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Study on Fungicide Sensitivity and Resistance in a Population of *Botryotinia fuckeliana* Collected from Table Grapes in Sicily (Southern Italy)

Ph.D. Thesis

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1. Viticolture in Sicily

Grapevine (*Vitis vinifera*) is certainly one of the most important crops, with great economic importance in all the areas in the Mediterranean Basin for the several ways of the use of its product (fresh fruit, musts, wine, dry grape, etc). Vineyards cover a total area of 10 million hectares and produce a total yield of about 65 million tons, of which 17 million tons are for table grapes (OIV International Organization of Vine and Wine, 2010).

Vines can be cultivated from temperate to tropical climates, but most vineyards are planted in temperate zones. The most concentrated cultures are in Europe. Italy, with its 1.3 million tons, is the largest table grape producer in the Europe (Istat, 2011). Despite the most of this production is destined for the domestic market, a substantial proportion goes to EU countries. The traditional countries, France, Germany, Belgium, Switzerland, have been joined by other Eastern European countries, most notably Russia and Poland. Occasionally table grapes are even exported to neighboring Arab countries and to Canada. According to OIV estimates, Italy ranks 6th in the world table grape production and 3rd as an exporter behind Chile and the United States. Most table grapes are grown in the south, especially in Apulia and Sicily, which account for nearly 65% and 25% of the total area respectively. The cultivars Italia, Victoria and Red Globe are the most widespread varieties in Italy, covering approximately 66% of the table grape area.

In Sicily the most vineyards of table grapes are located in the geographic area of Canicattì and Mazzarrone. The production zone of Canicattì includes numerous town districts in the provinces of Agrigento and Caltanissetta. Instead, the geographical area where Mazzarrone table grapes are grown lies on either side of the border between the provinces of Catania and Ragusa and comprises the municipalities of Caltagirone, Licodia Eubea and Mazzarrone in the province of Catania and the municipalities of Acate, Chiaramonte Gulfi and Comiso in the province of Ragusa.

The origins of table-grape cultivation in the Mazzarrone area can be traced back far into the past. In the 1930s and 1940s, various varieties of table grapes were grown in the areas mentioned. In the 1950s, major innovations in grape-growing concerned

both the range of varieties cultivated and the cultivation techniques, which, together with the land reforms carried out, contributed to the development of grape-growing throughout the district. At the same time, this district became much more specialised in grape-growing, in terms of both the use of innovative vine-training methods and the techniques used to advance or retard the ripening process. The production of table grapes has been a significant factor in the economic development and the commercial activity of the whole district. The production of Mazzarrone table grapes accounts for more than 90% of local agricultural production. The climatic and soil conditions, which are peculiarly suited to growing table grapes, combined with the district's specialization in this activity, gives the end product the quality, organoleptic and commercial characteristics that set it aside from table grapes from other areas. The training system used in Sicily for table grapes is that of “tendone”, consisting of a continuous overhead canopy under which the bunches are disposed (Fig. 1).

Figure 1. Sicily pergulate known as `tendone'.

In the “tendone” training system, the bunches receive some protection against wind and excessive light intensity and benefit from a microclimate characterized by moderate air temperature and diffuse solar radiation, thus favoring berry development and a more uniform ripening and skin colour. Moreover, this system allows a good separation between the vegetative and reproductive zones, that forms a continuous belt on each side of the vine “row”.

The specialised nature of production and the characteristics both organoleptic and commercial of Mazzarrone table grapes have given the product a confirmed reputation on Italian markets.

In Italy vineyards can be infected with a variety of temperate-climate fungal diseases, many of which are facilitated by each other or other vineyard pests. Downy mildew (caused by *Plasmopara viticola* Berliner & de Toni.), powdery mildew (caused by *Uncinula necator* (Schw.) Burr.) and botrytis rot (caused by *Botrytis cinerea* Persoon ex Fries) are the most common diseases, each of which can cause total crop loss in the absence of control (Agrios, 2005). To a lesser extent, phomopsis (*Phomopsis viticola* Sacc.) and black spot (*Elsinoe ampelina* de Bary) can also be important (Agrios, 2005). Often the development of botrytis rot in the bunches is associated by the presence of other diseases. The causative agents of these secondary rot are opportunist, weakly or not pathogenic fungi, belonging to the genera *Aspergillus*, *Penicillium*, *Cladosporium*, *Alternaria* and *Rhizopus* (Hellman, 2004). Among these fungi, *A. carbonarius* is particularly considered since it was identified as the largest producer of ochratoxin A (OTA) (Cabanes *et al*., 2002; Abarca *et al*., 2003, Hocking *et al*., 2007). A new disease causing vine canker of table grapes was first observed in the San Joaquin Valley, California, in 1989 on vigorous 1-year-old cv. Redglobe vines (*Vitis vinifera*) (Michailides *et al*., 2002). Subsequently, in 2003, vine cankers were observed in Italy (Sicily) on vigorous 1-year-old cv. Black Rose vines (Vitale *et al*., 2008). Based on molecular characterization and pathogenicity tests, the pathogen was identified as *Aspergillus* spp.(Vitale *et al*., 2012). Estimated losses for fungal disease development in Italy vineyards amount to 15-40% of harvests depending on climatic conditions. The major economic losses are caused by *B. cinerea.* Its ability to attack a wide range of crops in a variety of modes of

infection and its ability to develop under conditions prevailing during storage, shipment and marketing make its control a challenge.

2. *Botrytis cinerea*

Botrytis cinerea, the anamorph of *Botryotinia fuckeliana* (de Bary) Whetzel, is an ubiquitous fungus which can attack a wide number of host plants without showing any apparent specialization.

At the present, it is conceivable that *B. cinerea* parasitizes well over 200 host, including fruit trees, grapevine, horticultural plants like strawberry, tomato, pepper as well as ornamentals plants (Leroux, 2007). In many of these hosts the pathogen may infect flowers, leaves, buds, shoots, stems and/or fruits, often limiting plant development, fruit-set, yield and fruit quality in fruit crops (Maude, 1980; Nicholas *et al.*, 1994) and yield and crop quality in vegetables (Maude, 1980; Alfonso *et al.*, 2000). It may also attack seedlings, reducing establishment and so plant density in a new crop. As concerning grapevine, it can affect the plant at different stages of development and the infections by fungal conidia can occur during the whole growing season: inflorescence, flowering, ripening, vegetative stage and cluster (Kretschmer *et al*., 2007). It is also a saprophyte on senescent and dead plant material.

It can be found in open field so as in greenhouses, causing direct losses of production and determining the increase of production costs due to need to control the fungus.

B. cinerea is a fungus ubiquitous and its high capacity of adaptation to different climatic conditions allows it to develop in different periods in the year and in different countries. Coley-Smith (1980) referred to *Botrytis* spp. as temperate area pathogens perhaps because of the vast research that has been carried out in such areas or due to its importance on vineyard grapes. Nevertheless, species of the genus *Botrytis* occur wherever their host are grown, ranging from tropical and subtropical to cold areas. For example Anderson (1924) recorded *B. cinerea* in Alaska and Yunis and Elad (1989) dealt with this pathogen in warm and dry areas. A rapid rate of conidial germination, infection, mycelium growth and conidiation occur in many

Botrytis spp. under a wide range of microclimate conditions and pose severe disease management problem all around the world (Elad *et al.,* 2007).

The fungus must be considered always present in the vineyards. Several biological event, like sporulation of the pathogen, dispersal and germination of conidia, infection, variability, virulence and survival are involved in arising of epidemics of grey mould. Each of these biological events is more or less influenced by climatic and cultural factors. These include temperature, rain, relative humidity, wind, fertilization, phonological state of the host, density of plantation and cultivar susceptibility (Jarvis, 1980).

2.1. Taxonomy

Botrytis and its sexual form *Botryotinia* comprise 22 species and one hybrid (Hennebert, 1973; Yohalem *et al.,* 2003) and are classified within the family *Sclerotiniaceae* Whetzel (*Inoperculate Discomycetes*). The species of *Botrytis* have to date been delimited primarily on the basis of morphological and cultural characteristics coupled with host specificity (Hennebert, 1973; Jarvis, 1977, 1980). Features such as sclerotial size and form and conidium size are useful in delimiting some species, but many species are morphologically similar and growing conditions significantly influence variation. No key to all recognised species is available and identification of species based on traditional criteria can be fraught (Nielsen *et al*., 2001).

Some species have been also distinguished based on sexual crosses between them (Bergquist and Lorbeer, 1972). However, homothallism (self-fertilization) is not uncommon in *Botrytis*, which makes it difficult to ensure if progeny had two parents. Other *Botrytis* species apparently entirely lack sexuality, which further limits the use of the biological species concept for species discrimination (Staats, 2007).

Rather than on morphological and biological traits, species may be identified by phylogenetic analyses of variable nucleic acid sequences. In this approach, an evolutionary tree is used to model the relationships of a group of individuals. Phylogenetic species can be identified as terminal monophyletic clades. The internal transcribed spacer (ITS) region of the ribosomal DNA has been widely used for

species-level discrimination of fungal species, but variation in the ITS region within *Botrytis* is low, limiting its use in this genus (Holst-Jensen *et al.*, 1998; Nielsen *et al*., 2001).

The intergenic spacer region (IGS) rDNA region may offer better prospects, although its usefulness may be limited by recombination (Giraud *et al.,* 1997).

On the basis of the recent phylogenetic analysis, *B. cinerea* was proposed to be a species complex (Giraud *et al.,* 1997, 1999; Albertini *et al.,* 2002, Munoz *et al.,* 2002; Fournier *et al.,* 2003). Initially, two sympatric sibling species or transposon types were described: 1) *transposa,* that contained two transposons *Boty* and *Flipper* and 2) *vacuma*, which contained no transposons (Diolez *et al.,* 1995; Levis *et al.,* 1997; Giraud *et al.,* 1997). Recently, Fournier *et al.,* (2005) showed that genetic differentiation determined from multiple gene sequences was not concordant with either of the previously described transposon types (*transposa* or *vacuma*) and revised partitioning of *B. cinerea* into Group I and Group II phylogenetic cryptic species.

These cryptic species have also been shown to coincide with resistance to the fungicide fenhexamid, and synonymously known as FenR (resistant) = Group I and FenS (sensitive) = Group II (Albertini *et al.,* 2002). Diagnostic molecular markers for these groups have been developed based on cleaved amplified polymorphic sequence (CAPS) profiles of the Bc-*Hch* gene, a homologue of the *Neurospora crassa* vegetative incompa-tibility *hch* locus (Albertini *et al.,* 2002; Fournier *et al.,* 2003). To date, *vacuma*, *flipper*-only, and *boty* only transposon types have been detected with no *transposa* types in Group I and all transposon types have been detected in Group II (Giraud *et al.,* 1999; Albertini *et al.,* 2002; Fournier *et al.,* 2003; Ma and Michailides, 2005).

In grapevine pathology studies, *transposa* isolates were shown to be more virulent than *vacuma* isolates and changes in transposon type frequencies during crop development were possibly due to differences in their saprotrophic and pathogenic fitness (Martinez *et al.,* 2003, 2005). Thus, these observations supported the possibility of genetic differentiation between transposon types (Martinez *et al.,* 2003, 2005). Recently, other phenotypic differences between the two types *'vacuma'* and

'transposa' have been demonstrated: the *transposa* isolates are small if compared with the macroconidia of *vacuma* isolates and they are often resistant to vinclozolin and diethofencarb (Giraud *et al*., 1999); moreover, the *transposa* isolates grow slowly when inoculated on a medium rich in nutrients (Martinez *et al*., 2003).

2.2. Morphology

The *Botrytis* species produce colonies effuse, at first white to grayish, then dark brown (Fig. 2a). The mycelium of *B. cinerea* is olive brown in colour with cylindrical, septate hyphae, 11-23 μm in diameter (Pearson and Goheen, 1988). Macroconidia, usually called conidia, are produced in clusters from enlarged apical cells at the end of branched, slender, conidiophores (1-3 mm long) (Pearson and Goheen, 1988), which originate from enlarged basal cells (Jarvis, 1977, 1980) (Fig. 2b). They are smooth, single-celled, faintly ash-coloured structures, quite large (8-14 × 6-9 μm) and oval in shape (Willetts, 1997).

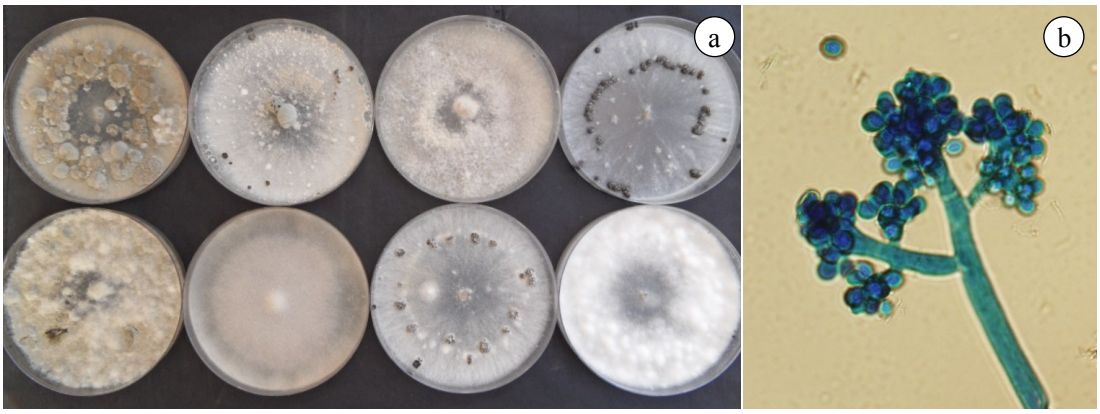

Figure 2. Morphological characteristics of colony (front side) (a), conidia and conidiophores (b) of eight isolates of *B. cinerea*.

Conidia of *B. cinerea* survive only for a short period in the vineyard. Their survival is influenced strongly by temperature, moisture, activity microbial and by exposure to sunlight. The conidia, when stored in a dry place, can survive for up to 14 months (Salinas *et al.,* 1989), but their survival in the conditions of the vineyard is

very limited. Studies conducted in a vineyard in New Zealand showed that only 50% of conidia survive after eight hours of exposure to sunlight (Pertot *et al.,* 2007).

In unfavourable environmental conditions, *B. cinerea* can produce sclerotia, that can be considered the most important structures for the survival of fungus. They are flat or convex, hard, adherent to the substrate or immersed in the tissues. They measure 2-4 x 1-2.5 mm; first whitish become black at maturity. Sclerotia may germinate producing mycelium, conidiophores and conidia or apothecia and ascospores.

Apothecia originated from sclerotia can arise singly or in group. *B. cinerea* apothecia are seldom found in nature (Lorbeer, 1980), although it is salutary to reflect that Anton de Bary described *Peziza* (*Botryotinia*) *fuckeliana* and *B. cinerea* from grapevine in Switzerland well over a century ago (Gregory, 1949). Despite the rarity of apothecia in nature, they can be readily obtained in the laboratory following protocols refined by Faretra and Antonacci (1987) and Faretra *et al.* (1988).

Apothecia are cupulate, stalked, brownish structures, with a 4-5 mm-long stipe. Ascospores are smooth, oval and single-celled measuring 7 × 5.5 μm (Pearson and Goheen, 1988).

2.3 Life cycle and epidemiology

B. cinerea survives saprophytically through the winter on a diverse range of host species (Bisiach *et al*., 1984; Sutton, 1991). Within the vineyard, several sources of overwintering inoculum have been identified, including sclerotia (Nair *et al*., 1995), grape vine prunings (Thomas *et al*., 1983) and other necrotic grape tissues in the vine (Emmett and Nair, 1991; Fowler *et al*., 1999; Elmer and Michailides, 2004) and on the ground (Seyb, 2004). Apothecia of the sexual state (*Botryotinia fuckeliana*) have been reported, but their occurrence is sporadic (Beever and Weeds, 2004).

Release of fresh conidia from these sources in the spring provides an abundance of inoculum for infection of leaves and tender young shoots (McClellan and Hewitt, 1973; Nair and Hill, 1992; Nair *et al*., 1995). Conidia may also infect blossoms, colonize dead flower parts and penetrate young grapevine berries. Senescing floral tissues are highly susceptible to *B. cinerea* and profuse sporulation is frequently

observed on these tissues when conditions favor pathogen development in the spring (Keller *et al*., 2003).

In berries, *B. cinerea* remains in a latent state until the postveraison (change of berry colour and commencement of berry ripening) period and until the fruit sugar content increases to a level that supports fungal growth (Pezet and Pont, 1992; Nunan *et al*., 1998; Holz *et al*., 2003; Pezet *et al*., 2003). Latent infection of berry pedicels, and to a lesser extent grape bunch rachii, also accounts for fruit infection at vintage (Michailides *et al*., 2000; Holz *et al*., 2003). Late-season berry infections can also arise, particularly in wounded or cracked berries, from direct infection by airborne conidia or from mycelia growing out from saprophytic bases within aborted flowers, aborted fruit lets and calyptras trapped within developing bunches (Nair and Parker, 1985; Nair and Hill, 1992; Latorre and Rioja, 2002; Seyb, 2004).

The conidia produced on every host plant by *B. cinerea* strains can be transported by wind over long distances before infecting the next host. Following attachment, conidia germinate under favorable conditions and produce a germ tube that penetrates the host surface. Whether true infection structures are produced during this process is a matter of debate. After surface penetration the underlying cells are killed and the fungus establishes a primary lesion, in which necrosis and defense responses may occur. In some cases this is the onset of a period of quiescence of an undefined length, in which fungal outgrowth is negligible (Prusky, 1996). At a certain stage the defence barriers are breached and the fungus starts a vigorous outgrowth, resulting in rapid maceration of plant tissue, on which the fungus finally sporulates to produce inoculum for the next infection. Under optimal conditions, one infection cycle may be completed in as little as 3-4 days, depending on the type of host tissue attacked.

During active growth, *B. cinerea* produces a range of hydrolytic enzymes and metabolites to facilitate penetration and colonization of host tissues, causing necrosis of the plant tissue (Pearson and Goheen, 1988). Conidia can be released either singly or in clusters (Coertze *et al.*, 2001). The quantity of enzymes secreted is much higher from a cluster than from a single spore, so likelihood of an infection also increases (Elad *et al.*, 2004). This was demonstrated by Coertze and Holz (1999) who observed

that single conidia were unable to infect ripe table grapes (cv. Dauphine), yet clusters are able to create infection on ripe berries (Nair and Allen, 1993; Broome *et al.*, 1995). Similarly, when primary inoculum levels are high, singular spores and clusters can accumulate on plant surfaces and increase the chance of infection (Coertze *et al.*, 2001). For this reason, secondary cycles, which can produce high numbers of spores, usually cause more disease than do primary ones (Nicholas *et al.*, 1994).

Conidia of *B. cinerea* are spread by wind, air currents in greenhouses, or insect (Jarvis, 1980). Dry conidia are generally dispersal by wind that is responsible of their liberation, transport and deposition on host plants (Aylor, 1990). Rain may be also involved in the release of conidia which are subsequently dispersed by rain splashing (Vercesi and Bisich, 1982). The role of wind blow and rain-splashed pieces of plant debris containing mycelia as dispersal propagules is probably under estimated. They provide a large inoculum in a saprophytic stage. Dispersal of *B. cinerea* can be also favoured by insects such as the grapevine moth *Lobesia botrana* (Fermaud and Gaunt, 1995), the flower thrips *Thrips obscuratus* (Fermaud and Gaunt, 1995), the vinegar fly *Drosophila melanogaster* (Louis *et al*., 1996) and the Mediterranean fruit fly *Ceratitis capitata* (Engelbrecht *et al*., 2004).

Among insects, *Lobesia botrana* has been shown repeatedly to be associated with *Botrytis* outbreaks in grapes. The first generation of the pest attacks flowers, the second feeds on immature berries promoting green berry rot (Fermaud and Giboulot, 1992) and the third generation damages ripe berries (Fermaud and Le Menn, 1992). Some studies revealed that numerous conidia contaminated the ornamentations of the cuticle segments of the larva and ingested conidia remained viable after passing through the insect's digestive system (Fermaud and Le Menn, 1989). Supplying viable conidia to second-generation larvae resulted in an increase in the proportion of injuries infected by *B. cinerea* in grape berries (Fermaud and Le Menn, 1992). The first instar larvae were attracted by *B. cinerea* infection on grape berries, possibly due to volatile kairomones produced by *Botrytis* (Mondy *et al.*, 1998) and a mutualistic relationship between the two partners was proposed. The presence of *B.*

cinerea infected grapes consistently increased insect fecundity and attracted females to oviposit (Mondy and Corio-Costet, 2000).

A number of other factors playing a role in predisposition of the severity of grey mould epidemics. The physiological status of the host plant is considered so that environmental conditions for optimal development of the pathogen.

The structure and thickness of the cuticle and the epidermal layers have long been regarded as major factors of resistance against *B. cinerea* infection (Commenil *et al*., 1997). Berry-to-berry contact, where the cuticle is absent or very thin, increases the susceptibility of grape berries to *B. cinerea* (Marois *et al*., 1986; Rosenquist and Morrison, 1989) and clones within the same cultivar (e.g. Chardonnay) characterized by tight clusters also develop more severe bunch rot (Vail and Marois, 1991; Vail *et al*., 1998). In general, stomata number (Bernard and Dallas, 1981) or natural openings were independent of susceptibility to *B. cinerea*. However, a recent study found that the number of stomata in the berry epidermis was negatively correlated, while the number and thickness of epidermal and hypodermal cell layers and cuticle and wax contents were positively correlated with resistance to *B. cinerea* in a wide range of table grape cultivars (Mlikota Gabler *et al*., 2003). Vines grown under UV screens had less cuticular wax and lower lipid oxidase (an indicator of membrane damage) than those grown under ambient light, suggesting that an increase in UV light could lead to thicker wax on the fruit and leaf tissues, which may reduce susceptibility to *B. cinerea* (Steel, 2001). Developing vegetative and floral tissues are highly susceptible to frost damage, but the role of freezing injury and early season build-up of *B. cinerea* epidemics have not been well studied in orchard crops, though profuse *B. cinerea* sporulation was visually observed on terminal grape shoots after frost injury prior to flowering in Chardonnay grapes (Elmer and Michailides, 2004).

Also plant nutrition has an important effect on *B. cinerea* epidemics. As a matter of fact, the effects of specific nutritionally relevant ions on host susceptibility and development of *B. cinerea* epidemics has long been well documented (Jarvis, 1980; Goodman *et al*., 1986).

Nitrogen (N) and calcium (Ca^{2+}) have been the two most studied (Elad and Shtienberg, 1995). Low N nutrition is a significant problem in viticulture, associated

with ‘stuck’ fermentations (Tromp, 1984; Conradie and Saayman, 1989) and deterioration of wine aroma (Marangoni *et al.*, 2001). An over-supply of N leads to excessive growth in terms of vine vigour, berry number, bunch compaction and cuticle thinning-factors all known to increase grey mould (Delas *et al*., 1984, 1991; Keller *et al*., 2001). Other studies report no adverse effect of N on wine quality (Conradie and Saayman, 1989) and no increase in grey mould (Chambers *et al*., 1993). Excessive N fertilization in kiwifruit in Italy did not increase plant growth or leaf number, but *B. cinerea* incidence in cool-stored fruits was higher (Pertot and Perin, 1999). In New Zeland, *Botrytis* incidence in cool storage was strongly linked to excessive N (Prasad *et al*., 1990; Prasad and Spiers, 1991), but a later study found no evidence of a link (Smith and Buwalda, 1994). *B. cinerea* populations pre-harvest were not measured in the studies described above and we suggest that the relationship between N and post-harvest *Botrytis* is indirect, perhaps leading to an increase in the susceptibility of leaves and shoots to physical damage, reducing disease resistance of leaves, as reported in related host-pathogen systems (Daane *et al*., 1995), thereby increasing inoculum potential in the canopy.

Regarding the ion Ca^{2+} , it has been demonstrated that it increases resistance to *B. cinerea* disease (Volpin and Elad, 1991; Conway *et al*., 1991), reduces leakage of exudates to the host surface thus, reducing their availability to the pathogen (Volpin and Elad, 1991) and modulates various cell functions (Conway, 1982; Elad *et al*., 1992a). In contrast, Ca^{2+} deficiency increases susceptibility to *B. cinerea* (Schwab *et al*., 1993). Grape cultivars differ in their response to Ca^{2+} and enzymatic degradation by *B. cinerea*, indicating that the relationship between Ca^{2+} and *B. cinerea* is complex (Chardonnet and Doneche, 1995). When Ca^{2+} was applied before veraison to a range of grape cultivars, infection was reduced. In contrast, Ca^{2+} applied after veraison had no effect on epidemic development (Doneche and Chardonnet, 1996). When Ca^{2+} applications were made to table grapes in the field, resistance to *B. cinerea* was increased and correlated with increased levels of cellulose and of both oxalate and alkali-soluble pectins (Miceli *et al*., 1999). Incubating *B. cinerea* conidia in increasing concentrations of $CaCl_2$, conidial germination and germ tube length decreased (Chardonnet *et al*., 2000). However, the inhibitory effect of $CaCl_2$ on *B.*

cinerea could be overcome by the addition of glucose to the medium (Wisniewski *et al.,* 1995). This has important implications since sugar leakage from grape berries increases postveraison, potentially neutralizing the beneficial effects of Ca^{2+}.

Generally, infections caused by *Botrytis* spp. occur in cool, wet and humid weather, conditions which favor sporulation, infection, and also predisposition of the host. Surface wetness and temperature often closely related in *Botrytis* disease and operate together in determining initial infection from spores and probably the transition of latent infections and nonaggressive lesion to aggressive lesions (Jarvis, 1980). *B. cinerea* can thrive under a range of temperatures between 2 and 30°C (Elad and Yunis, 1993; Yunis *et al.,* 1994). The optimum temperatures for the different growth phases range from 12-30°C.

Regarding wetness conditions, *Botrytis* spp. are regarded as high humidity pathogens and their conidia germinate at high humidity (Snow, 1949). Successful infection requires 93 to 100% relative humidity (Blakeman, 1980). In many patho-systems infection occurs in the presence of a film of water on the susceptible plant tissue. The role of water drops and nutrients in germination and infection have been long recognized (Brown, 1916). However, it is interesting that the pathogen is also able to infect plants when no film of water exists on the plant surfaces (Williamson *et al*., 1995; Elad, 2000a). The water film, as a matter of fact, has not been considered necessary for infection from conidia by various workers who report that high relative humidity suffice. Schein (1964), however, has questioned these reports because condensation occurs from near saturated and saturated air with small temperature changes.

2.4 Pathogenesis of *Botrytis*

Invasion of host tissue can be achieved by active penetration or passive ingress. *B. cinerea* is a renowned opportunist that can initiate infection at wound sites or at sites that have previously been infected by other pathogens. *B. cinerea* can also enter the host via stomata and other natural openings (Clark and Lorbeer, 1976; Fourie and Holz, 1995; Hsieh *et al.,* 2001).When conidia land on aerial parts of a plant, the first barrier to overcome is the cuticle, which covers the epidermal cells. Its major

structural component, cutin, is a polyester composed of hydroxylated and epoxidised C16- and C18-fatty acids (Martin and Juniper, 1970). Physical damage or mechanical penetration of the cuticle by *B. cinerea* is not usually observed (Williamson *et al*., 1995; Cole *et al*., 1996). Hence, cutinolytic activity is presumably required to penetrate this layer (Salinas and Verhoeff, 1995; van der Vlugt-Bergmans *et al*., 1997).

Studies with antibodies against cutinase (Salinas, 1992) and lipase (Comménil *et al.,* 1998) suggested that these enzymes play a role in the infection process of *B. cinerea*. Various cutinases and lipases were suggested to be involved in active penetration (van Kan *et al.,* 1997; Gindro and Pezet, 1999).

Salinas (1992) investigated whether a particular 18 kDa cutinase is important in this process and raised monoclonal antibodies against the enzyme. Application of the antibody to gerbera flowers prior to inoculation reduced lesion formation by 80%. A cutinase-deficient gene replacement mutant, however, did not have any discernible reduction in virulence on gerbera flowers nor on tomato fruits, as compared to the wild type (van Kan *et al.,* 1997). Although the observations of Salinas (1992) remain to be explained, it can be ruled out that this particular 18 kDa cutinase is essential in penetration.

It should be taken into account that the 18 kDa cutinase, like all other cutinases studied thus far in plant pathogenic fungi, is most likely an exo-hydrolase. Enzyme activity of cutinases is usually defined by their ability to release soluble fatty acid monomers from the water insoluble substrate (Purdy and Kolattukudy, 1973). It would be much more efficient for a pathogen to produce an endo-hydrolase, in order to create openings for penetrating a polymer by fungal hyphae. Such endo-cutinase, however, will not release water-soluble products from the insoluble cutin, since cleavage products most likely remain attached in the network. Hence, endo-cutinase activity is difficult to detect in biochemical assays.

One candidate for an enzyme with such activity is a 60 kDa lipase that is induced upon growth in liquid medium with apple cutin as the sole carbon source (Comménil *et al.,* 1998). This lipase possesses low but significant cutinolytic activity and it has clearly distinct kinetic properties from the 'typical' cutinases mentioned above. When

polyclonal antibodies raised against this lipase were applied prior to inoculation with *B. cinerea* conidia, germ tubes were no longer able to penetrate the cuticle. The antibodies did not affect germination (Commenil *et al.,* 1998). Whether the lipase plays an essential role in host tissue penetration should be assessed by cloning the corresponding gene, constructing a targeted lipase-deficient mutant and determining its virulence. Microscopic studies have shown that after penetration of the cuticle, hyphae of *B. cinerea* frequently invade the anticlinal wall between two epidermal cells. The concomitant swelling of the epidermal cell wall (Mansfield and Richardson, 1981) is indicative for the degradation of the pectin in the matrix of the epidermal wall, presumably as a result of water absorption.

Biochemical evidence suggested that also pectinases might be involved in primary infection since pectic materials are so widely distributed in the middle lamella and primary cell wall (Bateman and Basham, 1976) and enzymes of the pectic type are also responsible for maceration of plant tissue as well as for cell death (Basham and Bateman, 1975). At least one (basic) endopolygalacturonase (endoPG) is expressed constitutively and it was therefore proposed to be involved in early stages of the infection process (Van der Cruyssen *et al*., 1994).

Gene cloning revealed that *B. cinerea* contains an endoPG gene family, consisting of six members encoding basic as well as acidic isozymes (ten Have *et al.,* 1998; Wubben *et al*., 1999). Targeted deletion mutants were made in both genes encoding the basic endoPGs (*Bcpg*1 and *Bcpg*2) by gene replacement. Both types of mutants were still able to cause primary necrotic lesions on non-wounded tomato and bean leaves (ten Have *et al.,* 1998), excluding an essential role for BcPG1 and BcPG2 in host surface penetration. Movahedi and Heale (1990) detected extracellular aspartic protease (AP) activity in ungerminated conidia as well as during germination, prior to the appearance of pectinase activity. Application of the specific AP inhibitor pepstatin during inoculation markedly reduced infection of carrot slices, suggesting an important role for AP during primary infection (Movahedi and Heale, 1990). Recently, a gene was cloned encoding an aspartic protease, *BcAP*1, and targeted mutants were made to study its involvement in the infection of detached tomato leaf tissue. No discernible loss of virulence was

observed for the BcAP1-deficient mutant, indicating that this protease is not essential for virulence. Since *B. cinerea* probably contains at least one additional AP gene, the importance of aspartic proteases in pathogenesis cannot yet be excluded.

Recent studies have demonstrated that invasion of plant tissue by *B. cinerea* triggers nuclear condensation and plant membrane damage, two indicators for programmed cell death, in a ring of cells around the hyphae. These results imply that diffusible factors have a direct or indirect phytotoxic activity. Several phytotoxic compounds that have been proposed to play a role in killing host cells are evaluated. Botcinolide and botrydial are low molecular weight metabolites with a general phytotoxic activity that are secreted by *B. cinerea* (Cutler *et al*., 1993; Durán-Patrón *et al*., 2000; Colmenares *et al*., 2002). However, their role in pathogenesis is unknown. There is no evidence for the production of host-selective toxins (HSTs) by *B. cinerea*, which is in agreement with the broad host range of this species. HSTs are typically active only toward plants that serve as hosts for the specialized pathogens that produce them (Wolpert *et al*., 2002).

The production of toxins conferring host specificity was reported for *B. fabae* infecting *Vicia faba* (Harrison, 1980). Other specialized *Botrytis* species may also be equipped with HSTs, but this remains to be studied.

B. cinerea produces also oxalic acid, which may have direct toxic effect through acidification of the environment (Germeier *et al*., 1994). Furthermore, oxalic acid may be a co-factor in pathogenesis as several fungal cell wall degrading enzymes, such as endo- and exopolygalacturonases and pectin methylesterase, are most active at low pH values (ten Have *et al*., 2002).

The activities of many different pectinolytic and non-pectinolytic enzymes cause the breakdown of plant cell walls by which carbohydrates are released that form the major carbon source for consumption (ten Have *et al*., 2002). *B. cinerea* endopolygalacturonases are differentially expressed during pathogenesis on different hosts, which may contribute to the broad host range of this species (Wubben *et al.,* 2000; ten Have *et al*., 2001).

2.5 Symptoms of gray mould

B. cinerea causes a range of symptoms including spots and blight on leaf or petal tissues, crown rot, stem canker, cutting rot and damping-off. Storage tissues such as roots, corms, or rhizomes are also susceptible. Soft rots, accompanied by collapse and water soaking of parenchyma tissues, followed by a rapid appearance of grey masses of conidia are perhaps the most typical symptoms on leaves and soft fruit. On many fruits and vegetables the infection commonly begins on attached senescent flowers and then as a soft rot it spreads to affect the adjacent developing fruit (Williamson *et al.*, 2007). On grapevine, in early spring, buds and young shoots may be infected, turn brown and dry out. Large and irregular reddish brown necrotic patches appear on few leaves and are often localized at the edge of the lamina. The fungus may invade inflorescences which rot or dry out and fall off. At the end of bloom, *B. cinerea* frequently develops on the withered calyptras, stamens and aborted berries attached to or trapped in the clusters. From these sites the pathogen may attack pedicel and rachis forming small patches that are brown at first and then turn black. On these organs, infection is latent till summer, and can increase the dissemination of the propagules in the bunch before they close. In Mediterranean area, these infections are generally rare (Ciccarone, 1970) also if on some seedless cultivars symptoms can be observed on young and lignified branches, especially following the operation of leaf thinning or defoliation, where the fungus can penetrate through the wounds caused (Faretra *et al*., 1996). The inoculums present on flower residues can play a major role for the infection on bunches especially in presence oh humid summer and on grapevine varieties with serrate bunches. The fungus can infect bunches directly through the epidermis or through wounds that are present at the point of insertion of the pedicel on the berry.

B. cinerea is economically extremely important especially as a pathogen of grape berries. The pathogen may completely destroy grape berries, inflicting heavy crop losses as grey mould. Alternatively, under certain conditions, it may cause a slow decay permitting the berries to desiccate considerably. Such dry berries affected by “noble rot” are harvested and processed into valuable sweet wines. Grapes affected by the destructive grey mould are of low value for making wine not only because of

the weight loss but also because of interference with fermentation and changing the flavor and colour of the wine. Among all the many *Botrytis* plant hosts, grey mould management in vineyards is therefore the most important target for agrochemical companies and researchers (Elad *et al*., 2007).

The first signs of disease on grapes often appears during the bloom period, when the fungus attacks the flower parts. These early infection sites are a source of inoculum that can cause severe bunch rot near harvest, although not all fruit infections at harvest are the result of floral and latent infections during the bloom period. The infected fruit may become covered with grayish-tan conidia of the fungus. Berry stalks and cluster stems may be invaded, causing them to shrivel, and berries that have split or have been punctured are often attacked by other organisms (Elmer and Michailides, 2004).

Characteristic symptoms of botrytis rot on ripe berries include small, circular water-soaked spots that appear brown on white grape varieties and slightly clear on red grape varieties. At this stage of infection, rubbing causes the skin to slip over the inner pulp, a condition known as 'slip skin' (Pearson and Goheen, 1988). Berries then soften and the pulp turns brown. After periods of mild weather and high humidity, berries develop grey fluffy spores, initially in cracks in the skin, then over the entire infected area (Nicholas *et al.*, 1994) (Fig. 3). Infection can move from berry to berry either via spore dispersal or mycelium growth. If humidity is low, the infected berries dry to raisins, which usually remain attached to the vine (Nicholas *et al.*, 1994). In addition to this damage, the *Botrytis* rot lesions can act as entry points for secondary fungi, yeasts, bacteria and insect (Nicholas *et al.*, 1994).

Vineyards covered to delay the harvest are more susceptible to the attack by the fungus, and control requires a high number of fungicide sprays to the harvest (Faretra *et al*., 1996); as a consequence, the danger for leaving residues of fungicides in grapes and induction of resistance to fungicides in the fungus populations will dramatically increase.

Symptoms can be observed on table grapes also in post-harvest: numerous, small brown-violet lesions on berries can evolve and form a whitish mycelium generally with no sporifications (Droby and Lichter, 2004).

Figure 3. Symptoms of infection by *B. cinerea* on grape berries.

2.6 Disease management

The control of the disease is based on an integration of several cultural methods with the use of fungicides belonging to several group. Fungicides, biocontrol agents, modification of the greenhouse atmosphere, and cultural treatment are major control methods for gray mold disease. Effective disease management usually requires sanitation and other cultural practices to avoid introducing the pathogen, manipulation of environmental conditions to discourage disease development, and fungicide applications to prevent or limit disease spread (Jarvis, 1992).

2.6.1. Cultural practices

Cultural practices that alleviate the effects of grey mould are diverse and often specific to particular species and cropping systems. In perennial woody plants, such as grapevines, pruning to reduce excessive vegetative growth of the plant has been shown to be beneficial (Gubler *et al*., 1987). In addition, the orientation of rows, irrigation systems, soil drainage and fertilization plays an important role in the prevention of the disease. For example, excessive use of nitrogen fertilizer

encourages rapid vegetative growth and increases the risk of grey mould and other diseases. Some of the problems in soft fruit production caused by rainfall during the blossom period have been overcome by plastic rain shelters and tunnels, and facilitated a massive expansion in crop area for strawberries and raspberries. For example, 90% disease reductions in strawberries grown under plastic have been reported, compared with field-grown plants (Xiao *et al*., 2001). However, it is still important to encourage ventilation to reduce high relative humidity inside these structures and minimize wetting of foliage. When the plastic covers are removed in late summer there is still infection of leaves and stems, leading to over-wintering mycelium and sclerotia. Spectral modification of daylight by near-UV filters incorporated into plastic covers has been useful to reduce conidiation and infection in a number of crops (Reuveni and Raviv, 1992; Reuveni *et al*., 1989; West *et al*., 2000). In unheated greenhouses, the night temperature of plants can be lower than the air temperature due to irradiative cooling; heating briefly before sunrise to raise plant temperature above the ambient air temperature reduces dew formation on leaves and can control grey mould (Dik and Wubben, 2004).

A remarkable importance is dedicated to the selection of cultivars that are elusive to the pathogen. For instance some table grapes, like “Cardinal” and “Centennial Seedless” are less susceptible to *B. cinerea* because of their precocity in ripening, as compared to the mid or late maturating cultivar like “Italia”, “Regina” and “Red globe” that are harvested in periods favorable for the development of *Botrytis* bunch rot (Faretra *et a*l., 1996). Cultivar resistance was attributed to higher cuticle and wax contents and certain anatomical features rather than induced or constitutive antifungal host defence mechanisms (Mlikota Gabler *et al*., 2003). In some raspberry cultivars, the stigmatic fluid was inhibitory to *B. cinerea* thereby avoiding latent infections (Williamson and Jennings, 1992). These findings suggest that cultivar selection will play a major role in future *Botrytis* management strategies.

The choice of plantation sites, rootstocks and the training system, in addition to the cultural practices like thinning of berries and defoliation, the arrangement of the leaves and bunches on the tree in order to favor aeration of bunches, can help to

create less favorable conditions for the development of the pathogen. These practices help also the chemical treatments because bunches are well exposed to sprays.

The effect of rootstocks on *Botrytis* bunch rot of grapes has been well studied (Egger *et al*., 1979; Delas *et al*., 1984; Ferreira and Marais, 1987; Cristinzio *et al*., 2000) and generally is indirect in its nature, primarily affecting scion vigour and bunch compactness (Ferreira and Marais, 1987). The rootstock may impart a 'resistance factor' to the scion, for example leaves of cv. Falanghina produced smaller lesions on rootstock SO_4, compared to three other rootstocks (Cristinzio *et al*., 2000). However, inoculum production from such lesions and the nature of the resistance mechanism requires further investigation. Extensive research in France on Mèdoc and Graves soils on the impact of rooting depth and water up-take on skin splitting and grey mould have been made (Ribèreau-Gayon *et al*., 1980); deep rooted vines were much less susceptible to splitting and grey mould than shallow-rooted vines. Rootstocks also had a significant impact on the extent of fruit micro-cracking in sweet cherries and differences in soil moisture uptake by rootstocks were believed to be responsible.

Regarding the training systems, grapes grown in dense canopies are exposed to greater periods of wetness after rainfall, resulting in increased susceptibility to *B. cinerea* (Steel, 2001). A range of different vine training systems were evaluated in Italy on several grape cultivars to identify systems that were non-conducive to pathogen development. The highest incidence of *B. cinerea* was reported in the 'Pergola' system, while vines pruned to the 'Guyot' system had the lowest disease development (Cargnello *et al*., 1991). Vines trained in the horizontal bilateral cordon ('traditional Moser system') had improved exposure to light and lower incidence of *Botrytis* and powdery mildew (*Uncinula necator*), higher yields and better quality grapes than the 'high' cordon system supported by a one-wire trellis (Redl, 1988). Also, the practice of leaving 60 rather than 40 nodes per vine in vigorously grown Chenin Blanc grapes reduced bunch rot in spur- or cane-pruned systems, and the *Botrytis* reduction was attributable to less compact clusters (Christensen, 1981).

In Australia, the practice of 'lighter pruning' the vine canopy reduced berry-to berry-contact within the bunch and *B. cinerea* development (Martin, 1990).

Noncontact Riesling berries had 15.7 and 35% more epicuticular wax and cuticle compared to the contact samples, explaining the lower incidence of bunch rot (Percival *et al*., 1993). Similarly, reducing epicuticular waxes in grapes by spraying an adjuvant can increase bunch rot (Marois *et al*., 1987). Along with the training system itself, the bunch architecture can also affect development of the pathogen.

Infection of Cabernet Sauvignon clusters after veraison by *B. cinerea* was significantly influenced mainly by cluster compactness (Vail and Marois, 1991; Fermaud *et al*., 2001a, b); reduced *Botrytis* was correlated with less compact clusters, associated with lower berry number and reduced cluster weight. Thus, training and pruning systems adopted to reduce the risk of *Botrytis* at vintage may be cultivar-specific and dependent upon a range of other factors.

Vine 'hedging' is the practice of pruning off the over-hanging current season growth at veraison. Vines trained on a two-wire trellis, sprayed and hedged, had a 39% reduction in bunch rot as compared to vines sprayed and not hedged. Hedging improved air circulation in the bunch zone, reduced relative humidity in the canopy and exposed more fruit bunches to light (Savage and Sall, 1982). This practice has now been widely adopted in Australasian (Clingeleffer, 1984; Sommer *et al*., 1995) and in North American vineyards (Reynolds and Wardle, 1993) as a cost-effective alternative to hand pruning and as a cultural operation aiming to reduce bunch rot. A better practice was proposed subsequently, based on careful selection of node number at winter pruning, providing better shoot spacing and thus creating a canopy with optimal density (Smithyman *et al.,* 1997).

Different cultivars respond to pruning regimes quite differently. In seasons conducive to infection, the practice of removing or thinning 'distal' clusters just before veraison reduced infection in northern Italian vineyards. The level of cluster thinning depended on the particular cultivar, e.g., bunch rot incidence at harvest was 21% for no thinning, 10% for the 20% cluster thinning and 7% for the 40% cluster thinning level. In contrast, cluster thinning in Cabernet Sauvignon had no significant effect on bunch rot at vintage (Palliotti *et al.,* 2000).

Among cultural practice that can be utilized for reducing the presence of *B. cinerea*, we remind defoliation practice. Leaf removal from the fruiting zone of vines

('leaf plucking') has significantly reduced epidemics thereby improving *Botrytis* control in grapes in European (Zoecklein *et al*., 1992), Californian and Australian vineyards (Gubler *et al.,* 1987; Percival *et al.,* 1994). Leaf removal affects the microclimate (temperature, vapour pressure deficit, wind speed and wetness) in and around the receptive bunch, often reducing bunch rot at vintage. Increased wind speed after leaf plucking (English *et al*., 1989) increased the evaporative potential on the berry surface, thereby significantly reducing *B. cinerea* infection and development. In addition, stimulation of phytoalexin production by increased UV light has been reported as a result of leaf removal (Langcake, 1981). Following leaf removal, exposed berries of Riesling grapes had 19 and 35% more epicuticular wax and cuticle, respectively, compared to the shaded bunches resulting in significantly less grey mould (Percival *et al.,* 1993). Leaf removal has been adopted globally as an effective non-chemical practice to manage *B. cinerea* in vineyards.

A less well-adopted practice to manage *Botrytis* is the removal of potential substrates to reduce inoculum potential in the bunch early in the season. Removal of senescent floral tissues and aborted berries ('bunch trash') reduces *B. cinerea* by 30% in Merlot grapes (Jermini *et al*., 1986). The relationship between senescent floral debris retained in fruit clusters of Chardonnay and *Botrytis* bunch rot was investigated for three seasons in California. Compressed air was used to remove bunch trash at early or late fruit set. Removal of inoculum in bunch trash significantly reduced bunch rot in some, but not all vineyards, indicating that other factors besides bunch trash biomass may contribute to subsequent bunch rot at harvest (Wolf *et al*., 1997). In addition, in a Californian kiwifruit plantation, removal of flowers from male vines, a potent source of *B. cinerea*, reduced stem-end rot by 60% in neighboring female vines compared to fruit from vines where the male flowers were retained (Michailides and Elmer, 2000). The impact of removal of necrotic tissue on epidemics was also demonstrated in The Netherlands (Köhl *et al*., 1992). In this study, removal of up to 30-50% of necrotic tissues by hand reduced the number of *Botrytis* spp. conidia in the air by 34% and subsequently delayed the *Botrytis* epidemic. This finding was used as the rationale for developing a new biocontrol strategy, based upon saprophytic colonization of necrotic tissues by

selected antagonists (Köhl *et al*., 1995a). Use of compressed air to remove necrotic canopy and bunch tissue in grapes in New Zeland reduced the *B. cinerea* epidemic and at harvest, bunch rot incidence was reduced by 50% (Elmer and Michailides, 2004). These and other studies demonstrate the importance of necrotic tissue substrates for *B. cinerea* epidemics.

Harvesting earlier than scheduled is the commonest cultural practice used to limit losses of mature grapes by *Botrytis* (Nair, 1985). If conditions favour *Botrytis* development, it was demonstrated that the crops will be harvested at 18° Brix (soluble solids content) to limit pathogen development (Elmer and Michailides, 2004). Field and experimental data support that more mature fruit at harvest have increased levels of resistance to *B. cinerea* than less mature fruit (Pyke *et al.,* 1993). Also treatment against the grapevine moth (especially the second generation), thrips and other pest, reducing wounds on berries, can limit indirectly gray mold (Ciccarone, 1970). Wounds can be also caused by wind, birds and wasp; thus covering the vineyards by nets is a suitable practice in order to prevent infections by *B. cinerea*. In conclusion, in the context of integrated crop management (IPM) there is great merit in using the maximum effort to reduce pesticide residues by minimal chemical treatment, alternating chemical groups to reduce resistant build-up; application of biological control agent(s) appropriate for the temperature regime and humidity; scrupulous removal of dead crop material to remove inoculums; use of mulches to bury leaf litter; adequate plant spacing, good control of weeds to create open well-ventilated canopy and management of insect pests that wound the plant and act as vectors. Disease forecasting, especially when combined with accurate local weather data, has been successful in reducing serious crop damage by specifying timely treatment in grape (Broome *et al.,* 1995) or strawberry (Berrie *et al*., 2002).

2.6.2. Biological control

The development of biological control methods may be a good complement to control the disease and many biological control agents, including the fungal genera *Alternaria*, *Cladosporium*, *Epicoccum*, *Gliocladium*, *Trichoderma* and *Ulocladium*

have been described in the past years (Boff *et al*., 2002; Elad *et al*., 1998; Elmer and Reglinski, 2006; Helbig, 2002; Utkhede and Mathur, 2006). Many of these nonpathogenic microorganisms suppress the growth of plant pathogens through competition for nutrients, the production of inhibitory metabolites and/or parasitism, thereby naturally limiting plant disease in the environment. However, despite many reports of successful biocontrol of *B. cinerea* in laboratory conditions, only a small proportion of these have demonstrated field efficacy and an even smaller subset have been developed into commercial products. Moreover, biological control cannot be used as the unique way to control the pathogen due to particle effectiveness and broad variability of results obtained with the usage of microbial antagonists.

No other single fungal genus has received as much attention as the *Trichoderma* spp. for biocontrol of plant pathogens. Biocontrol research with *Trichoderma* spp. against *B. cinerea* in grapes commenced nearly three decades ago (Dubos *et al*., 1978, 1982) and the best results were achieved when disease pressure in the vineyard was low to moderate (Bisiach *et al*., 1985; Gullino and Garibaldi, 1988; Garibaldi *et al* ., 1989). An isolate of *T. harzianum* (T39), originally isolated from cucumber, was the first *Trichoderma* sp. to be specifically formulated into a commercial product for control of *B. cinerea* (Elad, 2001). Then, the efficacy of this product was comprehensively evaluated in 139 field experiments in commercial vineyards over 19 countries on 34 varieties between 1988 and 1994. The control efficacy was 36%, compared with 52% with standard botryticides, when applications were made at the four growth stages; end of flowering, bunch closure, veraison, 2–3 weeks postveraison, at a rate of 4 kg ha^{-1}. Efficacy declined when the interval between the last preharvest application and vintage was extended out to 5 weeks, indicating that late-season protection of ripening fruit was important for *B. cinerea* control (O'Neill *et al*., 1996). An isolate of *T. harzianum* was specifically selected for its ability to colonize senescent floral debris (stamens, calyptra and aborted fruitlets) and the green structural tissues of the grape bunch, since these were potential sites for latent *B. cinerea* infections (Holz *et al.,* 1997, 2003). Colonization capability of *T. harzianum* was superior to that of *Gliocladium roseum*, *Ulocladium atrum* and *Trichosporon pullulan*, in bunches of table (cv. Dauphine) and wine (cv.

Chardonnay) grapes in South African vineyards (Holz and Volkmann, 2002). Antagonist establishment fluctuated between vineyards and between seasons, but only *T. harzianum* effectively colonized monitored positions within bunches during the season. However, efficacy of botrytis bunch rot control was not possible due to the sporadic nature of the pathogen in the experimental vineyards over two growing seasons. The product T-22 (Bioworks Inc., USA) contains *T. harzianum*, and is primarily used to control soilborne pathogens and as a plant growth stimulant (Harman, 2000; Dissevelt and Ravensberg, 2002). In grapes, suppression of *B. cinerea* with T-22 was equivalent to a standard botryticide programme (Harman *et al*., 1996; Wilson, 1997).

T. harzianum isolates (code S10B and P1) have been evaluated on table grape cv. Thompson Seedless in Chile. Over a range of disease pressure conditions (1992-96), isolate P1 provided effective control of botrytis bunch rot and efficacy was equivalent to a botryticide programme based upon the dicarboximide fungicide, vinclozolin. The effectiveness of P1 as a formulated BCA was significantly better compared with the unformulated treatment (Latorre *et al*., 1997).

Mechanisms of *B. cinerea* suppression by different *Trichoderma* spp. are diverse and include antibiosis (Cutler *et al*., 1996; Cooney *et al*., 1997; Rey *et al*., 2001), competition (Elad *et al*., 1999), mycoparasitism (Dubos *et al*., 1982; Papavizas, 1985) and induction of plant defence mechanisms (Elad, 2000b; Hanson and Howell, 2004). Some isolates exhibit multiple modes of action: for example, the T39 isolate used in Trichodex is an effective nutrient competitor, but also interferes with *B. cinerea* pectolytic enzymes and induces host resistance (Elad and Stewart, 2004).

Among the antagonistic species belonging to *Ulocladium* genus, an isolate of *U. atrum* (U385) was first identified as an antagonist of *B. cinerea* over a decade ago (Kohl *et al*., 1993) and, since then, has been shown to suppress this pathogen in several field and glasshouse crop systems (Köhl *et al*., 1995b, 1995c, 2001). In field tests in grapes, applications of this isolate reduced botrytis bunch rot by up to 67% in different wine-growing regions in Germany (Lennartz *et al*., 1998; Schoene and Kohl, 1999; Schoene *et al*., 2000) and similar levels of botrytis bunch rot control were reported from studies with U385 in French vineyards (Roudet and Dubos,

2001). Subsequent field studies in German vineyards confirmed that, under moderate pathogen pressure, *U. atrum* (385) has the potential to control botrytis bunch rot of grapes. However, it was proposed that when vineyard conditions were highly conducive to infection, the efficacy of this biological control agents (BCAs) would decline to the point where it could not completely replace synthetic botryticides (Metz *et al*., 2002).

Several *Ulocladium* spp. isolates have been investigated as potential *B. cinerea* antagonists on necrotic grape leaf discs in New Zealand (NZ) laboratories (Stewart *et al*., 1998). One isolate (U13) suppressed *B. cinerea* conidiophore production by up to 90% on necrotic leaf discs that had been preinoculated with *B. cinerea*, then exposed to field conditions in a NZ vineyard (Stewart *et al*., 1998). In separate studies, U13 was field evaluated for suppression of *B. cinerea* overwintering inoculum potential on grape rachii in the canopy. Some reduction in *B. cinerea* inoculum potential was reported 2 months after harvest at one site, but the reduction in pathogen inoculum was not maintained through to early spring (Fowler *et al*., 1999).

Several other commonly occurring fungi (e.g. *Alternaria* spp., *Cladosporium* spp., *E. nigrum*) and basidiomycetous yeasts (e.g. *Aureobasidium pullulans*) have been isolated from grape tissues and found to be antagonistic to *B. cinerea* (Dugan *et al*., 2002). There have been many reports of successful suppression of *B. cinerea* with isolates of *E. nigrum* (syn. *E. purpurascens*) (Hill *et al*., 1999; Elmer *et al*., 2001; Szandala and Backhouse, 2001). In grapes, *E. nigrum* effectively suppressed *B. cinerea* on leaf discs that had been preinoculated with the pathogen in laboratory and vineyard assays (Stewart *et al.,* 1998). Application of *E. nigrum* to grape rachii significantly reduced *B. cinerea* inoculum production on these tissues over a range of incubation temperatures (10–20°C), indicating that this biocontrol agent had the potential to effectively reduce overwintering *B. cinerea* in the vineyard (Fowler *et al.,* 1999). Further experiments demonstrated that *E. nigrum* reduced *B. cinerea* inoculums on overwintering rachii 2 months after harvest, but the reduction in inoculum potential was not maintained through to early spring. Field applications of an isolate of *E. nigrum* (HRE2) suppressed botrytis bunch rot by 60% (cv. Chardonnay) when evaluated in a New Zeland vineyard in separate studies. Although

some isolates of *E. nigrum* produce antimicrobial metabolite(s) capable of completely suppressing germination of *B. cinerea* conidia, this isolate did not produce assay-detectable antimicrobials, and it was concluded that the primary mode of action of this isolate was aggressive saprophytic colonization of necrotic vine tissues (Elmer *et al.,* 2001).

Gliocladium roseum (reclassified as *Clonostachys rosea*) has effectively suppressed *B. cinerea* in both field and glasshouse crops (Sutton *et al.,* 1997; Kohl *et al*., 1998; Morandi *et al*., 2001). Published comparative field evaluations of this biocontrol agent in viticulture are sparse, but in one report *Gliocladium* spp. were reported to be less effective than *Trichoderma* spp. when tested on grapes (Machowicz Stefaniak, 1998). In contrast, an unnamed *Gliocladium* spp. was as effective as the dicarboximide fungicide vinclozolin against *B. cinerea* in vineyard tests (Cherif *et al.,* 1998). Mode of action studies indicate antibiosis, and mycoparasitism of conidia and germ tubes are important biocontrol mechanisms (Kohl *et al.,* 1997; Li *et al.,* 2002).

Among yeasts and yeast-like fungi, a diverse range of yeasts (e.g. *Rhodotorula glutinis*, *Candida* spp., *Pichia membranifaciens*, *Kloeckerea apiculata*, *Saccharomyces* spp.) and yeast–like species (e.g. *A. pullulans* and *T. pullulans*) have shown efficacy against *B. cinerea*. *Rhodotorula glutinis* (LS-11) and *Cryptococcus laurentii* (LS-28) were reported to be effective *B. cinerea* antagonists in vineyards. LS-28 was regarded as more promising based upon its biosuppression capabilities over a broad range of experimental conditions (Lima *et al*., 1998). These antagonists also demonstrated low sensitivities to copper oxychloride and dicarboximide fungicides, but were classed as being highly sensitive to the demethylation inhibitor (DMI) fungicides, penconazole (Topas) and tebuconazole (Folicur).

The formulated yeast product Saccharopulvin 25 PU (*Saccharomyces chevalieri*) was applied at the end of flowering, petal fall, berry formation, bunch closure and 3 weeks before harvest for three seasons (1995–97) at 6 × 106 CFU mL^{-1} (Sesan *et al*., 1999). Average botrytis bunch rot incidence was 40% in the untreated controls and Saccharopulvin treatment efficacy was 91% when averaged over three seasons. Zahavi *et al*. (2000) evaluated *Candida guilliermondii* A42 and *Acremonium*

cephalosporium B11 against *B. cinerea* in field-grown table and wine grapes. Two to five applications of A42, at 7- to 10-day intervals, to both crops from veraison (1996-1998), reduced the incidence of rots from *B. cinerea* and *Aspergillus niger* at harvest (wine grapes) and after postharvest storage (table grapes) in two of the three growing seasons: B11 reduced both rot-inducing pathogens in the wine grapes but not in the table grapes. Interestingly, in the 1996 growing season, none of the BCA treatments or the chemical controls effectively reduced incidence of rot in Sauvignon blanc grapes compared with the untreated controls. On this occasion *B. cinerea* may have been established in the bunches as a consequence of conditions favourable to the pathogen over the flowering period (Elmer and Michailides, 2004). Consequently, the postveraison BCAs and chemical treatments could only be expected to protect rapidly ripening berries from conidial infections and not from aggressive *B. cinerea* infections from a saprophytic base residing in floral debris and aborted fruitlets within the bunch.

The ascosporic yeast, *Metschnikowia pulcherrima* (anamorph: *Candida pulcherrima*) isolate 320, was identified as an effective antagonist against botrytis storage rot in table grapes (Nigro *et al*., 1999). An isolate of the yeast *M. fructicola* was also evaluated by Kurtzman and Droby (2001), and Keren-zur *et al*. (2002) reported good disease control against both *B. cinerea* and *A. niger*. A water dispersible granule with a shelf life of 1 year has been successfully formulated and marketed as Shemer and registered in Israel by AgroGreen Minrav Group. Also an isolate of *Pichia membranifaciens* (FY 101), isolated from grapes, was an effective antagonist of *B. cinerea* on grapevine plantlets grown *in vitro* and in coinoculation studies on grape berries (Masih *et al*., 2000, 2001). Similarly, two isolates of *A. pullulans* (L47 and LS-30) are reported to be highly effective against *B. cinerea* on table grapes (Lima *et al*., 1996, 1997; Castoria *et al*., 2001). Interestingly, there are very few reports of field-based evaluation of isolates of *A. pullulans* in wine grapes.

At the same time, many studies have investigated the potential of bacteria as *B. cinerea* antagonists in a wide range of fruit crops, including tomatoes (Daggas *et al*., 2002; McHugh *et al*., 2002), strawberries (Helbig, 2001; Guetsky *et al*., 2002), apples (Janisiewicz and Jeffers, 1997) and pears (Seddon *et al*., 2000; Nunes *et al*.,

2001). Bacterial BCAs with reported activity against *B. cinerea* on grape tissues include *Bacillus* spp. (Ferreira, 1990; Krol, 1998; Paul *et al*., 1998), *Bacillus circulans* (Paul *et al*., 1997), *Brevibacillus brevis* (formerly *Bacillus brevis*; Seddon *et al*., 2000), *Bacillus subtilis* (Esterio *et al*., 2000), *Pseudomonas fluorescens* (Krol, 1998), and *Serratia liquefaciens* (Whiteman and Stewart, 1998). Four applications of a new formulation of Serenade (*B. subtilis* strain QST-713) were compared with a traditional spray program used to treat table grapes (cv. Thompson Seedless) for *B. cinerea* management in Chile. Higher rates of the product (15 kg/ha) resulted in postharvest disease control equivalent to a traditional botryticide program (Esterio *et al*., 2000). Up to 90% disease control was reported also when green table grapes were artificially inoculated with *B. cinerea* and then treated with a suspension of *B. brevis* (Seddon *et al*., 2000). Several bacteria, such as *Enterobacter agglomerans*, *Serratia* spp. or *Pseudomonas* spp., described as potential biological control agents against *B. cinerea* produce the antibiotic pyrrolnitrin (3-chloro-4-(2'-nitro-3'-chlorophenyl)-pyrrole) (Chernin *et al*., 1996; Janisiewicz and Roitman 1988; Raaijmakers *et al*., 2002). This antibiotic has been reported to inhibit the growth of *B. cinerea* (Hammer *et al*., 1993).

A recurring problem encountered in the field with biological control against plant pathogens is the inconsistency of its efficacy (Elad and Stewart, 2004). According to Elad and Stewart (2004), this can be attributed to climatic variations encountered in field conditions, a lack of ecological competence of the biological control agents, and/or an unstable quality of the products. However, reduction of efficacy may also result from the variability of sensitivity of plant pathogens to biological control agents. The build-up of field resistance of biological control agents could arise if plant pathogens have the ability to produce natural mutants with reduced susceptibility under the selection pressure of products used by farmers. For instance, Li and Leifert (1994) have shown that after ten successive treatments on plants, the efficacy of the antibiotic-producing bacterium *B. subtilis* CL27 against *B. cinerea* dropped dramatically. Recently, Ajouz *et al*. (2010) have demonstrated that *B. cinerea* can become less sensitive to the antibiotic pyrrolnitrin. This resistance makes the pathogen less sensitive to a pyrrolnitrin producing bacterium *in vitro* tests (Ajouz

et al., 2010). Possible loss of efficacy of a biological control agent could also result from the selection of preexisting plant pathogen isolates with low susceptibility in natural populations.

A recent study has shown that *B. cinerea* can tolerate the antibiotic 2,4 DAPG produced by the bacteria *Pseudomonas* spp. (Schouten *et al*., 2008). Accordingly, despite the commonly reported assumption that resistance of plant pathogens to biological control agents will develop less frequently as compared to chemical control methods (Duffy *et al.,* 2003), one might fear a possible repercussion on the durability of efficacy of antibiotic-producing biological control agents.

2.6.3. Chemical control

Chemical control remains the main way to reduce the incidence and severity of grey mould and other *Botrytis* diseases on major crops. However, registration restrictions, tolerances established by import countries, and the development of resistant strains limit fungicide treatments in table grapes and in other fruit crops, especially at harvest or during post harvest (Latorre *et al*., 1994; Latorre *et al*., 2002; Errampalli and Crnko, 2004; Sallato and Latorre, 2006).

Fungicides can provide disease control through both pre- and post-infection activity. Pre-infection activity is commonly known as protectant (preventive) activity and post infection activity comprises a curative action that can involve both pre- and post-symptom expression activities.

Three preventative fungicide applications are recommended in the vineyards: firstly, between budding and pre-bloom, to protect susceptible inflorescences; secondly, during bloom to pea-size stage, to reduce inoculums in clusters and to prevent colonization of floral debris; and thirdly, at bunch closure, to reduce inoculums of *B. cinerea* at various positions of the inner bunch, especially for cultivars with tight bunches (Van Rooi and Holz, 2003; Van Schoor, 2004).

Laboratory studies (Van Rooi, 2001) have shown that when fungicides are properly applied to the susceptible target sites in bunches, the amount of *B. cinerea* at the various sites within bunches is reduced, and infection and symptom expression are prevented at all growth stages. The same efficacy is, however, not achieved with

the same fungicides when using conventional spraying methods in vineyards (Holz *et al*., 2003).

Botryticides were introduced in viticulture during the late 1950s. Sulphamides (dichlofluanid), pthalimides (captan, captafol, folpet) and dithiocarbamate (thiram) were the first fungicides used in many countries for *Botrytis* control. They were used until 1968 (Elad *et al*., 1995). At this point of time the efficacy of fungicidal treatments for *Botrytis* control ranged between 20 and 50 percent. All this fungicides were multi-site inhibitors, affecting many target sites in fungal cell and therefore acting as general enzyme inhibitors. In 1960s, first fungicides appeared which act primarily at one target site therefore referred to as single-site or site-specific and they more efficiently control pathogen.

Today, several families of synthetic site-specific botryticides are available. They can be classified according to their biochemical modes of action into five categories: 1) anti-microtubule toxicants (benzimidazoles); 2) compounds affecting osmoregulation (dicarboximides, fludioxonil); 3) inhibitors of methionine biosynthesis (anilinopyrimidines); 4) sterol biosynthesis inhibitors (fenhexamid) and 5) fungicides affecting fungal respiration (fluazinam, boscalid and multi-site inhibitors). The era of sigle-site or site specific fungicides begun in late 1960s with introduction of benzimidazoles (Dekker, 1977; Georgopoulos, 1979; Beever and O'Flaherty, 1985). Benzimidazole is a heterocyclic aromatic organic compound. This bicyclic compound consists of the fusion of benzene and imidazole. The most prominent benzimidazole compound in nature is N-ribosyl-dimethylbenzimidazole, which serves as an axial ligand for cobalt in vitamin B12. Representatives of this class of fungicides are benomyl, carbendazim and tiophanate-methyl. They act as inhibitors the mitosis; this inhibition is ascribed to complex formation between benzimidazoles and β-tubulin, which is required for microtubule formation (Davidse and Ishii, 1995). In *B. cinerea*, these fungicides do not prevent conidial germination, but at low concentrations, they inhibit hyphal growth and cause distortion of germtubes (Leroux *et al.,* 1999).

Only a few years later the new group of dicarboximides become available and they shadowed all previously used ingredients. Dicarboximides were introduced into

the market between 1975 and 1977 primarily for the control of *B. cinerea* in grapes (Beetz and Löcher, 1979). Due to good efficacy they were popularly named botryticides and it seemed that the problem of protection against *Botrytis* had been successfully solved. Dicarboximides or cyclic imides (e.g. chlozolinate, iprodione, procymidone, vinclozolin) are characterized by the presence of a 3,5-dichlorophenyl group. The activity of dicarboximides fungicides was first reported in the early 1970's with the three key commercial products being introduced within three years; iprodione in 1974 (Lacroix *et al*., 1974), vinclozolin in 1975 (Pommer and Mangold 1975), while procymidone was registered a year later (Hisada *et al*., 1976). They are typically protectant fungicides and although some claims to systemicity have been made (Hisada *et al*., 1976), they are best regarded as protectant materials. The mode of action of dicarboximides fungicides is not yet fully understood. Morphological disorders of hyphae and germ tubes upon treatment with the fungicides are often observed. Antifungal activity is reversed by free radical scavengers such as α-tocopherol suggesting that the mode of action of dicarboximides may relate to lipid peroxidation (Orth *et al*., 1993). Glutathione synthetase as a target enzyme of dicarboximides has been also proposed (Ellner, 1996).

In the mid-1990s a novel family of botryticides was arose, the anilinopyrimidines, with three representative ingredients: pyrimethanil, cyprodinil and mepanipyrim. The mode of action of anilinopyrimidines differs from other classes of fungicides (Gasztonyi and Lyr, 1995). First report of the mode of action of mepanipyrim (Miura *et al*., 1994) and pyrimethanil (Milling and Richardson, 1995) indicate that the compounds inhibit the secretion of hydrolyzing enzymes such as cutinase, pectinase and cellulose from fungal hyphae. These enzymes are required for the fungus to penetrate plant cells. Specific interference with the biosynthesis of methionine was also proposed as a mode of action of anilinopyrimidines (Masner *et al*., 1994; Gasztonyi and Lyr, 1995). More specifically, pyrimethanil seems to decrease the accumulation of methionine and to induce the accumulation of cystathionine. This observation indicates that pyrimethanil effects methionine biosynthesis by inhibition of cystathionine β-lyase (Fritz *et al*., 1997). Mepanipyrim and pyrimethanil exhibit a high activity against *B. cinerea,* while cyprodinil came in

combination with fludioxonil (phenylpyrroles) in protection of grapes. *In vitro* studies with *B. cinerea* revealed that anilinopyrimidines strongly inhibit germ tube elongation but effects on mycelial growth varied according to the composition of nutrient media (Leroux, 2007). Fungitoxicity was generally low with complex media, for instance those containing yeast extract. This phenomenon seems to be related to the ability of the fungus to obtain nutrients by methods that circumvent the mode of action of anilinopyrimidines. Several amino acids, particularly methionine, have been shown to antagonize the fungitoxicity of anilinopyrimidines. In particular, anilinopyrimidines fungicides inhibit the cystathionine β-lyase in biosynthesis of methionine and therefore they would not show activity of growth inhibition in media containing methionine (Leroux, 1994; Masner *et al*., 1994).

Although anilinopyirimidines showed to be highly effective against *B. cinerea,* a high risk of resistance build up was already evident in the laboratory investigations at preregistration phase (Birchmore and Forster, 1996). In spite of that they have been registered in most European winegrowing countries since 1994 but with recommendations for restricted use: once per season when anilinopyrimidines are applied alone and a maximum of two applications per season is proposed for the mixture cyprodinil + fludioxonil (phenylpyrrol) (Fabreges and Birchmore, 1998). Shortly after introduction of anilinopyrimidines, owing to its good light stability, in 1995 fludioxonil (phenylpyrroles) start to be used as a foliar fungicide in vineyards against *B. cinerea* (Rosslenbroich and Stuebler, 2000). Fludioxonil is a synthetic analogue of antibiotic pyrrolnitril (phenylphyrol), an antibiotic compound produced by a number of *Pseudomonas* spp. and is thought to play a role in biocontrol by these bacteria. Fludioxonil is used also as a foliar fungicide and in seed treatments to control *Fusarium*, *Tilletia* and other seed-borne pathogens. It belong to class of fungicides affecting osmoregulation; it has been hypothesized that phenylpyrroles interfere with the osmotic signal transduction pathway, resulting in an abnormal accumulation of glycerol (Leroux *et al*., 2002; Pillonel and Meyer, 1997). Therefore, pyrronitrin derivates may inhibit energy production by uncoupling of the oxidative phosphorylation in fungal respiration (Lambowitz and Slayman, 1972). Similarly to dicarboximides, phenylpyrroles inhibit both conidial germination and mycelium

growth, but the latter process is more sensitive. Fludioxonil appears 30-40 times more toxic than dicarboximides in *in vitro* effects on hyphal growth, but under field conditions the registered doses for both families are similar (i.e. 500 g fludioxonil/ha versus 750 g dicarboximide/ha in vineyards) (Leroux, 2007).

In 1999, firstly in Switzerland, a botryticide with novel botryticidial action was registered, the fenhexamid (Baroffio *et al*., 2003). Early investigations on the fenhexamid mode of action suggested that it has different mechanism from than of all other botryticides (Rosslenbroich and Stuebler, 2000). Fenhexamid is a 1,4-hydroxyanilide with a high preventive activity against *B. cinerea*. It is easily degraded and therefore presents a favourable toxicological profile and environmental behavior (Rosslenbroich *et al*., 1998; Rosslenbroich and Stuebler, 2000). It is characterized by a long duration action. Due to its lipophilic character, it shows rapid uptake into the plant cuticle and within the upper tissue layer limited but significant locosystemic redistribution occurs (Haenssler and Pontzen, 1999) and as a result the rain fastness of fenhexamid is very pronounced. Fenhexamid suppresses the germination of spores only at relatively high concentrations but it is highly effective in inhibiting subsequent stages of infections. After the initiation of spore germination, the fenhexamid inhibit the germ-tube elongations, germ-tubes collapse and die before they are able to penetrate plant surface. Also, treated hyphae frequently show a characteristic leakage of cytoplasm or cell wall associated material at the hyphal tip area (Haenssler and Pontzen, 1999; Debieu *et al*., 2001). It is a sterol biosynthesis inhibitor (Rosslenbroich and Stuebler, 2000). Sterols function as important components of cell membranes and are synthesized by a conserved pathway starting from acetate. The main sterols in mammals, plants and fungi are cholesterol, β-sitosterol and stigmasterol, and ergosterol, respectively. Sterols are not only important for structural strength of cell membranes but also for maintenance of appropriate membrane fluidity, regulation of membrane permeability and activity of membrane-bound enzymes (Darke *et al.,* 1972; Hall, 1987; Nes *et al*., 1978; Vanden Bossche and Marichal, 1993). In *B. cinerea*, fenhexamid reduced the content of ergosterol and induced accumulation of 3-keto compounds such as 4-α-methylfecosterone, fecosterone and episterone. This observation suggests that

fenhexamid inhibits the activity of 3-keto reductase involved in C4 demethylation (Debieu *et a*l., 2001).

Among the sterol biosynthesis inhibitors, also prochloraz and some triazoles, such as tebuconazole, are used against grey mould on various crops (Leroux, 2007). However, applications of higher rates of triazoles, which would allow better control of grey mould, may be limited by phytotoxicity (Leroux, 2007). This problem can be overcome by using mixtures with multi-site fungicides (e.g. tebuconazole + dichlofluanid) (Yunis *et al*., 1991). Additionally, strains with reduced sensitivity towards several sterol biosynthesis inhibitors including tebuconazole have been associated with poor grey mould control on vegetable crops in greenhouses (Elad, 1992).

In the same years (1999) another fungicide, the fluazinam (phenylpyridinamine), was introduced in Europe vineyards, although in Japan has been used since 1990 against grey mould in various crops. Fluazinam belongs to group of fungicides that affecting fungal respiration so, it shows multi-site activity probably related to uncoupling of mitochondrial oxidative phosphorilation. It is highly toxic to spores and mycelia. The intensive use of these site-specific inhibitors has led to a rapid selection of pathogen strains resistant to benzimidazoles, dicarboximides and anilinopyrimidines in many countries worldwide (Baroffio *et al*., 2003; Elad *et al*., 1992b; Fraile *et al*., 1986; Leroux *et al*., 1999; Moorman and Lease, 1992; Moyano *et al.,* 2004; Myresiotis *et al*., 2007; Pappas, 1997). For this raison, in the early 2000s two other new active fungicides, pyraclostrobin and boscalid, were introduced.

Pyraclostrobin belongs to the group of Quinone outside inhibitors (QoIs), a fungicide class that was developed from natural fungicidal derivatives such as strobilurin A and oudemansin A (Ammermann *et al*., 2000; Bartlett *et al*., 2002). QoI fungicides include kresoxim-methyl (Ammermann *et al*., 1992), azoxystrobin (Godwin *et al*., 1992) and trifloxystrobin (Margot *et al*., 1998). The mechanism of action of this fungicide class is the inhibition of mitochondrial respiration by binding at the Qo site of the cytochrome b, causing the blocking of electron transport between cytochrome b and cytochrome c_1. Inhibition of mitochondrial respiration leads to a disruption of the energy cycle (Bartlett *et al*., 2002). Pyraclostrobin

possesses an extremely broad spectrum of activity, including fungal species such as *B. cinerea* (Ammermann *et al*., 2000; Karadimos *et al*., 2005; Markoglou *et al*., 2006).

The pyridine carboxamide boscalid [2-chloro-N-(4'-chlorobiphenyl-2-yl)nicotinamide] is another new broad-spectrum fungicide recently introduced for the control of several fungi belonging to *Ascomycetes* and *Basidiomycetes*. It was first introduced in grapevines in 2004 year. In Italy, commercial formulations containing boscalid are allowed for use against several fungal species attacking fruit, vegetables and vines, including *Sclerotinia* spp., *Alternaria* spp., *Monilinia* spp. and *B. cinerea* (Matheron and Porchas, 2004; Stammler and Speakman, 2006). It is new generation of succinate dehydrogenase inhibitors (SDHIs, II generation) and it act as inhibitor of fungal respiration (McKay *et al*., 2011). In particularly, its mode of action is the inhibition of electron transport in mitochondrial respiration by binding to the complex II, also referred to as succinate deydrogenase (SDH) complex or succinate: quinine reductase (SQR). Like the other enzymatic complexes of the respiratory chain (I,III and IV), the enzyme is a component of the inner mitochondrial membrane. However, it does not function as a proton pump and consist of four nucleus-encoded sub-units: the flavoprotein (SDHA) and the iron-sulphur protein (SDHB) sub-units are located in the peripheral part, and act as the dehydrogenase catalytic portion oxidating succinate to fumarate in the tricarboxylic acid cycle; whereas two membrane-anchored protein sub-units (QPs), known as cytochrome b (SDHC) and CybS protein (SDHD), anchoring SDHA and SDHB to the membrane, are responsible for the quinine reductase activity (Hägerhäll, 1997). Boscalid prevents energy production and makes unavailable the chemical building blocks for the synthesis of essential cell components and, hence, disrupts fungal growth with deleterious effect on spore germination, germ tube elongation, mycelia growth and sporulation. In this respect, it resembles QoI fungicides, but there is no cross-resistance between the two grops of fungicides due to their different sites of action.

These two new fungicides, with modes of action distinct from those of botryticides already in use, could play in the future a significant role in the control of the gray mold and in the management of resistance developed to other fungicide

classes. Moreover, recent studies indicated a high risk for resistance development in this pathogen to pyraclostrobin (Markoglou *et al*., 2006) and a moderate risk for resistance development to boscalid (Zhang *et al*., 2007).

Fluopyram and penthiopyrad are two novel fungicides from the succinate dehydrogenase inhibitor (Generation II SDHIs) group that may be used for *B. cinerea* management (McKay *et al*., 2011). Fluopyram (*N*-{2-[3-chloro-5-(trifluoromethyl)-2-pyridyl]ethyl}-*α,α*-trifluoro-*o*-toluamide) is a fungicide belonging to the subgroup of pyridinyl ethylbenzamides, a chemical group within the class of succinate dehydrogenase inhibitors (SDHIs). It is biologically active against all the stages of fungal growth, from spore germination to spore production, and its activity spectrum includes several pathogens belonging to Ascomyctetes and Deuteromycetes, such as *Botrytis* spp., *Sclerotinia* spp. and *Monilinia* spp., on vegetable, pomes and stone fruit crops (Avenot and Michailides, 2010). Fluopyram has not been delivered to the market yet, and information relating to its activity against *B. cinerea* or the baseline sensitivity of the pathogen is limited. Recently, Veloukas and Karaoglanidis (2012) reported the efficacy of fluopyram both *in vitro* and *in vivo* assays. Fluopyram proved to be extremely active against both spore germination and germ tube elongation of *B. cinerea*, causing complete inhibition at very low concentrations. The effect of fluopyram on spore germination can be explained by the fact that germinating fungal spores respire actively, as it is a fungal developmental stage highly demanding in energy (Allen, 1965). Mycelial growth of *B. cinerea* was less sensitive to fluopyram than spore germination. Also *in vivo* assays, fluopyram provided excellent protective activity against *B. cinerea* when applied at 100 $\mu g mL^{-1}$ 96, 48 or 24 h before the artificial inoculation of the strawberry fruit. Similarly, fluopyram showed a high curative activity when it was applied at 100 $\mu g mL^{-1}$ 24 h post-inoculation, but, when applications were conducted 48 or 96 h post-inoculation, disease control efficacy was modest or low (Veloukas and Karaoglanidis, 2012). Also the penthiopyrad exhibited a high activity against fungicide-resistant strains of various diseases, such as gray mold, cucumber powdery mildew and apple scab (Yanase *et al*., 2006; Sakurai, 2007).

3. Fungicide resistance

The evolution of fungicide resistance has become a major problem worldwide, particularly in cases in which high resistance factors have been reported and the frequencies of mutant phenotypes in the population are high. This phenomenon may greatly decrease the efficacy of the active ingredient concerned, increasing the cost of chemical control and potentially resulting in damage to the environment if repeated treatment is required (Brent and Hollomon, 2007).

Fungicide resistance may be defined as the stable, inheritable adjustment by a pathogen to a fungicide, resulting in reduced sensitivity of the pathogen to the fungicide. Reduced sensitivity is thought to be a result of genetic mutations which occur at low frequencies or of naturally occurring sub-populations of resistant individuals (Bardas *et al*., 2008). Some people prefer to call this phenomenon 'insensitivity' or 'tolerance'. The former term is preferred by some plant pathologists, because they believe that fungicide resistance is easily confused with host-plant resistance to certain species or pathotypes. Some agrochemical companies have also tended to use "loss of sensitivity' or 'tolerance', because these sound less alarming than 'resistance'. However, two studies on terminology recommended that 'resistance' should be the preferred term (Anon, 1979; Delp and Dekker, 1985), because the expression 'tolerance' is used when the sensitivity to fungicides is due to non-genetic factors; in this case the reduced sensitivity is unstable and disappears rapidly in the absence of selective pressure of the fungicide (Brent and Hollomon, 2007).

The resistance trait may results from single gene or multiple gene mutations. Single-gene mutations that confer resistance to site-specific fungicides are more likely to develop than the simultaneous occurrence of mutations in multiple genes needed to confer resistance to multi-site inhibiting fungicides. These two types of fungicide resistance have been described for fungal populations as qualitative and quantitative resistance, respectively (Brent, 1986). When fungal isolates express qualitative resistance, increasing the rate of fungicide or decreasing spray interval will not affect the resistant isolates. On the contrary, populations with quantitative

resistance toward a fungicide can be controlled by higher rates or decreased spray intervals between applications. These two types differ in that qualitative resistance occurs when a single location in a gene is targeted (monogenic resistance), whereas quantitative resistance occurs when a few metabolic processes must be altered (polygenic resistance). The benzimidazole, phenylamide and strobilurin groups are subject to single-gene resistance and carry a high risk of resistance problems. Other fungicide groups with site-specific modes of action include dicarboximides and sterol demethylation inhibitors, but resistance to these fungicides appears to involve slower shifts toward insensitivity because of multiple-gene involvement (Heaney *et al*., 1994). Multi-site fungicides interfere with many metabolic processes of the fungus and are usually protective in activity. Typically, these fungicides inhibit spore germination and must be applied before infection occurs. Multi-site fungicides form a chemical barrier between the plant and fungus. The risk of resistance to these fungicides is low or absent.

Intensive and exclusive usage of at-risk fungicides increases the risk of resistance problems. Selection pressure is increased where repeated applications are required for disease control as with many foliar diseases. Selection pressure and the risk of resistance are low for seed treatments and for many soilborne diseases which require only one or two applications. The method and rate of application may also impact resistance development. Poor disease control resulting from inadequate spray coverage leads to a need for a more intensive spray program and the exposure of more individual in the fungus population to the fungicide. Using adequate rates in a manner that produces good disease control reduces the reproductive capacity of fungal pathogens, thus reducing selection pressure. Similarly, a protective spray program is less risky than a rescue program because selection pressure is applied to fewer individuals. Finally, an increase in selection pressure results from an excessive number of applications where a real need is not justified.

In *B. cinerea* the resistance phenomenon, as in other plant pathogenic fungi, becomes apparent with the site-specific fungicides. Site-specific or single-site fungicides act primarily at single target under responsibility of single major gene. Thus, just a single gene mutation can cause the target site to alter (monogenic

resistance), so as to become much less vulnerable to the fungicide (Brent, 1995). Therefore, within few years of intensive use of such fungicide, in populations of polycyclic pathogen with high propagation rate, can be found a high frequency of resistant mutants. The most common mechanism of fungicide resistance is based on alternations in the fungicide target protein. The resistance to multi-site fungicides, which effect many target sites in fungal cell, has been rarely reported. Multi-site fungicides have been considered as low-risk fungicide from the resistance point of view because they interfere with numerous metabolic steps and cause alternation of cellular structures.

Pathogen populations that develop resistance to one fungicide automatically and simultaneously become resistant to those other fungicides that are affected by the same gene mutation and the same resistance mechanism. Generally these have proved to be fungicides that bear an obvious chemical relationship to the first fungicide, or which have a similar mechanism of fungitoxicity. This is the phenomenon known as 'cross-resistance'. For example, pathogen strains that resist benomyl are almost always highly resistant to other benzimidazole fungicides such as carbendazim, thiophanate-methyl or thiabendazole. Sometimes cross-resistance is partial, even when allowance is made for the greater inherent activity of different members of a fungicide group.

The level of resistance to a fungicide can be measured in the laboratory by exposing a collection of members of a field population to the fungicide and measuring toxicity response. Toxicity responses are usually measured as inhibition of fungus growth, spore germination, or actual plant infection in cases where the fungus cannot be cultured. The effective concentration which inhibits growth, germination, or infection by 50% (EC_{50}) is then calculated for each sampled individual.

Mechanism of resistance differ depending principally on the mode of action of the fungicide and include: alteration of the biochemical target site so that it is no longer sensitive; reduced fungicide uptake; increased production of the target protein; developing an alternative metabolic pathway that bypasses the target site; detoxification or breakdown of the fungicide; exclusion or expulsion of the fungicide outside fungal cells through ATP-ase dependent transporter proteins. By far the

commonest mechanism appears to be an alteration to the biochemical target site of the fungicide. This could explain why many of the older products have not encountered resistance problems.

The development of fungicide resistance is influenced by complex interactions of factors such as the mode of action of the fungicide (how the active ingredient inhibits the fungus), the biology of the pathogen, fungicide use pattern, and the cropping system. Understanding the biology of fungicide resistance, how it develops, and how it can be managed is crucial for ensuring sustainable disease control with fungicides.

A high persistence of fungicide in the site of infection, a good coverage and a its frequent use entails a significant increase in the number of resistant strains. A repeated use of the fungicide exerts selection pressure on the population; the fungicide selectively inhibits sensitive strains, but allows the increase of resistant strains. This shift toward resistance occurs at different rates, depending on the number of genes conferring resistance. When single gene mutations confer resistance, a rapid shift toward resistance may occur, leading to a population that is predominantly resistant and where control is abruptly lost. When multiple genes are involved, the shift toward resistance progresses slowly, leading to a reduced sensitivity of the entire population.

Regarding pathogen characteristics, the development and the evolution of fungicide resistance in fungal populations are largely dependent on the fitness of the resistant fraction of the population and this has important implications on disease management (Peever and Milgroom, 1995; Skylakakis, 1987). Fitness can be defined as the survival and reproductive success of an allele, individual, or group (Pringle and Taylor, 2002). The development and the evolution of fungicide resistance would be lessened if resistant subpopulation had lower parasitic or saprophytic fitness. In contrast, absence of fitness costs in the resistant fraction of the population would lead to a stable resistance frequency in the absence of fungicide selection force or to rapid development and evolution of resistance under the fungicide selection force.

There are several experimental studies that evaluate the relationship between fitness and fungicide resistance, but results are quite contradictory (Hsiang and

Chastagner, 1991; Kadish and Cohen, 1988; Mérida and Loria, 1994; Moorman and Lease, 1992; Peever and Milgroom, 1994; Raposo *et al*., 1996).

Due to the high importance of fitness, development of resistance to fungicide classes such as the benzimidazoles and the dicarboximides by strains of *B. cinerea* was followed by reports regarding the fitness of the resistant strains compared with that of the sensitive ones. In most of these reports, development of resistance was associated with fitness costs and reduced sporulation (Hsiang and Chastagner, 1991; Raposo *et al*., 2000; Wang and Coley-Smith, 1986), while in another study development of resistance was not associated to fitness costs. These researchers found no differences in fungicide-sensitive phenotypes in the area under disease progress curve; in linear growth rates (Moorman and Lease, 1992) or in lesion growth rates and sporulation (Raposo *et al*., 1996). Attributes such as these mentioned, which are measured in a single reproductive cycle, define the predicted fitness (Antonovics and Alexander, 1988). Therefore, results support either a reduced or a similar parasitic fitness of dicarboximide-resistant isolates of *B. cinerea*.

As pointed out by Peever and Milgroom (Peever and Milgroon, 1993), possible reasons for the contradictions found in the literature are that these studies involved a few isolates possibly from different populations, and fitness differences between resistant and sensitive isolates may have been due to differences in the genetic background of the isolates rather than fitness costs. Recently, a procedure to measure correlations between fitness and resistance to fungicides has been described (Peever and Milgroon, 1993; 1994). This method describes procedures to separate genetic and environmental factors controlling resistance and fitness components and methods to estimate the genetic and phenotypic correlations between them. According to this method, the lack of correlation between fitness and dicarboximide resistance in *B. cinerea* isolates from Spanish greenhouses has been demonstrated (Raposo *et al*., 1996). In that study, the components of fitness measured on inoculated cucumber leaves were lesion growth rate and sporulation, characteristics related to the parasitic cycle of the pathogen. However, other attributes related to the nonpathogenic phase such as the ability to over season were not considered and may be correlated with the dicarboximide resistance.

In addition, studies on the fitness of pathogen fungicide-resistant strains have also been conducted prior to the introduction of novel fungicides such as pyraclostrobin, fenhexamid, and fludioxonil to determine the inherent risk for resistance development using isolates with laboratory-induced resistance (Markoglou *et al*., 2006; Ziogas *et al*., 2003, 2005).

Regarding cropping system, the production practices that favor increased disease pressure also promote resistance development by increasing the number of individuals exposed to selection pressure. Pathogens reproduce at higher rates on susceptible varieties compared to resistant or partially resistant varieties. Selection pressure also may be reduced for resistant varieties because fewer applications should be needed for effective disease control. Inadequate or excessive fertilization with nitrogen or excessive/frequent irrigation may increase disease incidence in some crops and so the resistance phenomenon. Also closed cropping systems such as greenhouses are particularly prone to resistance problems because plants are grown in crowded conditions that may favor severe disease development, rapid spread, and high selection pressure (Brent and Hollomon, 2007).

B. cinerea represents a classic "high risk" pathogen for fungicide resistance development due to its high genetic variability, the abundance sporulation, the short generation time, the wide host range, and the high number of fungicide applications required for its successful control (Leroux *et al.,* 2002; Petsikos-Panayotarou *et al.,* 2003; Yourman *et al.,* 2001). Moreover, *B. cinerea* has earned its reputation as a high-risk pathogen mainly because of its capacity to develop specific resistance to single-site fungicides based on target gene mutations. Specific resistance may emerge within a few years of release of a new fungicide group onto the market, and is usually associated with high resistance factors in laboratory test. Specific resistance has been described e.g. to benzimidazoles such as benomyl, thiophanate-methyl and carbendazim (Yarden and Katan, 1993; Yourman and Jeffers, 1999), dicarboximides such as iprodione and vinclozolin (Northover and Matteoni, 1986; Yourman and Jeffers, 1999), QoI fungicides (Bardas *et al*., 2010; Ishii *et al.*, 2009), anilinopyrimidines such as cyprodinil and pyrimethanil (Chapeland *et al*., 1999;

Myresiotis *et al*., 2007), carboxamides/SDHIs (Leroux *et al*., 2010) and the hydroxyanilide compound fenhexamid (Fillinger *et al*., 2008; Ziogas *et al.,* 2003).

Resistance to benzimidazole and dicarboximide fungicides has been described from a number of crops in the greenhouses and in the field (Beever and Brien, 1983; Bollen and Scholten, 1971; Faretra *et al*., 1989; Fletcher and Scholdfield, 1976; Gullino *et al*., 1982; katan, 1983; Miller and Fletcher, 1974; Moorman and Lease, 1992; Northover and Matteoni, 1986; Pappas *et al*., 1979; Pepin and Macpherson, 1982). Various *B. cinerea* benzimidazole-resistant isolates have been found in several crops throughout the world (Stehmann, 1996; Malandrakis *et al*., 2011). A 'negative correlated cross-resistance' between the benzimidazoles and the N-phenylcarbamates (Kato *et al*., 1984) led to the introduction of carbendazim and diethofencarb mixtures for the control of these strains. However, the intensive commercial use of this mixture resulted in the selection of strains resistant to both chemistries in several countries such as Greece, Israel, France and Spain (Faretra *et al*., 1989; Katan *et al*., 1989; Leroux and Gredt, 1989; Raposo *et al*., 1994; Laskaris *et al*., 1996). As a matter of fact, since these two fungicides are in relationship in negative cross-resistance, the use of diethofencarb has been increased against *B. cinerea* populations resistant to benzimidazole (Leroux *et al*., 2002).

In most cases, benzimidazole resistance is characterized by its high persistence in the field long after the interruption of fungicide applications (Georgopoul and Skylakakis, 1986). Genetic studies suggested at least three single gene allelic mutations responsible for an equal number of resistant phenotypes (Ziogas and Girgis, 1993). Over the years, β-tubulin amino acid substitutions leading to benzimidazole or double benzimidazole and N-phenylcarbamate resistance were detected (Yarden and Katan, 1993; Leroux *et al*., 2002).

Similarly, also the intensive use of dicarboximide fungicides and the high-risk character of *B. cinerea* for development of resistance have led to the rapid selection of strains resistant to this group of in many countries worldwide (Elad *et al*., 1992b; Fraile *et al*., 1986; Leroux *et al*., 1999; Leroux, 2004; Moorman and Lease, 1992; Myresiotis *et al*., 2007; Pappas, 1997). The first dicarboximide-resistant field isolates of *B. cinerea* were found in a German vineyard (Mosel growing area) at the end of

1978, 3 years after the first registration of dicarboximides. In 1980 many European vineyards were concerned, but due to the lack of good alternative fungicides, dicarboximides use continued and the number of resistant strains increased considerably in these regions in the early 1980s. Further reports of dicarboximide-resistant *Botrytis* strains are mainly concerned with strawberries, vegetables and greenhouse crops of a wide variety (Lorenz, 1988). Failures of control have been reported for instance on protected crops (Katan, 1983) or in vineyards (Leroux and Clerjeau, 1985), but sometimes only decreased efficacy was observed (Lorenz, 1988). Monitoring done on various crops generally shows a decline in frequency of dicarboximide-resistant isolates following discontinuation of fungicide applications (Gouot, 1988; Pak *et al*., 1990; Leroux, 1995; Pommer and Lorenz, 1995). This could represent a reduced fitness of dicarboximide-resistant strains. According to Raposo *et al.* (2000), such a phenomenon occurs during the saprophytic phase rather than the parasitic phase in the life-cycle of *B. cinerea*. Some authors also suggest that in *B. cinerea*, phenotype instability and heterokaryosis lead to a decrease in resistant after cessation of dicarboximide treatments (Faretra and Pollastro, 1993a; Yourman *et al.*, 2001).

Resistance to dicarboximides is caused by the polyallelic major gene *Daf1* coding for a histidine kinase, with at least five classes of alleles responsible for sensitivity, different levels of resistance variously accompanied by resistance to phenylpyrrole fungicides and reduced tolerance to high osmotic pressure (Faretra e Pollastro, 1991; 1993a,b,c; Leroux and Descotes, 1996; Oshima *et al.*, 2002; Vignutelli *et al*., 2002; Baroffio *et al.,* 2003).

As observed for *B. cinerea* in vineyards, the selection pressure for resistance is related to the number of dicarboximide sprays (Leroux and Clerjeau, 1985). As a guide, FRAC (Fungicide Resistance Action Committee) recommends that the number of dicarboximide-based treatments should be restricted to two or three per crop and per season. In addition to limiting the number of applications of dicarboximides, another anti-resistance strategy consists of mixing these botryticides with a fungicide with multi-site mode of action, such as chlorothalonil or thiram. In most cases the performance of the combination was better and more stable than

dicarboximides alone (Gullino and Garibaldi, 1982; Katan and Ovadia, 1985; Lorenz *et al.*, 1994). With respect to the selection pressure, full dosages of dicarboximides alone or in mixtures caused in general the same increase in the resistant population. On the other hand, mixtures with reduced dosages of dicarboximides often delayed the selection of resistant strains (Lorenz *et al.*, 1994; Leroux, 1995).

Also the intensive use of anilinopyrimidines can lead to resistance phenomenon. Monitoring in vineyards of various countries including Italy, France, Switzerland, Spain, Chile and Australia has detected *B. cinerea* anilinopyrimidine-resistant strains (Baroffio *et al*., 2003; Gullino *et al*., 2000; Gullino and Garibaldi, 2003; Chapeland *et al*., 1999; Leroux *et al*., 1999; Latorre *et al*., 2002; Moyano *et al*., 2004; Sergeeva *et al*., 2002). In most cases they were moderately to highly resistant to anilinopyrimidines in which ever *in vitro* assay was used. Resistance was also confirmed by *in vivo* methods (Birchmore and Forster, 1996; Leroux *et al*., 1999).

Genetic analysis of resistant strains from field populations of *B. fuckeliana* showed that resistance to anilinopyrimidines is caused by single mutations in at least three major genes, two of which cause multidrug resistance (MDR) also to dicarboximides, phenylpyrroles and several inhibitors of sterol biosynthesis (Chapeland *et al.,* 1999; Leroux *et al*., 1999, 2002). Three AP-resistant phenotypes (AniR1, AniR2 and AniR3) were detected in field populations of *B. cinerea* (Chapeland *et al*. 1999). AniR1 strains are moderately to highly resistant to APs and they respond like wild type (WT) isolates to other fungicides; the specific resistance in AniR1 might be related to a change at the target site. Low-level resistance in AniR2 and AniR3 was mainly noted at the germ-tube elongation stage, and this resistance extended to several other fungicide classes (multi-drug resistant phenotypes) (Kretschmer *et al*., 2009). EC_{50} values for isolates sensitive to pyrimethanil ranging from 0.03 to 0.08 μg ml^{-1} was reported by Korolev and collaborators (2011); these values ranged from 0.03 to 0.5 μg ml^{-1} in previous studies (Chapeland *et al*., 1999; Myresiotis *et al.,* 2007) with resistance factors of 10 to 200 for AniR1, and below 10 for AniR2 or AniR3 (Leroux, 2004).

High variability in fitness parameters among both AP-resistant and AP-sensitive isolates was observed in a recent study, and, as a group, resistant isolates showed

reduced mycelial growth and virulence (Bardas *et al*., 2008). Korolev and collaborators (2011) showed that AP-resistant isolates, that were also resistant to other fungicides, grew significantly more slowly than the WT isolates and formed smaller lesions on bean leaves. However, the isolates that were resistant to pyrimethanil only were no different from the WT isolates.

To date the detection of anilinopyrimidine-resistance within field populations of *B. cinerea* is based on either *in vitro* or *in vivo* methods (Birchmore and Forster, 1996). The fact that on complex media, there may be variability in response to anilinopyrimidines, it is essential to use well-defined synthetic nutrient media, especially in tests involving mycelial growth. Another alternative consists of testing the effects of anilinopyrimidines on germ tube elongation, for instance with pyrimethanil discriminatory concentrations between 1.0 and 2.5 mg/l allowing the detection of resistant isolates. As mentioned previously, the intensive use of anilinopyrimidines can lead to resistance (Forster and Staub, 1996; Latorre *et al*., 2002; Petsikos-Panayotarou *et al.*, 2003). Consequently, the first approach to resistance management consists of restricting the number of treatments involving anilinopyrimidines.

Shortly after the introduction of anilinopyrimidines, fludioxonil compound start to be used as a foliar fungicide in vineyards against *B. cinerea*. EC_{50} values for fludioxonil-sensitive isolates, as defined by the mycelial growth test, ranged from 0.001 to 0.016 μg ml−1 (Chapeland *et al.*, 1999; Förster *et al.*, 2007; Hilber *et al.*, 1995; Leroux *et al.*, 1999; Myresiotis *et al.,* 2007; Vignutelli *et al.*, 2002; Ziogas *et al.,* 2005). No report of high resistance to fludioxonil among field isolates has been published as yet (Baroffio *et al*., 2003; Förster and Staub 1996; Leroux *et al*., 1999; Vignutelli *et al*., 2002; Myresiotis *et al*., 2007; Weber and Entrop, 2011), although *B. cinerea* mutants sensitive to osmotic stress and highly resistant to phenylpyrroles, dicarboximides and aromatic hydrocarbons can be easily produced in the laboratory (Leroux 2004).

Among the sterol biosynthesis inhibiting fungicides (SBI), the most effective botrycide is fenhexamid. *B. cinerea* responds with varying sensitivity to *in vitro* treatments with fenhexamid at various stages of its development. The EC_{50} value for

conidial germination is greater than 10 μg ml^{-1}; whereas the corresponding value for the inhibition of subsequent germ-tube elongation and mycelial growth is less than 0.1 μg ml^{-1} (Hänßler and Pontzen 1999). In several survey studies, EC_{50} values for fenhexamid-sensitive isolates, as defined using a mycelial growth test, ranged from less than 0.01 to about 0.1 μg ml^{-1} (Esterio *et al*., 2007; Förster *et al*., 2007; Leroux *et al*., 1999; Ma and Michailides 2005; Myresiotis *et al*., 2007). A discriminatory dose of 0.1 μg ml^{-1} of fenhexamid was used by different researchers to distinguish between sensitive and resistant isolates, with isolates showing EC_{50} values of at least 0.1 μg ml^{-1} (Baroffio *et al*., 2003) or more than 0.1 μg ml^{-1} (Esterio *et al*., 2007) being regarded as resistant. The subdividing of isolates on resistant and sensitive categories based on the discriminatory dose is helpful in monitoring programs, but may not always correspond to the described resistant phenotypes. To date, four fenhexamid-resistant phenotypes have been recognized: multi-drug-resistant AniR3 (MDR2); "naturally" resistant HydR1 (recognized recently as *B. pseudocinerea*); HydR2, whose resistance seemed to be based on p450- mediated detoxification; and HydR3, whose resistance is based on reduced sensitivity of the target site (Fillinger *et al*., 2008; Kretschmer *et al*., 2009).

In a recent study, HydR3 isolates were detected in France at frequencies of up to 50%, and the fenhexamid treatments were still effective, suggesting that the fitness of these resistant isolates may be reduced (Fillinger *et al*. 2008). Fenhexamid-resistant field isolates have also been recovered in California, Chile, Greece, Switzerland and other regions (Baroffio *et al.,* 2003; Esterio *et al.,* 2007; Leroux 2004; Ma and Michailides 2005; Myresiotis *et al.,* 2007). No resistance was found in vineyards in Switzerland in the first 2 years, but there was a steady increase in the size of the resistant subpopulation over the next 3 years. In one of the vineyards, only resistant isolates were found in the fifth year, and there was a reduction in the efficacy of fenhexamid in that vineyard (Baroffio *et al.,* 2003). In other cited works, the proportion of resistant isolates in examined populations did not exceed 1-3%, confirming that baseline populations of *B. cinerea* often contain a small proportion of isolates with reduced sensitivity to fenhexamid and reduced fitness (Suty *et al.*, 1999). No highly fenhexamid-resistant isolates were found in vineyards located in

Israel; the few isolates with low level resistance to fenhexamid found showed low fitness and were controlled with fenhexamid similar to the sensitive isolates (Korolev *et al*., 2011).

Another important fungicide used against gray mould is the pyraclostrobin compound. It is a strobilurin fungicide, belonging to the group of quinone outside inhibitor (QoI) fungicides, which have become one of the most important groups of fungicides in agriculture (Bartlett *et al.*, 2002). Previous studies showed that QoI fungicides have a protective, curative, and eradicative effect by inhibiting spore germination, mycelia growth, and sporulation of the fungal pathogens; however, individual QoI fungicides may vary in their levels of activity against specific pathogens and diseases (Bartlett *et al.*, 2002). Because of their site-specific mode of action, risk for development of resistance to QoIs is considered high (Fungicide Resistance Action Committee-FRAC Code List - http://www.frac.info; Markoglou *et al*., 2006). Numerous fungal pathogens from various crops, including *B. cinerea*, have developed resistance to QoI fungicides (http://www.frac.info; Ishii *et al*., 2009; Jiang *et al*., 2009; Kim and Xiao, 2010). Biochemical and molecular studies have shown that resistance to QoI fungicides could appear either by a target site modification through point mutations in the Qo site of cytochrome b (Di Rago and Colson, 1989; Zheng and Köller, 1997; Zheng *et al.,* 2000) or by increased electron transfer through the alternative oxidase pathway (Ziogas *et al.,* 1997; Olaya and Köller, 1999a; Tamura *et al*., 1999). However, alternative respiration appears to play no significant role in pathogenesis on QoI-treated plants in natural populations of pathogens controlled by these fungicides (Ziogas *et al*., 1997; Olaya and Köller, 1999b) possibly because host flavones released during infection interfere with induction of this pathway (Zheng *et al*., 2000).

Boscalid is a relatively new broad-spectrum fungicide belonging to the class of succinate dehydrogenase inhibitor (SDHI) fungicides. It is biologically active against different stages of fungal development but it primarily inhibits spore germination and elongation of germ tubes of different fungi, including *B. cinerea* (Stammler *et al*., 2008). Although SDHIs fungicides are consider medium risk for the development of resistance in fungal pathogens (http://www.frac.info), resistance to boscalid has been

found in field isolates of several fungal pathogens, including *Alternaria alternata* (Avenot and Michailides, 2007), *Corynespora cassiicola* (Miyamoto *et al*., 2008), *Didymella bryoniae* (Keinath *et al*., 2009), and *Podosphaera xanthii* (McGrath *et al*., 2009). In *B. fuckeliana*, the baseline sensitivity to boscalid was determined in laboratory tests (Stammler and Speakman 2006). Boscalid-resistant mutants were obtained by UV treatment (De Miccolis Angelini *et al.* 2006, 2007; Stammler *et al.* 2008; Zhang *et al.* 2007), whereas field resistance has been restricted to few European countries (Stammler 2008; Stammler *et al.* 2008). Gene sequence analysis of the four sub-units of the boscalid-target protein, the succinate dehydrogenase enzyme, revealed that single or double point mutations in the highly conserved regions of the iron-sulphur protein (Ip) gene were associated with resistance. Mutations resulted in proline to leucine or phenylalanine replacements at position 225 (P225L or P225F) in high resistant mutants, and in a histidine to tyrosine replacement at position 272 (H272Y) in low resistant mutants (Stammler *et al*., 2008; De Miccolis Angelini *et al*., 2010).The use of botryticides is an efficient way to protect crops against *Botrytis* spp., but the development of resistant strains limit fungicide treatments, especially at harvest or during postharvest. Strategies for managing fungicide resistance can be useful at delaying its development. Therefore, a management strategy should be implemented before resistance becomes a problem. The only way to absolutely prevent resistance is to not use an at-risk fungicide. This is not a practical solution because many of the modern fungicides that are at risk for resistance problems provide highly effective, broad-spectrum disease control. By delaying resistance and keeping its level under control, resistance can be prevented from becoming economically important. Because practical research in the area of fungicide resistance management has been limited, many of the strategies devised are based in the theory of expected responses of a pathogen population to selection pressure. For the most part, evaluations of the effectiveness of these strategies have not been based on research, but rather on observations made where the fungicides have been used commercially on a large scale. Specific strategies for resistance management vary for the different fungicide groups, the target pathogen(s), and the crop. However, some strategies are generally effective. Monitoring resistance levels

in pathogen populations is essential for assessing risk and evaluating management practices. Moreover, resistance management should integrate cultural practices and optimum fungicide use patterns. The desired result is to minimize selection pressure through a reduction in time of exposure or the size of the population exposed to the at-risk fungicide. Probably the most important aspect of optimizing use patterns is the deployment of tank mixtures and alternating sprays or blocks of sprays of the at-risk fungicide with an unrelated companion fungicide. The comparative merits of tank-mixing compared to alternating sprays has been debated. Some theorize that tank-mixing reduces selection pressure only when the partner fungicide is highly effective and good coverage is achieved. Alternating fungicides is thought to act by reducing the time of exposure. In practice, examples can be cited for the effectiveness of both approaches. Both practices are much more effective when cultural practices are implemented to reduce disease pressure.

The effectiveness of alternating blocks of sprays is probably less effective that the other use patterns unless an equal number of applications of the partner are made.

The proper choice of a partner fungicide in a resistance management program is critical. Generally, good partner fungicides are multi-site inhibitors that have a low resistance risk (e.g. chlorothalonil, mancozeb, etc.) and are highly effective against the target pathogen. However, the use of an unrelated at-risk fungicide with no potential for cross-resistance problems also may be effective.

4. Objectives of research

B. cinerea is an ubiquitous plant pathogenic fungus which can infect more than 265 plant species, including ornamentals, vegetables, fruits and grapevines. In favorable conditions, grey mould becomes one of the most economically important diseases of grapes, causing serious damage to quality and significant yield losses. The control of the disease is based on an integration of several cultural methods with the use of fungicides belonging to several groups. Current disease management strategies aim to reduce the initial inoculums source of *B. cinerea*, preventing flower infection by fungicide applications (Holz *et al*., 2003; Walter *et al*., 2005; Zitter and Wilcox, 2006). However, intensive use of fungicides has led to a raid selection of pathogen strain resistant to botryticides. *B. cinerea* is a classical "high-risk" pathogen, and development of resistance to several classes of fungicides has been frequently reported worldwide (Leroux *et al*., 1999; Baroffio *et al*., 2003).

The development of field resistance to fungicides can seriously affect the effectiveness of chemical control of phytopathogenic fungi, as frequently experienced for several fungicide-pathogen combinations (Brent and Hollomon, 2007). The anti-resistant strategies and careful monitoring of the fungal population are important for preservation the efficacy of a fungicide in time.

For these reasons, the first objective of this research was to determine the occurrence of resistance to fungicides belonging to different groups in populations of *B. cinerea* on vineyards located in various agricultural areas of eastern and southeastern Sicily. In particular, the *B. cinerea* isolates were characterized for their level of sensitivity to modern fungicides fenhexamid, fludioxonil and boscalid as well as to older fungicides carbendazim, pyrimethanil and iprodione.

At the same time, we also evaluated the effects of fluopyram, a novel SDHI fungicide, on development of *B. cinerea* isolates collected from vineyards that never been exposed to use of this fungicide. The introduction of new fungicides belonging in different chemical groups with no cross-resistance with botryticides already in use into spray programs could be a convenient solution for overcoming limitations in disease control caused by fungicide resistance. Fluopyram is an excellent candidate

for managing fungicide resistance development because it shows no cross-resistance with other chemical classes such as strobilurins, benzimidazoles or anilinopyrimidines (Stammler *et al*., 2007; Zhang *et al*., 2007; Avenot *et al*., 2008). It was developed to effectively combat various plant diseases caused by fungal pathogens including diseases such as gray mold, powdery mildew, sclerotinia and monilia diseases, but information relating to its activity against *B. cinerea* or the baseline sensitivity of the pathogen is limited. Therefore, biological activity of this novel fungicide was investigated in order to obtain baseline sensitivity which will serve as a starting point in future fungicide resistance management.

Determination of sensitivity to older and modern fungicides was first conducted *in vitro* on media amended with the above-mentioned fungicides at different concentrations. As a matter of fact, response of fungi to fungicides generally is reported as the effective concentration that inhibits conidia germination or mycelium growth by 50% (EC_{50}) on fungicide-amended medium. According to some researchers (Yourman and Jeffers, 1999), to determine the accuracy of this method for assessing fungicide sensitive phenotypes of isolates of *B. cinerea*, results from the *in vitro* assays should be correlated with those from an *in vivo* seedling assays. For this reason, fungicide sensitivity phenotypes determined by mycelium growth was verified *in vivo* on leaves of seedlings. In particular, bean plants were used because they are easy to grow and have a large cotyledon which is susceptible to infection by *B. cinerea* and on which it is easy to measure the diameter of the resultant lesion. Fungicide sensitivity of *B. cinerea* isolates was subsequently determined also on leaves of grapevine potted plants and the data were compared with those obtained *in vitro* assays.

Genetic analysis of *B. cinerea* representative isolates, which had been found sensitive or resistant to boscalid and carbendazim, was subsequently conducted to confirm the results obtained *in vitro* assays. Resistance to fungicide is often associated with point mutations in some definite genes that result in altered aminoacid sequences at the fungicide binding site. In particular, resistance to boscalid and to benzimidazoles is due to mutations in *Sdh* and in β-tubulin genes, respectively. Therefore, to identify the mutations correlated with resistance to these

fungicides, the *Sdh*B subunit and the β-tubulin gene of representative isolates were compared with the corresponding gene sequences of reference sensitive strains.

The second objective of this study was to verify the efficacy of the fungicides in controlling gray mould on detached grape berries and to determine whether their effectiveness was compromised by fungicide-resistant isolates of *B. cinerea*. For this motivation, at least two sensitive isolates and two isolates with decreased sensitivity were chosen and tested for their ability to produce infections on fruits previously treated with fungicides at label rate. The efficacy of pyrimethanil was determined also on apple fruits cv. Golden Delicious.

5. Materials and methods

5.1. Pathogen isolates

A total of 146 isolates of *B. cinerea* were recovered from 15 different commercial vineyards located in Catania and Ragusa provinces. In detail, the towns most interested by the collection of *B. cinerea* isolates were Mazzarrone and Chiaramonte Gulfi (Table 1; Fig. 4). The isolates were collected from diseased grape berries from October to November between 2009 and 2011.

Table 1. Location, source and number of *B. cinerea* isolates collected from diseased grape berries in Sicilian vineyards and used in this study

Province	Location (main street)	Cultivar	No. of isolates	Year of isolation
Agrigento	Ravanusa (Poggio Rotondo)	Red Globe	12	2010
Catania	Caltagirone (Croce Rossa)	Italia	8	2010
Catania	Licodia Eubea (Donna)	Italia	4	2009
Catania	Licodia Eubea (Donna)	Italia	6	2010
Catania	Licodia Eubea (Sciri Sopra)	Italia	5	2010
Catania	Mazzarone (Molinia)	Italia	7	2009
Catania	Mazzarone (Piano Maenza)	Italia	8	2009
Catania	Mazzarone (Bidine)	Italia	5	2010
Catania	Mazzarone (Piano Maenza)	Italia	7	2010
Catania	Mazzarone (Stella)	Italia	6	2010
Catania	Ramacca (Palmeri)	Italia	1	2010
Ragusa	Acate (Torrevecchia)	Italia	4	2009
Ragusa	Comiso (Comuni)	Italia	4	2009
Ragusa	Chiaramonte Gulfi (Mazzaronello)	Italia	3	2009
Ragusa	Chiaramonte G. (Fegotto, vineyard 1)	Italia	4	2009
Ragusa	Chiaramonte G. (Mazzaronello)	Italia	26	2010
Ragusa	Chiaramonte G. (Fegotto, vineyard 2)	Italia	8	2010
Ragusa	Chiaramonte G. (Mortilla)	Italia	2	2010
Ragusa	Chiaramonte G. (Mortilla)	Red Globe	7	2010
Ragusa	Chiaramonte G. (Mortilla)	King's ruby	4	2010
Ragusa	Chiaramonte G. (Mazzaronello)	Italia	10	2011
Ragusa	Chiaramonte G. (Mazzaronello)	Victoria	5	2011

Figure 4. Illustration map of the southern part of Sicily showing the monitored sites marked by yellow stars.

Grapes exhibiting the characteristic symptoms of *Botrytis* infection were detached from plants and transferred to the laboratory in individual polyethylene bags to prevent cross-contamination. Sometimes the grape berries were placed in a 90-mm-diam Petri dish on moistened filter paper and incubated at room temperature (22°C to 23°C) to obtain abundant conidia.

Conidiophores were transferred onto PDA (Potato Dextrose Agar, Oxoid) plate and incubated at 22°C for 5-7 days. Then, all isolates were purified by single spore. Small pieces of mycelia were placed in a falcon containing 10 ml of sterile water. After 15s of agitation using a vortex, 10µl of spore suspension was spread onto 2% water agar plates and incubated in the dark at 20°C for 16 h. Pieces of agar containing only one spore were removed from water agar plates and placed on PDA amended with 0.1 g/l of tetracycline to avoid bacterial contamination.

Identification of *B. cinerea* was verified by examination under a compound microscope. Morphological characteristics such as conidiophores length, conidial

shape and dimensions of sclerotia were examined. The collections were stored on PDA slants at 4°C.

5.2. Fungicides

Fungicides used in the study were the commercial formulations of boscalid (Cantus, BASF group, Ludwigshafen, Germany), carbendazim (Bavistin, BASF group, Ludwigshafen, Germany), fenhexamid (Teldor, Bayer Crop Science AG Dormagen, Germany), fludioxonil (Geoxe 50 WP, Syngenta Crop Protection SA Monthey, Switzerland), iprodione (Rovral Plus, BASF Agri Production, Genay, France), pyrimethanil (Scala, Bayer Crop Science, Wolfenbüttel, Germany) and fluopyram (Luna Privilege, Bayer Crop Science AG Francoforte, Germany). The carbendazim compound was employed only *in vitro* assays and it has been replaced by thiophanate methyl (Enovit Metil Fl, Salerano sul Lambro, Italy) for the *in vivo* assays.

Stock solutions of fungicides were prepared in sterilized distilled water, with exception of fluopyram which was dissolved in ethanol.

5.3. Sensitivity tests

The sensitivity of all isolates to chemical compounds was assessed by measuring the radial growth on solid medium plates amended with various concentrations of fungicides.

All of the fungicides were tested on PDA medium except for pyrimethanil, which was tested on minimal medium containing 10g glucose, 1.5g K_2HPO_4, 2g KH_2PO_4, 1g $(NH_4)_2SO_4$, 0.5g $MgSO_4 \cdot 7H_2O$ and 12.5g agar (Oxoid) per liter (Myriesotis *et al*., 2007). Autoclaved agar media were cooled to 50°C and amended with appropriate volumes of the fungicide stock solutions to obtain the final following doses: 0.05, 0.5, 1, 5 μg ml^{-1} for boscalid; 0.01, 0.1, 1 and 10 μg ml^{-1} for carbendazim; 0.001, 0.005, 0.01, 0.05, 0.1, 1 μg ml^{-1} for fenhexamid and fludioxonil; 0.1, 1, 5, 10 and 20 μg ml^{-1} for iprodione; 0.01, 0.05, 0.1, 1 and 5 μg ml^{-1} for pyrimethanil and 0.1, 1, 10, 100 μg ml^{-1} for fluopyram. Control media were not amended with the fungicides.

Media were poured into Petri plates and a plug of mycelium, cut from the edge of an actively growing culture on PDA, was inverted and placed upside down on the center of each fungicide-amended or -unamended plate. Cultures were incubated at 20°C in the dark for 3 days. For each concentration, three plates were used and the experiment was performed once.

For each plate, colony diameter was measured in two perpendicular directions with the original diameter of the mycelia plug (6 mm) subtracted. Mean colony diameter, growth reduction relative to untreated control, EC_{50} values (effective concentration that reduces the mycelial growth by 50%) and discriminating dose (DD) were defined.

The DD was defined as the concentration at which *B. cinerea* isolates could be separated in two groups: those inhibited in the presence of the fungicide and those not inhibited. The discriminatory concentrations were as follows: 1 µg ml^{-1} for boscalid, carbendazim, iprodione, pyrimethanil and 0.1 µg ml^{-1} for fenhexamid and fludioxonil, as determined in previous studies (Baroffio *et al*., 2003; Faretra and Pollastro, 1991; Latorre and Torres, 2012; Leroux *et al*., 1999; Myresiotis *et al*., 2007; Yourman and Jeffers, 1999).

The fungicide sensitivity categories (sensitive and resistant) were defined according to the estimated EC_{50} values as follows: boscalid, carbendazim, iprodione and pyrimethanil resistant if $EC_{50} \geq 1$ µg ml^{-1}; fenhexamid and fludioxonil if $EC_{50} \geq 0.1$ µg ml^{-1}.

5.4. Assays on bean seedling

Fungicide sensitivity of phenotypes determined by mycelium growth was first verified on bean seedlings (*Phaseolus vulgaris*). For each fungicide, at least two sensitive isolates and/or two isolates with decreased sensitivity were chosen for this assay.

Bean seedlings were grown in plastic pots containing a 2:1 mixture of peat and perlite. When cotyledons expanded fully, approximately 10 to 14 days later, seedlings were sprayed with the seven fungicides tested. The control was sprayed

with sterile water. The concentrations of active ingredients employed in this study were reported in table 2.

Fungicides and water were applied with hand-pumped until cotyledons were thoroughly wet. Cotyledons were allowed to dry completely before preparing plants for inoculation. After 5 h, the leaves were detached and were placed in Petri dishes on a filter paper soaked with 2 ml of sterile water to maintain high humidity.

Table 2. Concentrations of the active ingredients applied to bean seedlings

Fungicide	Formulate	Concentration*
Boscalid	Cantus	100 g/hl
Fenhexamid	Teldor	100 g/hl
Fludioxonil	Geoxe	100 g/hl
Iprodione	Rovral Plus	150 ml/hl
Pyrimethanil	Scala	200 ml/hl
Thiophanate methyl	Enovit Metil	100 g/hl
Fluopyram	Luna Privilege	50 ml/hl

* These concentrations were the same as those registered to be employ against grey mold in vineyard

Each leaf was inoculated with *B. cinerea* isolates. Six mycelial plugs were removed, with the aid of a 6 mm diameter cork borer, from the colony margins of a growing 5-days-old culture on PDA and were placed on the surface of each cotyledon. Leaves were incubated at 23-24°C in the dark for 3 days. For each fungicide, each isolate was tested on five cotyledons.

Lesion development was determined by measuring two diameters at right angles. Isolates were considered resistant if leaves showed rotting after 3 days as occurred in the control.

5.5 Assays on leaves of grapevine

The sensitivity of isolates to boscalid, fenhexamid, fludioxonil, iprodione, pyrimethanil and thiophanate methyl was determined also on potted three-week-old

plants of grapevine (*Vitis vinifera*) cv. Italia grown in a growth chamber at 25°C and 70% relative humidity with a photoperiod of 16 h. The plants were sprayed up to run-off with an aqueous suspension of each formulated fungicide at the recommended commercial rates reported in table 2.

After a few hours, the leaves of these plants were inoculated with *B. cinerea* isolates. The fungal isolates tested were the same as those employed on bean seedling. Three mycelial plugs removed from the margin of the colonies growing on PDA were placed on the surface of each leaf. Three leaves were used for each isolate. The control plants were sprayed with distilled water and then received PDA plugs containing the *B. cinerea* mycelia.

To create favorable conditions for infection, inoculated plants were covered with plastic bags and incubated in the moist chamber at 25°C with a photoperiod of 16 h. The diameters of the developing lesions were measured 4 days after inoculation.

Isolates were considered resistant if leaves showed a rotting level similar to the control. The experiment was repeated once.

5.6. Molecular analysis

To identify the mutations correlated with resistance to boscalid and carbendazim, the coding sequences of *sdhB* subunit and the coding sequences of β-tubulin gene extracted from six representative *B. cinerea* isolates, selected on the basis of their funcigides sensitivity, were compared with the corresponding gene sequence of the reference sensitive strains B05.10 and SAS56. Four isolates resistant to boscalid, three of which resistant also to carbendazim, and two sensitive isolates were used. Genomic DNA was extracted and purified from mycelium of *B. cinerea* isolates grown on PDA for 5 days in the dark. Mycelia were harvested and washed in sterile water, frozen in liquid nitrogen, and lyophilized. DNA from each isolate was extracted using the kit Wizard® Magnetic DNA Purification System for Food (Promega, USA). The purified DNA was eluted in a final volume of 100 µl and evaluated by electrophoresis on 0.8% agarose gel. The concentration and purity of DNA extracted was determined by the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Scientific Instruments).

According to the known complete sequence of the β-tubulin gene in *B. cinerea* (GenBank accession number U27198), the PCR primer pair Bcb-F (5'-CACTGAGGGTGCTGAGCTTGT-3')+ Bcb-R (5'-AGCGGCCATCATGTTCTTA-3') was designed to amplify the β-tubulin gene fragment containing the codon 198 and 200 relevant to identify the resistant strains to benzimidazoles (Zhang *et al*., 2010). The primers B1189/2346F (5'-CCCACTACCCCACACCTATG-3') + B1189/2346R (5'-ACAAGCATCGGTTTTGGAAC-3') were instead used to amplify the *sdhB* sequence and determined the resistance of isolates to boscalid (De Miccolis Angelini *et al*., 2010).

The PCR products were purified with EXOSAP, a mixture of exonuclease I and alkaline phosphatase used to remove unincorporated dNTPs and primers present in the PCR products and then they were sequenced using BigDye Terminator V3.1 Cycle Sequencing Ready Reaction Kit (Applera, USA).

Sequencing was performed on an ABI PRISM 3730 Genetic Analyzer (Applera, USA) and the amplicon sequences were aligned using BioNumerics 5.1 (Applied Maths, Belgium) software to locate and identify the base changes.

5.7. Assays on detached grape berries

The effectiveness of the fungicides used in this study for the control of *B. cinerea* was determined on detached grape berries cv. "Italia". For each fungicide, at least two sensitive isolates and two isolates with decreased sensitivity were chosen for inoculation tests. These isolates were the same as those employed *in vitro* assays.

Single detached berries were prepared from freshly harvested grapes by clipping the berries from the rachis, so as to leave a portion of the stem on the berries to avoid making a large wound when they were detached. Berries with the pedicel intact were superficially disinfected with 2% sodium hypochlorite for 2 min and rinsed twice in sterile distilled water.

After drying, four punctures (1-2 mm deep) were made with the aid of a sterile hypodermic needle before being sprayed with fungicide solutions. The concentrations of active ingredients used on these assays were the same as those registered to be employ against grey mold in vineyard. They were reported in table 2.

The pyrimethanil and fluopyram compounds were also tested at concentrations of 5 μg ml^{-1} and 1, 10 μg ml^{-1}, respectively.

Control berries were sprayed with sterile water. Fungicides and water were applied with hand-pumped until berries were thoroughly wet.

After 6 h, the berries were inoculated by placing at the surface of the wounds a 20 μl drop of the conidial suspension obtained by the sensitive or fungicide-resistant isolates. Fungal suspension was prepared by flooding 10 day-old sporulating cultures in PDA with sterile distilled water. The final concentration was adjusted to 1-2x10^5 conidia/ml with the aid of a hemacytometer.

Berries were placed in separate rows (40 mm apart) on expanded metal sheets in clear plastic-covered cages. Each cage contained a stainless steel tray at the bottom in which a thin layer of water was poured to maintain a high relative humidity. For each isolate, lesion diameter (severity of decay) on each fruit and the number of infected berries per treatment was recovered after six days of incubation at 24-25°C.

Thirty berries were used for each treatment and the experiment was performed once. Isolates were considered resistant if the lesion diameters on fruits treated were not significantly different with those observed on control berries.

5.8. Assays on apple fruits

The level of resistance of *B. cinerea* isolates to pyrimethanil and the efficacy of the latter were determined also on apple fruits cv. Golden Delicious. For this experiment, two sensitive isolates and three isolates with decreased sensitivity were used. The fruits were surface-disinfected by immersing them for 2 min in 2% sodium hypochlorite solution and rinsed twice in sterile distilled water. After drying, the apples were wounded (4 wounds for apple) into 3 mm in depth with a 4 mm diameter finishing-nail head. Fifty microliters of fungicide solution at concentrations of 5 and 20 μg ml^{-1} were pipetted into each wound site. The pyrimethanil was dissolved in dimethyl sulfoxide (DMSO) (0.1 mg of pyrimethanil technical grade was dissolved in 2 ml of DMSO and subsequently in distilled water to obtain the final concentrations used). The control apples were treated with sterile water. After allowing the fungicide drops to dry, the same wounds were inoculated with 50μl of

B. cinerea suspension (1-2x10^5 conidia/ml). The apples were placed into plastic box with 200 ml water in the bottom to create high humidity. The percentage of infected wounds (incidence of disease) and the diameter of rots on apples were recorded after 6 days of incubation at 24-25°C.

Three apples were inoculated for each isolate. The experiment was carried out once. Isolates were considered resistant if the incidence and the severity of decay on fruits treated were not significantly different with those observed on apple control.

5.9. Statistical data analysis

The concentration causing a decrease of 50% in fungal growth (EC_{50} value) was always calculated for each active ingredients (a.i.) using a non linear but polynomial equation adjusted with values from the couple measures replicated for each of discriminatory rates employed in *in vitro* fungicides assays. Subsequently, the EC_{50} range and the relative mean were determined for each chemical compound.

The analysis of variance (ANOVA; Statistica 10, Statsoft Inc., Analytical Software for Windows) was performed both to examine the effects of a.i. concentrations in reducing mycelial growth in *"in vitro"* assays and to compare the efficacy of different concentrations of fluopyram in "*in vivo*" assays on detached grape berries. The corresponding mean values were compared and separated by Fisher's least significant difference test (P <0.05 or 0.01) for the adopted randomized complete block design (RCBD). As concerning the "*in vitro*" tests, arithmetic means of mycelial growth (± standard error of the mean = SEM), including the reduction relative to untreated control are presented.

Otherwise, for all "*in vivo*" assays the data on a.i. efficacy (referred to reduction of lesion diameter caused by *B. cinerea* isolates on leaves of bean seedling and grape, on detached grape berries and on apple fruits) were analyzed according to non-parametric approach (Statistica 10) using one-way analysis according to Mann-Whitney test. For both sensitive isolates and ones with decreased sensitivity, the z value was always calculated, according to Mann Whitney procedure and *P*-level associated in all pair wise comparisons between control and relative used concentrations.

6. Results

6.1. Sensitivity tests

The sensitivity of 146 *B. cinerea* isolates to fungicides and the frequency distribution of EC_{50} values for each chemical compound are shown in table 3 and in figures 5, 6 and 7. The inhibition of mycelial growth on media amended with the fungicides at different concentrations is presented in table 4.

Boscalid The 146 *B. cinerea* isolates tested showed a bimodal distribution of EC_{50} values to boscalid, which varied from 0.04 to more than 5 μg ml^{-1} (Fig. 5). The isolates were arbitrary classified in categories with EC_{50} values <0.05, 0.05 to 0.074, 0.075 to 0.099, 0.1 to 0.29, 0.3 to 0.49, 0.5 to 0.74, 0.75 to 0.99, 1 to 5, >5 μg ml^{-1}.

Using this discriminatory dose (1 μg ml^{-1}), 140 isolates were classified as sensitive to boscalid, having EC_{50} values ranging from 0.04 to 0.98 μg ml^{-1}. Their growth was significantly reduced by boscalid beginning from the concentration of 0.05 μg ml^{-1} (mean growth reduction of 32.4%) and the EC_{50} values for the majority of them (36.3% of isolates) varied from to 0.05 to 0.074 μg ml^{-1} (Fig. 8).

The other six isolates (4.1%) showed a decreased sensitivity to boscalid, having EC_{50} values >1μg ml^{-1}. Among these less sensitive isolates, 4 had an EC_{50} value >5 μg ml^{-1} (Table 3, Fig. 5).

Carbendazim The EC_{50} values of the 146 *B. cinerea* isolates had a bimodal distribution and ranged from 0.01 to more than 10 μg ml^{-1} (Fig. 5). The isolates were arbitrary classified in categories with EC_{50} values from 0.01 to 0.024, 0.025 to 0.049, 0.05 to 0.074, 0.075 to 0.099, 0.1 to 0.24, 0.25 to 0.49, 0.5 to 0.75, >10 μg ml^{-1}. Similarly to boscalid, 140 isolates were sensitive to carbendazim, having EC_{50} values ranging from 0.01 to 0.54, with a mean EC_{50} of 0.13 μg ml^{-1}. These isolates did not grow on media supplemented with carbendazim concentrations of 1 μg ml^{-1} or more and 41.8% of them showed EC_{50} values falling in the range of 0.05-0.074 μg ml^{-1}. Among the 146 isolates tested, only 6 isolates had an EC_{50} value higher than 10 μg ml^{-1} and were considered to be benzimidazole resistant (Table 3, Fig. 5).

Fenhexamid The EC_{50} values for 146 isolates varied in a bimodal way, ranging from 0.005 to 0.092 μg ml^{-1}, with a mean EC_{50} of 0.025 μg ml^{-1} (Table 3; Fig. 5).

EC_{50} values of the isolates had a roughly normal distribution and the majority of them (54.1%) had EC_{50} values between 0.025 and 0.049 μg ml^{-1}. The other isolates were more sensitive and the EC_{50} values were less than 0.01 and 0.025 μg ml^{-1} for 22% and 20.5% of the isolates, respectively. No isolate grew on the media amended with fenhexamid at concentration >0.1 μg ml^{-1}. Therefore, these results suggested that there was no resistant population among the isolates used in the study.

Fludioxonil The EC_{50} values for most of the 146 *B. cinerea* isolates had a roughly normal distribution, ranging from 0.001 to 0.03 μg ml^{-1}, with a mean EC_{50} value of 0.008 μg ml^{-1} (Table 3). The other isolates showed EC_{50} values less than 0.001 μg ml^{-1} and consequently appeared more sensitive (Fig 6). No isolates grew on the media amended with fludioxonil at concentration >0.05 μg ml^{-1}; therefore, all isolates were considered sensitive to fungicide.

Iprodione The isolates were arbitrary classified in categories with EC_{50} values from 0.1 to 0.19, 0.2 to 0.29, 0.3 to 0.39, 0.4 to 0.49, 0.5 to 0.59, 0.6 to 0.69, 0.7 to 0.79, 0.8 to 0.89, 0.9 to 0.99, >1 μg ml^{-1}. The EC_{50} values of 146 *B. cinerea* isolates ranged from 0.11 to 2.1 μg ml^{-1}, with a mean EC_{50} value of 0.47 μg ml^{-1} (Table 3).

A group of 141 isolates showed a weak growth retardation on PDA amended with 0.1 μg ml^{-1} of the iprodione and was strongly suppressed on media amended with fungicide at concentration of 1 μg ml^{-1}. Thus, they were classified as sensitive isolates. These isolates showed a normal distribution of EC_{50} values, which varied from 0.11 to 0.71 μg ml^{-1}, with a mean EC_{50} value of 0.42 μg ml^{-1} (Fig. 6). Five of 146 isolates (3.4%) showed a decreased sensitivity to iprodione. These isolates grew on media amended with iprodione at concentrations of up to 1 μg ml^{-1}, without any significant reduction in colony diameter. The EC_{50} values for them varied from 1.16 to 2.1 μg ml^{-1}, with a mean EC_{50} value of 1.76 μg ml^{-1}. None of the isolates grew on media amended with the concentrations above to 5 μg ml^{-1} (Table 3, Fig. 6).

Pyrimethanil The EC_{50} values of the 146 *B. cinerea* isolates had a multimodal distribution and ranged from 0.05 to more than 5 μg ml^{-1} (Table 3, Fig. 6). Seventy-five percent of the 146 isolates were sensitive to pyrimethanil, having EC_{50} values ranging from 0.05 to 0.9 μg ml^{-1}, with a mean EC_{50} of 0.29 μg ml^{-1}. The sensitive isolates showed a bimodal distribution of EC_{50} values. All of them grew on media

amended with pyrimethanil at concentrations ≤ 0.05 μg ml^{-1}, without any significant reduction in colony diameter compared to the control (Fig. 9). On the contrary, a notable reduction on their growth was observed on pyrimethanil amended-media at concentrations of 0.1 and 1 μg ml^{-1} and the EC_{50} values for 22.4 and 37.4% of the isolates felled in the range of 0.075-0.099 μg ml^{-1} and 0.25-0.49 μg ml^{-1}, respectively. Thirty-seven of 146 isolates (25.4%) grew on media amended with pyrimethanil at concentrations ≥1 μg ml^{-1} without any significant reduction in colony diameter, and thus they were regarded as resistant (Fig. 9). Different levels of resistance to pyrimethanil were found within the resistant population studied: 5 isolates (3.4% of 146 isolates) had EC_{50} values ranging from 1 to 2.5 μg ml^{-1}, 22 isolates (15%) showed EC_{50} values ranging from 2.5 to 5 μg ml^{-1} and 10 isolates (6.8%) had an EC_{50} value higher than 5 μg ml^{-1} (Table 3, Fig. 6).

Fluopyram The frequency distribution of the EC_{50} values was an unimodal curve and it was shown in figure 7. The EC_{50} values for fluopyram ranged from 0.084 to 3.48 μg ml^{-1} and a mean EC_{50} value of 0.98 μg ml^{-1} was measured within the population tested. The fungicide strongly reduced the growth of all *B. cinerea* isolates tested at concentrations of 1 and 10 μg ml^{-1}. The majority of them (52%) showed EC_{50} values falling in the range of 0.75-0.99 μg ml^{-1}, whereas a high number of isolates (39%) had EC_{50} values higher than 1 μg ml^{-1}. A complete inhibition of mycelial growth was observed at 100 μg ml^{-1}.

Table 3. Sensitivity of *B. cinerea* isolates to fungicides

Fungicides	EC_{50} (μg ml^{-1}) range		EC_{50} mean[a]	No. of isolates		Resistant isolates (%)
	Sensitive	Resistant		Sensitive	Resistant	
Boscalid	0.04 - 0.98	1.02 - >5	0.25 / -	140	6	4.1
Carbendazim	0.01 - 0.54	>10	0.13 / -	140	6	4.1
Fenhexamid	0.005 - 0.092	-	0.025	146	0	0.0
Fludioxonil	< 0.001 – 0.030	-	0.008	146	0	0.0
Iprodione	0.11 - 0.71	1.16 – 2.1	0.42 / 1.76	141	5	3.4
Pyrimethanil	0.05 - 0.90	1.18 - >5	0.29 / -	109	37	25.4

[a] Numbers are means of EC_{50} values of the *B. cinerea* sensitive / resistant isolates

Table 4. *In vitro* fungicide efficacy on mean mycelial growth (mm) and growth reduction (%) of 146 *B. cinerea* isolates on media amended with the seven active ingredients (a.i.) at different concentrations

Fungicide concentration (μgml^{-1})	Colony diameter (mm) [a]	Growth reduction %)
Boscalid		
0	70.9 (± 0.8) a	-
0.05	47.9 (± 0.6) b	32.4
0.5	32.7 (± 0.5) c	53.9
1	28.4 (± 0.6) d	59.9
5	23.6 (± 0.7) e	66.7
Carbendazim		
0	70.0 (± 0.8) a	-
0.01	66.9 (± 0.1) b	4.5
0.1	25.0 (± 1.3) c	64.2
1	2.9 (± 0.06) d	95.8
10	2.7 (± 0.04) d	96.1
Fenhexamid		
0	71.9 (± 0.8) a	-
0.01	51.0 (± 1.2) b	29.1
0.05	17.1 (± 1.1) c	76.2
0.1	11.5 (± 1.0) d	84.0
1	1.8 (± 0.4) e	97.5
Fludioxonil		
0	70.7 (± 0.8) a	-
0.01	25.4 (± 0.9) b	64.0
0.05	9.2 (± 1.4) c	87.0
0.1	4.5 (± 0.8) d	93.7
1	0.3 (± 0.1) e	99.6
Iprodione		
0	71.9 (± 1.0) a	-
0.1	55.8 (± 0.9) b	22.3
1	17.1 (± 0.9) c	76.3
5	2.3 (± 0.0) d	96.9
10	0.0 (± 0.0) d	100.0
20	0.0 (± 0.0) d	100.0
Pyrimethanil		
0	62.3 (± 2.1) a	-
0.01	64.8 (± 2.0) a	0.0
0.05	63.2 (± 2.2) a	0.0
0.1	45.2 (± 3.1) b	27.5
1	16.1 (± 1.4) c	74.2
5	7.5 (± 0.9) d	88.0
Fluopyram		
0	70.0 (± 1.2) a	-
0.1	56.9 (± 0.9) b	18.7
1	34.3 (± 0.7) c	51.1
10	13.9 (± 0.7) d	80.0
100	3.5 (± 0.3) e	95.0

[a] Colony diameter was determined after 3 days of incubation. Numbers are means ± standard error of the mean (SEM) of 3 replicates each formed by 146 values. Means followed by the same letter are not significantly different according to Fisher's least significance difference test ($P<0.01$).

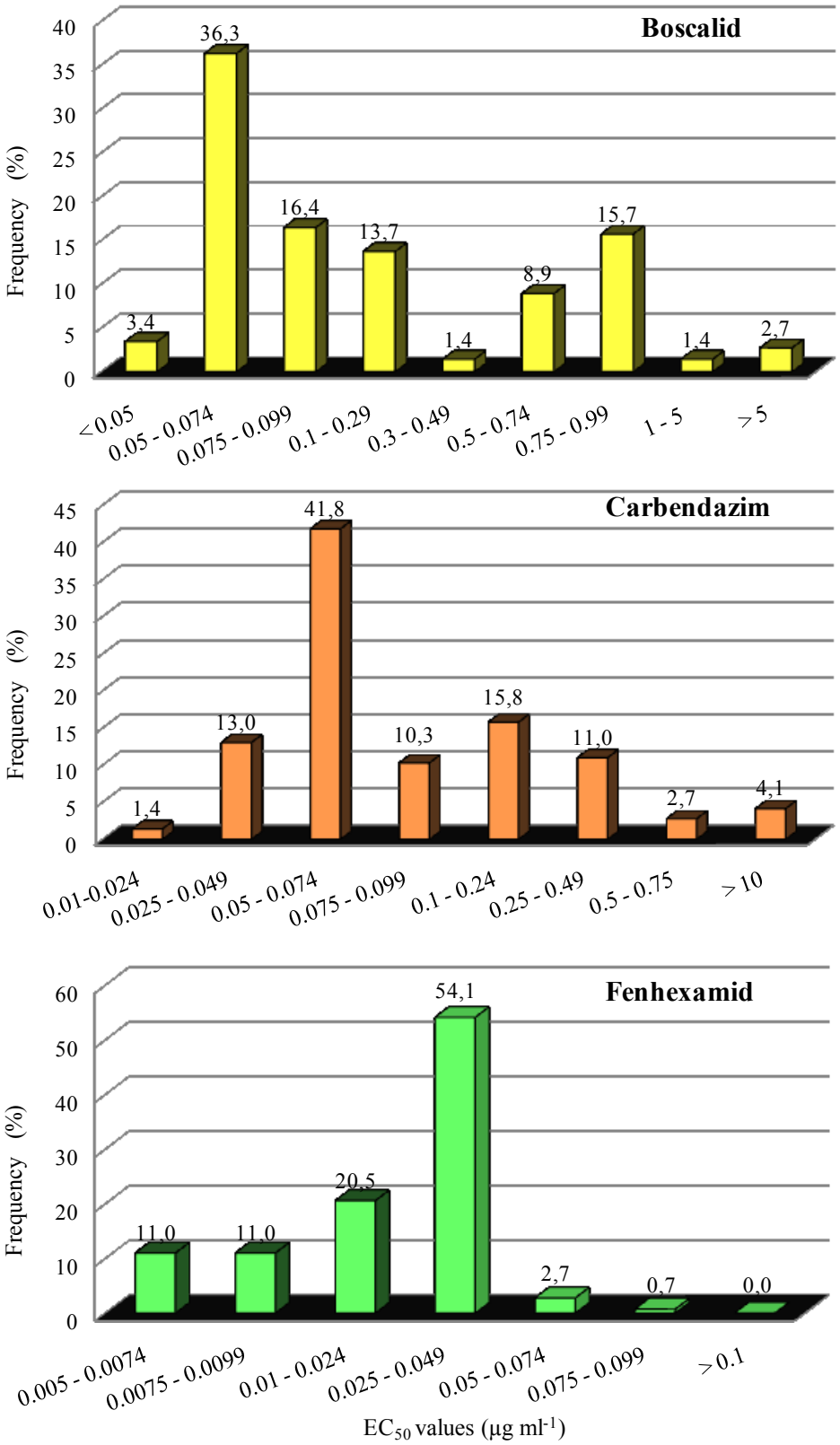

Figure 5. Frequency distribution of EC_{50} values for boscalid, carbendazim and fenhexamid among 146 isolates of *B. cinerea* collected in Sicily from different vineyards.

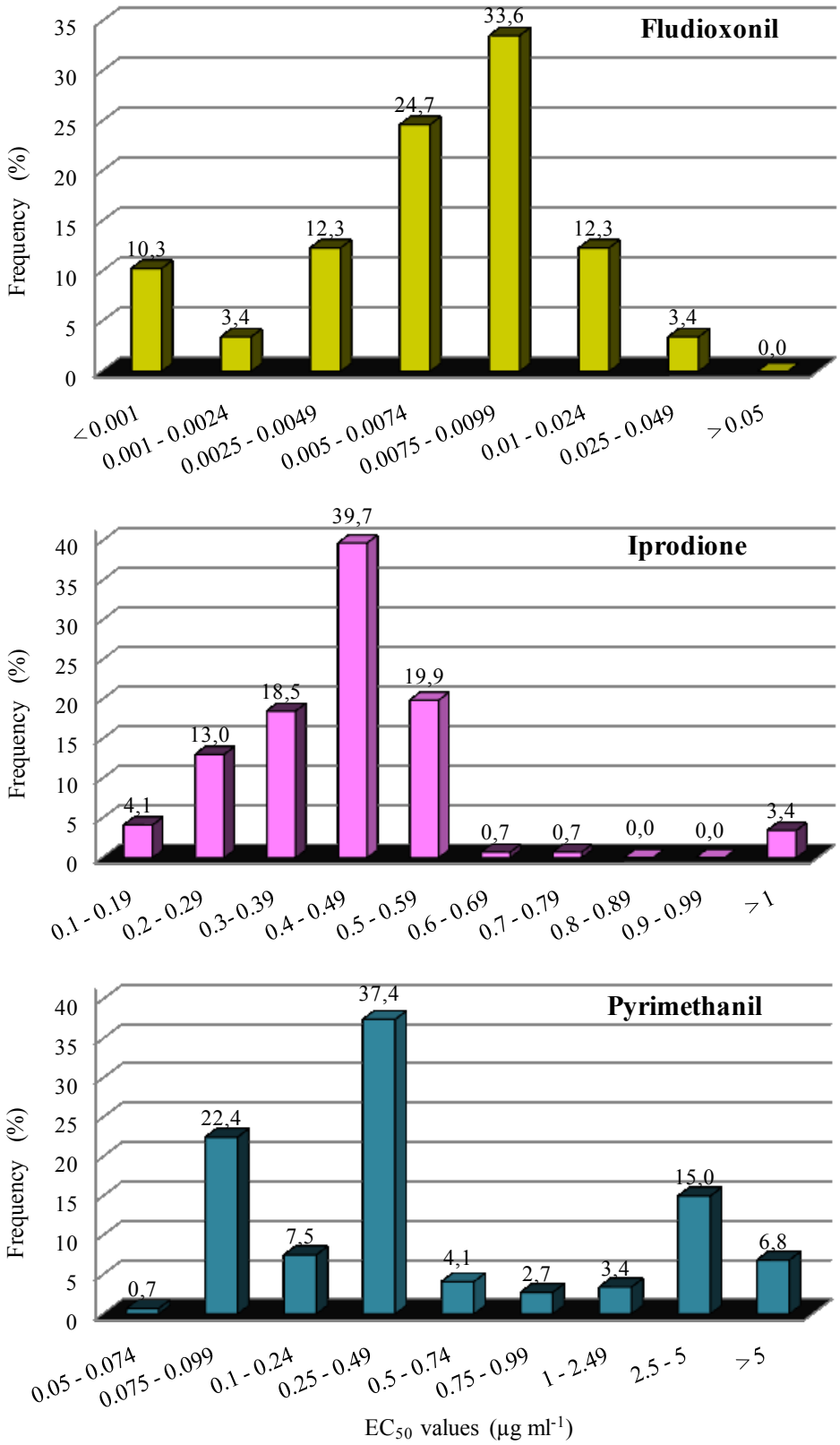

Figure 6. Frequency distribution of EC_{50} values for fludioxonil, iprodione and pyrimethanil among 146 isolates of *B. cinerea* collected in Sicily from different vineyards.

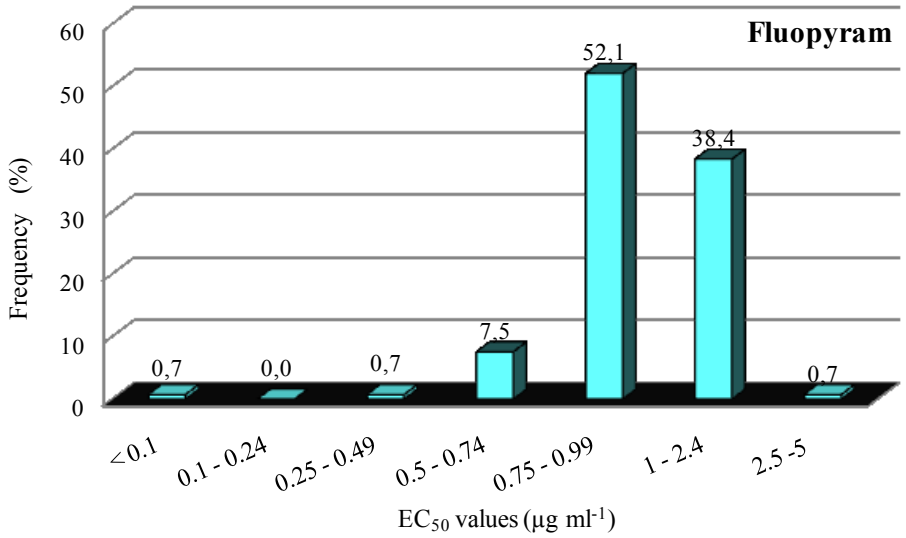

Figure 7. Frequency distribution of EC_{50} values for fluopyram among 146 isolates of *B. cinerea* collected in Sicily from different vineyards.

As shown in table 5, the majority of isolates showing a decreased sensitivity to boscalid (4 of 6 isolates) and all isolates resistant to carbendazim (4.1% of 146 *B. cinerea* isolates) and to iprodione (3.4% of 146 isolates) were found in just one vineyard, which was located in Chiaramonte Gulfi, Mazzaronello street (Ragusa). On the contrary, the isolates resistant to pyrimethanil (25.4% of 146 isolates) were found in different vineyards located in both Catania and Ragusa provinces. Thirty-eight percent of them came from 3 of the 8 vineyards located in Catania, whereas 60% of the pyrimethanil-resistant isolates were present in 3 of the 6 vineyards located in Ragusa. Within Ragusa province, a high number of pyrimethanil-resistant isolates came from the vineyard located in Chiaramonte Gulfi, Mazzaronello street.

Among the 39 isolates found to be resistant, eight isolates (about 2%) exhibited resistance to two or more fungicides simultaneously. Two isolates (SR1, SR5) were resistant to both boscalid and pyrimethanil and two (MZ1, MZ2) to both carbendazim and iprodione. One isolate (MZ11) was simultaneously resistant to boscalid, carbendazim and pyrimethanil, whereas three isolates (MZ4.1, MZ4.2, MZ4.3) were resistant to all four fungicides employed (Table 5).

Table 5. Fungicide profiles of the *B. cinerea* resistant isolates collected in Sicilian vineyards

Isolates	Location	Mean street	Boscalid	Carbendazim	Iprodione	Pyrimethanil
RV 6	Ravanusa	Poggio Rotondo	- [a]	-	-	+ [a]
DN 1	Licodia Eubea	Donna	-	-	-	+
DN 2	Licodia Eubea	Donna	-	-	-	+
DN 3	Licodia Eubea	Donna	-	-	-	+
DN 4	Licodia Eubea	Donna	-	-	-	+
DN 5	Licodia Eubea	Donna	-	-	-	+
DN 6	Licodia Eubea	Donna	-	-	-	+
SR1	Licodia Eubea	Sciri Sopra	+	-	-	+
SR 2	Licodia Eubea	Sciri Sopra	-	-	-	+
SR 3	Licodia Eubea	Sciri Sopra	-	-	-	+
SR 4	Licodia Eubea	Sciri Sopra	-	-	-	+
SR 5	Licodia Eubea	Sciri Sopra	+	-	-	+
PM 1	Mazzarone	Piano Maenza	-	-	-	+
PM 3	Mazzarone	Piano Maenza	-	-	-	+
PM 4	Mazzarone	Piano Maenza	-	-	-	+
FG 1a	Chiaramonte G.	Fegotto	-	-	-	+
FG 1b	Chiaramonte G.	Fegotto	-	-	-	+
FG 2	Chiaramonte G.	Fegotto	-	-	-	+
FG 3a	Chiaramonte G.	Fegotto	-	-	-	+
FG 3b	Chiaramonte G.	Fegotto	-	-	-	+
FG 4	Chiaramonte G.	Fegotto	-	-	-	+
MZ 1	Chiaramonte G.	Mazzaronello	-	+	+	-
MZ 2	Chiaramonte G.	Mazzaronello	-	+	+	-
MZ 3.5	Chiaramonte G.	Mazzaronello	-	-	-	+
MZ 3.7	Chiaramonte G.	Mazzaronello	-	-	-	+
MZ3.12	Chiaramonte G.	Mazzaronello	-	-	-	+
MZ 3.13	Chiaramonte G.	Mazzaronello	-	-	-	+
MZ 4.1	Chiaramonte G.	Mazzaronello	+	+	+	+
MZ 4.2	Chiaramonte G.	Mazzaronello	+	+	+	+
MZ 4.3	Chiaramonte G.	Mazzaronello	+	+	+	+
MZ 4.5	Chiaramonte G.	Mazzaronello	-	-	-	+
MZ 5.3	Chiaramonte G.	Mazzaronello	-	-	-	+
MZ 11	Chiaramonte G.	Mazzaronello	+	+	-	+
MT 3	Chiaramonte G.	Mortilla	-	-	-	+
MT6	Chiaramonte G.	Mortilla	-	-	-	+
MT 7	Chiaramonte G.	Mortilla	-	-	-	+
MTK1	Chiaramonte G.	Mortilla	-	-	-	+
MTK 2	Chiaramonte G.	Mortilla	-	-	-	+
MTK 3	Chiaramonte G.	Mortilla	-	-	-	+

The sensitivity of *B. cinerea* isolates to boscalid, carbendazim, iprodione and pyrimethanil was determined using the discriminatory concentrations of 1 µg ml^{-1}

[a] "+" indicates resistant isolate and "-" indicates sensitive isolate.

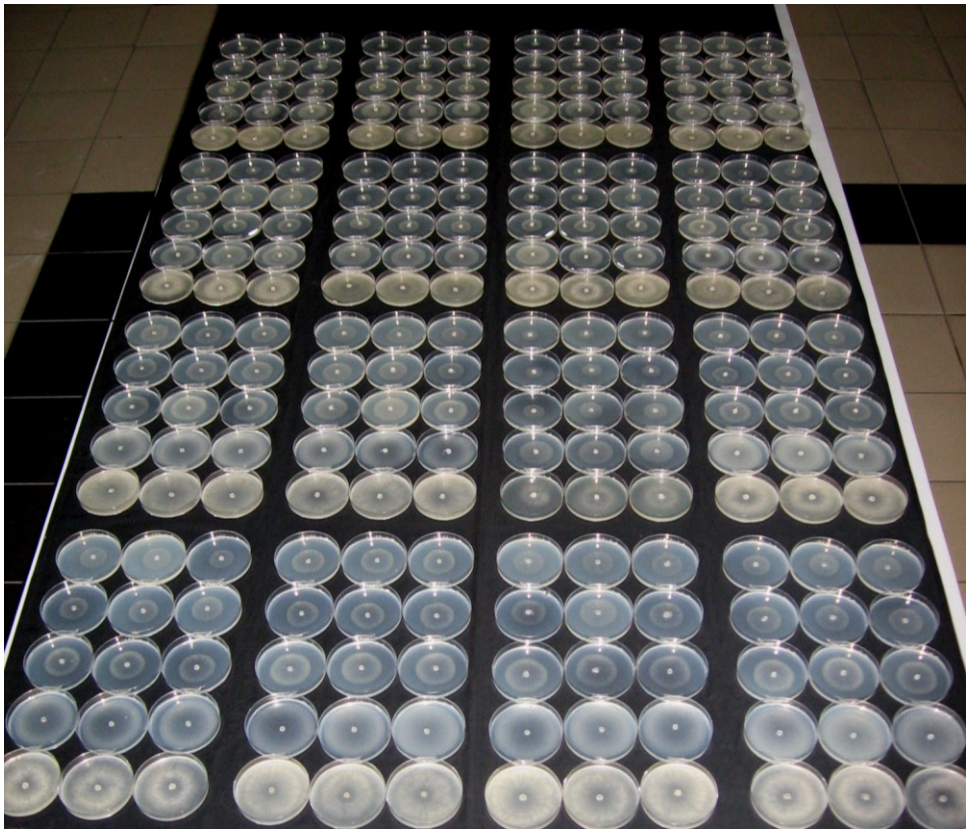

Figure 8. Effect of boscalid at different concentrations (0, 0.0.5, 0.5, 1 and 5 µg ml^{-1}) on the mycelial development of various *B. cinerea* isolates on PDA after 3 days of incubation.

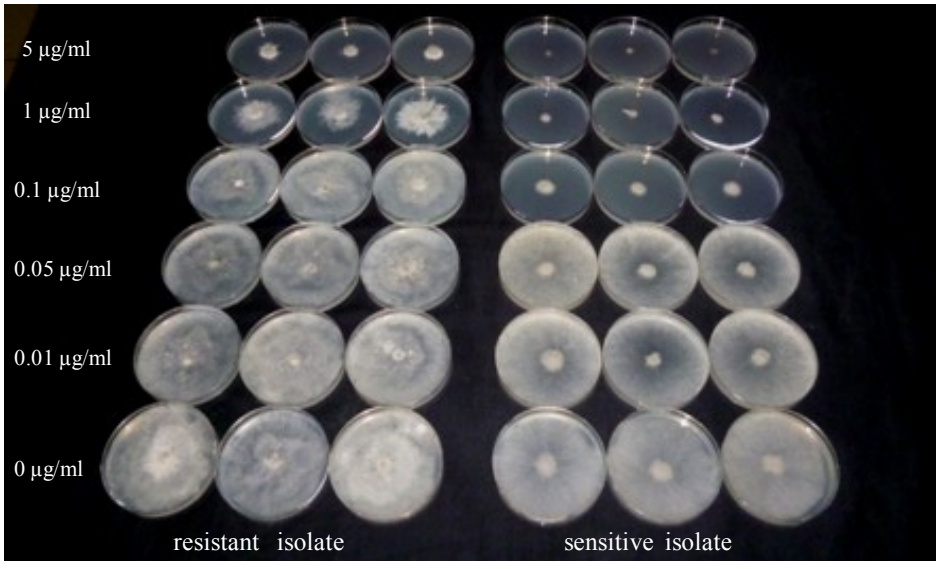

Figure 9. Effect of pyrimethanil at different concentrations on the mycelial development of *B. cinerea* isolates. The pyrimethanil-sensitive isolate (BN1) and the pyrimethanil-resistant isolate (FG4) were compared 3 days after inoculation.

6.2. Assays on bean seedling

The fungicide sensitivity phenotypes determined by mycelium growth was first verified *in vivo* on bean seedlings (Fig. 10).

As shown in table 6, all isolates considered resistant by the *in vitro* tests caused visible lesions on bean leaves previously treated with boscalid. However, only one of them, the isolate MZ11, produced lesions which did not differ in diameter from those formed on control leaves (0.0% of disease reduction). A low level of boscalid resistance was detected in the other isolates previously considered as resistant (48.5 and 52.5% of disease reduction) as well as in the sensitive isolates (69.5 and 85.9% of reduction). Fenhexamid and fludioxonil markedly controlled infection caused by all *B. cinerea* strains tested on bean seedlings (percentage of disease reduction between 66.3 and 100%). The diameter of lesions on leaves treated with the fungicides and subsequently inoculated with the pathogens ranged from 0.0 to 6.6 mm and thus all isolates tested were considered sensitive, as found in previous tests.

Bean seedling treated with iprodione at label rate and then inoculated with sensitive isolates were protected from infection (number of infected sites on leaves treated = 0; 100% of disease reduction), whereas those inoculated with resistant isolates were not protected and showed in all of their leaves disease symptoms (n. of infected sites = 30). However, in these leaves, the iprodione weakly reduced gray mould development (64.3 and 73.6% of disease reduction) and the lesion diameters were significantly less than those observed on the control; thus, the isolates were considered weakly resistant to iprodione in this assay.

As concerning thiophanate methyl and pyrimethanil, all isolates tested considered resistant by the *in vitro* test infected bean leaves, producing extensive lesions which were comparable to those observed on the untreated control leaves. No sensitive isolate caused severe symptoms of rotting on leaves (reduction of disease between 67 and 99.2%). These results confirmed the level of fungicide sensitivity determined by mycelium growth assays. Similarly to fenhexamid and fludioxonil, also fluopyram showed a strong antifungal activity in "*in vivo*" assay, because no isolate determined wide rots on treated plants (Table 6).

Table 6. Number of infected sites and diameter of lesion observed on bean seedlings treated with fungicides and subsequently with sensitive or resistant isolates of *B. cinerea*

Fungicides	Isolates	N. of infected sites (n=30)	Lesion diameter (mm)			
			Control [b]	Treated [b]	Significance	Reduction (%)
Boscalid						
S[a]	CR 6	12	17.7 a[c]	5.4 b	z = 6.65*	69.5
S	BN 5	8	17.3 a	2.4 b	z = 6.65*	85.9
R	MZ 11	30	16.8	17.3	z = -0.78^{ns}	0.0
R	SR 1	30	19.0 a	9.8 b	z = 6.47*	48.5
R	SR 5	30	21.7 a	10.3 b	z = 6.65*	52.5
Fenhexamid						
S	CR 5	1	4.3 a	0.4 b	z = 6.65*	90.6
S	PM 3	14	19.7 a	6.6 b	z = 6.65*	66.3
Fludioxonil						
S	RV 4	0	23.4 a	0.0 b	z = 6.65*	100.0
S	PS 4	1	23.3 a	0.4 b	z = 6.65*	98.4
Iprodione						
S	DN 1	0	17.9 a	0.0 b	z = 6.65*	100.0
S	CR 5	0	4.3 a	0.0 b	z = 6.65*	100.0
R	MZ 1	30	19.1 a	6.8 b	z = 6.65*	64.3
R	MZ 2	30	12.4 a	3.3 b	z = 6.15*	73.6
T. methyl						
S	MT 6	1	16.4 a	0.1 b	z = 6.65*	99.2
S	MT 4	2	17.2 a	0.4 b	z = 6.15*	97.9
R	MZ 1	30	19.6	18.1	z = 1.89^{ns}	7.8
R	MZ 2	30	12.6	14.0	z = -1.23^{ns}	0.0
R	MZ 11	30	18.0	18.4	z = -0.54^{ns}	0.0
Pyrimethanil						
S	BN 1	15	12.1 a	4.0 b	z = 6.31*	67.0
S	PM 1	2	6.0 a	0.3 b	z = 4.91*	95.6
S	MZ 3.1	1	7.4 a	0.2 b	z = 6.62*	97.7
R	FG 4	30	13.9	11.1	z = 1.78^{ns}	20.6
R	MT 6	30	18.3	13.9	z = 1.85^{ns}	23.7
R	SR 5	30	7.0	7.8	z = -0.61^{ns}	0.0
Fluopyram						
	BN 5	2	13.8 a	0.8 b	z = 6.65*	94.4
	ML 2	0	9.8 a	0.0 b	z = 5.77*	100.0
	SR 5	2	12.0 a	0.9 b	z = 6.65*	92.2
	MZ 3.5	5	17.5 a	1.8 b	z = 6.65*	89.5
	MT 6	1	14.3 a	0.2 b	z = 6.65*	98.6
	ST 5	5	19.8 a	2.1 b	z = 6.65*	89.2

[a] S = sensitive isolate and R = resistant isolates based on *in vitro* tests

[b] Numbers are means of 30 values (6 mycelial plugs per 5 leaves) corresponding to same infection sites

[c] Within rows, mean values with different letters and the symbol * denote significant differences at $P \leq 0.001$ according to Mann Whitney non parametric rank test (z parameter); ns: not significant.

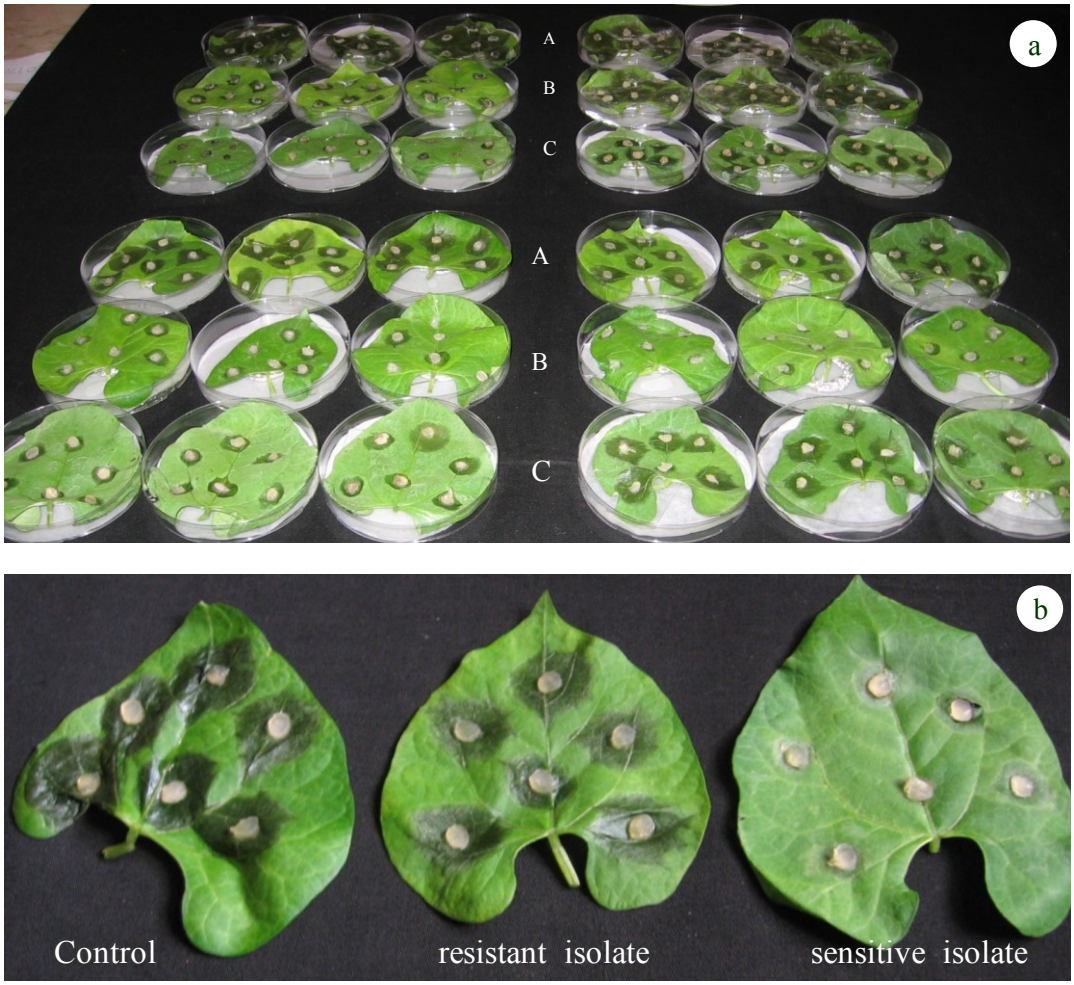

Figure 10. Infections induced by different isolates of *B. cinerea* on leaves of bean seedlings treated with water (control, A) boscalid (B) and pyrimethanil (C) after 3 days of incubation (a). The pyrimethanil-resistant isolate (FG4) and the pyrimethanil-sensitive isolate (MZ3.1) were compared with the control 3 days after inoculation (b).

6.3. Assays on leaves of grapevine

Inoculation of grapevine plants previously treated with fungicides or water confirmed phenotypes determined by the assay on bean seedling. Isolates were considered resistant if leaves showed a rotting level similar to the control (Fig. 11).

As well as found on been seedling, all isolates considered resistant by the *in vitro* tests infected grapevine leaves, producing extensive lesions which were significantly greater than those produced by sensitive isolates. As shown in table 7, there were no significant differences in diameter among the lesions caused by the resistant isolates in treated and non-treated leaves and their resistance was accompanied by the

complete or significant failure of fungicides to provide disease control (reduction of disease between 0.0 and 26.5%). We did not find any isolates that were resistant to fenhexamid or to fludioxonil (Table 7).

Table 7. Number of infected sites and diameter of lesion observed on grapevine leaves treated with fungicides and subsequently with sensitive or resistant isolates of *B. cinerea*

Fungicides	Isolates	N. of infected sites (n=9)	Lesion diameter (mm)[b]			
			Control	Treated	Significance	Reduction (%)
Boscalid						
S[a]	CR 6	0	3.7 a	0.0 b	z = 3.58***	100.0
S	BN 5	4	20.6 a	7.6 b	z = 2.96**	63.2
R	MZ 11	9	23.1	22.8	z = 0.66^{ns}	1.4
R	SR 1	9	24.0 a	19.0 b	z = 2.52*	20.8
R	SR 5	9	25.1 a	14.8 b	z = 2.65**	41.1
Fenhexamid						
S	CR 5	0	25.4 a	0.0 b	z = 3.58***	100.0
S	PM 3	5	23.1 a	9.2 b	z = 2.43**	60.2
Fludioxonil						
S	RV4	0	25.4 a	0.0 b	z = 3.58***	100.0
S	PS4	2	23.1 a	1.7 b	z = 3.58***	92.8
Iprodione						
S	DN 1	0	23.2 a	0.0 b	z = 3.58*	100.0
S	CR 5	0	4.0 a	0.0 b	z = 3.58*	100.0
R	MZ 1	8	21.9 a	12.1 b	z = 3.53*	44.7
R	MZ 2	5	21.0 a	5.8 b	z = 3.53*	72.5
T. methyl						
S	MT 6	1	20.3 a	1.0 b	z = 3.58***	95.1
S	MT 4	1	23.3 a	1.0 b	z = 3.58***	95.7
R	MZ 1	9	21.9	21.2	z = 0.75^{ns}	3.1
R	MZ 2	9	21.0	20.9	z = 0.00^{ns}	0.5
R	MZ 11	9	23.1	21.2	z = 1.28^{ns}	8.2
Pyrimethanil						
S	BN 1	4	14.1 a	5.7 b	z = 3.53*	59.8
S	PM 1	3	14.0 a	5.1 b	z = 3.53*	63.6
S	MZ 3.1	3	12.3 a	4.2 b	z = 3.44*	65.7
R	FG 4	9	22.0	22.4	z = -0.40^{ns}	0.0
R	MT 6	NT[d]	NT	NT	NT	NT
R	SR 5	9	25.1 a	18.4 b	z = 2.34*	26.5

[a] S = sensitive isolate and R = resistant isolates based on *in vitro* tests
[b] Numbers are means of 9 values (3 mycelial plugs per 3 leaves) corresponding to same infection sites
[c] Within rows, mean values with different letters and the symbols *, **, *** denote significant differences at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively according to Mann Whitney non parametric rank test (z parameter); ns: not significant.
[d] NT = not determined

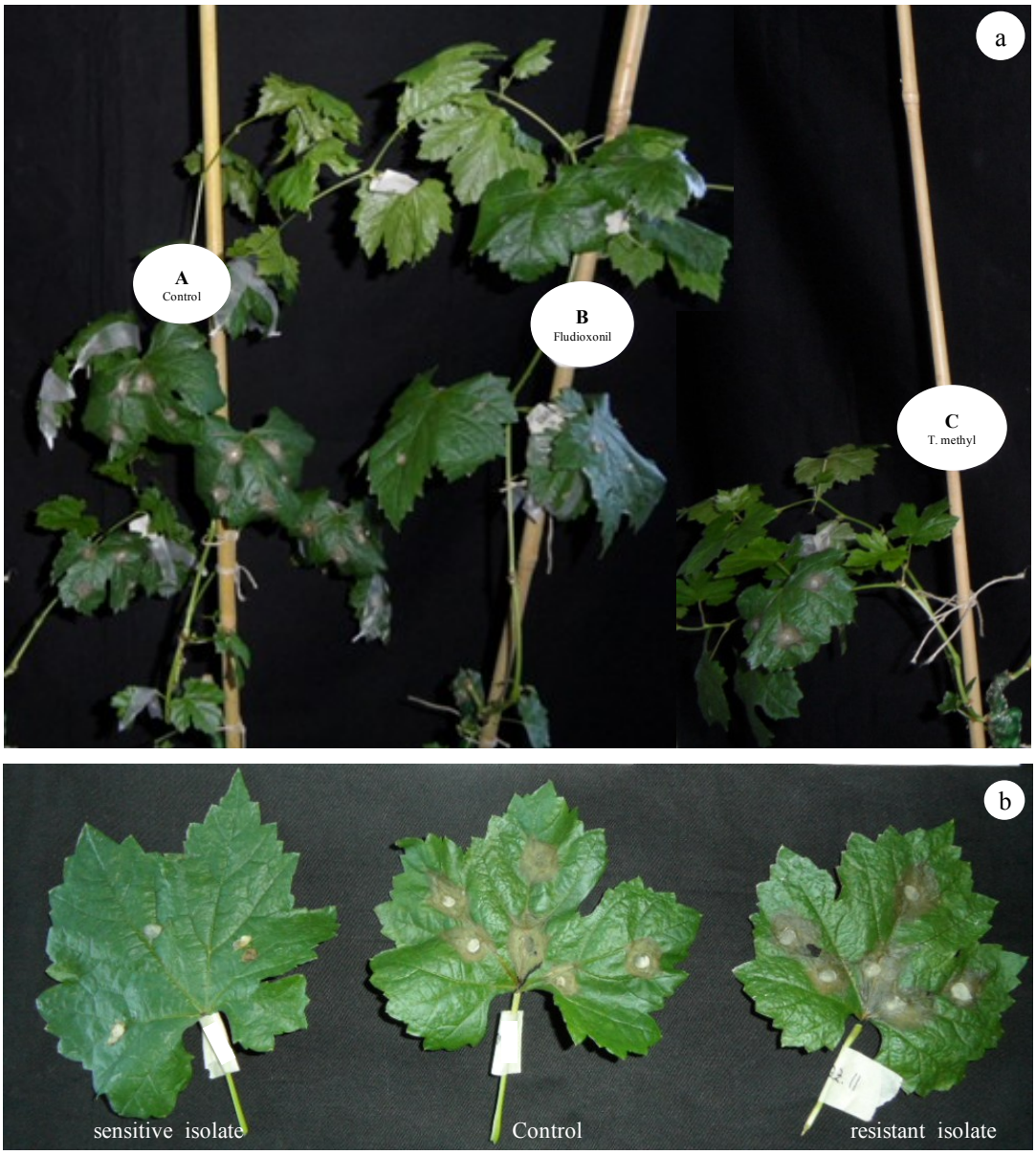

Figure 11. Infections induced by different isolates of *B. cinerea* on leaves of grapevine treated with water (control, A) fludioxonil (B) and thiophanate methyl (C) after 4 days of incubation (a). The thiophanate methyl-sensitive isolate (MT4) and the thiophanate methyl-resistant isolate (MZ11) were compared with the control 4 days after inoculation (b).

6.4. Molecular analysis

Nucleotide sequences from boscalid and/or carbendazim resistant isolates (MZ4.1, MZ4.2, MZ4.3 and SR1 isolates) were compared with the corresponding

nucleotide sequences belonging to the sensitive isolates (DN1 and ST4 isolates) and to the reference B05.10 and SAS56 strains.

A single-nucleotide substitution in the *Sdh*B gene coding the Fe-S protein sub-unit (Ip) of the succinate dehydrogenase was detected in the most of the boscalid-resistant isolates tested. In particular, three of the four boscalid-resistant isolates tested were modified into the codon 272 with TAC instead to CAC. The nucleotide change from C to T led to the substitution of tyrosine with histidine within the third cysteine-rich cluster-Ip sub-unit.

The nucleotide sequences of *Sdh*B gene were identical in the two boscalid-sensitive strains tested and in the reference strain B05.10. No isolate was found to possess the mutation into the codon 225, responsible of proline with leucine substitution (Fig. 12).

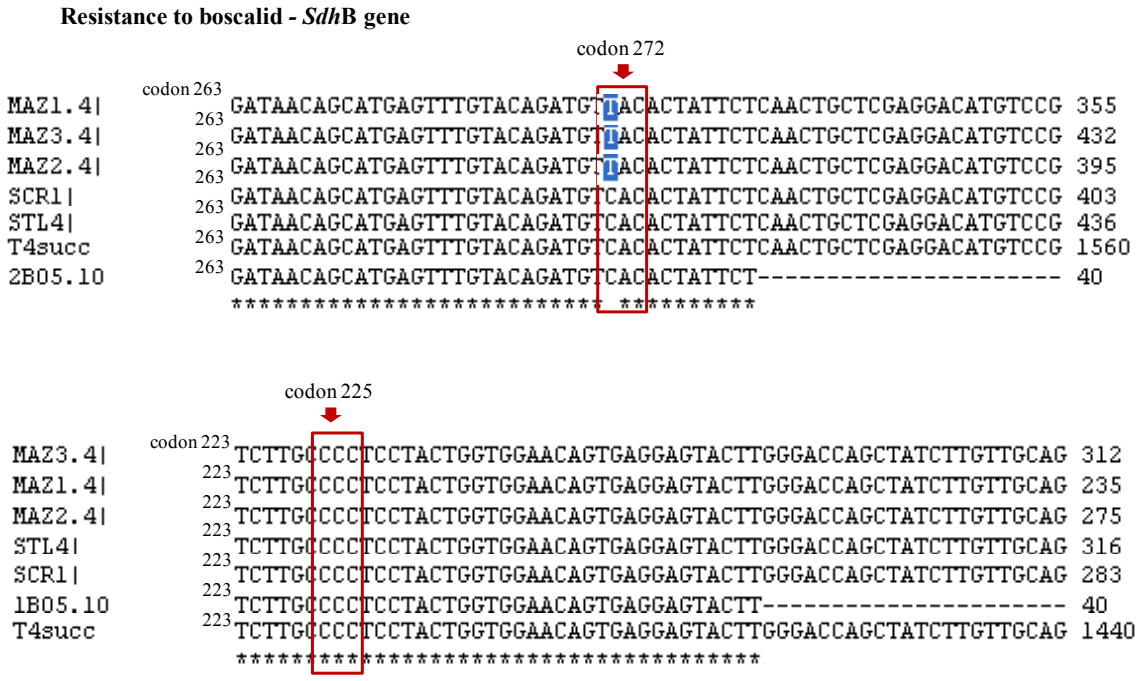

Figure 12. Comparison of partial nucleotide sequences of the *Sdh*B gene amplified with PCR primers from *B. cinerea* resistant and sensitive isolates.

As concerning the resistance to carbendazim, mutations in the nucleotide sequences were observed in all of the resistant isolates tested. The resistance, in this case, was correlated with a point mutation at codon 198 in the β-tubulin gene.

In this codon, these isolates had GCG rather than GAG, which resulted in the substitution of glutamic acid by alanine. Molecular analysis of the sensitive isolates did not reveal any mutations at this β-tubulin gene fragment (Fig. 13).

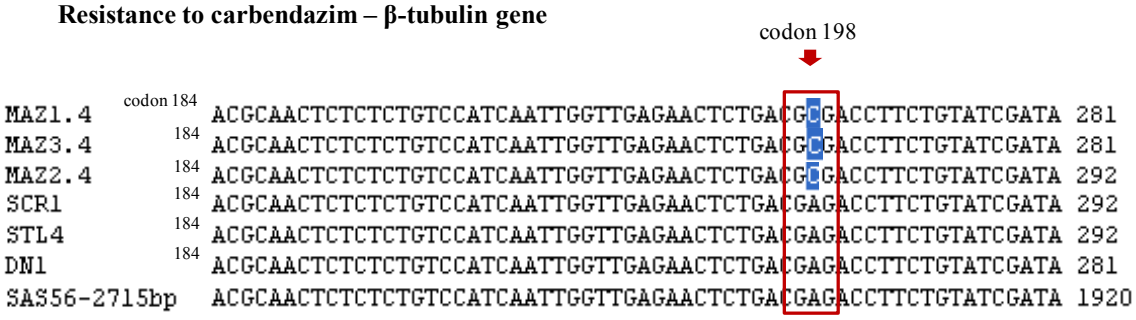

Figure 13. Comparison of partial nucleotide sequences of the β-tubulin gene amplified with PCR primers from *B. cinerea* resistant and sensitive isolates.

6.5. Assays on detached grape berries

The effectiveness of the fungicides used in this study for the control of *B. cinerea* was determined on detached grape berries cv. "Italia" (Fig. 14).

The fungicides provided effective control with decay reduction >69% for boscalid, >83% for fenhexamid and fludioxonil and >65% for iprodione and thiophanate methyl when grape berries were inoculated with sensitive isolates of *B. cinerea* (Table 8, Fig. 15 a, b, c).

The effectiveness of boscalid, iprodione and thiophanate methyl decreased considerable on fruits inoculated with resistant isolates (percentage of disease reduction between 0.0 and 32.3%) (Fig 15 a, c).

The resistance phenotypes identified in the mycelium inhibition test were confirmed with inoculation assays on detached grape berries and the lesion diameters caused by them on fruits treated were not significantly different with those observed on control berries.

Table 8. Lesion observed on detached grape berries treated with fungicides and subsequently inoculated with sensitive or resistant isolates of *B. cinerea*

Fungicides	Isolates	Lesion diameter (mm)[b]			
		Control	Treated	Significance	Reduction (%)
Boscalid					
S[a]	CR 6	12.2 a	4.5 b	z = 5.71***	63.5
S	BN 5	25.5 a	7.8 b	z = 4.16***	69.6
R	MZ 11	15.0	16.6	z = -0.71^{ns}	0.0
R	SR 1	21.0	24.8	z = -1.88^{ns}	0.0
R	SR 5	28.6	26.8	z = 0.11^{ns}	6.5
Fenhexamid					
S	CR 5	8.3 a	0.6 b	z = 5.24***	93.2
S	PM 3	23.6 a	4.0 b	z = 6.08***	83.2
Fludioxonil					
S	RV 4	14.1 a	1.7 b	z = 4.95***	87.9
S	PS 4	27.6 a	4.5 b	z = 6.34***	83.7
Iprodione					
S	DN 1	20.0 a	5.7 b	z = 5.44*	71.6
S	CR 5	8.3 a	2.9 b	z = 3.34*	65.5
R	MZ 1	18.4	20.1	z = -1.95^{ns}	0.0
R	MZ 2	21.7	26.1	z = -1.95^{ns}	0.0
T. methyl					
S	MT 6	23.6 a	3.6 b	z = 4.13***	84.8
S	MT 4	23.8 a	5.4 b	z = 4.16***	77.2
R	MZ 1	18.4	12.4	z = 1.95^{ns}	32.3
R	MZ 2	18.1	22.7	z = -1.95^{ns}	0.0
R	MZ 11	13.6	16.7	z = -1.26^{ns}	0.0

[a] S = sensitive isolate, R = resistant isolates based on *in vitro* tests
[b] Numbers are means of 30 values
[c] Within rows, mean values with different letters and the symbols *, **, *** denote significant differences at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively according to Mann Whitney non parametric rank test (z parameter); ns: not significant.

Figure 14. Arrangement of grape berries cv. "Italia" before treatments with fungicides

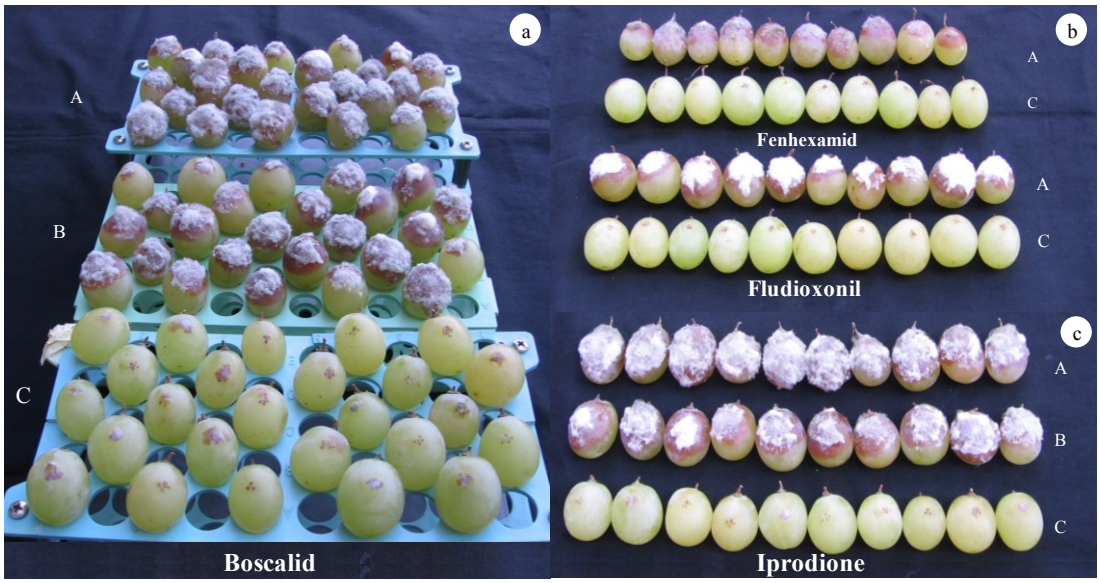

Figure 15. Infections induced by different isolates of *B. cinerea* (fungicide-resistant/sensitive isolates) on grape berries treated with boscalid (a), fludioxonil, fenhexamid (b) and iprodione (c) after 6 days of incubation (A = control; B = fungicide-resistant isolate; C = fungicide-sensitive isolate).

Treatments with pyrimethanil significantly reduced the *Botrytis* fruit rot on grape berries inoculated with *B. cinerea* sensitive isolates beginning from the lowest concentration tested (89.7 and 100% of disease reduction) (Table 9). No lesion was observed on berries inoculated with these isolates when pyrimethanil was applied at label rate (100% of reduction).

In contrast, the pyrimethanil failed to control satisfactorily the development of decay caused by *B. cinerea* resistant isolates. The diameters of lesions on the grape berries treated with pyrimethanil at 5 µg ml^{-1} and inoculated with the resistant strains did not differ significantly with those observed on fruit control and a low disease reduction was always observed (26.5-34.2%). The activity of pyrimethanil against these three isolates improved when this fungicide was applied at label rate (37.9 - 65.4% of disease reduction). However, the results showed that pyrimethanil-resistant isolates maintained their ability to cause infection on fruits, because the lesions caused by them were significantly greater than those caused by *B. cinerea* sensitive isolates on berries treated with pyrimethanil at the same concentration (Table 9).

Table 9. Lesion observed on detached grape berries treated with pyrimethanil at different concentrations and subsequently inoculated with sensitive or resistant isolates of *B. cinerea*

Concentrations	**Isolates**	**Lesion diameter (mm)**[b]			
		Control	Treated	Significance	Reduction (%)
5 µg ml-1					
S[a]	BN 1	17.6 a	1.8 b	z = 4.09***	89.7
S	MZ 3.1	4.8 a	0.0 b	z = 3.33***	100.0
R	FG 4	11.7	8.7	z = 0.86[ns]	34.2
R	SR 5	24.5 a	19.9 b	z = 2.56*	26.5
R	MT 6	22.7	16.3	z = 1.91[ns]	28.2
Field label rate					
S	BN 1	17.6 a	0.0 b	z = 4.44***	100.0
S	MZ 3.1	4.8 a	0.0 b	z = 3.33***	100.0
R	FG 4	15.1 a	5.2 b	z = 4.22***	65.4
R	SR 5	26.6 a	15.1 b	z = 5.71***	43.2
R	MT 6	22.7 a	14.1 b	z = 3.80***	37.9

[a] S = sensitive isolate, R = resistant isolates based on *in vitro* assays

[b] Numbers are means of 30 values

[c] Within rows, mean values with different letters and the symbols *, **, *** denote significant differences at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively according to Mann Whitney non parametric rank test (z parameter); ns: not significant.

Applications of fluopyram at 1 µg ml^{-1} were not effective in reducing gray mould infections on grape berries (0.2-10.7% of disease reduction), while they moderately controlled the development of *B. cinerea* isolates at concentration of 10 µg ml^{-1} (28.3-53.9% of disease reduction) and at label rate (28.6-61.4% of disease reduction) (Fig. 16).

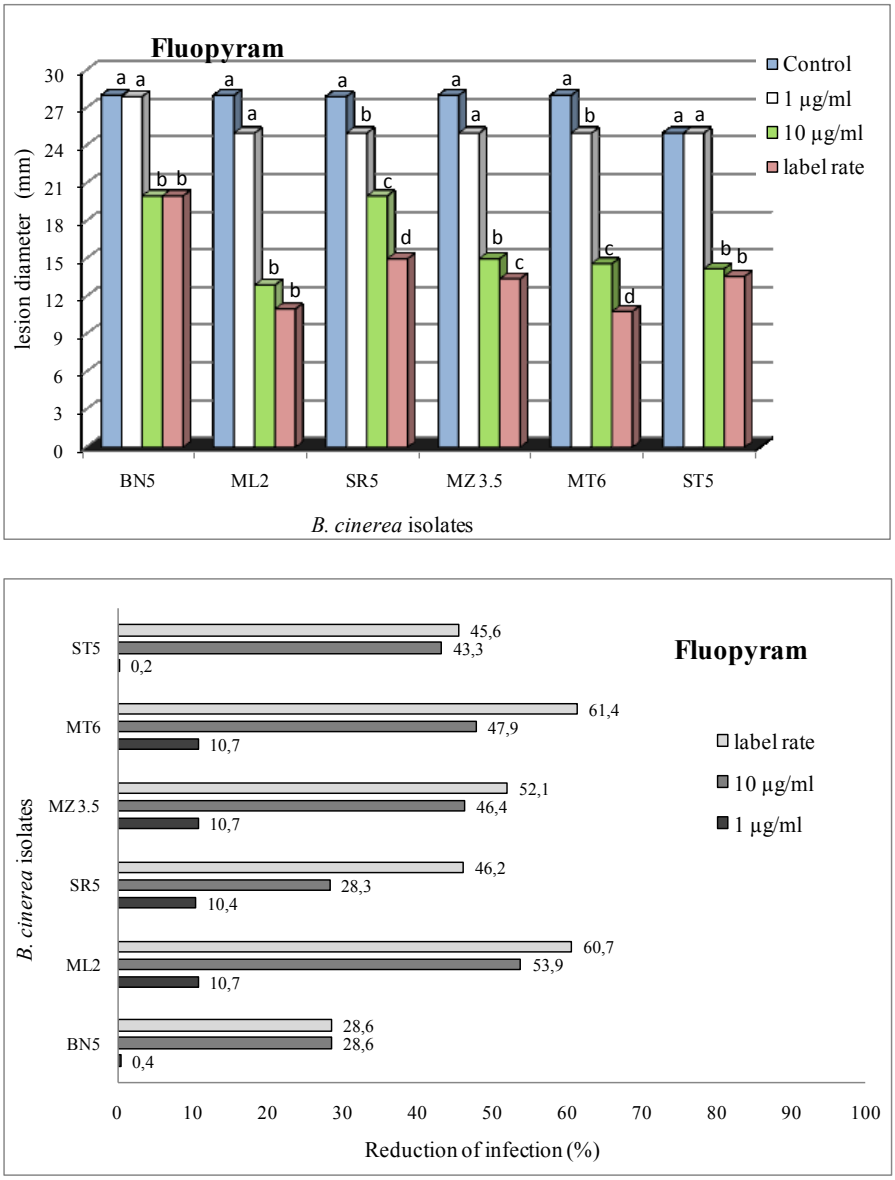

Figure 16. Efficacy of fluopyram at different concentrations in reducing gray mould (lesion diameter mm) and reduction of infections (%) on grape berries after 6 days of incubation. Columns marked with different letters are significantly different using Fisher's least significant difference test at P <0.01.

6.6. Assays on apple fruits

The effect of pyrimethanil on disease progress on apple fruits is shown in the figure 17 and 18. The fungicide at 5 µg ml^{-1} significantly suppressed the disease caused by pyrimethanil-sensitive isolates (the control efficacy had values higher than 90%), whereas it showed a decrease of effectiveness against the pyrimethanil-resistant isolates (FG4, SR5 and MT6 isolates). In this case, the control efficacy had values ranging from 13.4 to 45.6%.

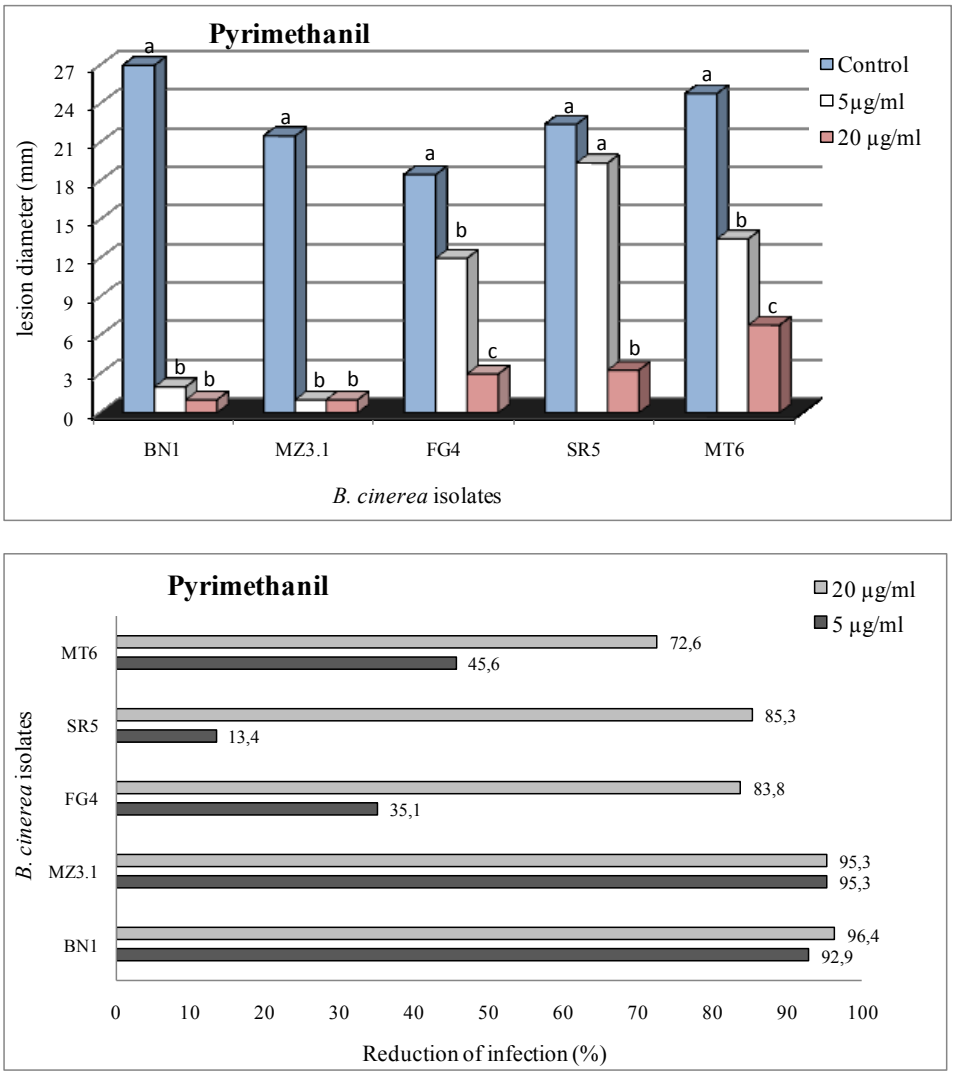

Figure 17. Efficacy of pyrimethanil at different concentrations in reducing gray mould (lesion diameter mm) and reduction of infections (%) on apple fruits after 6 days of incubation. Columns marked with different letters are significantly different using Mann Whitney non parametric rank test (z parameter).

In contrast, apples treated with pyrimethanil at 20 µg ml^{-1} were always protected from infections (72.3-96.4% of disease reduction). However, some differences in sensitivity to pyrimethanil were observed among the isolates tested and the lesions caused on apples by the *B. cinerea* resistant isolates were greater than those caused by the sensitive isolates (Fig. 17, 18).

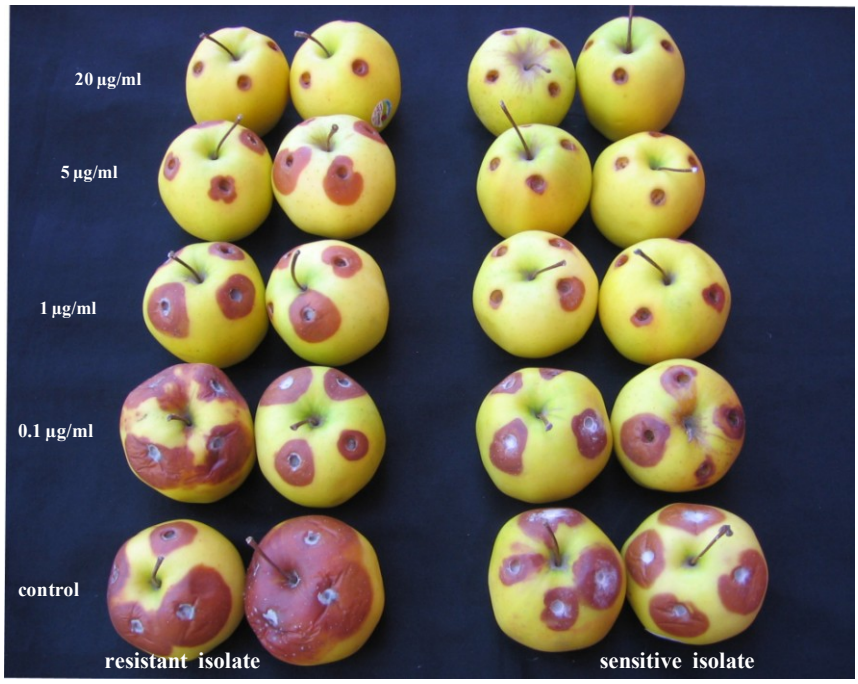

Figure 18. Lesions caused by *B. cinerea* isolates on apples fruits treated with pyrimethanil at different concentration (0.1, 1, 5 and 20 µg ml^{-1}) after 6 days of incubation. A pyrimethanil-sensitive isolate and a pyrimethanil-resistant isolate were compared with their respective controls.

7. Discussion

In the past, the most frequently used fungicides for controlling grey mould in the Italian regions are dicarboximides and pyrimethanil. More recently, anilinopyrimidines, phenylpyrroles and the hydroxanilide fenhexamid have also been developed to control gray mould. For *B. cinerea*, resistance has developed to benzimidazoles, dicarboximides and anilinopyrimidines worldwide (Katan, 1983; Noethover and Matteoni, 1996; Beever *et al*, 1989; Latorre *et al*., 1994; Zhou *et al*., 1994; Pappas, 1997; Leroux *et a*l., 1999; Diánez *et al*., 2002; Baroffio *et al*., 2003; Zhang *et al*., 2006). Also phenylpyrroles and hydroxyanilides face the possibility of resistance development, as has already been shown by several studies carried out with laboratory mutants of the pathogen (Faretra and Pollastro, 1993b; Ziogas *et al*., 2003). For these reasons, in this study, we wanted to determine the sensitivity of 146 *B. cinerea* single-spore isolates obtained from grape berries to fungicides belonging to several different families (benzimidazoles, dicarboximides, anilinopyrimidines, phenylpyrroles and hydroxanilide) used currently to control gray mould.

Our study report the first extensive data on fungicide resistance to benzimidazoles, dicarboximides, anilinopyrimidines, phenylpyrroles and the hydroxanilide fenhexamid on *B. cinerea* isolates, recently collected in Sicily. In addition in this study, we also determined the baseline sensitivity of *B. cinerea* isolates to fluopyram, a novel SDHI fungicide.

The survey conducted in Sicily vineyards between 2009 and 2011 did not revealed a high presence of *B. cinerea* strains resistant to fungicides studied, except for the pyrimethanil.

Among the 146 *B. cinerea* isolates tested, only 6 isolates (4,1% of population) appeared resistant to boscalid, having EC_{50} values >1µg ml^{-1}. In literature, few cases of field resistance have been described for this fungicide on grape berries. In the United States, boscalid resistance has been reported for *B. cinerea* populations on other crops (Kim and Xiao, 2011; Yin *et al*., 2011; Fernandez-Ortuno *et al*., 2012) whereas, in Europe, the phenomenon has been restricted to few countries (Leroux *et al*., 2010; Bardas *et al*., 2010). Therefore, our results confirm findings of the latter

studies reporting limited occurrence of boscalid-resistant isolates of *B. cinerea* and makes boscalid an excellent candidate for the control of grey mould disease. Boscalid-sensitive isolates showed EC_{50} values ranging from 0.04 to 0.98 μg ml^{-1}. Their growth was significantly reduced by the fungicide beginning from the concentration of 0.05 μg ml^{-1}. Among the resistant isolates recovered, two had EC_{50} values ranging from 1 and 5 μg ml^{-1} and four showed EC_{50} values >5 μg ml^{-1}.

Analysis of partial sequences of the iron sulfur subunit of succinate dehydrogenase gene in *B. cinerea* (*SdhB*) from 4 boscalid resistant-isolates and 2 boscalid-sensitive isolates showed that point mutations in *SdhB* leading to amino acid substitution at the codon position 272 from histidine to tyrosine (H272Y) were correlated with boscalid resistance. Results of molecular analysis were in agreement with those obtained through the mycelial growth assays because point mutations in *SdhB* were observed in the majority of boscalid-resistant isolates (3 of 4 isolates) but not in boscalid-sensitive isolates. Recently, H272Y has been reported to be the most frequent genotype in boscalid-resistant populations of *B. cinerea* in French and German vineyards (Leroux *et al*., 2010). Previous studies also indicated that proline at position 225 could be replaced by leucine, phenylalanine, or threonine in laboratory boscalid-resistant mutants of *B. cinerea* (De Miccolis *et al*., 2010; Leroux *et al*., 2010; Stammler, 2008). De Miccolis *et al.* (2010) reported that point mutations into the codon 272 (H272Y) and 225 (P225L or P225F) were correlated with low and high boscalid resistance in laboratory mutants of *B. cinerea,* respectively. In our study, no isolate showed to possess the mutation into the codon 225 among the 4 boscalid-resistant isolates tested and, consequently, a low resistance level can be attributed to these isolates.

As concerning the carbendazim, the benzimidazoles were not widely used for many years. Since the resistance to benzimidazole are stable, benzimidazoles-resistant isolates remain in pathogen populations years after their has been dismissed. In Italy, in the past, benzimidazoles-resistant strains were found in vineyards where the fungicide was not used for years (Gullino and Garibaldi, 2003; Gullino *et al*., 2000). However, a recent survey carried out in Italian vineyards showed a reduction in the frequency of benzimidazoles-resistant mutants of *B. cinerea* (Bertetti *et al*.,

2008). Similarly, a restricted occurrence of *B. cinerea* carbendazim-resistant isolates was detected in vineyards located in southern Sicily. In particular, only 6 isolates (4.1% of 146 isolates) were considered to be benzimidazole resistant, showing EC_{50} values >10 μg ml^{-1}. We considered these isolates moderately resistant to carbendazim in "*in vitro"* assays on the basis of EC_{50} values reported by others for benzimidazole family. Faretra and Pollastro (1991) considered the isolates as sensitive to benomyl when EC_{50} growth was less than to 1 μg ml^{-1}, while they reported a high resistance level when EC_{50} values were >100 μg ml^{-1}. In another study, three different levels of benzimidazole resistance were detected: isolates with a low resistance, that grow on 5 μg ml^{-1} but not on 10 μg ml^{-1} carbendazim or thiophanate-methyl (EC_{50} values ranging from 1 to 8.2 μg ml^{-1}); isolates with moderate resistance, that grow on 50 μg ml^{-1} but not on 100 μg ml^{-1} carbendazim or thiophanate-methyl (EC_{50} values ranging from 15.4 to 22.6 μg ml^{-1}); isolates with a high resistance, that grow on 200 μg ml^{-1} carbendazim or thiophanate-methyl (EC_{50} values >50 μg ml^{-1}) (Zhang *et al*., 2010). To determine if our resistant isolates possess a high level of resistance, further *"in vitro"* assays have to be conducted using the carbendazim also at concentration of 100 μg ml^{-1}.

Resistance to this fungicide has been reported many times and is related to point mutations in the β-tubulin gene (Davidse and Ishii, 1995). In benzimidazole-resistant strains, mutations at codon 198 from GAG to GCG/GTG, resulting in an alanine/valine replacing the glutamic acid, and at codon 200 from TTC to TAC, resulting in a tyrosine replacing the phenylalanine, were detected (Zhang *et al*., 2010). In this study, all *B. cinerea* carbendazim-resistant isolates used in molecular analysis had GCG rather than GAG, which resulted in the substitution of glutamic acid by alanine. Molecular analysis of the sensitive isolates did not reveal any mutations at this β-tubulin gene fragment. Therefore, these results confirm the data obtained in "*in vitro*" assays.

In this study, the "*in vitro*" results showed that there was no resistant population to fenhexamid and fludioxonil among the isolates used. A reduced sensitivity of *B. cinerea* isolates to fenhexamid has been recently reported also in the Chilean and South Italy (Esterio *et al*., 2010; Rotolo *et al*., 2010). Fenhexamid-resistant field

isolates have instead been recovered in California, Greece, Switzerland, France and other regions (Baroffio *et al*., 2003; Leroux, 2004; Ma and Michailides, 2005; Myresiotis *et al*., 2007; Billard, 2012). In other works, the proportion of resistant isolates in examined population did not exceed 1-3%, confirming that baseline populations of *B. cinerea* often contain a small proportion of isolates with reduced sensitivity to fenhexamid (Suty *et al*., 1999; Korolev *et al*., 2011). In several survey studies, EC_{50} values for fenhexamid-sensitive isolates, as defined using a mycelial growth test, ranged from less than 0.01 to about 0.1 μg ml^{-1} (Esterio *et al*., 2007; Leroux *et al*., 1999; Myriesotis *et al*., 2007; Korolev *et al*., 2011), which is similar to our data. The EC_{50} values for the 146 *B. cinerea* isolates used in this study varied from 0.005 to 0.092 μg ml^{-1} and the majority of them had EC_{50} between 0.025 and 0.05μg ml^{-1}. No isolate grow on media amended with fenhexamid at concentration ≥ 0.1 μg ml^{-1}.

Similarly, no resistance to fludioxonil was found in Sicily among the isolates tested, as it was found in vineyard located in northern Italy (Gullino *et al*., 2000). A low level of resistance to fludioxonil for populations of *B. cinerea* has been also reported in previous works (Baroffio *et al*., 2003; Forster and Staub, 1996; Leroux *et al*., 1999; Vignutelli *et al*., 2002; Korolev *et al*., 2011), although *B. cinerea* mutants with high resistance to phenylpyrroles can be easily produced in the laboratory (Leroux, 2004). In contrast, a relatively high frequency of fludioxonil-resistant *B. cinerea* isolates was recently found in Chilean vineyards, exhibiting EC_{50} values for mycelial growth inhibition between 1 and > 5 μg ml^{-1} (Latorre and Torres, 2012). In Italy, to the best of our knowledge, *B. cinerea* isolates showing a slightly decreased sensitivity to fludioxonil were recovered with a frequency up to 50% in vineyards located in southern Italy (Rotolo *et al*., 2009). In literature, EC_{50} values or fludioxonil-sensitive isolates, as defined by the mycelial growth test, ranged from 0.001 to 0.016 μg ml^{-1} (Forster *et al*., 2007; Myriesotis *et al*., 2007; Vignutelli *et al*., 2002, Ziogas *et al*., 2005). In our tests, EC_{50} values for the majority of *B. cinerea* isolates had a normal distribution and ranged from 0.001 and 0.03 μg ml^{-1}, with a mean EC_{50} value of 0.008 μg ml^{-1}.

As well as observed for boscalid and carbendazim, also the frequency of dicarboximide-resistance observed in the 15 Sicilian vineyards investigated appears to be very low. As a matter of fact, the majority of the isolates collected in 2009-2011 years showed EC_{50} values ranging from 0.11 to 0.71 μg ml^{-1} and only 5 isolates (3.4% of the population) showed a decreased sensitivity to iprodione, having EC_{50} values ranging from 1.16 and 2.1 μg ml^{-1}. We considered these isolates weakly resistant on the basis of previous studies that reported a low level of resistance for *B. cinerea* isolates with EC_{50} values ranging from 1 to 1.5 μg ml^{-1} (Myresiotis *et al*., 2007). In the past, the existence of field isolates with a low level of resistance to dicarboximides was also reported in other Italian vineyards (Gullino *et al*., 2000; Faretra and Pollastro, 1991; Faretra and Gullino, 2000).

As concerning pyrimethanil, results of this study showed a significant presence of pyrimethanil-resistant isolates, which reach a frequency of 25.4% (37 of 146 isolates). Different levels of resistance to pyrimethanil were found within the resistant population studied: 5 isolates (3.4% of 146 isolates) had EC_{50} values ranging from 1 to 2.5 μg ml^{-1}, 22 isolates (15%) showed EC_{50} values ranging from 2.5 to 5 μg ml^{-1} and 10 isolates (6.8%) had an EC_{50} value higher than 5 μg ml^{-1}. Resistance to pyrimethanil has developed worldwide and a high percentage of anilinopyrimidines-resistant isolates has been reported in Italy, France, Switzerland, Greece, China, Chile and Australia, suggesting that there is a high risk for the occurrence of anilinopyrimidines resistance in *B. cinerea* (Baroffio *et al*., 2003; Chapeland *et al*., 1999; Leroux *et al*., 1999; Latorre *et al*., 2002; Gullino *et al*., 2000; Gullino and Garibaldi, 2003; Rotolo *et al*., 2009, 2010, Sergeeva *et al*., 2002; Sun *et al*., 2010).

Among the 37 isolates found to be resistant to pyrimethanil, the majority of them (84%) were only resistant to anilinopyrimidines, showing a single specific resistance. On the contrary, the isolates showing a decreased sensitivity to boscalid, carbendazim and iprodione exhibited their resistance to two or more fungicides simultaneously. Two isolates were resistant to both boscalid and pyrimethanil and two to both carbendazim and iprodione, showing a double-resistance. One isolate was simultaneously resistant to boscalid, carbendazim and pyrimethanil, showing a

triple resistant, whereas three isolates were resistant to all four fungicides employed, showing a quadruple-resistance. Isolates of *B. cinerea* with multiple resistance to compounds have also been detected in Germany (Leroch *et al*., 2011; Weber, 2011), Greece (Myresiotis *et al*., 2007), Spain (Moyano *et al*., 2004), China (Sun *et al*., 2010) and in Chile (Latorre and Torres, 2012). The phenomenon of simultaneous resistance observed in this study could be explained by the hypothesis put forth by Köller and Wilcox (2001), which was that resistance development to one fungicide class accelerates resistance development to another unrelated fungicide class. The mechanism of this simultaneous resistance could be a decreased accumulation of the compounds in mycelium due to an energy-dependent efflux of the fungicides. This mechanism could be generated by the over-expression of ATP-binding cassette (ABC) transporters that may result in overproduction of encoded proteins and in increased pump capacity responsible for the energy-dependent efflux of fungicides (De Waard, 1997; Leroux et al., 1999).

The majority of isolates resistant to boscalid (4 of 6 isolates), a high number of isolates resistant to pyrimethanil (10 of 37 isolates) and all isolates resistant to carbendazim and to iprodione were found in just one vineyard, which was located in Chiaramonte Gulfi (Ragusa). This phenomenon could be due to a not correct use of fungicides for the control of gray in this vineyard. A careful monitoring of the fungal population and some anti-resistance strategies (limitation of the number of treatments per growing season, use of the fungicide at the recommended dose when strictly necessary, alternating fungicides with different modes of action and use of mixtures of two or more fungicides having different modes of action) are important for preservation the efficacy of a fungicide in time and must be carried out always.

The assays on leaves of bean seedling and grapevine plants previously treated with fungicides confirmed the level of fungicide sensitivity determined in mycelium inhibition test. The leaves treated with the fungicides at the label rate and then inoculated with sensitive isolates were protected from infection, whereas those inoculated with the isolates having a decreased sensitivity to fungicides were not protected and showed symptoms of disease after 3-4 days of incubation. However, the leaves inoculated with the resistant isolates showed some differences in disease

severity. In particular, the isolates having the highest EC_{50} values produced lesions which did not differ in diameter from those observed on control leaves, whereas the isolates having EC_{50} values close to 1µg ml^{-1} (discriminatory dose) produced lesions less extensive than the latter but always greater than those caused by the sensitive isolates. These representative resistant isolates displayed a considerable ability to infect also wounded grape berries pretreated with the respective fungicides at their label concentrations and their resistance was accompanied always by a complete or significant failure of fungicides to provide disease control. The sensitive isolates tested were always effectively controlled by all fungicides employed in this study.

On apple fruits inoculated with the pyrimethanil-resistant isolates, the pyrimethanil was not able to control satisfactory gray mold when used at 5 µg ml^{-1}, whereas it suppressed the disease when applied at 20 µg ml^{-1}. However, the lesions caused on apples by the *B. cinerea* resistant isolates were greater than those caused by the sensitive isolates. Therefore, the level of resistance to pyrimethanil identified in the mycelium inhibition test was confirmed with inoculation assays on leaves of bean seedling and grapevine, on grape berries but not on apple fruits. Unlike other authors (Forster and Muller, 1996; Lachaise, Lydie Sita, Bayer CropScience), in our opinion, this *in vivo* method could not be suitable for studying the sensitivity of *B. cinerea* isolates to fungicides. However, further studies have to be conducted to confirm our opinion.

In this study, we also determined the effects of fluopyram, a novel SDHI fungicide, on development of *B. cinerea* isolates collected from vineyards that never been exposed to use of this fungicide. Fluopyram strongly reduced the growth of all *B. cinerea* isolates at concentrations of 1 and 10 µg ml^{-1} and their EC_{50} values ranged from 0.08 to 3.48 µg ml^{-1}. These results were similar with those reached by Tanovic *et al*. (2012) that reported EC_{50} values ranging from 0.02 to 6.7 µg ml^{-1}. In another study, fluopyram proved to be extremely active against both spore germination and germ tube elongation of *B. cinerea* and the mycelial growth was less sensitive to fluopyram than spore germination (Veloukas and Karaoglanidis, 2012). On grape berries, applications of fluopyram moderately controlled the development of *B. cinerea* isolates at concentration of 10 µg ml^{-1} and at the label rate.

In conclusion, although the frequency of *B. cinerea* strains resistant to the fungicides used currently in field was low in the different Sicilian vineyards surveyed, management strategies for gray mold should focus on integrated disease management including the use of new low-risk fungicides with different modes of action. Regular monitoring of the fungal population about the development of resistance phenomenon in our region is always needed.

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