G. 1993. Silver staining of DNA in polyacrylamide gels. *Applied Biochemistry and Biotechnology* 42(2-3):181-188.
- Bayon, C., Pei, M.H., and Riuz, C. 2007. Genetic structure and spatial distribution of the mycoparasite *Sphaerellopsis filum* on *Melampsora larici-epitea* in a short-rotation coppice willow planting. *Plant Pathology* 56:616-623.
- Bayraktar, H, Dolar F.S., and Maden S. 2008. Use of RAPD and ISSR markers in detection of genetic variation and population structure among *Fusarium oxysporum* f. sp. *ciceris* isolates on chickpea in Turkey. *Journal of Phytopathology* 156:146-154
- Bell, A. A., and Wheeler, M. H. 1986. Biosynthesis and function of fungal melanins. *Annual Review of Phytopathology* 24:411-451.
- Berto, P., Belingheri, L., and Dehorter, B. 1997. Production and purification of a novel extracellular lipase from *Alternaria brassicicola*. *Biotechnology Letters* 19:533-536.
- Bock, K.R., 1964. Purple blotch (*Alternaria porri*) of onion in Kenya. *Ann. of Applied Biology* 54:303-311.
- Brown, A.E., Muthumeenakshi, S., Sreenivasaprasad, S., Mills, P.R., and Swinburne, T.R. 1993. A PCR primer-specific to *Cylindrocarpon heteronema* for detection of the pathogen in apple wood. *FEMS Microbiology Letters* 15:117-120.
- Bulajic, A., Filajdic, N., Babovic, M., and Sutton, T.B. (1996) First report of *Alternaria mali* on apples in Yugoslavia. *Plant Disease*: 80:709.
- Campbell, R., 1970. An electron microscope study of exogenously dormant spores, spore germination, hyphae and conidiophores of *Alternaria brassicicola*. *New Phytologist* 69(2):287-293.
- Chadha, S., and Gopalakrishna, T. 2007. Comparative assessment of REMAP and ISSR marker assays for genetic polymorphism studies in *Magnaporthe grisea*. *Current Science* 93:688-692.
- Choi, T.J., Chung, H.S., and Lee, Y. W. 1989. Occurrence of tomato stem canker in Korea and phytotoxin production by the pathogen. *Korean J. Plant Pathology* 5:32-39.
- Conn, K.L., and Tewari, J.P. 1989. Interactions of *Alternaria brassicae* conidia with leaf epicuticular wax of canola. *Mycological Research* 93(2):240-242
- Covert, S.F. 1998. Supernumerary chromosomes in filamentous fungi. *Current Genetics* 33:311-319.

- Dice, L.R. 1945. Measures of the Amount of Ecologic Association Between Species. *Ecology* 26(3): 297-302.
- Douglas, D.R. 1972 The effect of light and temperature on the sporulation of different *Alternaria solani*. *Can. J.Bot.* 50:629-634.
early blight of tomato. *Indian Phytopathology* 60: 180-187.
- Elliot, J. 1917. Taxonomic characters of the genera *Alternaria* and *Macrosporium*. *American Journal of Botany* 4:439-476.
- Ellis, M. B. 1971. Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew, New England.
- Ellis, M. B. 1976. More Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew, New England. *Enzymology* 266: 427-449.
- Eshel, D., Ben-Arie, R., Dinoor, A., and Prusky, D., 2000. Resistance of gibberellin-treated persimmon fruit to *Alternaria alternata* arises from the reduced ability of the fungus to produce endo-1,4- β -glucanase. *Phytopathology*. 90: 1256–1262
- Eshel, D., Lichter, A., Dinoor, A., and Prusky, D. 2002a. Characterization of *Alternaria alternata* glucanase genes expressed during infection of resistant and susceptible persimmon fruits. *Mol. Plant Pathology* 3: 347–358.
- Eshel, D., Miyara, I., Ailing, T., Dinoor, A., and Prusky, D. 2002b. pH regulates endoglucanase expression and virulence of *Alternaria alternata* in persimmon fruit. *Molecular Plant-Microbe Interaction* 15:774–779.
- Everts, K.L. and Lacy, M.L. 1990 the influence of dew duration, relative umidity, and leaf senescence on conidial formation and infection of onion by *Alternaria porri*. *Phytopathology* 80:1203-1207.
- Excoffier, L., Smouse, P.E., and Quattro, J. M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131:479-491.
- Fan, C.-Y. and Köller, W. 1998. Diversity of cutinases from plant pathogenic fungi: differential and sequential expression of cutinolytic esterases by *Alternaria brassicicola*. *FEMS Microbiology Letters* 158:33–38.
- Farr, D.F.; Bills, G.F.; Chamuris, G.P., and Rossman, A.Y. 1989. *Fungi on plants and plant products in the United States*. American Phytopathological Society, St. Paul, USA.
- Feng, D.F., and Doolittle, R.F. 1987. Progressive sequence alignment as a prerequisite to correct phylogenetic trees. *Journal of Molecular Evolution* 25:351-360.
- Filajdic, N. and Sutton, T.B. 1991. Identification and distribution of *Alternaria mali* on apples in North Carolina and susceptibility of different varieties of apples to alternaria blotch. *Plant Disease* 75:1045-1048.
- Frisvad, J.C. 1983 A selective and indicative medium for groups of *Penicillium viridicatum* producing different mycotoxins on cereals. *Journal of Applied Bacteriology* 54: 409–416.

- Fu, Y.X., and Li, W.H.. 1993. Statistical tests of neutrality of mutations. *Genetics* 133:693–709.
- Fujiwara, T., Oda, K., Yokota, S., Takatsuki, A., and Ikehara, Y. 1988. Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *The Journal of Biochemical Chemistry* 263:18545–18552.
- Fulton, C.E., and Brown, A.E. 1997. Use of SSU rDNA group-I intron to distinguish *Monilinia fructicola* from *M. laxa* and *M. fructigena*. *FEMS Microbiol. Lett.* 157:307-312.
- Gannibal, P.B., Klemsdal, S.S., and Levitin, M.L. 2007. AFLP analysis of Russian *Alternaria tenuissima* populations from wheat kernels and other hosts *European Journal of Plant Pathology.* 119(2):175-182.
- Gobber, M., Mattedi, L., Forno, F., Cappello, S., Piva, U., Marschall, K., and Rizzolli, W. 2004. Nuova malattia del melo causata dal fungo *Alternaria alternata*. *Terra Trentina* 6: 31-36.
- Godwin, I.D., Aitken, A.B., and Smith, L.W. 1997. Application of inter simple sequence repeat (ISSR) markers to plant genetics. *Electrophoresis* 18:1524-1528
- Grogan, R.G., Kimble, K. A., and Misaghi, I. 1975. A stem canker disease of tomato caused by *Alternaria alternata* f.sp. *lycopersici*. *Phytopatology* 65:880-886.
- Gupta, M., Chyi, Y-S., Romero-Severson, J. and Owen, J.L. 1994. Amplification of DNA markers from evolutionary diverse genomes using single primers of simple sequence repeats. *Theoretical and Applied Genetics* 89: 998-1006.
- Halldén, C., Hansen, M., Nilsson, N-O. and Hjerdin, A. 1996. Competition as a source of errors in RAPD analysis. *Theoretical and Applied Genetics* 93:1185–1192.
- Hammer, Ø., Harpe, D.A.T., and Ryan, P. D. 2001. PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontologia Electronica* 4:9. http://palaeo-electronica.org/2001_1/past/issue1_01.htm.
- Hansen, M., Halldén, C., and Säll, T. 1998. Error rates and polymorphism frequencies for three RAPD protocols. *Plant Mol Biol Rep.* 16:139-146.
- Hartill, W.F.T. 1968. Brown spot of tobacco. *World Crops* 20:54-59.
- Hatta, R., Ito, K., Hosaki, Y., Tanaka, T., Tanaka, A., Yamamoto, M., Akimitsu, K., and Tsuge, T. 2002. A conditionally dispensable chromosome controls host-specific pathogenicity in the fungal plant pathogen *Alternaria alternata*. *Genetics* 161:59–70.
- Hatzipapas, P., Kalosak, K., Dara, A., and Christias C. 2002. Spore germination and appressorium formation in the entomopathogenic *Alternaria alternata*. *Mycological Research* 106(11):1348-1359.
- Haug, R. and Levy, Y. 1994. Aggressiveness of *Alternaria brassicicola* to Broccoli. *Phytoparasitica* 22(2):109-114.
- Hayami, C., Otani, H., Nishimura, S., and Kohmoto, K. 1982. Induced resistance in pear leaves by spore germination fluids of nonpathogens to *Alternaria alternata* Japanese pear

- pathotype and suppression of the induction by AK-toxin. *Journal of the Faculty of Agriculture Tottori Univ.* 17:9.
- He, C., Rusu, A.G., Poplawski, A.M., Irwin, J.A. and Manners, J.M. 1998. Transfer of a supernumerary chromosome between vegetatively incompatible biotypes of the fungus *Colletotrichum gloeosporioides*. *Genetics*. 150:1459–1466.
- Higgins, D.G., and Sharp, P.M. 1988. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. *Gene* 73:237-244.
- Hong, G.S., and Pryor, B.M. 2004. Development of selective media for the isolation and enumeration of *Alternaria* species from soil and plant debris. *Canadian Journal of Microbiology* 50:461-468.
- Hong, H., Maccaroni, M., Figuli, P.J., Pryor, B. M., and Bellisario, A. 2006 Polyphasic classification of *Alternaria* isolated from hazelnut and walnut fruit in Europe. *Mycological Research* 110(11):1290-41. *Compendium of potato diseases*. Aps press, usa, 125 pp.
- Hong, S.G., Liu, D., Pryor B.M. 2005. Restriction mapping of the IGS region in *Alternaria* spp. reveals variable and conserved domains *Mycological Research* 109(1):87–5.
- Howard, R.J., and Valent, B. 1996. Breaking and entering: host penetration by the fungal rice blast pathogen *Magnaporthe grisea*. *Annual Review of Microbiology* 50:491–512.
- Humpherson-Jones, F.M. 1989. Survival of *Alternaria brassicae* and *Alternaria brassicicola* on crop debris of oilseed rape and cabbage. *Annals of Applied Biology* 115:45-50.
- Huys, G., Coopman, R., Janssen, P., and Kersters, K. 1996. High-resolution genotypic analysis of the genus *Aeromonas* by AFLP fingerprinting. *International Journal of Systematic Bacteriology* 46, 572–580.
- Isshiki, A., Akimitsu, K., Yamamoto, M., and Yamamoto, H. 2001. Endopolygalacturonase is essential for citrus black rot caused by *Alternaria citri* but not brown spot caused by *Alternaria alternata*. *Molecular Plant-Microbe Interaction* 4: 749–757.
- Janssen, P., Maquelin, K., Coopman, R., Tjernberg, I., Bouvet, P., Kersters, K., and Dijkshoorn, L. 1997. Discrimination of *Acinetobacter* genomic species by AFLP fingerprinting, *International Journal of Systematic Bacteriology* 47:1179–1187
- Johnson, L.J., Johnson, R.D., Akamatsu, H., Salamiah, A., Otani, H., Kohmoto, K., and Kodama, M. 2001. Spontaneous loss of a conditionally dispensable chromosome from the *Alternaria alternata* apple pathotype leads to loss of toxin production and pathogenicity. *Current Genetics* 40:65–72.
- Johnson, R.D., Johnson, L., Itoh, Y., Kodama, M., Otani, H., and Kohmoto, K. 2000. Cloning and characterization of a cyclic peptide synthetase gene from *Alternaria alternata* apple pathotype whose product is involved in AM-toxin synthesis and pathogenicity. *Molecular Plant-Microbe Interaction* 13:742–753.
- Johnson, R.D., Johnson, L., Kohmoto, K., Otani, H., Lane, C.R., and Kodama, M. 2000b. A Polymerase Chain Reaction-Based Method to Specifically Detect *Alternaria alternata* Apple Pathotype (*A. mali*), the Causal Agent of Alternaria Blotch of Apple. *Phytopathology* 90(9):973-6.

- Jolliffe, I.T. 1986. Principal Component Analysis. Springer-Verlag. pp.487.
- Joly, P. 1964. Le Genre *Alternaria*. Recherches Physiologiques, Biologiques, et Systematiques. P. Lechevalier, Paris.
- Kang, H.W., Lee, B.R., and Yu, S.H. 2003. Analysis of Genetic Relatedness in *Alternaria* species Producing Host Specific Toxins by PCR Polymorphism. Plant Pathology Journal 19(5):221-226.
- Kawamura, C., Moriwaki, J., Kimura, N., Fujita, Y., Fuji, S., Hirano, T., Koizimi, S., and Tsuge, T. 1997 The melanin biosynthesis genes of *Alternaria alternata* can restore pathogenicity of the melanin deficient mutants of *Magnaporthe grisea*. Molecular Plant-Microbe Interaction 10:446-453.
- Keller N. P., and Hohn, T. M. 1997. Metabolic pathway gene clusters in filamentous fungi. Fungal Genetic Biology 21:17-29.
- Khare, U.K., and Nema, K.G. 1981 Studies on purple blotch of onion – Sporulation on host and dispersal of conidia. Indian Phytopathology 34:214-218.
- Kimura, N., and Tsuge, T. 1993. Gene cluster involved in melanin biosynthesis in the filamentous fungus *Alternaria alternata*. Journal of Bacteriology. 175:4427-4435.
- Kirk, M. P., Cannon, P.F., Minter, D. W., Stalpers, J.A. 2008 Dictionary of the Fungi. Edited by P M Kirk, International Mycological Institute, Egham, UK, P F Cannon, CABI, UK, J A Stalpers, CBS, The Netherlands
- Kishore, P., Singh, V.S. and Gandhi, S.K. 2007. Variability among *Alternaria solani* isolates causing early blight of tomato. Indian Phytopathology 60:180-187.
- Kohmoto, K., and Otani, H. 1991. Host recognition by toxigenic plant pathogens. Experientia 47:755-764.
- Kohmoto, K., Taniguchi, T., and Nishimura, S. 1974. Correlation between the susceptibility of apple cultivars to *Alternaria mali* and their sensitivity to AM-toxin I. Ann. Phytopathol. Soc. Jpn. 43:65-66.
- Kothera, R.T., Keinath, A.P., Dean, R.A. and Farnham, M.W. 2003. AFLP analysis of a worldwide collection of *Didymella bryoniae*. Mycological Research 107:297-304.
- Kuninaga, S., and Yokusawa, R. 1987. Studies on the taxonomy of plant pathogenic fungi by a comparison of DNA homology. I. Genetic relatedness among species in the genus *Alternaria*. (In Japanese.) Ann. Phytopathol. Soc. Jpn. 53:368-369.
- Kusaba, M. and Tsuge, T. 1994. Nuclear ribosomal DNA variation and pathogenic specialization in *Alternaria* fungi known to produce host-specific toxins. Applied and Environmental Microbiology 60:3055–3062.
- Kusaba, M. and Tsuge, T. 1997. Mitochondrial DNA variation in host-specific toxin-producing pathogens in the genus *Alternaria*. Ann. Phytopathol. Soc. Jpn. 63:463–469.
- Kusaba, M., and Tsuge, T. 1995. Phylogeny of *Alternaria* fungi known to produce host-specific toxins on the basis of variation in internal transcribed spacers of ribosomal DNA. Current Genetics. 28:491-498.

- Laech, C.M., 1967. Interaction of the near-ultraviolet light and temperature on sporulation of the fungi *Alternaria*, *Cercospora*, *Fusarium*, *Helminthosporium*, and *Stemphylium*. Canadian Journal of Botany 45:1999-2016.
- Lucas, G.B. 1971. I *Alternaria alternata* (Fries) Keissler, the correct name for *A. tenuis* and *A. longipes*. Tobacco Science 15:229-243.
- Maddison, W. P., and Maddison, D. R. 1992. MacClade: Interactive analysis of phylogeny and character evolution. Sinauer Assoc, Sunderland, Mass.
- Maddison, W.P., and Maddison, D.R. 2009. Mesquite: a modular system for evolutionary analysis. Version 2.72 <http://mesquiteproject.org>
- Majer, D., Lewis, B.G., and Mithen, R. 1998. Genetic variation among field isolates of *Pyrenopeziza brassicae*. Plant Pathology 47:22-28.
- Majer, D., Mithen, R., Lewis, B.G., Vos, P., Oliver, R.P. 1996. The use of AFLP fingerprinting for the detection of genetic variation in fungi. Mycological Research 100:1107-1111.
- Malathrakis, N.E. 1983. *Alternaria* stem canker of tomato in Greece. Phytopathologia Mediterranea 22:33-38.
- Mantel, N. 1967. The detection of disease clustering and a generalized regression approach". Cancer Research 27(2) 209-220.
- Markham, J.E. and Hille, J. 2001. Host-selective toxins as agents of cell death in plant-fungus interactions. Molecular Plant Pathology 2: 229-239.
- Marschall, K., Rizzolli, W., and Bradlwarter, M. 2004. La lenticellosi da *Alternaria*-la difesa. Frutta e Vite 3:85-87.
- Marschall, K., and Bertagnoll, M. 2006. Patotipo di *Alternaria alternata* agente di maculatura lenticellare su frutti e di necrosi fogliari su melo in Alto Adige. Atti Giornate Fitopatologiche(2):93-96.
- Masunaka, A., Tanaka, A., Tsuge, T., Peever, T.L., Timmer, L.W., Yamamoto, M., Yamamoto, H., and Akimitsu, K. 2000. Distribution and characterization of AKT homologs in the tangerine pathotype of *Alternaria alternata*. Phytopathology. 90:62-768.
- Meng, X. and Chen, W. 2001. Applications of AFLP and ISSR techniques in detecting genetic diversity in the soybean brown stem rot pathogen *Phialophora gregat.a* Mycological Research Volume 105(8):936-940.
- Meronuck, R.A., Steele, J.A., Mirocha, C.J., and Christensen, C.M. 1972. Tenuazonic acid, a toxin produced by *Alternaria alternata*. Applied Microbiology 23:613-617.
- Meyer, W., Mitchell, T.G., Freedman, E.Z., and Vilgalys, R. 1993. Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*. Journal of Clinical Microbiology 31: 2274-2280.

- Misaghi, J., Grogan R.G., and Duniway, J.M. 1978. Influence of Environment and Culture Media on Spore Morphology of *Alternaria alternata*. *Phytopathology* 68:29-34.
- Moore, W.D. 1943. Some cultural practices that increment the development of *Alternaria solani* on tomato seedlings. *Phytopathology* 33:1176-84.
- Moore, W.D., and Thomas, H. R. 1942. Some factors affecting of on tomato seedlings. By *Alternaria solani* *Phytopathology* 32:399-403.
- Mount, D.W. 2001. Phylogenetic prediction. *Bioinformatics*, chap. 6, pp. 237-280.
- Neergaard, P. 1945. Danish Species of *Alternaria* and *Stemphylium*. Oxford university Press, London.
- Nei, M. 1972. Genetic distance between populations. *American Naturalist* 106:283-292.
- Niessen, M.L., and Vogel, R.F. 1998. Group specific PCR-detection of potential trichothecene-producing *Fusarium*-species in u.re cultures and cereal samples. *Systematic and Applied. Microbiology* 21:618-631.
- Nishimura, S. 1980. Host-specific toxins from *Alternaria alternata*: problems and prospects. *Proceedings of the. Japan Academy* 56(B):362-366.
- Nishimura, S. and Kohmoto, K. 1983. Host-specific toxins and chemical structures from *Alternaria* species. *Annual Review of. Phytopathology* 21:87–116.
- Nishimura, S., and Kohmoto, K. 1983. Host-specific toxins and chemical structures from *Alternaria* species. *Annual Review of Phytopathology* 21:87-116.
- Nishimura, S., Kohmoto, K., Otani, H., Ramachandran, P., and Tamura, F. 1982. Pathological and epidemiological aspects of *Alternaria alternata* infection depending on a host-specific toxin. Pages 199-214 in: *Plant Infection: The Physiological and Biochemical Basis*. A. Asada, W. R. Bushnell, S. Ouchi, and C. P. Vance, eds. Japan Scientific Societies Press, Tokyo/Springer-Verlag, Berlin, Heidelberg, New York.
- Nobles, M.K. 1948. Studies in forest pathology. VI. Identification of cultures of wood-rotting fungi. *Canadian Journal of Forest Research* 26:281-431.
- O'Hanlon, P.C., and Peakall, R. 2000. A simple method for the detection of size homoplasy among amplified fragment length polymorphism fragments. *Molecular Ecology* 9: 815-816.
- Okuno, T., Ishita, Y., Sawai, K., and Matsumoto, T. 1974. Characterization of alternariolide, a host-specific toxin produced by *Alternaria mali* Roberts. *Chemistry Letters* 1974:635-638.
- Ortalda, E. 2006. Indagine sulla presenza di *Alternaria alternata* su melo in Piemonte. *Annali del Settore Fitosanitario Regionale Piemonte*:55-57.
- Otani, H., Kohmoto, K., and Kodama, M. 1995. *Alternaria* toxins and their effects on host plants. *Canadian Journal of Botany* 73: S453–S458.
- Ozgonen, H., and Karaca, G.2006. First report of *Alternaria mali* causing necrotic leaf spot of apples in Turkey. *Plant Pathology* 55:578.

- Park, M.S., Romanoski, C.E., and Pryor, B.M. 2008. A re-examination of the phylogenetic relationship between the causal agents of carrot black rot, *Alternaria radicina* and *A. carotiincultae*. *Mycologia* 100(3):2008:511–527.
- Park, P., Tsuda, M. Hayashi, Y., and Ueno, T. 1977. Effect of host specific toxin (AM.toxin1) produced by *Alternaria mali*, an apple pathogen, on the ultrastructure of plasma membrane of cells of apple and Japanese pear leaves. *Canadian Journal of Botany* 55:2383-2387.
- Peakall, R., and Smouse, P.E. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6:288-295.
- Peever, T.L., Su, G., Carpenter-Boggs, L., and Timmer, L.W. 2004. Molecular systematics of citrus-associated *Alternaria* species. *Mycologia* 96(1):119-134.
- Pryor, B.M., and Bigelow, D.M. 2003. Molecular characterization of *Embellisia* and *Nimbya* species and their relationship to *Alternaria*, *Ulocladium*, and *Stemphylium*. *Mycologia* 95:1139-1152.
- Pryor, B.M., and Gilbertson, R.L. 2000. Molecular phylogenetic relationships among *Alternaria* species and related fungi based upon analysis of nuclear ITS and mtSSU rDNA sequences. *Mycological Research* 104(11):1312-1321.
- Pryor, B.M., and Michaelides, T.J. 2002. Morphological, pathogenic and molecular characterization of *Alternaria* isolate associated with *Alternaria* late blight of Pistachio. *Phytopathology* 92 (4):406-415.
- Rabouam, C., Comes, A.M., Bretagnolle, V., Humbert, J-F., Periquet, G., and Bigot, Y. 1999. Features of DNA fragments obtained by random amplified polymorphic DNA (RAPD) assays. *Molecular Ecology* 8:493–503.
- Rehnstrom, A.L., and Free, S.J. 1996. The isolation and characterization of melanin-deficient mutants of *Monilinia fructicola*. *Physiology and Molecular Plant Pathology* 49:321-330.
- Ridgway, R. 1912. *Color Standards and Color Nomenclature*. Published by the Author. Washington, DC.
- Roberts, J.W. 1924. Morphological characters of *Alternaria mali* Roberts. *Journal of Agricultural Research* 27:699-708.
- Roberts, R. G., Reymond, S. T., and Andersen, B. 2000. RAPD fragment pattern analysis and morphological segregation of small-spored *Alternaria* species and species groups. *Mycological Research* 104:151-160.
- Robeson, D.J., and Strobel, G.A. 1981. Alpha-beta-dehydrocurvularin and curvularin from *Alternaria cinerariae*. *Zeitschrift für Naturforschung*. 36:1081–1083.
- Rosendahl, S., and Taylor, J.W., 1997. Development of multiple genetic markers for studies of genetic variation in arbuscular mycorrhizal fungi using AFLP. *Molecular Ecology* 6:821–829.
- Rotem, J., 1994. The genus *Alternaria*. Biology, Epidemiology and Pathogenicity. American Phytopathological Society Press, Saint Paul, Minnesota (USA).

- Rotem, J., Blickle, W., and Kranz, J. 1989. Effect of environment and host on sporulation of *Alternaria macrospora* in cotton. *Phytopathology* 79:263-266.
- Rozas, J., and Rozas, R. 1997. DnaSP version 2.0: a novel software package for extensive molecular population genetics analysis. *Computer Applications in the Bioscience* 13:307-311.
- Saitu, N. 1996. Reconstruction of gene trees from sequence data. *Methods in Enzymology*, 266: 427-449.
- Sanger, F., Nicklen, S., and Coulson, A.R. 1977. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* 74:5463-5467.
- Sawamura, K. 1972. Studies on apple *Alternaria* blotch caused by *Alternaria mali* Roberts. In: *Faculty of Agriculture Hirosaki University Bulletin* 18:152-235.
- Sawamura, K. 1990. *Alternaria* blotch. Pages 24-25 in: *Compendium of Apple and Pear Diseases*. A. L. Jones and H. Aldwinckle, eds. American Phytopathological Society, St. Paul, MN.
- Scheffer, R.P., and Livingston, R.S. 1984. Host-selective toxins and their role in plant diseases. *Science* 223:415-419
- Schimmer, C.F. 1953. *Alternaria brassicicola* on summer cauliflower seed. *Plant Pathology*. 2(1): 16-17.
- Serdani, M., Kang, J.C., Peever, T.L., Andersen, B., and Crous, P.W. 2002 Characterisation of *Alternaria* species groups associated with core rot of apples in South Africa, *Mycological Research* 106:562-570
- Simmons, E.G. 1992. *Alternaria* themes and variations (63-72). *Mycotaxon* 48:91-107.
- Simmons, E.G., and Roberts, R.G. 1993. *Alternaria* themes and variations (73). *Mycotaxon* 48:10-140.
- Simmons, E.G. (1995). *Alternaria* themes and variations (112-114). *Mycotaxon* 55: 55-163.
- Simmons, E.G. (1999). *Alternaria* themes and variations (236-243). *Mycotaxon* 70: 325-369.
- Simmons, E.G. 2007. *Alternaria: an identification manual*, CBS Biodiversity
- Simpson, E.H. 1949. Measurement of diversity. *Nature* 163:688.
- Singh, B.P., Prasad, B., and Walia, M.S. 1987. Effect of plant age and environmental factors on potato leaf spot. *Indian Phytopathology* 40:70-75.
- Soleimani, M.J., and Esmailzadeh, M. 2007. First report of *Alternaria mali* causing apple leaf blotch disease in Iran. *Australasian Plant Disease Notes* 2:57-58.

- Steele, J.A., Durbin, R.D., Uchytel, T.F., and Rich, D.H. 1978. Tentoxin—uncompetitive inhibitor of lettuce chloroplast coupling factor-1. *Biochimica and Biophysica Acta* 501: 72–82.
- Stenglein S.A. & Balatti P.A. 2006. Genetic diversity of *Phaeoisariopsis griseola* in Argentina as revealed by virulence and molecular markers. *Physiological and Molecular Plant Pathology*. 68: 158-167.
- Strider, D.L. 1963. Control of *Alternaria* blight carrot. *Plant Dis. Rep.* 47:66-69.
- Swofford, D.L. 2000. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sunderland, Massachussets: Sinauer Associates.
- Swofford, D.L., Olsen, G.J., Waddell, P.J., and Hillis, D.M. 1996. Phylogenetic Inference. In Hillis DM, Moritz D, and Mable BK, editors, *Molecular Systematics*, pp. 407-514. Sinauer Associates, Sunderland, Massachusetts.
- Tajima, F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* 123:585-595.
- Tanabe, K., Tsuge, T. and Nishimura, S. 1990. Potential application of DNA restriction fragment length polymorphisms to the ecological studies of *Alternaria alternate* Japanese pear pathotype. *Annals of Phytopathological Society of Japan* 55: 361-365.
- Tanaka, A. and Tsuge, T. 2000. Structural and functional complexity of the genomic region controlling AK-toxin biosynthesis and pathogenicity in the Japanese pear pathotype of *Alternaria alternata*. *Molecular Plant-Microbe Interaction* 13:975–986.
- Tanaka, A., Shiotani, H., Yamamoto, M., and Tsuge, T. 1999. Insertional mutagenesis and cloning of the genes required for biosynthesis of the host-specific AK-toxin in the Japanese pear pathotype of *Alternaria alternata*. *Molecular Plant-Microbe Interaction* 12:691–702.
- Tewari, J.P., 1983. Cellular alterations in the blackspot of rapeseed caused by *Alternaria brassicae*. *Phytopathology*, 73, 831.
- Thomma, B., P., H., J. 2003. *Alternaria* spp.: from general saprophyte to specific parasite. *Molecular Plant Pathology* 4(4):225-236.
- Thompson, J.D., Higgins, D.G., and Gibson T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 11:22(22):4673-80.
- Thuleau, P., Graziana, A., Rossignol, M., Kauss, H., Auriol, P., and Ranjeva, R. 1988. Binding of the phytotoxin zinniol stimulates the entry of calcium into plant-protoplasts. *Proc. Natl Acad. Sci. USA*, 85, 5932–5935.
- Tisdale, W.B., and Wadkins, R.F. 1931. brown spot of tobacco caused by *Alternaria longipes* (E.& E.), n. comb. *Phytopathology* 21:641-660.
- toxins on the basis of variation in internal transcribed spacers of ribosomal DNA. *Curr. Genet.* 28, 491–498.

- Tymon, A.M. and Pell, J.K. 2005. ISSR, ERIC and RAPD techniques to detect genetic diversity in the aphid pathogen *Pandora neoaphidis*. *Mycological Research* 109: 285-293
- Ueno, T., Nakashima, T., Uemoto, M., Fukami, H., Lee S.N., and Izumiya, N. 1977. Mass spectrometry of *Alternaria mali* toxins and related cyclodepsipeptides. *Biomedical Mass Spectrometry* 4(3):134-142.
- Vos, P., Hogers, R., Bleeker, M., Reijans, M., Vandeele, and T., Hornes, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* 23:4407-4414.
- Vos, P., and Kupier, M. 1997. AFLP analysis. DNA markers: Protocols, applications and overviews. Editor: Caetano-Anoll6s. G. & Cresshoff, P.F., Wiley-VCH Verlag, Nelw York:115-131.
- Waals Vander, J.E., Korstn, L., and Slippers, B. 2004. Genetic diversity among *Alternaria solani* isolates from potatoes in South Africa. *Plant Disease* 88:959-964.
- Walton, J. D., and Panaccione, D. G. 1993. Host-selective toxins and disease specificity: perspective and progress. *Annual Review of Phytopathology* 31:275-303.
- Walton, J.D. 1996. Host-selective toxins: agents of compatibility. *Plant Cell* 8:1723-1733.
- Walton, J.D. 2000. Horizontal gene transfer and the evolution of secondary metabolite gene clusters in fungi: an hypothesis. *Fungal Genetics and Biology* 30:167-171.
- Wang, H., Li, J., Bostock, R.M. and Gilchrist, D.G. 1996b. Apoptosis: a functional paradigm for programmed plant cell death induced by a host-selective phytotoxin and invoked during development. *Plant Cell*. 8:375-391.
- Wang, S., Miao, X., Zhao, W., Huang, B., Fan, M., Li, Z., and Huang, Y. 2005. Genetic diversity and population structure among strains of the entomopathogenic fungus, *Beauveria bassiana*, as revealed by inter-simple sequence repeats (ISSR). *Mycological Research* 109(12):1364-72.
- Wang, W., Jones, C., Ciacci-Zanella, J., Holt, T., Gilchrist, D.G., and Dickman, M.B. 1996a. Fumonisin and *Alternaria alternata lycopersici* toxins: sphinganine analog mycotoxins induce apoptosis in monkey kidney cells. *Proceedings of the. National Academy of Science USA*, 93:3461-3465.
- Weir, T.L., Huff, D.R., Christ, B.J., and Romaine, C.P. 1998. RAPD-PCR analysis of genetic variation among isolates of *Alternaria solani* and *Alternaria alternata* from potato and tomato. *Mycologia* 90:813-821.
- Weising, K., Atkinson, R.G. and, Gardner, R.C. 1995. Genomic fingerprinting by microsatellite-primed PCR: a critical evaluation. *Genome Research* 4: 249-255
- Witsch, H., and Wagner, F. 1955. Beobachtungen über den Einfluss des Lichtes auf Mycel- und Coinidiebildung bei *Alternaria brassicae* var. *dauci*. *Archives of Microbiology*. 22:307-312.
- Wolpert, T.J., Dunkle, L.D., and Ciuffetti, L.M. 2002. Host-selective toxins and avirulence determinants: what's in a name? *Annual Review of Phytopathology* 40:251-285.

Yamagishi, D., Otani, H., and Kodama, M. 2006. G protein signaling mediates developmental processes and pathogenesis of *Alternaria alternata*. *American Phytopathological Society* 19(11):1280–1288.

Yao, C. and Köller, W. 1995. Diversity of cutinases from plant pathogenic fungi: Different cutinases are expressed during saprophytic and pathogenic stages of *Alternaria brassicicola*. *Molecular Plant-Microbe Interaction* 8:22–130.

Yoder, O.C. 1980. Toxins in pathogenesis. *Annual Review of Phytopathology*. 18:103-29.

Zietkiewicz, E., Rafalski, A., and Labuda, D., 1994. Genomic fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20:176-183.

Zhou, S., Smith, D.R., and Stanosz, G.R. 2001. Differentiation of *Botryosphaeria* species and related anamorphic fungi using Inter Simple or Short Sequence Repeat (ISSR) fingerprinting. *Mycological Research* Volume 105(8):919-926.