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Sensitivity to fungicides and genetic structure of *Botrytis cinerea* populations isolated in Lombardy.

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1. INTRODUCTION

Vitis vinifera L. is a species indigenous of Eurasia and is suggested to have first appeared 65 millions years ago (de Saporta, 1879). Nowadays two forms still co-exist in Eurasia and in North Africa: the cultivated form, *V. vinifera* subsp. *vinifera* (or *sativa*) and the wild form *V. vinifera* subsp. *silvestris* (or *sylvestris*), sometimes referred to as a separate subspecies based on morphological differences. This separation is rejected by many Authors since it is based on morphological differences probably resulting from domestication (Zohary, 1995). The wild grapevine is a heliophilous liana growing generally along river banks, and in alluvial and colluvial deciduous and semi-deciduous forest (Levadoux, 1956; Arnold *et al*., 1998). The present distribution of the wild grapevine is highly fragmented, in disjoint micro-populations or meta-populations, with few individuals, at least in the western part of the Mediterranean Basin. Anthropogenic pressure on their natural habitats and pathogens introduced from North America during the second part of the 19th century, may explain the progressive decline of wild grape populations (Arnold *et al*., 1998).

It is the single *Vitis* species that acquired significant economic interest over time; some other species, for example the North American *V. rupestris* Scheele, *V. riparia* Michaux or *V. berlandieri* Planchon, are used as breeding rootstock due to their resistance against p*hylloxera*.

The cultivation and domestication of grapevine occurred between the seventh and the fourth millennia BC, in a geographical area between the Black Sea and the Caspian Sea, known as Caucasus (Mc Govern, 2003; Costantini *et al*., 2005,2006; Forni 2005, 2006). From this area, cultivated forms would have been spread by humans in the Near East, Middle East and Central Europe. As a result, these areas may have constituted secondary domestication centers (Grassi *et al*., 2003; Arroyo-Garcia *et al*., 2006).

The domestication of grapes seems linked to the discovery of wine and associated with evident, sometimes dramatic, changes to ensure enhanced sugar content for better fermentation, greater yield and regular production. In particular the berry and bunch size enlarged and only hermaphrodite plants were cultivated while dioecius vines persist only in the wild population. It is still debated if these changes occurred gradually through sexual crosses and human or natural selection or quickly through mutations, selection and vegetative propagation. Moreover the period and the place of wine domestication or of secondary independent domestications are still uncertain. Wild grapes were present in many places in Europe during the Neolithic period, but archaeological and historical evidences suggest that wine was domesticated for the first time in the Near East. In particular the earliest evidence of wine production was found in Iran at the Hajji

Firuz Tepe site in the northern Zagros mountains about 7,400 – 7,000 BP (Before Present), but seeds of domesticated grapes were detected 8,000 BP in Georgia and in Turkey (McGovern, 2004). Remains of grape seeds in Neolithic and bronze age sites were discovered also in Western Europe and in particular in France.

From the earliest domestication sites the wine cultivation spread to Egypt and lower Mesopotamia and later all around the Mediterranean sea, following the main civilizations. The ancient Greeks, in particular, greatly contributed to the diffusion of wine growing across the Mediterranean basin: southern Italy proved to be particularly suitable for grape cultivation and was known as Enotria, land of wine. Grape cultivation and wine making techniques, extensively described by the Greek poet Hesiodus, were further improved by the Etruscans and Romans, as reported in various literary works written by Cato the Elder, Marcus Terentius Varro, Virgilius and Columella. During the Roman Empire, *V. vinifera* reached numerous European temperate regions and was cultivated as far north as Germany. The Romans mention many cultivars, difficult to relate to the modern ones, and clearly distinguish between wine and table grapes. In Middle Age, due to the recurrent barbarian invasions, viticulture was practiced only near the main towns and mainly in monasteries. The Catholic Church spread grape cultivation to new regions and in particular to Northern Europe, while the extension of Islam to North Africa, Spain and Middle East enabled the diffusion of table varieties in the Mediterranean countries. During the Renaissance the vineyard global surface greatly increased and moreover *V. vinifera* colonized the New World, where it was not indigenous. The missionaries took the grape seeds and cuttings from their countries to both Northern and Southern America to produce their own wine. In South Africa, a vineyard was planted in 1655 by the Dutch governor of the Cape, but only after the setting thirty years later of the French Huguenots the wine making began to flourish. At the end of 18th century, vine reached Australia, imported by governor Phillip from South America, and, later on, New Zealand.

During the 19th century, *Daktulosphaira vitifoliae* Ficht, commonly called phylloxera, and the agents of downy and powdery mildews, *Plasmopara viticola* (Berk. *et* Curt.) Berl. and De Toni and *Erysiphe necator* Schwein, were inadvertently imported in Europe from Northern America and devastated the European vineyards, due to the great susceptibility of *V. vinifera.* The diversity of the European grapevine was strongly reduced, since the most susceptible genotypes did not survive. European viticulture introduced several indigenous Northern American species that were used as rootstocks resistant to phylloxera and for breeding disease resistant interspecific hybrids. These hybrids were widely used until the middle of 20th century, but are now very uncommon. A

few cultivars such as Chardonnay, Merlot and Cabernet Sauvignon, are at the moment grown worldwide and represent the main source of the wine making industry (This *et al.*, 2006). They are highly susceptible to downy and powdery mildew, the main diseases of *V. vinifera,* which are usually controlled by applying chemical treatments, particularly numerous if suitable climatic conditions occur in vineyard. Moreover, severe losses, both in yield and quality, are caused by the aetiological agents of bunch rots and in particular by *Botrytis cinerea* Pers*.,* responsible for the grey rot on both the table and wine varieties.

1.1.1 *Botrytis cinerea* Pers.

Botrytis cinerea Pers. Fr. (synonym *Botryotinia fuckeliana* (de Bary) Whetzel) belongs to the cosmopolitan genus *Botrytis* that contains 20 recognized species. *B. cinerea* (Leroch *et al*., 2013) and forms a small clade with three other species, *B. calthae*, *B. fabae* and *B. pelargonii* (Staats *et al*, 2005). New *Botrytis* species have recently been described, such as *B. fabiopsis* (Zhang *et al*., 2010), B*. sinoallii* (Zhang *et a*l., 2010b), *B. caroliniana* (Li *et al*., 2012) and the cryptic species *B. pseudocinerea* (also named *B. cinerea* Group I; Choquer *et al*. , 2007; Walker *et al*. , 2011).

This fungus is an haploid, filamentous, heterothallic plant pathogen that can be found all over the world and can infect more than 200 cultivated and wild species (Giraud *et al*., 1997; Williamson *et al*., 2007) causing disease often referred as grey mould, a common disease of many economically important plants in temperate areas of the world. *B. cinerea* has very unique characteristics: it can behave pathogenically colonizing many organs including leaves, stems and fruits, but also saprophytically on senescent and dead plant material; moreover it can be very devastating in some crops but it can be of some benefit under certain conditions. *B. cinerea* is most destructive on mature or senescent tissues of dicotyledonous hosts, but it usually gains entry to such tissues during earlier stages in crop development and remains quiescent for a considerable period before rapidly rotting tissues, when the environment is conducive and the host physiology changes. *B. cinerea* can also cause latent infection of floral parts of the fruits during the early growing season leading to calyx-end grey mold during storage (Lennox and Spotts, 2004)

Rosslenbroich and Stuebler (2000) supposed that since the genus *Botrytis*, erected by Micheli in 1729, was named after the Greek word for “bunch of grape berries”, the grey mould symptoms in grape must have been known since many years. The name *Botrytis cinerea* appeared for the first time in *Synopsis Methodica Fungorum* (1771), written by von Haller and published in Zurich,

Switzerland. Finally in 1866, de Bary discovered the genetic connection between *B. cinerea* and its sexual stage *Botryotinia fuckeliana* (de Bary) Whetzel.

B. cinerea is responsible for a very wide range of different symptoms on various plant organs and tissues. Soft rots, often accompanied by a rapid appearance of grey tufts of conidia, are perhaps the most typical symptoms on soft fruits and, less frequently, on leaves. On many fruits and vegetable 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 bunch, grey rot caused by *B. cinerea* is one of the major fungal diseases. The pathogen seriously reduces the quantity and quality of the crop by causing premature cluster drop and pre and post harvest fruit rot. In wine grape production, a very serious damage results from modified chemical composition of diseased berries resulting in wines that have off-flavors, are fragile, and are more sensitive to oxidation and bacterial contamination. However, in some cultivars and under certain weather conditions, specific form of *Botrytis* cluster rot, known as "noble rot", is a desired rot that contributes to the production of sweet wines (Fournier *et al.*, 2013).

B. cinerea can infect herbaceous shoots, leaves and berries. Young shoots may be infected in early spring, turn brown, and then wither in dry climates or rot completely during rainy or humid periods (Figure 1.1.1).

Figure 1.1.1 Young shoots infected by *B. cinerea*.

At the end of spring, but before bloom, large, irregular reddish brown patches can appear on a few grapevine leaves, usually localized near the edge of the leaf blade or along the major veins (Figure 1.1.2). During humid or wet weather, these lesions may be covered by abundant grey conidial masses, characteristic of *B. cinerea*.

Before bloom the pathogen may invade the inflorescences, and may cause entire clusters to dry out and fall off. At the end of bloom, the fungus develops on the caps, stamens, and aborted berries trapped or still attached to the cluster. From the infected tissues the pathogen reaches the pedicel or the rachis, forming small brown lesions that eventually turn completely black. Near the end of summer, these lesions completely surround the pedicel or rachis and portions of the cluster below the necrotic drop off (Figure 1.1.3).

Figure 1.1.2 *B. cinerea* symptom on leaf.

Figure 1.1.3 *B. cinerea* symptom on rachis.

From the beginning of veraison onwards, the berries are infected directly through the epidermis or through the lesions caused by both biotic and abiotic factors. Starting from the first colonized berries, the pathogen progressively invades the entire cluster. Grey rot develops more quickly in compact clusters, where berries are tightly compressed together. The first symptoms caused by *B. cinerea* infection in mature grape berries are small circular water-soaked spots. Infected white grapes turn brown and red grapes become reddish purple. In dry weather, berries colonized by *B. cinerea* dry out, while in wet periods they tend to burst and a brown greyish sporulation appear at their surface (Figure 1.1.4). In certain grape varieties and in alternating dry and humid conditions, the fungus causes the 'noble rot', during which infections take a milder course and allow the berries to dry out gently, concentrating their sugar and flavours in the process. The resulting wine is much more sweeter and richer than normal table wine: the most famous wines obtained from clusters affected by 'noble rot' are the French Sauternes and the Hungarian Tokaji.

Figure 1.1.4 Grey rot on clusters.

Figure 1.1.5 *B. cinerea* sporulation on canes.

Poorly hardened canes can be invaded in fall by the fungus which can colonise the buds and inhibit their development during the next spring (Figure 1.1.5). *B. cinerea* causes serious losses on table grapes during storage: the clusters are completely colonized and develop a soft rot, sometimes associated with sporulation.

1.1.2 Life cycle and epidemiology of *B. cinerea*

B. cinerea is a polycyclic pathogen, surviving mainly as sclerotia or mycelium during winter and able to infect the vegetative and reproductive structures of numerous host plants (Figure 1.1.6).

Dormant or metabolically inactive fungal structures play a central role in the life cycle and epidemiology of *B. cinerea*, as in all *Botrytis* species. Sclerotia, probably the most important structures involved in the survival of the fungus, develop within or on dying host tissues, survive adverse environmental conditions and develop to originate tufts of conidia or apothecia after fertilization due to microconidia acting as spermatia (Coley-Smith, 1980). The melanized rind walls and the continuous matrix of β-glucans encasing the internal mycelium protect sclerotia from desiccation, UV radiation and microbial attack over long periods (Backhouse and Willets, 1984). Apothecia of *B. fuckeliana*, developing from fertilized sclerotia, are relatively rare in nature, especially in warm and dry climates. *B. fuckeliana* is heterothallic, although some single ascospore progeny are homothallic (Faretra *et al*., 1988; Debuchy *et al*., 2010). Often sclerotia produce conidiophores and multinucleate conidia: both ascopores and conidia can act as primary inoculum in the host at the beginning of the vegetative season.

Survival can be due also to the mycelium within infected dead host tissues left as crop debris and inside some seeds. The mycelium colonizing buds and the remains of rotten clusters is the main overwintering structure of *B. cinerea* in vineyards located in Northern and Central Italy (Garibaldi *et al*., 1982).

Conidia, produced by the overwintering mycelium and/or sclerotia both on host tissues and on the soil surface, are considered the most important infective units of *B. cinerea* in late winter and early spring. Additionally, numerous alternative hosts exist in vineyards, including herbicide-treated or senescing weeds and other infected crops in the vicinity. Over 200 host species were identified as sources of *B. cinerea* inoculum (Sutton *et al*., 1991). Surveys from a wide range of fruit growing regions consistently rank conidia of *B. cinerea* as an important part of the aerial microflora (Bisiach *et al*., 1984). Conidia, generated as source of primary inoculum, follow a well-defined diurnal cycle of initiation, production and dissemination that is regulated by fluctuation in temperature and humidity: a rapid decline in humidity associated with a rise in temperature in early morning causes drying of conidiophores and the subsequent ejection of conidia into air currents, either individually or in small clumps (Jarvis, 1962; Harrison and Lowe, 1987). Water droplets can also disperse conidia, but this is probably not a major dispersal method (Jarvis, 1962). Conidia formation is stimulated by specific wavelengths of light (Epton and Richmond,

1980) and near UV is now generally used to induce sporulation in *in vitro* culture. However, some isolates can sporulate in darkness.

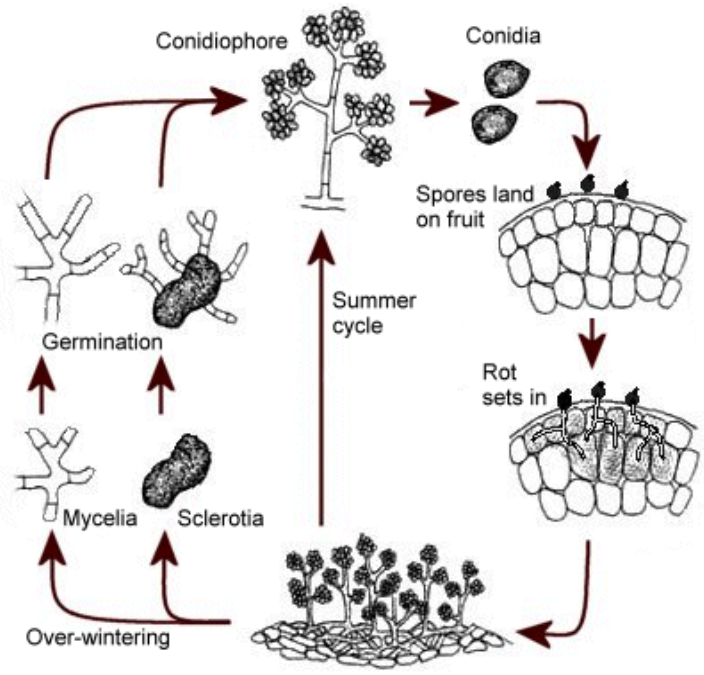


Figure 1.1.6. Life cycle of *B. cinerea.*

In temperate regions, an ample inoculum potential, ready for the infection, is available by early spring in the host environment. Morphologically distinct cultural types of *B. cinerea,* usually grouped as mycelial, sporulating (conidial) and sclerotial (Lorbeer, 1980), are frequently found in the host environment, although sub-types have been recognised by other Authors (Martinez *et al*., 2003). It is obvious that mycelia, conidia and sclerotia have different abilities for survival and dispersal, and the relative roles of these structures greatly vary depending on ecosystem and season. Sclerotial types are more frequently found on perennial hosts during winter and conidial type on annual hosts with abundant susceptible flowers.

The infection process of *B. cinerea* is usually described by the following stages: penetration of the host surface, killing of host tissue and formation of primary lesion, lesion expansion and tissue maceration and finally sporulation (van Kan, 2006).

1.1.3 *B. cinerea* penetration through undamaged host tissue

B. cinerea conidia germination and adhesion on plant surface represent the crucial events occurring before host penetration and colonization, whereby sensing and recognition of the host surface characteristics, including hydrophobicity and sugar sources, seem essential (Döhlemann *et*

al., 2006). Germlings of *B. cinerea* secrete an extracellular matrix responsible for their attachment to the host surface through hydrophobic interactions and might facilitate their penetration by breaching the physical barriers of the plant (Doss *et al*., 1995). *B. cinerea* enters the host mainly by producing degrading enzymes and causing an oxidative burst rather than using physical pressure. Appressorium-like structures, characterised by swollen tips and frequently observed in *B. cinerea* (Tenberge, 2004), do not produce highly melanized wall and are not always separated from the germ tube by a septum. Both wall melanization and septum formation are essential for generating the high osmotic pressure required for direct penetration as observed in appressoria of the rice blast fungus *Magnaporthe grisea* (T. T. Herbert) M. E. Barr (Howard and Valent, 1996). Therefore it seems unlikely for *B. cinerea* appressoria to penetrate host tissue by physical pressure alone. When infection is caused by mycelium, the hyphae growing on the host surface heavily ramify and form structures very similar to the complex appressoria typically differentiated by the closely related species *Sclerotinia sclerotiorum* (Lib.) de Bary.

Penetration involves breaking through the host cuticle that covers all aerial parts of the plant. The cuticle consists of cutin, a polyester of hydroxylated and epoxidised C_{16} and C_{18} fatty acids, in many cases covered with a hydrophobic wax layer consisting of fatty alcohols. The surface hydrophobicity due to the wax layer is reduced by surfactants produced by *B. cinerea,* while fungal cutinases degrade the wax components providing access to the underlying cutin polymer. The polysaccharide cinerean, covering *B. cinerea* germ tubes might act as surfactant. Alternatively, the reduction of host surface tension may be achieved enzymatically. Cutinase, serine esterase, lipase and other non specific esterase are reported to be involved in the adhesion of several plant pathogenic fungi (Köller *et al*., 1995; Yao and Köller, 1995; Berto *et al*., 1997; Pascholati *et al*., 1993; Clement *et al*., 1993).

The mechanism utilized by *B. cinerea* to penetrate the cutin is still not well known: in the 1980s and early 1990s several research groups presented evidence that cutinase was important for cuticle penetration in different pathogenic fungi (Rogers *et al*., 1994; Köller *et al*., 1995). In *B. cinerea* cutinase seems to be not essential in penetration: mutants that lacked the *BccutA* gene, the cutinase gene, remain able to penetrate intact cuticle surface (Van Kan *et al*., 1997).

1.1.4 *B. cinerea* penetration through wounds

Wound infection occurs when *B. cinerea* germ tubes, produced by germinating conidia, or hyphae enter the host tissue through a lesion. The great majority of experimental inoculations by fresh wounds, carried out with conidial suspensions of various *Botrytis* species especially *B.*

cinerea, resulted in establishment of infection. Little is known about the relationship between the inoculum dosage in air and incidence of wound infection, and how the relationship is influenced by environmental, wound and host factors.

B. cinerea conidia usually firmly adhere to the cuticle at the fruit surface (Spotts and Holz, 1996). Therefore, infection through a wound in the host tissue is possible only if newly arrived conidia land in or near the lesion and penetrate into the host tissues through it. On the other hand, propagules of *B. cinerea* may occur at various growth stages in the wound site. Firstly, there may be conidia in a dormant state adhering to the skin. Secondly, there may be germlings that had penetrated the skin, but where localized by host defence. Lesions occurring near a dormant conidium adhering on a dry surface, break the cuticle and supply the spores with necessary moisture and nutrients to germinate and to infect. The germling that had penetrated the skin, but recognized by the host defence, is provided by wounding with the nutrients necessary to escape the host defence barrier and cause the tissue to rot (Holtz *et al*., 2004).

Since a series of synchronized events is necessary for successful wound infection, *i.e.* fresh wounds, freshly dispersed conidia and free water on the host surface, some Authors concluded that penetration through lesion is not frequent in field conditions (Coertze and Holz, 2002).

Insects may play a very important role in determining disease outbreaks in the field. Conidia or, less frequently, mycelium portions are transported by the insects and deposited near lesions (Engelbrecht, 2002; Coertze and Holz, 2002). Moreover, larvae of grape moths feeding on berries cause wide lesions, easily colonised by *B. cinerea.*

1.1.5 *B. cinerea* tissue invasion and colonisation

Following penetration of the outer epidermal wall, the mycelium grows in the sub-epidermal tissue inter- and intra-cellularly to establish the infection. The pathogen produces different kinds of enzymes and metabolites that enable the fungus to invade the host tissues, kill host cells and eventually convert host tissue into fungal biomass. Many of these enzymes and metabolites act extracellularly at the plant-fungus interface, or even in the host tissue at some distance from the growing hyphae.

Invasion of plant tissue by *B. cinerea* triggers processes indicative of programmed cell death at some distance from the hyphae, implying that diffusible factors have a direct or indirect phytotoxic activity. The inducing factors may be proteins or low molecular weight compounds secreted by the fungus. The induction of programmed cell death facilitates *B. cinerea* invasion and may in fact be essential for successful infection (Govrin and Levine, 2000).

Phytotoxic metabolites

B. cinerea can produce a spectrum of non-specific phytotoxins of low molecular weight. The best studied compound secreted by the pathogen is the sesquiterpene botrydial (Colmenares *et al*., 2002). Botrydial was isolated from liquid cultures and for a long time it was unclear whether it was produced during the infection at a level that was sufficient to exert toxic effects. Using spectroscopic methods, botrydial was subsequently detected in infected plant tissue (Deighton *et al*., 2001) at a concentration above the toxicity threshold. Botrydial induces chlorosis and cell collapse, and both effects facilitate the pathogen colonization of host tissues. Deletion of genes belonging to the biosynthetic pathway genes for botrydial resulted in severe reduction of virulence in some strains but not in others indicating that this non-specific phytotoxin is a strain-dependent virulence factor (Siewers *et al*., 2004),.

A second class of phytotoxic metabolites derived from botcinic acid have been isolated from some *B. cinerea* aggressive strains (Reino *et al*., 2004).

Oxidative burst

B. cinerea actively triggers an oxidative burst during cuticle penetration (Tenberge, 2004; Tenberge *et al*., 2002) and primary lesion formation (Lyon *et al*., 2004). An oxidative burst occurs in many plant-pathogen interactions during a hypersensitive reaction (HR) that confers resistance toward biotrophic pathogens (Rivas and Thomas, 2005): HR, a type of programmed cell death (PCD), limits access of the pathogen to nutrients and water. In necrotrophs such as *B. cinerea*, PCD seems to promote the growth of the pathogen and may even be required to achieve full pathogenicity (Govrin and Levine, 2000). *B. cinerea* produces reactive oxygen species (ROS) and in particular H_2O_2, both in the host tissues and in the extracellular sheath covering the surface of fungal hyphae (Schouten *et al*., 2002). Infection is associated with the accumulation of free radicals, in infected and uninfected tissue (Muckenschnabel *et al*., 2001; Muckenschnabel *et al*., 2003), culminating in lipid peroxidation (Deighton *et al*., 1999; Muckenschnabel *et al*., 2001) and depletion of antioxidants (Muckenschnabel *et al*., 2002). Altogether, these oxidative processes cause massive perturbation of the redox status in and around the infection tissue, thereby promoting disease progress (Lyon *et al*., 2004).

During the penetration of the cuticle by the appressorium, *B. cinerea* secretes a superoxide dismutase (BcSOD1), probably involved in oxidative burst determinism. Deletion of the *Bcsod1* gene results in reduced virulence on multiple hosts (Rolke *et al*., 2004).

Oxalic acid

Oxalic acid (OA) is produced by fungi belonging to various taxonomic classes (Dutton and Evans, 1996). Pathogenesis of *S. sclerotiorum* (Godoy *et al*., 1990), seems to be associated with OA production since its mutants, deficient in OA production, were unable to infect *Arabidopsis* plant (Dickman and Mitra, 1992) and the deficiency could be restored by supplementing inoculum with OA. The oxalate mediated acidification facilitates induction of gene expression by the ambient pH-dependent regulator *pac1*, which is required for virulence of *S. sclerotiorum* (Rollins, 2003).

B. cinerea produces OA both *in vitro* (Germeier *et al*.,1994) and *in planta* (Verhoeff *et al*., 1988) and the size of lesions induced by different strains of *B. cinerea* on grapevine and bean leaves can be correlated with the amount of OA that these strains secreted *in vitro* (Germeier *et al*., 1994). OA is more likely a cofactor than a primary phytotoxic agent. Production of numerous enzymes is enhanced in low pH media and, moreover, pectin hydrolysis, due to the endopolygalacturonases produced by the pathogen, may be increased by the occurrence of OA, which acts sequestering Ca^{2+} ions released from the Ca-pectates in the middle lamella.

1.1.6 Conversion of host tissue into fungal biomass

Plant cell walls, representing the host barriers to biotic and abiotic agents, are made up of different types of polysaccharides: the primary cell wall consists of cellulose and hemicellulose, while the middle lamella has a high pectin content. Pectin, a complex network of various polygalacturonans, also extends into the primary wall. Following penetration of the epidermal cell wall, *B. cinerea* grows through the middle lamella and produces numerous cell wall-degrading enzymes (CWDEs). Enzymatic breakdown of the plant cell wall is essential for the release of carbohydrates, which represent a major carbon source for consumption. Cell wall and middle lamella degradation by *B. cinerea* is mediated by pectinase, cellulase and hemicellulase. Each of these CWDEs has specific features and is involved in different steps of host tissue colonisation and maceration.

Endopolygalacturonases are endo-enzymes catalysing the hydrolysisi of homogalacturan, resulting in polymer fragmentation. These enzymes have a main, if not exclusive, role in tissue colonisation and maceration. *B. cinerea* contains at least six endopolygalacturonase genes which show differential regulation *in vitro* (Wubben *et al*., 2000). Expression of the endopolygalacturonase gene family during host infection depends on the plant species, tissue type and incubation conditions applied (Ten Have *et al*., 2001), suggesting some degree of

versatility in the pectinolytic complex. Deletion of two endopolygalacturonase genes, separately, resulted in pronounced reduction of virulence on multiple host plants (Kars *et al*., 2005a).

The role of pectin methylesterases (PMEs) is controversial. It is generally assumed that endopolygalacturonases cannot efficiently depolymerize highly methylated pectin, hence demethylation by PMEs facilitates the action of endopolygalacturonases. Therefore PMEs are important for fungal growth when highly methylated pectin is the sole carbon source and for virulence on plant tissues such as leaves containing mainly highly methylated pectin, but not on tissues such as fruit with low pectin methylation. Inactivation of *Bcpme1* gene induced a strong reduction in virulence on several host plant (Valette-Collet *et al*., 2003). However, results with single and double mutants in two *Bcpme* genes, including the same *Bcpme1* gene, do not support this hypothesis. Surprisingly, the wild-type strain and the *Bcpme* deficient mutants grew better on 75% methylated pectin than on non-methylated polygalacturonic acid, suggesting that pectin demethylation by PMEs is not important for its depolymerization *in vivo* (Kars *et a*l., 2005b). Other cell wall degrading enzymes produced by *B. cinerea*, such as cellulases and hemicellulases, have only recently been studied. Deletion of a cellulase gene did not affect virulence (Espino *et al*., 2005), whereas the deletion of a β-1,4-xylanase gene delayed lesion formation and reduced lesion width by more than 70% (Brito *et al*., 2006).

1.1.7 Host defence system

During the interaction between *B. cinerea* and its host, the plant vigorously attempts to prevent pathogen invasion and colonisation by activating multiple defence pathways, including the production of antifungal metabolites and pathogenesis related proteins (Van Baarlen *et al*., 2004). Antimicrobial metabolites are usually classified into two distinct categories: phytoalexins and phytoanticipins (Van Etten *et al*., 1994). Phytoanticipins are preformed, while phytoalexins are induced by pathogen infection. The resistance of grapevines, and in particular of *V. vinifera*, to penetration by *B. cinerea* seems to be due in part to an induced resistance mechanism involving the rapid production, through the plant secondary phenolic metabolism, of several stilbenic compounds, namely resveratrol (3,5,4' – trihydroxystilbene) (Figure 1.7). *Trans*-resveratrol, one of the most abundant stilbene in grapevine, represents one of the major components in wood (Langcake and Pryce, 1976) and acts as phytoalexin in leaves (Langcake and Pryce, 1977a; Langcake, 1981). Beside *trans*-resveratrol, numerous other stilbenes have been characterized in grapevine, including a 3-O-β-glucoside of resveratrol called piceid (Romero–Perez *et al*., 1999) and a dimethylated derivative of resveratrol, named pterostilbene (Pezet and Pont, 1988). Stressed or

Botrytis-infected leaves also accumulate oligomers of resveratrol termed viniferins, the most abundant of which is *trans*-ε-viniferin, a resveratrol dehydrodimer believed to result from the oxidative dimerization of resveratrol by a plant peroxidase (Langcake and Pryce, 1977b; Langcake, 1981). *Cis*-isomers of resveratrol, piceid and viniferins have been detected in mature fruit and wine.

The antifungal activity of stilbenes is due to the rapid inhibition of fungal cells respiration, since they probably act as uncoupling agents and form protein-phenol complexes (Hart, 1981). Based on the structural similarity of hydroxystilbenes and aromatic hydrocarbons, it was inferred that their mode of action may involve lipid peroxidation by blocking cytocrome c reductase and monooxygenase (Pezet and Pont, 1995).

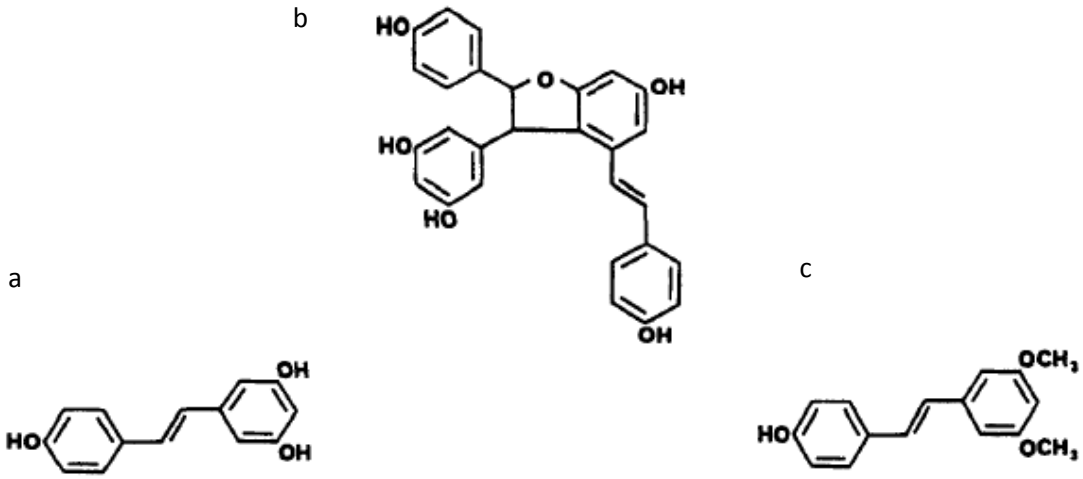


Figure 1.1.7: Chemical structures of *trans*-resveratrol (a), *trans*-pterosilbene (b) and ε-viniferin (c).

Pathogenesis related protein represent a large array of proteins coded by the host plant that are co-ordinately expressed under pathological or related situations. They have been characterized in over 70 plant species and 13 plant families including mono- and dicotyledonous plants. They are extremely diverse in terms of enzymatic and biological activity and have been grouped into diverse protein families based on primary structure and serological relationship (Van Loon, 1999). One of these family is represented by polygalacturonase inhibiting proteins (PGIPs), leucine rich repeat-containing proteins that may inhibit endopolygalacturonases produced by plant pathogenic and non-pathogenic fungi (Juge, 2006) by direct physical interaction between the two proteins. PGIPs display *in vitro* specificity towards different fungal

endopolygalacturonases (Leckie *et al*., 1999). Expression of PGIPs from different sources in transgenic plants resulted in a quantitative increase of resistance to *B. cinerea* (Agüero *et al*., 2005; De Lorenzo and Ferrari, 2002; Joubert *et al*., 2006; Powell *et al*., 2000). Recent research has shown that *in vitro* studies of PGIP–polygalacturonases interactions only partially reveal the potential of PGIPs in increasing resistance. It was generally considered that among the family of PGIPs described thus far, the most potent *in vitro* inhibitors would be the most beneficial proteins to express in plants for achieving optimal resistance to *B. cinerea.* However, a grapevine PGIP, VvPGIP1, which did not display any detectable *in vitro* interaction with the *B. cinerea* endopolygalacturonase BcPG2, interacted *in planta* with the pathogen enzyme and conferred partial protection from damage caused by BcPG2 action (Joubert *et al*., 2007). To be a successful pathogen on many host species, *B. cinerea* must be able to cope with plant defence compounds. The *Arabidopsis* phytoalexin camalexin is one example of a potent antifungal compound that contributes to basal resistance to *B. cinerea*, as evidenced by the increased susceptibility of camalexin-deficient mutants (van Baarlen *et al*., 2007; Kliebenstein *et al*., 2005). Differences in aggressiveness between *B. cinerea* isolates were attributed partly to the ability to detoxify camalexin (Kliebenstein *et al*., 2005). Other examples of the ability of *B. cinerea* to counteract the activity of antifungal plant metabolites are provided by the enzymatic degradation of alpha tomatin (Quidde *et al*.,1999) and the active secretion of plant defence compounds by ABC (ATP-binding cassette) or MFS (major facilitator superfamily) transporters. These proteins are able to excrete a spectrum of chemically unrelated toxic metabolites from the cytoplasm and serve as membrane pumps with a broad substrate range that expel chemically heterogeneous antifungal compounds at the expense of ATP or proton extrusion (de Waard *et al*., 2006).

In grapevine the structure of berries, their chemical composition, the ability to contrast the penetration and the colonisation by the synthesis of antifungal compounds determine the evolution of the infection process in the different phenological phases. Early in the season the thickness of the berry cuticle, the scarcity of lesions and the synthesis of antifungal substance, tend to decrease the infection probability. In the case of penetration, the composition and the structure of the pectins, which are highly methylated and cross-linked with calcium, together with the high level of defence compounds, prevent or brake the pathogen growth. The infections ocurring in the early phases of the season remain in general latent. During veraison, the chemical composition of berries changes with an accumulation of sugars and the ability of synthesize defence compounds decreases. These events allow the colonisation by *B. cinerea* and the degradation of pectins that results in the appearance of rot symptoms (Vercesi *et al*., 1996).

1.2.1 *B. cinerea* taxonomy and genetic variation

Botrytis cinerea Pers. Fr. (synonym *Botryotinia fuckeliana* (de Bary) Whetzel) belongs to the subkingdom *Eumycota*, *phylum Ascomycota*, class *Leotiomycetes*, order *Helotiales*, family *Sclerotiniaceae*. The genus *Botrytis* contains 20 recognized species. *B. cinerea* (Leroch *et al*., 2013) and forms a small clade with three other species, *B. calthae*, *B. fabae* and *B. pelargonii* (Staats *et al*, 2005). Recently new *Botrytis* species have been described, such as *B. fabiopsis* (Zhang *et al*., 2010), B*. sinoallii* (Zhang *et a*l., 2010b), *B. caroliniana* (Li *et al*., 2012) and the cryptic species *B. pseudocinerea* (also named *B. cinerea* Group I; Choquer *et al*. , 2007; Walker *et al*., 2011).

B. cinerea is closely related to *Sclerotinia* and indeed findings with the rDNA internal trascribed spacer (ITS) region challenge the generic delimitation and provide support for a broad concept for *Sclerotinia* (Holst-Jensen *et al*., 1998). The association of host-specific taxa with primarily northern hemisphere hosts indicates that *Botrytis* is a northern hemisphere genus that has probably been widely distributed by human activity including movement to southern temperate regions.

Botrytis species have been distinguished primarily on the basis of morphological and cultural characteristics coupled with host specificity (Hennebert, 1973; Jarvis, 1980). Features such as sclerotial size and conidium shape and size are useful in delimiting some species, but many species are morphologically similar and growing conditions can influence variations. The ITS rDNA region 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 (Nielsen *et al*., 2001). The intergenic spacer region (IGS) rDNA may offer better prospect (Giraud *et al*., 1997), although its usefulness may be limited by recombination. Another approach to defining biological species involves determining the distribution of allelic variation to define populations that are inter-breeding (Fournier *et al*., 2003).

B. cinerea is characterized by multinucleate macroconidia and uninucleate microconidia acting as male spermatia. Shirane and coworkers (1988) reported 16 chromosomes at mitotic metaphase and the same number of chromosomes was found in developing asci (Faretra and Grindle, 1992). Apothecia of *B. fuckeliana* are rare in the field, on the other hand other *Botrytis* spp. show often sexual structures. Most *B. cinerea* strains are heterothallic, carrying one or other allele of the mating type locus *MAT1-1* or *MAT1-2* (Faretra *et al*., 1988). However, there are also field isolates with dual mating strains (Faretra *et al*., 1988, 1996; Amselem *et* al., 2011).For these reasons *B. cinerea* could be considered an unusual example of dual mating fungus (Bin and van Kans, 2014)

Based on multiple gene genalogies, Fournier *et al*. (2005) postulated that *B. cinerea* is a species complex comprising two phylogenetic species (*B. cinerea* Group II or *B. cinerea sensu stricto* and *B. cinerea* Group I recently re-named *B. pseudocinerea* by Walker *et al.* 2011), but this hypothesis has not been fully adopted by the *Botrytis* community (Williamson *et al*., 2007).

B. cinerea Group I is characterized by natural resistance to fenhexamid and the absence of transposable elements (TEs), while Group II is sensitive to fenhexamid and divided into 4 subpopulations on the basis of the presence/absence of the TEs: *transposa* type bears both TE *Boty* and *Flipper*; *Boty* type contains only the *Boty* element; *Flipper* type contains only the *Flipper* element; the last type, *vacuma*, lacks for TEs (Giraud *et al.,* 1997; Giraud *et al.*, 1999; Fournier *et al*., 2003; Fournier *et al.*, 2005). In particular, Fournier and collaborators (2005) pointed out that the genetic diversity was lower within Group I, as revealed by DNA polymorphism and vegetative incompatibility tests. Groups I and II exhibited phenotypic differences in their phenology, host range, size of conidia and vegetative compatibility. All these morphological and molecular aspects suggest that *B. cinerea* Groups I and II may be different cryptic species, isolated for a long time. Fertile crosses were obtained between strains within each group, including *vacuma* and *transposa* types in the case of Group II, but not between the groups, indicating that they were reproductively isolated (Fournier *et al*., 2005).

Recently, Fekete and colleagues (2012) found only moderate differentiation between the two groups in Hungarian populations and the presence of *Boty* and one *Flipper* molecular types in the Group I strains. Furthermore, *B. cinerea* Group I was found at low frequencies in French vineyards (Giraud *et al*. 1997; Martinez *et al*., 2005; Walker *et al*. 2011), on strawberry and rape plant in Hungary (Fekete *et al*., 2012), two strains were found in a German vineyard (Kretschmer and Hahn, 2008) and one strain on rooibos in the Western Cape of South Africa (Wessels *et al*., 2013).

Leroch and collaborators in 2013 found the first evidence that *B. cinerea* strawberry isolates are genetically distinct from *B. cinerea* and that they belong to a novel clade, called *Botrytis* group S.

1.2.2 Extrachromosomal elements in *B. cinerea*

Fungi in general, and *B. cinerea* in particular, possess a variety of extrachromosomal genetic elements including the chromosomes of mitochondria, viruses, plasmids and TEs (Rosewich and Kistler, 2000). While mitochondrial chromosomes are clearly essential to cell growth, the others are generally considered dispensable and to behave as selfish genetic elements, although TEs integrate into chromosomes and may play an important long-term role in evolution (Kidwell and Lisch, 2001).

Apart from encoding genes involved in their own existence, such genomics parasites may influence their host in various ways. Despite the wide distribution of TEs, relatively little is known about the mechanism and regulation of transposition. This is probably due to the fact that many TEs appear to be remnants of transposons and because in some sexual strains mechanism exist that inactivate repeated DNA sequence. Although some fungal TEs contain most of the genetic information necessary for their own transposition, demonstration of transposition has been obtained only in a few cases.

Eukaryotic TEs are divided into two main classes by their mode of transposition and structural organization. Class I elements, or retroelements, transpose by a “copy-and-paste” mechanism by the reverse transcription of an RNA intermediate. This class can be subdivided into LTR retrotransposons, which are flanked by long terminal repeats (LTRs) sharing an overall organization similar to retroviruses, and non-LTR retroelements, which have structural features of long and short interspersed nuclear elements (LINEs and SINEs, respectively).

Class II TEs, also called DNA transposons, are flanked by two terminal inverted repeats (TIRs) and transpose directly through a DNA form by a “cut-and-paste” mechanism. Both classes are subdivided into distinct superfamilies on the basis of structural features, internal organization, the size of the target site duplication (TSD) generated upon insertion, and sequence similarities at the DNA and protein levels. Most but not all TE families include both autonomous and nonautonomous elements. Proteins supplied by autonomous elements, generally belonging to the same family, can transactivate nonautonomous elements.

TEs have a remarkable potential to cause a variety of changes in the genome of their hosts. By transposing into or near genes, class I and class II TEs contribute to partial or total gene inactivation. Insertion may also place a gene under the control of TE regulatory sequences. Owing to their ability to excise from a given site, class II transposons can generate a wide degree of variation in DNA sequence and phenotype (Kidwell and Lisch, 2001; Daboussi and Capy, 2003).

Two TEs have been found in strains of *B. cinerea. Boty* is a 6-kb putative Class I retrotransposon, characterized by a long terminal repeat (LTR), present in multiple copies in different regions of the genome (Diolez *et al*., 1995; Giraud *et al.*, 1999). *Flipper* is a 1842 bp Class II trasposon, present in up to 20 copies per genome, and is known to be mobile by its insertion into nitrate reductase during spontaneous mutant selection (Levis *et al*., 1997). Initially dot blot methods were used to detect these elements, but more recently PCR methods have been developed (Muñoz *et al*., 2002). When first reported, both elements were found in the same strain (*transposa* type), whereas *vacuma* strains lacked both elements. However, strains

containing only the *Boty* elements have been detected in Europe (Giraud *et al*., 1999; De Miccolis *et al*., 2003; Rajaguro and Show, 2010; Samuel *et al*., 2012; Fekete *et al.,* 2012; Asadollahi *et al.,* 2013; Vercesi *et al*., 2014), America (Muñoz *et al*., 2002; Ma and Michailides, 2005; Esterio *et al*., 2011) and Australia (Isenegger *et al*., 2008), while strains harbouring the *Flipper* TE alone have been isolated only in northern, southern and eastern Europe (Albertini *et al*., 2002; De Miccolis *et al.,* 2003; Milicević *et al*., 2006; Kretschmer and Hahn, 2008; Vaczy *et al*., 2008; Rajaguro and Shaw, 2010; Fekete *et al.,* 2012; Asadollahi *et al.,* 2013; Samuel *et al*., 2012; Vercesi *et al*., 2014), Tunisia (Ahmed and Hamada, 2005) and Bangladesh (Isenegger *et al*., 2008). *Vacuma* strains were predominant on green peas in France (Giraud *et al*., 1999), apple and kiwifruit in Greece (Samuel *et al.*, 2012) and on grape in northern Italy during grapevine growing season (Vercesi *et al*., 2014).

In vineyards, *transposa* strains are predominant (Muñoz *et al*., 2002; Ma and Michailides, 2005; Kretschmer and Hahn, 2008; Vaczy *et al*., 2008; Esterio *et al*., 2011; Samuel *et al*., 2012), particularly at harvest (Giraud *et al*., 1997; Martinez *et al*., 2005; Vercesi *et al*., 2014), while *vacuma* strains are detected mainly on floral residues and strongly decrease during summer (Martinez *et al*., 2005). The higher isolation frequency of *vacuma* strains from senescent tissues has been attributed to marked saprophytic capability linked to rapid growth on highly nutritive media and reduced pathogenicity (Martinez *et al.*, 2005). *Transposa* isolates are characterized by greater virulence on grape berries, and are more frequently resistant to the fungicides vinclozolin and carbendazim (Giraud *et al.*, 1997; Martinez *et al.,* 2003). Phenotypic and molecular diversity has been intensively investigated in *B. cinerea* populations isolated mainly from diseased organs of different plants or at various host developmental stages.

1.2.3 Population structure of *B. cinerea*

The great morphological and physiological variability exhibited by *B. cinerea* has been attributed for long time to parasexuality and, at the genetic level, the pathogen has been considered a clonal species, due to the great production of conidia and the rare sexual structures observed in natural conditions (Elad *et al*., 2004). This view, in the last ten years, was partially revised by several studies focused on the structure of the populations isolated from different hosts.

Genetic variation in *B. cinerea* populations has been studied using a variety of molecular techniques, including restriction fragment length polymorphism (RFLP) analysis of PCR-amplified loci (Giraud *et al*., 1997), polymerase chain reaction (PCR) detection of TEs (Diolez *et al* ., 1995; Levis *et al*., 1997a), random amplified polymorphic DNA (RAPD) fingerprinting (Kerssies *et al*.,

1997; van der Vlugt-Bergmans *et al*., 1993), amplified fragment length polymorphism (AFLP) analysis (Moyano *et al*., 2003), fingerprinting of repetitive sequences by microsatellite primed (MP)-PCR (Ma and Michailides, 2005), PCR amplification of microsatellite loci (Fournier *et al* ., 2002; Fournier *et al*., 2005; Walker *et al*., 2014) and DNA sequencing of gene regions (Albertini and Leroux, 2004; Albertini *et al* ., 2002; Fournier *et al* ., 2003).

The RAPD techniques first applied to *B. cinerea* showed, using a set of 50 primers resulting in 139 markers, that all the *B. cinerea* isolates analysed were genetically different (Van der Vlugt-Bergmans *et al.*, 1993). No grouping of isolates by their host, year of isolation, geographical origin or mating type were observed. Since then, further studies have been carried out on *B. cinerea* populations using analogous techniques. Kerssies and coworkers (1997) analysed 29 isolates of *B. cinerea* from the Netherlands with 11 primers and 70 markers and found that, with few exceptions, almost all the isolates tested were genetically different. Alfonso and coworkers (2000) studied 40 strains from Spain and found out that the population was highly heterogeneous: this result was confirmed by another research concerning Spanish strains carried out by Moyano and coworkers (2003). High genotypic diversity was found also in Chile (Muñoz *et al*., 2002) and in South Carolina (Yourman *et al*., 2000). Giraud and coworkers (1997) used for their population studies different molecular markers including presence or absence of the TEs *Boty* and *Flipper*, a suite of PCR-RFLP (based on the rDNA intergenic spacer region, nitrate reductase, ATP synthase and ADP-ATP) and fungicide resistance. The strains isolated from grape in the France region of Champagne, showed extensive genotypic diversity that indicates limited clonal propagation and a significant role for recombination. An analogous result (Giraud *et al*., 1999) was obtained by studying isolates from various host plants.

The population structure of *B. cinerea* was further investigated by analysing the amino acid polymorphism in two genes, *Cyp51* (14α-demethylase gene) and Bc-*hch* (*B. cinerea* het-c homolog). The polymorphism detected at these loci (Albertini *et al*., 2002; Fournier *et al*., 2003) as previously described allows to differentiate two separate groups, Group I and Group II, within the population.

More recently the genotypic diversity and the role of regular occurrence of sexual reproduction of *B. cinerea* was investigated by using microsatellite markers developed by Fournier *et al*. (2002), by the mating type ratios and by the use of rigorous genetic tests such as linkage disequilibrium (LD).

The nine microsatellite markers characterized in the fungus *B. cinerea* (Fournier *et al*., 2002) have been applied to strain fingerprinting in population genetic studies (Karchani-Balma *et al*.

2008; Vaczy *et al*. 2008; Decognet *et al*. 2009). Microsatellite markers or simple sequence repeats, (SSR) are short tandem repeated tracts of DNA composed of units that are 1-6 base pairs long, spread throughout the genome and used as genetic markers in genome mapping or population genetics studies because of their high level of polymorphism (Jarne and Lagoda 1996). Ajouz and collaborators (2010) tested the stability overtime of the nine microsatellite markers developed for this fungus and observed no changes in the allele size during some *B. cinerea* generations.

Random mating and recombination in the heterothallic *B. cinerea* should result in high genotypic diversity, 1:1 mating-type ratios, and random associations of alleles, or linkage equilibrium, at different loci. Recombination resulting from sexual reproduction leads to new genotypes that permit organisms to better adapt to changing conditions and purges genomes of the accumulation of deleterious mutations . Many hypothesis were done on the advantages and disadvantages of sexual reproduction in the evolution. Sexual reproduction indeed have also costs, such as breaking up favourable allele combinations in stable environments (Barton and Charlesworth, 1998), as well as the costs of finding a suitable mate and engaging in mating (Agrawal, 2006).

In *B. cinerea,* the apothecia have rarely been found in nature (Polach and Abawi, 1975) but genetic analysis of the structure of the populations frequently reveled genetic diversity, probably arising from sexual recombination (Faretra *et al.*, 1988; Giraud *et al*., 1997; Fournier and Giraud, 2007; Isenegger *et al*., 2008; Karchani-Balma *et al*., 2008 Vaczy *et al*., 2008; Wessels *et al*., 2013; Walker *et al*., 2014).

Contrasting results were obtained in *B. cinerea* populations both on the presence/absence of differentiation among transposon genotypes (Giraud *et al*., 1997; Giraud *et al*., 1999; Munoz *et al*., 2002; Martinez *et al*., 2003; Ma and Michailides, 2005; Samuel *et al*., 2012) and on differentiation between *B. cinerea* cryptic species (Diooez *et al*., 1995; Levis *et al*., 1997; Giraud *et al*., 1997; Fekete *et al*., 2012), host specificity (Ma and Michailides, 2005), geography (Karchani Balma *et al*., 2008; Fournier *et al*., 2013) and mode of recombination (Giraud *et al*., 1997; Vaczy *et al*., 2008; Wessels *et al*., 2013).

1.3.1 Control of *B. cinerea* diseases

Epidemics caused by *B. cinerea*, especially during long wet or rainy periods, can be severe and economically damaging on many agricultural and horticultural crops and in particular on grapevine. During the past 30 years there have been major developments in the chemical control of fungal diseases, and viticulturists have access to a wide range of antifungal compounds.

However, it has become clear that there are serious risks associated with over-reliance on chemical methods of plant protection. The main risks associated with fungicide use concern the occurrence of toxic residues in foodstuffs and environmental contamination. For these reasons pesticide application has been regulated in many countries. In response to very important quality and safety requirements, in the last years the concept of integrated pest management (IPM) was established. IPM is a pest control strategy that consists of a variety of complementary methods including the utilization of mechanical, physical, genetic, biological tools and cultural practices, in order to keep the crop loss due to the pathogens/pests under a fixed economic threshold and, at the same time, minimize the number of treatments (Oliver and Hewitt, 2014).

Control of *B. cinerea* is best achieved through IPM: in fact the successful control of grey mould depends on overall management of the disease and should take into account all the biotic and abiotic factors affecting both the infection probability and the host susceptibility (Oliver and Hewitt, 2014).

1.3.2 Cultural management of *B. cinerea* diseases

B. cinerea spores require known environmental conditions for the germination and the subsequent fungal growth: control can be obtained by creating a canopy microclimate (*i.e*. temperature, vapour pressure deficit, wind speed and wetness) less conducive to fungal development. The objective is to increase exposure of the grape clusters to air and light so that they dry out more quickly after wetting.

Canopy management can be directed either to the canopy or to the overall growth of the vine. Direct measures include trellis systems, pruning methods, shoot positioning, shoot thinning, hedging or leaf removal. All these methods aim to increase the penetration of air and light to the cluster. A range of different vine training system were evaluated in Italy on several grape cultivars to identify systems that were non-conducive to the 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 incidence (Cargnello *et al*., 1991).

Direct measures, also applied to vineyards after establishment, can still achieve disease control. The practice of vine “hedging”, that consists in pruning off the over-hanging current season growth at veraison, is often used to maintain an upright pattern of shoot growth that will enhance air movement directly to the clusters. 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).

Another good practice to reduce *Botrytis* epidemics is the leaf removal from the fruiting zone of vines. Leaf removal ("leaf plucking") affects the microclimate in and around the receptive bunch, often reducing bunch rot at harvest. Increased wind speed after leaf removal increased the evaporative potential on the berry surface, thereby significantly reducing *B. cinerea* infection and development (Elmer and Michailides, 2004). In addition, stimulation of phytoalexin production by increased UV light was obtained as a result of leaf removal (Langcake, 1981). Along with the leaf plucking, the removal of potential substrate for the pathogen is a recommended practice to reduce inoculum potential in the bunch early in the season. In fact, clusters left on vines or on the vineyard floor from the previous season can be source of inoculum in the following spring.

Indirect measures may involve irrigation, fertilizer strategies, rootstock and cultivar selection and planting density. These indirect practices affect the canopy density by altering the number of shoots, shoot length, lateral growth and the number of leaf layers and may also affect cluster tightness by altering the cluster length, number of berry set and size. The correct fertiliser applications and timing are very important. If too much nitrogen is applied, especially in spring, it may result in excessively vigorous and therefore dense canopies. The type of irrigation system, the irrigation frequency and timing are other factors that may play a role in grey mould control. Overhead irrigation is very conducive to *Botrytis* infection since the foliage remains wet for long periods, and should therefore preferably be eliminated, if possible. Excessive irrigation furthermore results in dense canopies, more compact bunches and thinner berry skins, with the accompanying harmful effects.

Along with the cultural practice, also the choice of the cultivar is an important variable that affects grey mould epidemics in grapes. The morphological, anatomical and chemical characteristics of 42 cultivars with a range of resistance to *B. cinerea* were measured to establish whether resistance was linked to specific characteristics. Cultivar resistance was attributed to thicker cuticle and enhanced wax content rather than induced or constitutive antifungal host defence mechanisms (Mlikota Gabler *et al*., 2003). In addition, the bunch architecture can also affect development of the pathogen. Infection by *B. cinerea* can be influenced by cluster compactness and a reduction of *Botrytis* development is correlated with less compact clusters, associated with lower berry number and reduced cluster weigh.

1.3.3 Chemical control of *B. cinerea* diseases

Beside preventive control methods, such as the cultural practices described above, several agrochemicals are available to control the pathogen and new ones are being developed. Several fungicides with preventive or/and curative effects have successfully supported vine growers to reduce losses caused by *B. cinerea*.

In the early 1970s, fungicide treatments were applied, especially in France, whenever favourable climatic conditions were recorded in vineyard. Infection of inflorescences and berries requires, according to the French researchers Bulit and Lafon (1972), not less than 15 hours of temperature ranging from 15 to 20 °C and saturated relative humidity or wetness. Baldacci and co-workers (1962) pointed out that infection risk varies during the grapevine phenological stages: at fruit set young berries are easily infected by the mycelium of *B. cinerea* developing on withered caliptras and stamina, while the lesions frequently occurring on these organs from veraison onwards represent favourable infection sites for the pathogen. Therefore four treatments are recommended: at the end of flowering (A phase), just before the bunch closure (B phase) in order to protect the inner parts of clusters, especially on compact bunches, at the beginning of veraison (C phase) and before harvest (D phase). The treatment at the end of flowering can be omitted in dry seasons, and the fungicide application at the beginning of veraison delayed until the occurrence of rain or long wetness periods.

The first fungicides used in vineyards against grey mould have multisite mode of action such as thiram and captan. Currently dithiocarbamates, thiram, captan and chlorothalonil, are revoked. Their preventive activity relies on the spore germination, through the inhibition of several thiol-containing enzymes involved in germling formation. In the early 1970s new systemic antifungal active substances, belonging to the benzimidazole class and exhibiting a broad spectrum of activity, were introduced. Shortly afterwards, several cases of resistance were recorded in European vineyards and after only three to four seasons the use of benzimidazoles in northern Europe vineyards was severely curtailed (Leroux and Clerjeau, 1985). Dicarboximides or cyclic imides superseded the benzimidazoles in the late 1970s and the early 1980s, but their intensive use led to practical resistance. This phenomenon was related to the selection of *B. cinerea* strains moderately resistant to dicarboximides. From the middle 1990s, new compounds with excellent *B. cinerea* activity came to the market: the anilinopyrimidines, phenylpyrrol, the carboximides and hydroxyanilide.

Nowadays, *B. cinerea* active substances are characterized by six different mode of action: interference with the osmotic signal transduction pathway (dicarboxamide iprodione and

phenylpyrroles fludioxonil); inhibition of the synthesis of methionine (the anilinopirimidines mepanipyrim, cyprodinil and pirymethanil); disruption in energy production in the fungus (fluazinam); inhibition of the sterol biosynthesis (fenhexamid e fenpyrazamine); and inhibition of respiration at complex II - succinate dehydrogenase (boscalid and fluopyram). All this substances are used alone, with the only exception of the mixture of cyprodinil+fludioxonil.

Because *B. cinerea* is the classical high-risk pathogen in the true sense of resistance management, also the new active substances are endangered by resistance development, which was demonstrated by many authors (Leroux *et al*., 2002; Walker *et al*., 2013). However, while in the past *B. cinerea* treatments mainly relied on the dicarboximides alone, the new botryticides belonging to different chemical groups and different mode of actions represent effective options for *B. cinerea* control and anti-resistance management strategies.

Anilinopyrimidines

Active substances belonging to the anilinopyrimidine class were firstly patented as fungicides in 1981 by VEB Fahlberg-List in the ex German Democratic Republic. During 1980s new investigations on these molecules resulted in the introduction into the market of three novel fungicides belonging to the anilinopyrimidines class: pyrimethanil in 1992 (Figure 1.4.1), cyprodinil in 1993 (Figure 1.4.2) and mepanipyrim in 1994 (Figure 1.4.3).

The spectrum of fungicidal activity of anilinopyrimidines is limited to ascomycetes, including a broad range of pathogens such as *B. cinerea* on grapes, fruits, vegetables and ornamentals, *Venturia inaequalis* (Cooke) G. Winter on apple and *Alternaria* spp. and *Monilia* spp., causing leaf spot diseases and rot on a range of vegetables and fruits (Muller *et al*., 1998). Cyprodinil is also active on several pathogens infecting cereals, such as *Tapesia* spp., *Pyrenophora teres* Drechsler*, Blumeria graminis* (Dc.) Speer and *Rhynchosporium secalis* (Oudesm.) Doris*,* while pyrimethanil has additional activity against *Ascochyta* spp. on legumes and *Aspergillus* spp. and *Penicillium* spp. on fruits and grapevine.

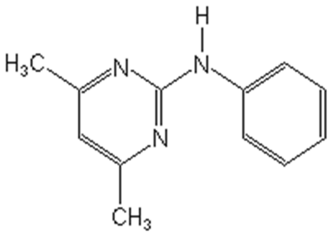


Figure 1.4.1. Chemical structure of pyrimetanil.

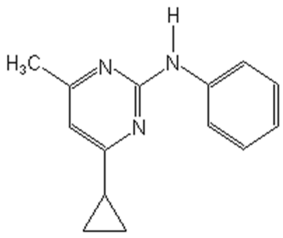


Figure 1.4.2. Chemical structure of cyprodinil.

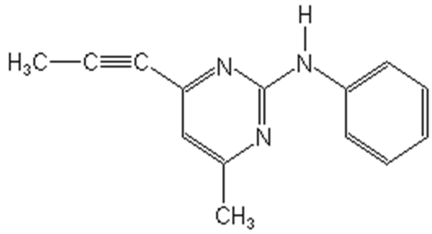


Figure 1.4.3 Chemical structure of mepanypirim.

The preventive activity of anilinopyrimidines is due to the inhibition of germ tube elongation during spore germination, of appressoria formation and of mycelial growth. Moreover the penetration and infection process of the pathogen into the host tissue is affected, presumably through inhibition of secretion of hydrolytic enzymes during pathogenesis (Sierotzki and Gisi, 2003). Anilinopyrimidines are systemic fungicides, translocated in apoplast of leaves, resulting in the inhibition of later stages in the pathogenesis such, as the formation of haustoria in powdery mildew, intercellular growth of mycelium and sporulation.

Several amino acids, particularly methionine, have been shown to reverse the fungitoxicity of anilinopyrimidines in *B. cinerea* (Masner *et al*., 1994). Biochemical studies indicated that the mycelial level of radiolabelled methionine and homocysteine were lower after a treatment by pyrimethanil; at the same time a slight increase in radiolabelled cystathionine was recorded (Fritz *et al*., 1997). These results suggested that the primary target site of anilinopyrimidines may be the cystathionine β-lyase (encoded by *BcmetC*) or cystathionine-γ-sinthase (encoded by *BcmetB*), the enzymes that catalyse the transformation of cysteine in homoserine in the methionine biosynthesis pathway. No mutation was found in *BcmetC* (Fritz *et al.,* 2003), and the mutations detected in *BcmetB* (S24P and 164V) were also found in susceptible strains (Sierotzki *et al*., 2001). Further studies are therefore required to identify the primary target of anilinopyrimidines and the associated resistance mechanism. It has been suggested that they could affect protein secretion pathways involving the Golgi complex (Leroux *et al*., 2002).

Resistance to anilinopyrimidines has already been detected in several pathogens, including *B. cinerea.* Sensitivity distribution of *B. cinerea* populations to anilinopyrimidine fungicides suggests a qualitative, disruptive selection for resistance, due to a monogenetic factor (Hilber and Hilber-Bodmer, 1998). Resistant isolates show significantly lower mycelial growth and virulence, but higher spore production in *vivo*. However, it seems that resistance to anilinopyrimidine is not associated with a significant reduction of fitness (Bardas *et al.,* 2008).

FRAC (Fungicide Resistance Action Committee) classified the anilinopyrimidine as a class with a medium risk of resistance.

Succinate Dehydrogenase Inhibitor (SDHI)

The Succinate Dehydrogenase Inhibitors (SDHIs), initially called carboxamides fungicides, were developed in the 1960s. The first compound in this class was carboxin, developed in 1966 and used mainly as a seed treatment against basidiomycetes (Von Schmeling and Kulka 1966). In the last decade, several novel SDHIs exhibiting a wide spectrum of activity have been discovered. Among them, boscalid (Figure 1.4.4) was the first pyridinecarboxamide to be introduced in 2003 and is effective against ascomycetes, including grey mould and powdery mildew agents.

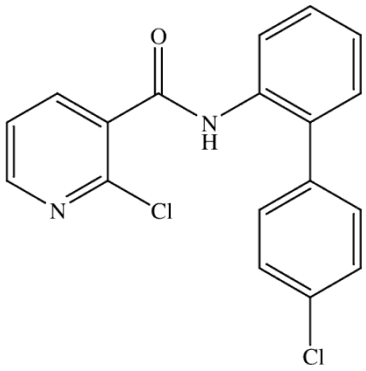


Figure 1.4.4 . Chemical structure of boscalid.

In recent years, several new SDHIs fungicides have been launched. Currently the “overall” spectrum of SDHI fungicides is extremely broad, being comparable with the QoI spectrum, with the exception of oomycete activity, which is still lacking (Sierotzki and Scalliet, 2013).

Carboxin and related anilides or carboxamides are systemic fungicides inhibiting the succinate dehydrogenase (SDH) of Krebs cycle that catalyzes the oxidation of succinate to fumarate, coupled to the reduction of ubiquinone to ubiquinol. Succinate dehydrogenase enzyme is a mitochondrial heterotetramer composed of four subunits (SDHA, SDHB, SDHC, SDHD) (Cecchini, 2003). The SDHA and SDHB subunits are exposed to the matrix of the mitochondria and SDHC and SDHD are integral membrane proteins anchoring the SDHAB dimer to the internal membrane of the mitochondria. In contrast to other dehydrogenases of the TCA cycle, the SDH enzyme transfers succinate-derived electrons directly to the ubiquinone pool of the respiratory chain and not to soluble NAD+ intermediates. For this reason, SDH is considered to be an essential component of the respiratory chain and is also termed complex II (Figure 1.4.5). SDHIs can be classified into two main categories: those binding to the succinate-binding pocket (e.g., malonate)

and those binding to the ubiquinone-binding pocket (e.g., carboxamides). All crop protection SDHIs target the ubiquinone-binding pocket, structurally defined by the interface between the SDHB, -C, and -D subunits. Although a few amino acid residues important in the catalysis of ubiquinone reduction are strictly conserved across species, most SDHC and SDHD residues display a high degree of variation across species (Cecchini, 2003). This feature is largely responsible for the diversity in chemical structures and diversity of biological spectrum displayed across SDHIs. SDHIs bind strongly to the ubiquinone-binding (Qp) site; upon binding, they physically block access to the substrate which consequently prevents further cycling of succinate oxidation.

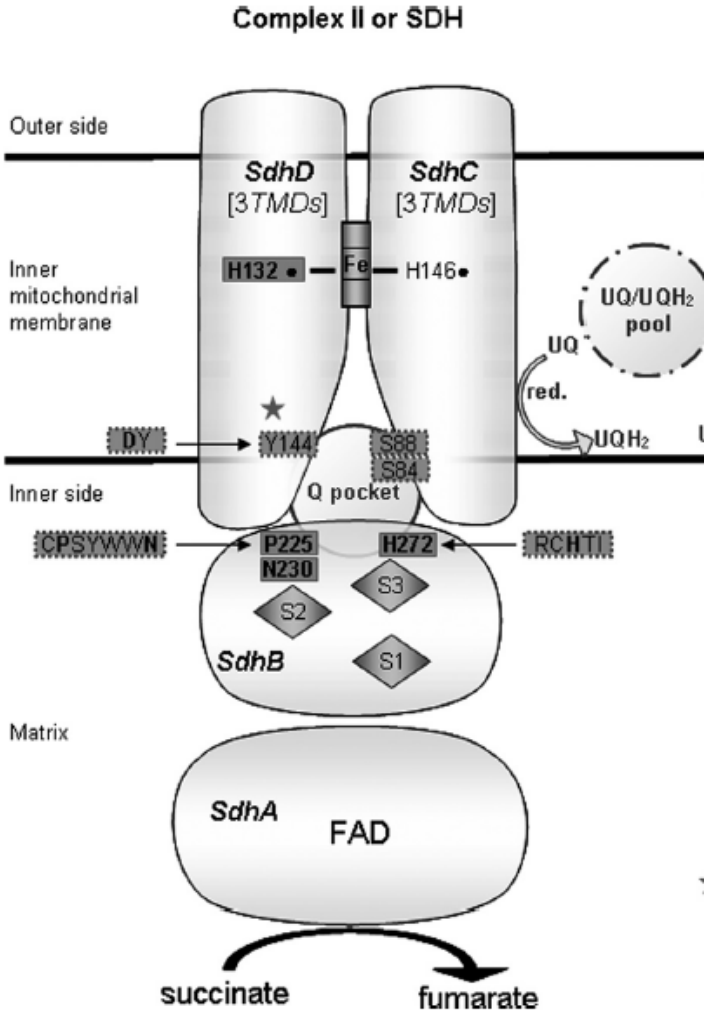


Figure 1.4.5. Schematic structure of mitochondrial complexes II indicating the main amino acids of the different subunits involved in binding of inhibitors and in resistance. Complex II contains four subunits and in subunit *SdhB* are present 6 aminoacidic substitutions.

After the increased usage of SDHIs as foliar fungicides, resistance to them has been reported in *Alternaria alternata* (Avenot *et a*l., 2008), *Didymella brioniae* (Stevenson *et al*., 2008), *Corynespora cassiicola* (Mayamoto *et al*., 2009), and *Podosphaera xanthii* (Mayamoto *et al*.,

2010). In few years resistance to boscalid has been reported also in field strains of *B. cinerea* obtained from grape, strawberry, blackberry, apple and kiwifruit (Strammler *et al*., 2008; Bardas *et al*., 2010; Leroux *et al*., 2010; Kim and Xiao, 2010; Leroch *et al*., 2011; Stammler *et al*., 2011; Veloukas *et al*., 2011; Walker *et al*., 2011; Yin *et al*., 2011; Amiri *et al*., 2013; Amiri *et al*., 2014; De Miccolis Angelini *et al*., 2014a; Li *et al*., 2014) and in laboratory mutants (De Miccolis *et al*., 2010). For this reason the Fungicide Resistance Action Committee (FRAC) classified the SDHI as a class of fungicides with a medium to high risk of resistance.

In a recent study exploring the mechanisms of resistance in boscalid-resistant strains, seven different amino acid substitutions in the SDHB subunit were found and correlated with resistance to boscalid. The most frequent mutation is an histidine (H) replaced with a tyrosine (Y) at codon 272 (H272Y), followed by other amino acids substitutions such as arginine (R), leucine (L) or valine (H272R, H272L H272V). A nucleotide change resulting in an asparagine (N) to isoleucine (I) substitution was found at codon 230 (N230I), while two different amino acid changes were found at codon 225, where proline (P) is replaced by phenylalanine (F) or leucine (L) (P225F and P225L) (Sierotzki and Scalliet, 2013; De Miccolis Angelini *et al*., 2014a).

Phenylpyrroles

The best known molecule belonging to the phenylpyrrole class is fludioxonil (Figure 1.4.6). This active substance was developed in 1990 by the synthetic optimization of the natural product pyrrolnitrin, a simple secondary metabolite isolated from *Pseudomonas pyrrocinia* Imanaka, Kousaka, Tamura and Arima that exhibited a strong antifungal activity.

Fludioxonil is highly effective against *B. cinerea* on grapes, fruits, vegetables and ornamentals. In addition to the very efficient *Botrytis* control, the active substance also controls *Penicillium* spp., *Aspergillus* spp. and *Trichothecium* spp. on grapes. Due to its high efficacy against different agent of rot, fludioxonil is widely used for pre- and postharvest treatments in grape and other crops. The major part of the applied fludioxonil is recovered from the host surface suggesting that the fungicide does not penetrate in the plant tissues.

Fludioxonil strongly inhibits germination of *B. cinerea* conidia *in vitro* and *in vivo,* as shown by experiments carried out with *B. cinerea* on grape leaves (Errampalli, 2004). The relevant effect on germination of *B. cinerea* conidia on the leaf surface explains the excellent efficacy of fludioxonil after preventive application. In order to broaden the range of controlled pathogens and at the same time to manage resistance problems, fludioxonil is mainly applied in mixture with the anilinopyrimidine cyprodinil.

The mechanism of action of phenylpyrroles is not completely clarified, but data obtained with laboratory mutants (Fujimura *et al*., 2000) suggest that phenylpyrroles, like dicarboximides, might interfere with the osmotic signal transduction pathway, resulting in an abnormal accumulation of glycerol. The regulation of this pathway is due to the phosphorylation or the dephosphorylation of an histidine kinase, a protein characterized by a kinase domain fused to the response regulator domain. Under conditions of normal osmolarity the histidine kinase is phosphorylated and inactive. The dephosphorylation of the protein in high osmolarity conditions leads, by a cascade reaction, to the transcription of enzymes involved in glycerol production that allows the cell to compensate for the high external osmotic pressure.

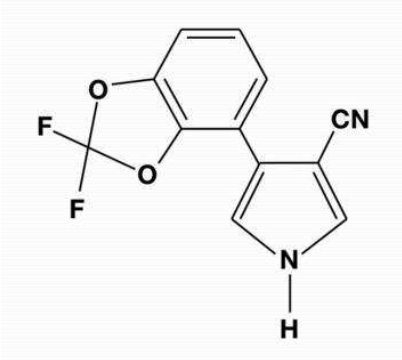


Figure 1.4.6 Chemical structure of fludioxonil.

In *B. cinerea* dicarboximide and phenylpyrrole resistant laboratory strains and less sensitive field isolates were usually found to be mutated in *Daf1*, the histidine kinase gene, but experimental results suggested the involvement of more than one gene in dicarboximide and phenylpirroles resistance (Ziogas *et al.,* 2005). Moreover, dicarboximide resistance segregates separately from phenylpirrole resistance, suggesting that different genes regulate resistance for the two fungicide classes (Corran, 2007). Structure modelling of the cytoplasmic linker domains (also named HAMP domains because of their presence in Histidine kinaseAdenyl cyclases, Methyl accepting proteins and Phosphatases) recently revealed that the replacements of hydrophobic residues within these domains generally affected their helical structure, probably eliminating signal transduction (Fillinger *et al*., 2012).

The fungicide resistance action committee (FRAC) classified phenylpyrroles as fungicides with low to medium risk of resistance. Resistance management is required even if resistance has been found only sporadically.

Hidroxyanilides

Hydroxyanilides belong to the third class of sterol biosyntesis inhibitors fungicides (SBI) and in particular of a 3-keto reductase, an enzyme involved in sterol C-4 demethylation (Debieu *et al*., 2001). Fungal cell membranes are characterized in most pathogens belonging to ascomycetes and basidiomicetes, by a common dominant sterol component, namely ergosterol. Lack of ergosterol prevents the synthesis of new membrane and leads to deterioration of the existing ones.

SBI fungicides have been the most important group of specific fungicides world-wide for over two decades. This success is due to their biochemical mechanism of action: fungi have specific sterols that differ from those produced in plants and animals, giving the chance to develop selective inhibitors. SBIs show a broad spectrum of activity against ascomycetes and basidiomycetes but not against oomycetes, since species belonging to the last taxonomic group are, in fact, unable to perform the full synthesis of sterols but can metabolize exogenous precursors derived from plants.

The fungicidal activity of hydroxyanilides was discovered during the development of some herbicides compounds when the biological activity of the synthesis intermediates were tested against fungi and insects. Some of them showed a stable activity against *B. cinerea.* In particular, 1,4-hydroxyanilides proved to be of particular interest as the starting point for chemical research. The molecule, in fact, depending on the properties of the aromatic substituent, is easily degraded and thus potentially shows a favourable toxicological profile and environmental behaviour. The chemical optimization of 1,4-hydroxyanilides led to the development of a new efficient fungicide named fenhexamid (Figure 1.13). Fenhexamid does not affect spore germination, but inhibits germ tube elongation and mycelial growth. Unless the SBI fungicides, fenhexamid has a quite narrow spectrum of biological activity since it is efficient against *B. cinerea* and other *Botrytis* spp. and against species belonging to the related genera *Sclerotinia* and *Monilia*. The activity against other fungi belonging to the ascomycetes and basidiomycetes is observed only at high concentrations under *in vitro* conditions.

Four different phenotypes exhibiting *in vitro* resistance to fenhexamid have been detected in field isolates of *B. cinerea*. The first phenotype, Ani R3, was initially detected because of their resistance towards anilinopyrimidines and show multi drug resistance mainly at the germ-tube elongation. Four other phenotypes with in vitro resistance to the hydroxyanilide fenhexamid have been detected in French field populations of *B. cinerea*: *HydR1*, *HydR2*, *HydR3*$^{-}$ and *HydR3*$^{+}$ (Leroux *et al*., 2002; Fillinger *et al*., 2008). In *HydR1* and *HydR2*, the resistance was mainly expressed i*n vitro* mycelial growth tests, whereas the germ tubes produced by these strains

appeared sensitive to fenhexamid. *HydR1* quickly metabolizes fenhexamid when grown in liquid media. The main route corresponded probably to hydroxylation at various positions in the cyclic ring. Synergism of fenhexamid activity in *HydR1* strains of *B. cinerea* (and not in wild-type strains) by cytochrome P450 inhibitors such as DMI fungicides (DeMehylation Inhibitors, belonging to SBI fungicides, class I) provided indirect evidence of the involvement of a cytochrome P450 monooxygenase (P450) in fenhexamid detoxification. The negative cross resistance to DMI in *HydR1* strains suggests that the same P450 is responsible for detoxification of fenhexamid. Moreover *HydR1* strains contain two mutations in *CYP51* gene, encoding eburicol 14 α-demethylase. These differences can explain why *HydR1* strains responded differently to DMIs in *in vitro* tests. This result, combined with other observations such as the existence of morphological differences, the failure to cross *HydR1* and non-*HydR1* strains and the somatic incompatibility between these two groups, suggested that they corresponded to two distinct genetic entities.

HydR3^{+} strains are the greatest cause of concern in practice because they display high levels of resistance both at germ tube elongation and during mycelial growth (Leroux *et al*., 2004).

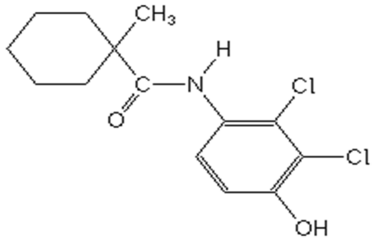


Figure 1.4.7 Chemical structure of fenhexamid.

Resistance to fenhexamid in *B. cinerea* has been reported in southern Italian, French, Israel, German and Chilean vineyards (Albertini and Leroux, 2004; Esterio *et al*., 2007; Fillinger *et al*., 2008; Korolev *et a*l. 2011, Leroch *et al.*, 2011; 2010; De Miccolis Angelini *et al*., 2014a); in strawberry fields in Florida and in north and south Carolina (Amiri *et al*., 2013; Grabke *et al*., 2013); in lettuce in Greece (Chatzidimopoulos *et al*., 2013); and in a single case from different vegetables in Greece (Myresiotis *et al*., 2007). Resistance to fenhexamid in *B. cinerea* has been linked to a point mutation at codon 412 in the *erg27* gene encoding for the 3-KR enzyme (Fillinger *et al*., 2008). This mutation was found to be deleterious to mutants carrying it in comparison to the wild-type isolates under laboratory conditions (Billard *et al*., 2011). The extent to which these isolates can persist in the field and cause control failures is unknown.

FRAC classified the hidroxyanilides as low to medium risk of resistance even if resistance management is required.

2. AIMS OF THE WORK

The Directive 2009/128/EC aims at achieving a sustainable use of pesticides by adopting integrated protection management (IPM) strategies and avoiding harmful active substances (a. s.). Thus, numerous a.s., and in particular various multisite fungicides, will be probably banned in the near future. Unfortunately, multisite fungicides play a key role in preventing or moderating the selection of resistant strains of many fungal pathogens. Therefore, in the Directive 128 specific activities concerning the monitoring of fungal populations responsible for serious epidemics in many important crops are strongly recommended. On grapevine, the attention is focused mainly on *P. viticola,* the aetiological agent of downy mildew, on *Erysiphe necator*, the agent of powdery mildew and on *B. cinerea* which causes grey mould and can severely reduce both the quality and the quantity of the yield. In northern Italian vineyards, generally only two treatments against *B. cinerea* are carried out with single site fungicides. Despite the low selection pressure exerted on the fungus, *B. cinerea* strains resistant to numerous a.s., namely boscalid, vinclozolin, carbendazim, fenhexamid, anilinopyrimidines and fludioxonil were found in Piedmont (Vercesi *et al*., 2014). In the same region, increased resistance to cyprodinil have been reported also in untreated vineyards (Lopez *et al*., 2011; Gullino *et al*., 1998). In southern Italian vineyards, a higher number of fungicide treatments are often applied particularly on table grape. Higher frequencies of strains resistant to anilinopyrimidine, fludioxonil, fenhexamid, boscalid, QoI were detected in *B. cinerea* populations isolated from Apulain sites (De Miccolis Angelini *et al*., 2014a)

The aims of the present work were to characterize the *B. cinerea* populations associated with numerous vineyards located in Lombardy and to evaluate the possible presence of resistant strains or a reduction in sensitivity to a.s. of the pathogen populations. Standardized resistance monitorings activities were performed in order to investigate in three subsequent vegetative seasons the sensitivity of *B. cinerea* strains towards the four a. s. most frequently used in vineyard, with specific single-site modes of action: cyprodinil (anilinopyrimidines), fenhexamid (hydroxyanilides), fludioxonil (phenylpyrroles) and boscalid (pyridine carboxamides).

Furthermore, due to increasing application of boscalid, recently introduced in the market (2003-2004 vegetative season) and classified by the Fungicide Resistance Action Committee (FRAC; http://www.frac.info/) as a medium to high risk of resistance product, the presence and the impact of the main point mutations found in the *SdhB* gene, more frequently found on grapevine isolates (H272Y and H272R) (Leroux *et al*., 2010; De Miccolis Angelini *et al*., 2014a; De Miccolis Angelini *et al*., 2014b) were evaluated in the boscalid resistant strains.

The genetic variability of *B. cinerea* populations includes also the presence/absence of transposable elements (*Boty* and *Flipper*) (Diolez *et al*., 1995 Levis *et al*., 1997). So the occurrence and frequency of the *transposa* (carrying the two transposable elements) and *vacuma* (devoid of the two transposable elements) strains were assessed in order to detect possible differences in the distribution of *B. cinerea* strains belonging to different molecular classes and populations and relationships between the four molecular classes of transposons and a reduction in sensitivity through the four tested fungicides.

Apothecia of *Botryotinia fuckeliana,* the sexual stage of *B. cinerea,* have been rarely found in nature (Polach and Abawi, 1975), therefore the occurrence of recombination in the pathogen populations has been for a long time matter of debate. Nowadays, rigorous genetic tests for random mating such as the evaluation of the mating type ratio, which in populations randomly mating is expected to be 1:1, or the assessment of the genotypic diversity and index of association of the populations (linkage disequilibrium) can be used in order to make hypotheses on the mode of reproduction of *B. cinerea* populations (Milgroom, 1996). In this study, the assessment of the mating type ratio and the Linkage disequilibrium have been used to verify if the *B. cinerea* populations in Lombardy exhibit relevant recombination or they are clonal populations. The mating composition of *B. cinerea* populations will gain also insight on the possible influence of sexual reproduction on the transmission of resistance characters.

Finally, in Italy no informations were present on the genetic variability of the populations of *B. cinerea*. The genetic structure and diversity of the populations of *B. cinerea* isolated from different northern Italian vineyards was assessed by six microsatellite markers to verify the mode of reproduction and even the possible geographic or temporal differentiation among populations.

3. MATERIALS AND METHODS

3.1 Sites and sampling methods of *B. cinerea* strains

Samplings of diseased berries showing grey mould symptoms were carried out at harvest (September or October) in 36 vineyards located in the main viticultural districts of Lombardy, and precisely in the provinces of Sondrio, Brescia, Mantova and Pavia. In 2011 16 vineyards were sampled: 4 located in Oltrepo Pavese in the province of Pavia, 4 in Colli Mantovani and Basso Mantovano in the province of Mantova, 4 near the lake of Garda and in Franciacorta in the province of Brescia and 4 in Valtellina in the province of Sondrio. In 2012, 12 vineyards, 3 per province, were sampled. In 2013 8 vineyards were sampled: 6 located in Franciacorta, 1 in Valtellina and 1 in Oltrepo Pavese. All the vineyards were treated at least with an active substance against grey mould, with the exception of the Tirano vineyard (SO) sampled in 2011. Moreover treatments against *Plasmopara viticola* (Berk. *et* Curt.) Berlese and De Toni and *Erysiphe necator* Schw., the causal agents respectively of downy and powdery mildew, were carried out using fungicides listed in the Integrated Pest Management (IPM) protocols (Piano di Sviluppo Rurale 2007-2013, Regione Lombardia), with no side effects against *B. cinerea*. In order to have a comparison between years, Sirmione (BS), Chiuro (SO) and Torrazza Coste (PV) vineyards were sampled each year (Table 3.1).

Vineyard N°	Municipality	Cultivar	Treatments 2011	Treatments previous years	I%I
1	Rovizza di Sirmione (BS)	Trebbiano di Lugana	fenhexamid in B	Anilinopyrimidines twice per year for 7-8 years	5,4
2	Sirmione (BS)	Trebbiano	boscalid in B	cyprodinil + fludioxonil in B for 4 years	1,9
3	Adro (BS)	Cabernet sauvignon	pyrimethanil in A and B	pyrimethanil and cyprodinil + fludioxonil	<1
4	Corte Franca (BS)	Cabernet	pyrimethanil in C	AP in C	<1
5	Tirano (SO)	Chiavennasca	Untrated for two years	cyprodinil + fludioxonil until 2009	<1
6	Chiuro (SO)	Chiavennasca	cyprodinil + fludioxonil in D	2009 cyprodinil + fludioxonil in D and pyrimethanil in C; 2010 cyprodinil + fludioxonil in D	<1
7	S. Anna (SO)	Nebbiolo	fenhexamid in D	cyprodinil + fludioxonil in D for 6 years	<1
8	Dossi salati (SO)	Nebbiolo	boscalid in C; cyprodinil + fludioxonil in D	2007-2008 procimidone in B; 2009-2010 boscalid in B; 2007-2010 cyprodinil + fludioxonil in D	<1
9	Retorbido (PV)	Merlot	fenhexamid in B	fenhexamid in B for 4 years	5
10	Codevilla (PV)	Reisling Renano	pyrimethanil in C	2008 and 2010 cyprodinil + fludioxonil in B	31,9
11	Torrazza Coste	Barbera	iprodione in B; fluazinam in C	2005 procimidone in C; 2006 mepanypirimin B, procimidone in D; 2007-2008- 2010 mepanipyrim in C; 2010 iprodione in B, mepanypirim in C	7,9
12	Torrazza Coste	Merlot	iprodione in B; fluazinam in C	2005 procimidone in C; 2006 mepanypirimin B, procimidone in D;2007-2008-2010 mepanipyrim in C; 2010 iprodione in B, mepanypirim in C	6%
13	Monzambano Visconti (MN)	Merlot	pyrimethanil in C	2008-2010 anilinopyrimidines in A/B, fenhexamid in C/D	3,1
14	Cavriana (MN)	Merlot	cyprodinil + fludioxonil in B; fenhexamid in D (+ 2 boscalid)	2008-2010 AP in A /B, fenhexamid in C/D	<1
15	Gonzaga (MN)	Lambrusco	cyprodinil + fludioxonil in B	2008-2010 pyrimethanil / cyprodinil + fludioxonil in B	2
16	Mantova	Lambrusco viadanese	cyprodinil + fludioxonil in B and C	2008-2010 cyprodinil + fludioxonil in B and C	<1

Vineyard N°	Municipality	Cultivar	Treatments 2012	Treatments previous years	I%I
17	Torrazza Coste (PV)	Barbera	fluazinam in B	iprodione in B for 3 years, fluazinam in C for 2 years, mepanipyrim in C for 5 years	10
18	Sirmione (BS)	Trebbiano	thiohanate methyl in A, cyprodinil+fludioxonil in B, boscalid in C	cyprodinil+fludioxonil for 4 years, boscalid for 3 years	2
19	Pozzolengo (BS)	Trebbiano	boscalid in B, cyprodinil+fludioxonil in C	boscalid in A for 3 years, pyrimethanil in C for 7 years, cyprodinil+fludioxonil in B for 4 years	5
20	Adro (BS)	Cabernet	mepanipyrim in A and B	pyrimethanil in A and B, for 6-7 years	2
21	Torrazza Coste (PV)	Pinot	mepanipyrim in A, cyprodinil+fludioxonil in B	pyrimethanil in A, cyprodinil+fludioxonil in B for 3 years	2
22	Casteggio (PV)	Barbera	fluazinam in B	cyprodinil+fludioxonil for 2-3 years	30
23	Chiuro (SO)	Chiavennasca	cyprodinil+ fludioxonil in C	cyprodinil+fludioxonil in B/C for 3 years	2
24	Berbenno (SO)	Chiavennasca	pyrimethanil in C	Anilinopyrimidines in C for 3 years	25
25	Berbenno (SO)	Chiavennasca	pyrimethanil in C, cyprodinil+fludioxonil in D	pyrimethanil in C, cyprodinil+fludioxonil in D for 2 years	35
26	Gonzaga (MN)	Lambrusco	cyprodinil+fludioxonil in B	cyprodinil+fludioxonil in B for 4 years	10
27	Cavriana (MN)	Merlot	cyprodinil+fludioxonil in B	cyprodinil + fludioxonil in B, fenhexamid in D	50
28	Ponti sul Mincio (MN)	Garganega	cyprodinil+fludioxonil in B	cyprodinil+fludioxonil in B for 4 years	40

Vineyard N°	Municipality	Cultivar	Treatments 2013	Treatments previous years	I%I
29	Cazzago San Martino (BS)	Chardonnay B	cyprodinil; mepanipyrim	cyprodinil; mepanipyrim; fenhexamid	5,3
30	Rodengo Saiano (BS)	Chardonnay B	no one	cyprodinil + fludioxonil for 2 years	11,9
31	Erbusco (BS)	Chardonnay B	2 boscalid; cyprodinil + fludioxonil	boscalid; cyprodinil + fludioxonil; pyrimetanil	9,3
32	Cortefranca (BS)	Chardonnay B	2 boscalid; cyprodinil + fludioxonil	boscalid; ciprodinil + fludioxonil; pyrimethanil	5,4
33	Adro (BS)	Chardonnay B	boscalid	boscalid for 4 years; fenhexamid; cyprodinil + fludioxonil for 2 years	16,5
34	Sirmione (BS)	Trebbiano	pyrimethanil; boscalid	cyprodinil+fludioxonil for 5 years, boscalid for 4 years	1
35	Torrazza Coste (PV)	Barbera	fluazinam in B	iprodione in B for 3 years fluazinam in C for 3 years mepanipyrim in C for 5 years	1
36	Chiuro (SO)	Chiavennasca	cyprodinil+ fludioxonil in C	cyprodinil+fludioxonil in B/C for 4 years	1

Table 3.1: Sampled vineyards, their localization, previous and present treatments and percentage infection index (I%I) calculated according to the formula proposed by Townsend and Heuberger (1943). The untreated vineyard of Tirano, sampled in 2011, is highlighted in grey, while the three vineyards repeated over the years are in the black square. The letters beside the a.s. are referred to the phenological phase in which the treatment was carried out: A is the end of flowering, B is before the bunch closure, C is the veraison, D is before harvest.

Each vineyard was divided in four plots composed by three rows. Sampling of diseased berries was carried out in four subplots in the central row of each plot and at the four apexes of the vineyard (Figure 3.1). The samples were placed in sterile containers, kept at 4 °C and rapidly transported to the laboratory.

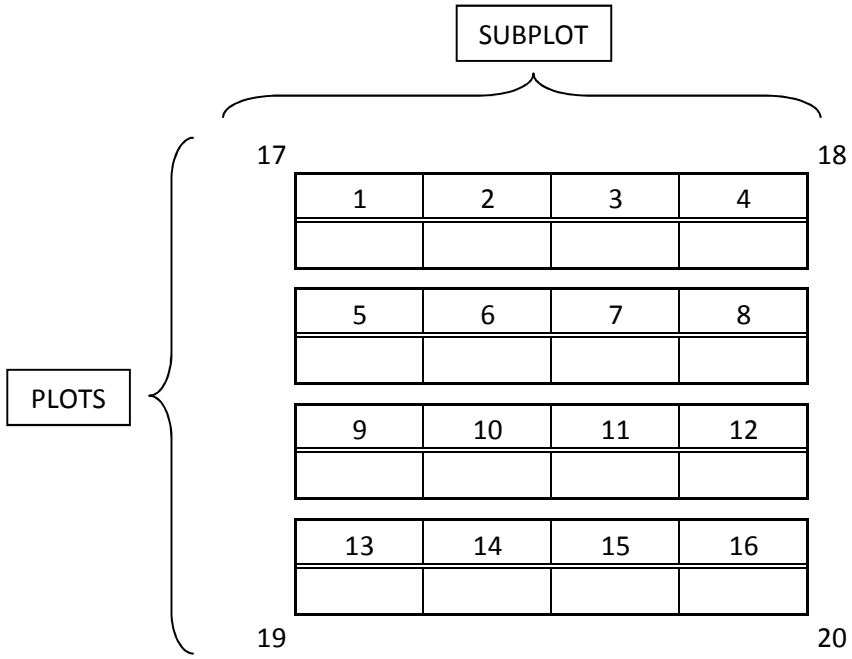


Figure 3.1: Plots and subplots scheme. The double line represents the sampled rows and the numbers represent the diseased berries sampled.

3.2. Isolation of monoconidial strains of *B. cinerea*

In order to obtain monoconidial *B. cinerea* strains, symptomatic samples showing abundant conidial production were processed immediately. Conidial tufts were collected from the infected plant material with a sterile loop and suspended in 1 mL solution containing sterile distilled water and 0.001% of surfactant (Tween). Afterwards, 100 µL of the corresponding conidial suspension were inoculated in Petri dishes of 9 cm in diameter containing 2 % water agar (WA, Bacto Agar, Difco®, Becton & Dickinson Company, Sparks, MD, USA) previously sterilized in autoclave at 1 atm for 20 minutes at 120 °C. The dishes were incubated for 12 hours at 20 °C in the dark and the germinated conidia were identified using an Orthoplan Leitz microscope (Ernst Leitz GmbH, Wetzlar, HEL, Germany) at 10x, collected with a sterilized knife and inoculated on Petri dishes (Ø 6 cm) containing Potato Dextrose Agar (PDA, Potato Dextrose Agar, Difco®) with a final pH of 5.6 and sterilized as previously described. All the operations were carried out in a laminar flow hood.

Twenty *B. cinerea* strains per vineyard were isolated for a total of 720 monoconidial strains (320 strains were isolated in 2011, 240 in 2012 and 160 in 2013).

For long term conservation, 10 agar plugs were collected from the sporulating culture of each monoconidial strain with a sterilized cork borer and put in a 2 ml sterile Eppendorf tube containing a water and glycerol (20%) solution and kept at -80 °C. For short term conservation, the same number of of agar-mycelium plugs was placed in a 2 ml sterile Eppendorf tube containing water and glycerol (20%) solution and kept at -20 °C.

3.3 Preparation and storage of *B. cinerea* conidial suspension

In order to perform the fungicide sensitivity assays, conidial suspension of *B. cinerea* strains were prepared. Conidia of *B. cinerea* were collected from 7 days old PDA cultures grown in the dark at 20 °C. The conidia were harvested using a sterilized loop and suspended in a sterile 1.5 mL Eppendorf tube (Eppendorf AG, Hamburg, DE) containing 1 mL of a sterile glycerol solution (20 %). Conidial concentration was assessed with a Kova counting grid (Hycor Biomedical Inc., Garden Grove, California, USA) and adjusted in order to obtain the concentration of $2x10^4$ conidia/ml using the Orthoplan Leitz microscope. Four replicates of the conidial suspension per each *B. cinerea* strain were stored at -20 °C.

3.4 Fungicide sensitivity assay of *B.cinerea* strains

The automated quantitative method proposed by Raposo and coworkers (1995) was used to perform the fungicide sensitivity assays. The active substances and the concentrations of each fungicide were chosen according to the data published by several authors (Bardas *et al*., 2010; Korolev *et al*., 2011, Billard *et al*., 2011; FRAC monitoring method, http://www.frac.info/) and are listed in the Table 3.2.

Active substances	Class	Concentrations (mg/L)							
Boscalid	Pyridine carboxamides	0	0.005	0.05	0.25	0.5	2.5	5	25
Fludioxonil	Phenylpyrroles	0	0.00025	0.0025	0.025	0.05	0.25	2.5	5
Fenhexamid	Hydroxyanilides	0	0.0005	0.005	0.025	0.05	0.25	0.5	5
Cyprodinil	Anilinopyrimidines	0	0.00025	0.0025	0.025	0.5	0.25	2.5	5

Table 3.2: Active substances, class and concentrations used in the sensitivity assays of *B. cinerea* strains.

The active substances were dissolved in different amounts of dimethylsulphoxide (DMSO, Carlo Erba Reagenti S.p.A., Rodano, Milano, Italy) in order to obtain a 0.3% (v/v) final concentration of DMSO in the growth medium utilized for the sensitivity assays. Previous studies demonstrated that 0.3% DMSO has no significant inhibitory effect on the fungal growth (Randhawa and Aljabre, 2007). For each fungicide a stock solution (10 g/L) was prepared and serially diluted in sterile, distilled water obtaining concentrations 2 times higher than those listed in table 3.2.

B. cinerea sensitivity to the chosen fungicides was tested in the minimal cultural medium (MCM) proposed by Myresiotis and coworker (2007). MCM was sterilized in autoclave for 30 minutes at 120 °C and stored at 4 °C.

Minimal cultural medium (MCM)

Glucose	10 g
K_2HPO_4	1.5 g
KH_2PO_4	2 g
$(NA_4)_2SO_4$	1 g
$MgSO_4 . 7H_2O$	0.5 g
Yeast exract Difco®	2 g
Distilled water	1 L

The medium utilized for the assays concerning the anilinopyrimidine cyprodinil was prepared omitting the yeast extract, since anilinopyrimidines inhibit the biosynthesis of methionine and some other aminoacids which are contained in the yeast extract (Masner *et al*., 1994). All the operations were carried out in a chemical hood.

Sensitivity assays were carried out in 96 wells microtiter plates (Sero-wel, Bibby Sterilin LTd, Stone UK) (Figure 3.2). 50 µL of conidia suspensions ($2x10^4$ conidia/ml), 50 µL of the active substances and the MCM were mixed in the wells of a microtiter plate by using an automated liquid handler (Ep-motion 5070, Eppendorf srl, Milano, Italy) in laminar flow hood. Two wells were used as replicates for each concentration of active substance and *B. cinerea* strain. In addition, all the strains were grown in two separate well in MCM not amended with fungicides and used as untreated controls (row A, Figure 3.2). The inoculated plates were incubated at 20 °C for 72 hours in the dark (Myresiotis *et al*., 2007).

FINAL CONCENTRATION OF FUNGICIDE		STRAIN 1		STRAIN 2		STRAIN 3		STRAIN 4		STRAIN 5		STRAIN 6	
		1	2	3	4	5	6	7	8	9	10	11	12
0	A												
0,005	B												
0,05	C												
0,25	D												
0,5	E												
2,5	F												
5	G												
25	H												

Figure 3.2: Scheme of the microtiter plate used for the *B. cinerea* sensitivity assays.

The absorbance of each well was measured with a spectrophotometer at 492 nanometers wavelength in a Sunrise™ Absorbance Reader (Tecan Group Ltd, Männerdorf, CH) immediately before and after the incubation period. The actual growth of each *B. cinerea* strain can be obtained by comparing the absorbance values assessed at the beginning and at the end of the incubation period. For each *B. cinerea* strain the growth inhibition percentage (GIP) which is the growth inhibition caused by each concentration of the active substances was calculated according to the following formula:

$$GIP = \frac{\left[\left(A_{t72} - A_{t0}\right)_{untreated}\right] - \left[\left(A_{t72} - A_{t0}\right)_{treated}\right]}{\left(A_{t72} - A_{t0}\right)_{untreated}} X100$$

where A_{t72} and A_{t0} are the absorbance of the treated or untreated control measured at the end and at the beginning of the incubation period respectively.

GIPs were used to calculate the EC_{50} which is the effective concentration of fungicide that reduces mycelial growth by 50%. The EC_{50} value was used to assess the sensitivity of *B. cinerea* strains towards different active substances. The EC_{50} values were compared with those available in literature and high EC_{50} values was attributed to a lower level of *B. cinerea* sensibility. The EC_{50} values were calculated through probit analysis (Finney, 1971), the most utilized and reliable methodology used in dose-response biological assays.

Finally the resistant factor (Rf) was calculated as the ratio between the EC_{50} of a single *B. cinerea* strain and the mean value of EC_{50} obtained for sensitive strains isolated in 2011 from the untreated vineyard of Tirano (SO). *B. cinerea* strains with resistance factor greater than 10 were considered resistant (Oliver and Hewitt, 2014).

3.5 Mycelial growth rate assays and phenotypic characterization of *B. cinerea* strains resistant to boscalid

The mycelial growth rate of 2012 and 2013 *B. cinerea* strains resistant to boscalid and the mycelial growth of some sensitive strains were assessed at three different temperatures (15, 20, 25 °C) on Malt Agar (MA, Oxoid Ltd., Basingstoke, UK).

Malt Agar (MA):

Malt extract Oxoid	15 g
Agar Oxoid n° 1	20 g
Distilled water	1 L

The *B. cinerea* strains were cultured on PDA for four days at 20 °C in the dark. Afterwards, mycelial plugs (Ø, 5 mm) of B*. cinerea* strains grown on PDA were excised with a sterile cork borer from the edges of the actively growing cultures and transferred to the center of MA dishes (Ø ,90 mm) for radial growth measurements (Martinez *et al*., 2003). MA dishes were incubated at 15 °C for 8 days in the dark and at 20 and 25 °C for 5 days in the dark. For each strain and cultural conditions combinations, three replicate plates were prepared and two perpendicular colony diameter per dishes and the mean colony diameter were measured.

B. cinerea colonies grown on MA at 20 °C were incubated for further two weeks. After the incubation period phenotypic observations concerning mycelia aspect, sporulation and scleriotial production were carried out for each colony.

The mycelium of each strains was classified in four categories:

- mycelial characterized by an evident sporulation (Figure 3.3 a);
- mycelial characterized by the presence of mycelial masses (Figure 3.3 b);
- mycelial characterized by the absence of sporulation (Figure 3.3 c);
- sclerotial, characterized by the production of sclerotia (Figure 3.3 d).

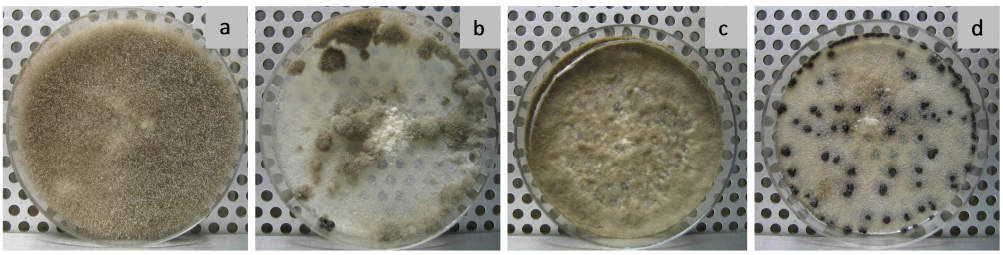


Fig.3.3: The main phenotypes of *B. cinerea* a) sporulated mycelia; b) mycelia with mycelial masses c) mycelia without sporulation and d) scelerotial.

3.6 Molecular characterization of *B. cinerea* strains

B. cinerea strains isolated in 2011, 2012 and 2013 were analyzed through molecular analyses. The detection of transposable elements was carried out also on four standard tester strains: *Boty* (I25), *Flipper* (C2), *vacuma* (PM10) and *transposa* (SAN4) kindly provided by prof. Francesco Faretra, Dipartimento di Scienze del Suolo, della pianta e degli Alimenti (DiPSSA), Università di Bari, Italy. The detection of the two mating types was carried out using, as controls, two reference strains, *MAT1-1* (SAS405) and *MAT 1-2* (SAS56), kindly provided by Dr Rita Milvia De Miccolis Angelini, Dipartimento di Scienze del Suolo, della pianta e degli Alimenti (DiPSSA), Università di Bari, Italy. All the used primers were synthesized by PRIMM (Milano, Italy).

3.6.1 DNA extraction and purification protocol

Genomic DNA was extracted using a modified extraction method developed by De Miccolis *et al.* (2010). The DNA was extracted from *B. cinerea* mycelium obtained from colony actively growing in Petri dishes (Ø, 90 mm) on 20 mL of Malt Extract Agar (MEA).

Malt Extract Agar (MEA)

Malt Extract Oxoid	20 g
Bacteriological Peptone Oxoid	1 g
Glucose	20 g
Agar Oxoid	20 g
Distilled water	1 L

The agar surface was covered with a sterile layer of Chellopane disk (BCL Cellophane, Bridgewater, UK) in order to facilitate the collection of the mycelium. Cellophane disks were previously boiled for 30 minutes in order to eliminate any plasticiser. After 72 hours of incubation at 20 °C in the dark, the mycelium was collected with a sterile loop and placed in a 2 mL sterile Eppendorf tube. The *B. cinerea* mycelium was lyophilized for 48-60 hours and then frozen with liquid nitrogen and pulverized with a sterile micropestle. Finally, 1 mL of CTAB buffer was added to all the samples.

CTAB buffer:

Tris HCl	1 M pH 8.0		
NaCl	1.4 M	EDTA (Ethylenediaminetetraacetic acid)	20 nM pH 8.0
2-mercaptoethanol	0.2 % (v/v)	CTAB (cetyltrimethyl ammonium bromide)	2 % (v/v)
dd H_2O			

The suspension was incubated at 65 °C for one hour and an half, then 660 µL chloroform:isoamyl alcohol 24:1 were added and the suspension was mixed for 15 minutes and then centrifuged for 12 minutes at 13,200 rpm. The supernatant was recovered in a 2 mL sterile Eppendorf tube and the suspension was added with 500 µL of CTAB buffer and 500 µL of chloroform:isoamyl alcohol 24:1. The suspension was mixed for 15 minutes and then centrifuged for 12 minutes at 13,200 rpm. The supernatant was recovered again in a 1,5 ml sterile Eppendorf tube. The suspension was added with 500 µL of isopropanol and the tubes were mixed by inversion six times. The DNA was then precipitated by centrifugation at 13,200 rpm for 12 minutes. The tube was then overturned to remove the supernatant and the precipitate was rinsed with 500 µL of 70 % ethanol pipetting delicately down the side of the tube. The suspension was centrifuged for 13 minutes at 12,300 rpm and then the supernatant was removed. The tubes were placed at 50 °C in order to let the ethanol evaporate and then the DNA was re-suspended in 200 µL of TE buffer incubated at 65 °C for 3 minutes.

TE buffer:

Tris HCl	10 nM
EDTA (Ethylenediaminetetraacetic acid)	1 nM pH 8

The quality and quantity of extracted DNA was evaluated using NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), diluted to a final concentration of 25 ng/µL and kept at -20 °C.

All chemicals were purchased from Sigma Aldrich (Milano, Italy).

3.6.2 Detection of transposable elements *Boty* and *Flipper*

The presence or absence of transposable elements (TE), *Boty* and *Flipper* was evaluated by PCR using two primer pairs: B_{1830}/B_{2800} for *Boty* (Topolovec-Pintarić *et al.*, 2004), and F_{300}/F_{1550} for *Flipper* (Giraud *et al*, 1999):

B_{1830}/B_{2800}: 5'-ATAAAGAAGCAACCGGATGG-3' / 5'-AGTCTATCGGGTCCATCCTT-3'

F_{300}/F_{1550}: 5'-GCACAAAACCTACAGAAGA-3' / 5'-ATTCGTTTCTTGGACTGTA-3'

Boty primer amplified a 970 bp sequence and *Flipper* a 1250 bp sequence.

Multiplex PCR reactions were carried out in an Eppendorf Mastercycler® ep (Eppendorf, Germany) in a 20 µL final volume containing:

- 2 x Thermo Scientific *Dreem Taq Greeg PCR Master Mix*	10 µL
- 0.1 µM primer *Boty* (forward and reverse)	0.3 µL
- 0.6 µM primer *Flipper* (forward and reverse)	1.8 µL
- fungal DNA	1 µL
- RNA free water	4.8 µL

PCR was performed using the following conditions: 4 initial cycles of denaturation at 95 °C for 3 minutes followed by 35 cycles at 94 °C for 1 minutes, annealing at 60 °C for 1 minutes and 72 °C for 1 minutes and 30 seconds, with a final extension at 72 °C for 5 minutes. Positive (the DNA of a *transposa* strain B^+/F^+) and negative (ddH_2O RNase free) controls were included in each experiment.

The PCR products were separated by electrophoresis on 1.5 % agarose gels in Tris-acetate buffer (TAE) for 120 minutes at 60 V, stained with 1 µg/mL Realsafe Nucleic Acid Staining Solution (20.000x) and visualized under UV light (Gel Doc 2000, BioRad Laboratories inc., Hercules, CA, USA) (Figure 3.3).

TAE buffer:

Tris-acetate	20 nM
EDTA (Ethylenediaminetetraacetic acid)	1 nM pH 8

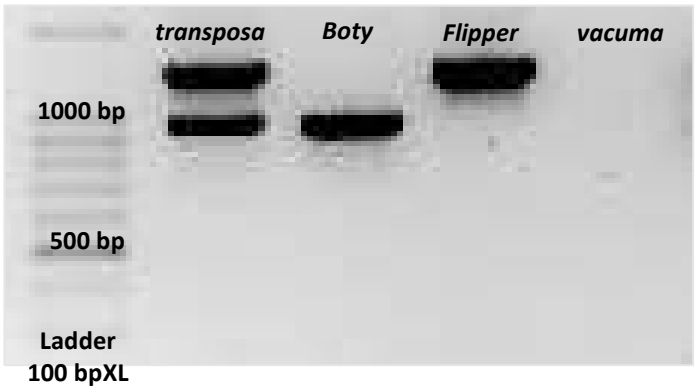


Figure 3.3 PCR products from genomic DNA of *B. cinerea* tester strains *transposa*, *Boty, Flipper* and *vacuma*.

3.6.3 Detection of mating type

The distribution of the mating types was detected using primers developed by Faretra and coworkers (personal communication).

The *MAT 1-1* primer pair amplified a 627 bp sequence and the *MAT 1-2* primer pair a 958 bp sequence. Multiplex PCR reactions were carried out in an Eppendorf Mastercycler in a 25 µL final volume containing:

- 2 x Thermo Scientific *Dreem Taq Greeg PCR Master Mix* 12.5 µL
- 0.1 µM primer *MAT 1-1* 1.2 µL
- 0.6 µM primer *MAT 1-2* 1.2 µL
- fungal DNA 1 µL
- RNA free water 6.7 µL

PCR was performed using the following conditions: an initial cycle of denaturation at 95 °C for 10 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60 °C for 45 seconds and 72 °C for 1 minutes, with a final extension at 72 °C for 2 minutes. Positive (the DNA of the *B. cinerea* strains SAS405 for *MAT1-1* and SAS56 for *MAT 1-2*) and negative controls (ddH_2O RNase free) were included in each experiment.

The PCR products were separated by electrophoresis on 1.5 % agarose gels in Tris-acetate buffer (TAE) for 120 minutes at 60 V, stained with 1 µg/mL Realsafe Nucleic Acid Staining Solution (20.000x) and visualized under UV light (Figure 3.4).

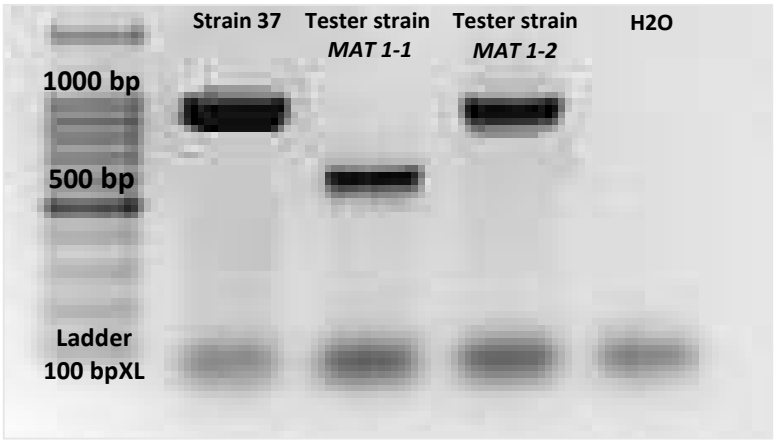


Figure 3.4 PCR products from genomic DNA of *B. cinerea* tester strains *MAT 1-1* and *MAT 1-2*.

3.6.4 Detection of the main point mutation in the SdhB subunit

The presence of the two main mutations linked with resistance to the SDHI boscalid (H272R or –Y) was assessed in 22 *B. cinerea* strains resistant to boscalid. 7 sensitive strains were used as negative controls. The primer pairs H272R-F/H272R-rev developed by Yin *et al*. (2011) and H272Y-fw/H272Y-rev, developed by Amiri *et al*. (2014) were used to amplify a fragment of the *sdhB* gene harboring respectively the B^{H272R} and B^{H272Y} mutations:

H272R-fw: 5'-AAGGAGGATCGTAAGAAGCTTG-3'

H272R-rev:5'TCGAGCAGTTGAGAATAGTGC-3'

H272Y-fw: 5'-ATCTTGCCCCTCCTACTGGT-3'

H272Y-rev:5'-CTCCAGCAGTTGAGAATAGTGTA-3'

The H272R-F/H272R-rev amplified a 232 bp sequence and the H272Y-fw/H272Y-rev a 170 bp sequence. The PCR reaction was carried out in an Eppendorf Mastercycler in a 25 µL final volume containing:

2 x Thermo Scientific *Dreem Taq Greeg PCR Master Mix*	12,5 µL
Primer fw	1 µL
Primer rev	1 µL
fungal DNA	2 µL
RNase free water	8,5 µL

PCR was performed using the following conditions: an initial cycle of denaturation at 95 °C for 3 minutes followed by 35 cycles of a second denaturation at 95°C for 40 seconds, annealing at 55 °C for 40 seconds and 72 °C for 1 minutes with a final extension at 72 °C for 5 minutes.

The PCR products were separated by electrophoresis on 2.5 % agarose gels in Tris-acetate buffer (TAE) for 85 minutes at 70 V, stained with 1 µg/ml Realsafe Nucleic Acid Staining Solution (20.000x) and visualized under UV light (Figure 3.5). Positive (the sequenced strain 361 isolated in Pozzolengo, BS in 2012 for the point mutation H272Y) and negative controls were included in each experiment.

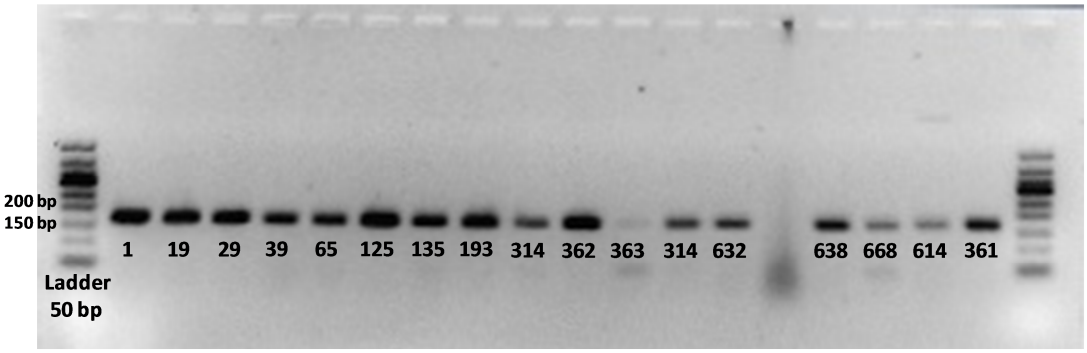


Figure 3.5 PCR products from genomic DNA of some *B. cinerea* strains carring the H272Y mutation.

3.6.5 Sequencing and bioinformatic analysis

In order to exactly confirm the presence of the mutation H272Y and the size of the amplicon, the product of the amplification of a resistant *B. cinerea* isolated strain (BC361), obtained using the primers H272Y rev / fw H272Y, was sequenced by the company Primm srl (Milan, Italy).

The sequence obtained was compared using the on-line software BLAST nucleotide with those present in the database (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

3.6.6 Microsatellite amplification

Six *B. cinerea* microsatellites loci described by Fournier and coworkers (2002) were analyzed (Bc2, Bc3, Bc5, Bc6, Bc7, Bc10) for a total of 317 strains, arbitrarily chosen from the populations. Loci Bc9 and Bc4 were excluded because Bc9 differed only 70 bp from Bc10 and Bc4 had low allele polymorphism. Thus, six different primer pairs were used to analyze the genetic structure of *B. cinerea* populations (Fournier *et al*., 2002). The microsatellite Bc6 carries a diagnostic allele (86 bp) for *B. pseudocinerea* (Fournier and Giraud 2008; Walker *et al*., 2011) and is also used to discriminate between the two species, *B. cinerea sensu stricto* and *B. pseudocinerea*.

The PCR reactions were carried out with forward primers conjugated with fluorescent dyes (Table 3.6.3).

Locus	Repeat motif	T*a* (°C)	Fluorescent dyes
Bc2	(AC)12 AT(AC)4	52	VIC
Bc3	(GA)10	54	6-FAM
Bc5	(AT)12	52	VIC
Bc6	(CA)10	54	6-FAM
Bc7	(TA)9	52	6-FAM
Bc10	(AC)13	54	NED

Table 3.6.3 Some characteristics of the six microsatellite loci: locus name, core repeat, annealing temperature (T*a*) and fluorescent dye used.

Four PCR amplifications for each strain were performed in an Eppendorf Mastercycler in a 12.5 µL final volume. Bc2 and Bc7 and Bc6 and Bc10 were multiplexed in a unique reaction composed of:

- Amplitaq Gold 1000U 0.0625 µL
- Buffer II 1.25 µL
- $MgCl_2$ 0.85 µL
- dNTP 2 µL
- 0.3 µM primer Bc forward 0.75 µL
- 0.3 µM primer Bc reverse 0.75 µL
- 0.3 µM primer Bc forward 0.75 µL
- 0.3 µM primer Bc reverse 0.75 µL
- 20 ng of fungal DNA 1 µL
- RNA free water 4.3375 µL

The PCR reactions with microsatellite Bc3 and Bc5 were performed in two different reactions in a 12.5 µL final volume, composed of:

- Amplitaq Gold 1000U 0.0625 µL
- Buffer II 1.25 µL
- $MgCl_2$ 0.85
- dNTP 2 µL
- 0.3 µM primer forward (0.1 µM for Bc 1) 1.5 µL
- 0.3 µM primer reverse (0.1 µM for Bc 1) 1.5 µL
- 50 ng of fungal DNA 1 µL
- RNA free water 5.8375 µL

PCR was achieved using the following conditions: an initial cycle of denaturation at 95 °C for 10 minutes followed by 40 cycles of a second denaturation at 95°C for 15 seconds, annealing for 45 seconds and 72 °C for 1 minutes with a final extension at 72 °C for 2 minutes. The temperature of annealing was 52 °C for Bc 2-5-7 and 54 °C for Bc 3-6-10.

Fragment size analysis was performed in an automated single capillary genetic analyzer 3730xl DNA analyzer (Applied Biosystems®).

3.6.7 Data analyses

The SPSS statistical package for Windows, Version 22.0 (SPSS Inc., Chigago, IL, US), was used for all the statistical analyses. The normal distribution and homogeneity of variances of means of quantitative variables were verified using respectively the Shapiro-Wilk test and the Levene's test.

The EC_{50} values were calculated through probit analysis (Finney, 1971), the most utilized and reliable methodology used in dose-response biological assays. The distribution of EC_{50}, differences among them and the distribution of EC_{50} in relation to the number of treatment carried out in vineyards were visualized by box plot graphs.

Differences between the averages values of EC_{50} among years, provinces and vineyards and the growth rate were analyzed by non parametric analysis of variance by ranks, the Kruskal-Wallis test, because the normal distribution of these data could not be assumed. Differences between temperatures of sensitive/resistant strains to boscalid were tested with a Mann Whitney non parametric U test.

Friedman two way analysis of variance was used to test the effects of year and site (province) on the EC_{50} related to the four tested fungicides.

The differences among frequencies of the four molecular classes of transposons (*vacuma*, *transposa*, *Boty* and *Flipper*) and of the two mating types (*MAT1-1* and *MAT1-2*) within *B. cinerea* populations isolated in different provinces, vineyards and years were analyzed using χ^2. In all cases, the differences were considered significant at $P < 0.05$.

Population genetic analysis

B. cinerea strains with the same alleles at all loci were considered to be clones of the same multilocus genotype (MLG). To estimate the contribution of asexual reproduction to the epidemic, the clonal fraction was calculated for each and the total population. The clonal fraction was calculated as the occurrence and frequency of clones within a population, ((N−G)/N), where N is the sample size and G is the number of MLGs present. Genotypic diversity, the probability that two individuals taken at random have unique MLG, was estimated for each population with Multilocus Ver 1.3b (Agapow and Burt, 2001) according to the following formula:

$$\frac{n}{n-1}\left(1-\sum_i p_i^2\right)$$

where p_i is the frequency of the i^{th} multilocus genotype and n is the sample size for each population (Stoddart and Taylor, 1988; McDonald *et al.,* 1994). This value is 0 when all individuals are the same, and 1 when all individuals are different.

The gene flow was calculated as the average number of individuals migrating (Nm) between populations per generation (Wright 1951).

Allele frequencies, unbiased gene diversity (h) and gene flow were calculated for all loci with Genalex 6.501 (Peakall and Smouse, 2006).

Analiysis for random mating

Mating-type distributions of uncorrected and clone-corrected samples were tested for deviation from expected ratios of 1:1 using chi-square goodness-of-fit tests.

The degree of association among alleles within a population was calculated through the index of association I_A ($I_A = (V_O/V_E - 1)$ which is a generalized measure for linkage disequilibrium (Brown *et al*., 1980; Haubold *et al.,* 1998; Maynard-Smith *et al.,* 2006) and the standardized index of association, $\bar{r}_d$ (Agapow and Burt, 2001; Giraud *et al*., 2006), on subset of uncorrected and clone-corrected data using Multilocus v.1.3b. These indexes give information on whether two different individuals sharing an allele at one locus are more likely to share an allele at another (Karchani-

Balma *et al*., 2008). The $\bar{r}_d$ is independent of the number of loci considered, and varies between 0 (complete panmixia) and 1 (no recombination). *P* values were estimated by 1.000 random permutations of the data. I_A is not significantly different from 0 when the population is in linkage equilibrium thus the null hypothesis could be rejected. Moreover, the estimates of I_A and $\bar{r}_d$ are higher in populations with a high frequency of clones.

Population structure analysis

Nei's genetic identity (*I*) (Nei, 1972) and genetic differentiation (Φ_{PT}, an analog of F_{ST} measured via analysis of molecular variance) (Peakall *et al.,* 1995) were determined among *B. cinerea* populations samples using GenAlEx v.6.41 on full data sets. Significant differentiation between populations was determined by comparing observed values to the distribution of 1000 randomizations of the data. Principal coordinates analysis (PCoA) was carried out using GenAlEx v.6.501 on pairwise genetic distances between all genotypes in order to visualize the major patterns of variation within and among populations. Nei genetic identity is the normalized identity of genes between two populations and varies between 0 (the compared populations are different), and 1 (the compared populations are identical).

The existence of a population structure in the total sample was further investigated using the Bayesian approach implemented in Structure Version 2.3.4 (Pritchard *et al*., 2000; Falush *et al*., 2003). This clustering algorithm assumes a model in which there are populations (where *K* may be unknown), each of which is characterized by a set of allele frequencies at each locus. Individuals in the sample are assigned probabilistically to these *K* populations, or jointly to two or more populations if their genotypes indicate that they are admixed, without consideration of their region or year of sampling. Loci are assumed at linkage equilibrium within the *K* populations, but this approach proved to be robust to deviations from these assumptions (Falush *et al*., 2003). The model with admixture, recommended when little is known about the existence of admixture (Falush *et al*., 2003) was used in this study. *K* varied from 1 to 8, each with 10 independent simulations to check the consistency of the results. Each simulation consisted in 1 000 000 Monte-Carlo Markov Chain (MCMC) iterations preceded by a burn-in period of 500 000 iterations. According to Evanno *et al*. (2005) the best estimation of *K* was that associated with highest ΔK, an ad hoc quantity related to the second order rate of change of the log probability (likelihood) of the data and was calculated by Structure Harvester (Earl and vonHoldt, 2012).

Finally on the genotyped *B. cinerea* strains, dendrograms were drawn using Mega4 (Tamura *et al.*, 2007) under the clustering rule of the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm.

4 RESULTS

4.1 *B. cinerea* strains

During three consecutive years 720 monoconidial *B. cinerea* strains were isolated at harvest from 36 vineyards located in Lombardy: 320 strains were isolated in 2011, 240 in 2012 and 160 in 2013 (Table 4.1.1). Overall, 260 strains were isolated from the province of Brescia, 160 from the provinces of Sondrio and Pavia and 140 from the province of Mantova.

	Year		
N° of strains per province	2011	2012	2013
Brescia	80	60	120
Sondrio	80	60	20
Pavia	80	60	20
Mantova	80	60	
		Total	720

Table 4.1.1: Number of *B. cinerea* strains isolated over three years of study in the main viticultural provinces of Lombardy.

4.2 Fungicide sensitivity assay

The growth inhibition percentage of *B. cinerea* strains showed always a proportional increase associated with the increasing fungicide concentrations so in the next paragraphs only the results related to the EC_{50} are reported.

4.2.1 First year of sampling, 2011

In 2011, 281 strains were analyzed for sensitivity to boscalid, 279 to fenhexamid, 269 to cyprodinil and 280 to fludioxonil. The average EC_{50} values of the 2011 untreated vineyard of Tirano (SO) was used to calculated the Rf of all the strains (average EC_{50} of boscalid=0.242; average EC_{50} of cyprodinil=0.617; average EC_{50} of fenhexamid=0.098; average EC_{50} of fludioxonil=0.023).

Boscalid

The EC_{50}s concerning boscalid in each vineyard were characterized by low average values, varying from 0.07 and 1.06 mg/L of active substance (Table 4.2.1). Significant differences were found among the median values of EC_{50} of the vineyards according to the Kruskal Wallis test (H=37.15; P<0.001; df=15) (Table 4.2.1). The vineyards located in Sirmione (BS) and Dossi salati

(SO), treated with boscalid in 2011, showed a significantly different EC_{50} distribution compared to that found in Mantova (MN), not treated with boscalid.

Vineyard	EC_{50}	KW*	Vineyard	EC_{50}	KW*
1 Rovizza di Sirmione (BS)	0.140	abc	**9 Retorbido (PV)**	0.129	abc
2 Sirmione (BS)	0.295	c	**10 Codevilla (PV)**	0.621	abc
3 Adro (BS)	0.257	bc	**11 Torrazza Coste (PV) cv Barbera**	0.608	abc
4 Corte Franca (BS)	0.154	abc	**12 Torrazza Coste (PV) cv Merlot**	0.069	ab
5 Tirano (SO)	0.242	abc	**13 Monzambano Visconti (MN)**	0.095	abc
6 Chiuro (SO)	1.064	abc	**14 Cavriana (MN)**	0.212	abc
7 S. Anna (SO)	0.177	abc	**15 Gonzaga (MN)**	0.561	abc
8 Dossi salati (SO)	0.473	c	**16Mantova (MN)**	0.076	a

Table 4.2.1: Average values of EC_{50} concerning boscalid, 2011. *Different letters correspond to significant differences among the EC_{50} medians according to Kruskal Wallis test.

The distribution of the EC_{50} for boscalid associated with the examined *B. cinerea* strains ranged from 0.0001 and 0.9 mg/L (Table 4.1.1, Figure 4.2.1). Several outliers showed EC_{50} higher than 0.9 mg/L but only 7 strains were characterized by Rf higher than 10. These *B. cinerea* strains were isolated in:

- Codevilla (PV): strains BC1 (EC_{50}= 5 mg/L; Rf= 20.9) and BC19 (EC_{50}= 4.3 mg/L; Rf=17.8);
- Torrazza Coste cv barbera (PV): strains BC29 (EC_{50}= 4.6 mg/L; Rf= 19.2) and BC39 (EC_{50}= 3.4 mg/L; Rf=14.1);
- Gonzaga (MN): strains BC125 (EC_{50}= 5 mg/L; Rf= 20.8) and BC135 (EC_{50}= 3.7 mg/L; Rf=15.3);
- Chiuro (SO): strain BC314, characterized by a particularly high Rf (Rf=63.8) due to an EC_{50} of 15.2 mg/L (Figure 4.2.1).

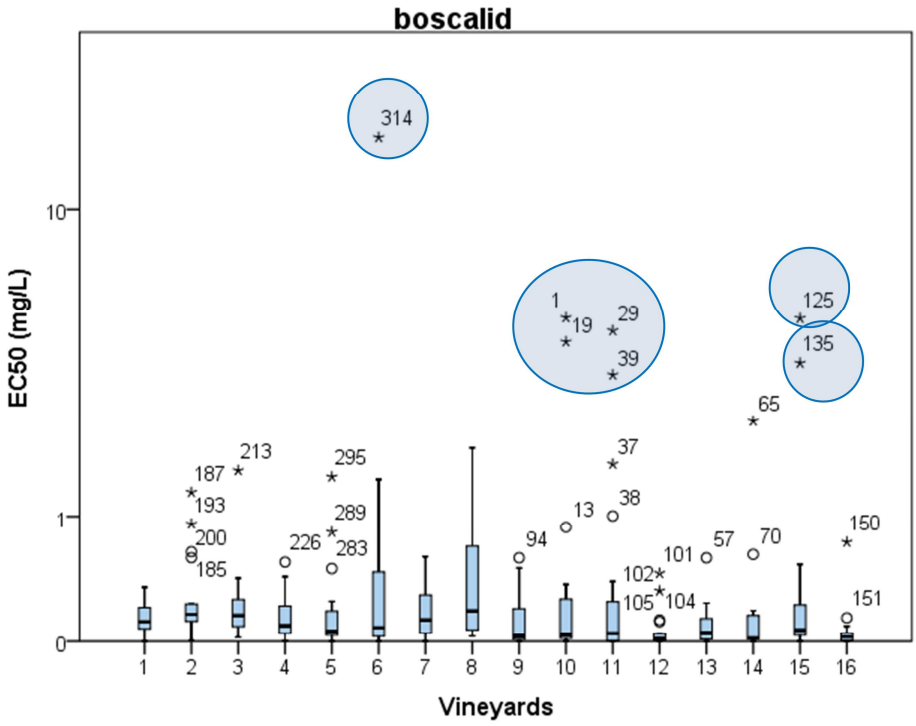


Figure 4.2.1: Distribution of boscalid EC_{50}s assessed for *B. cinerea* strains isolated in 2011 in different vineyards. The asterisks and the circles represent the outlier strains. The blue circles indicate the strains characterized by Rf >10.

The EC_{50}s of the strains isolated from vineyards treated with boscalid showed a slightly wider distribution and a higher median value than those observed for the vineyards were boscalid has non been applied during grapevine growing season(Figure 4.2.2). However, numerous outliers with high resistance factors were found in the latter case. Therefore it seems likely that changes in sensitivity can not be explained solely by the usage of the fungicide in vineyard in a single grapevine growing season.

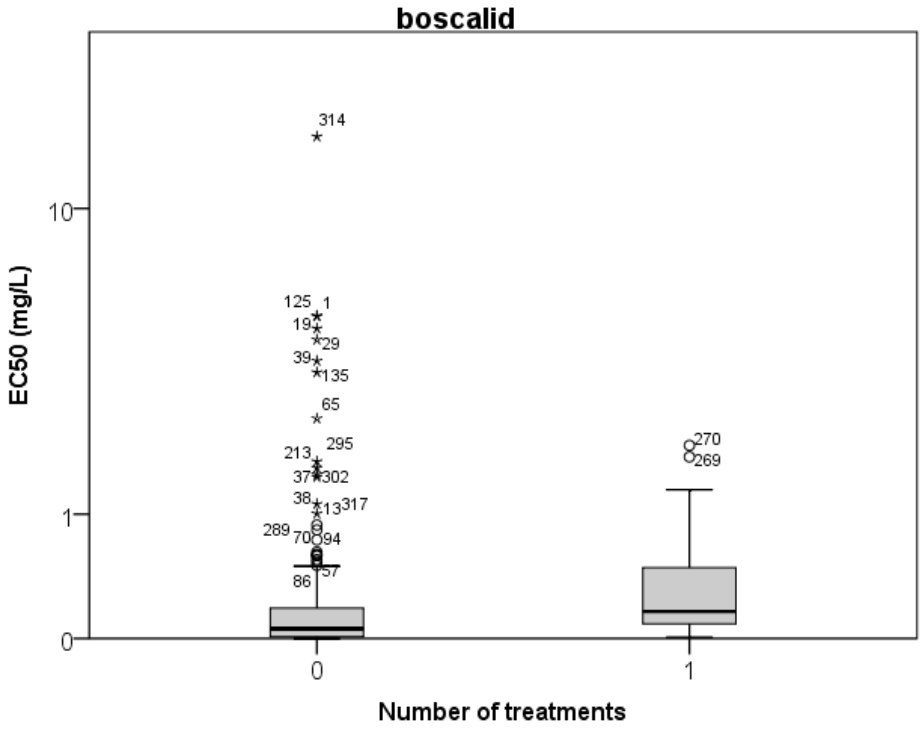


Figure 4.2.2: Distribution of boscalid EC_{50} related to the number of treatments carried out in 2011 with the same active substance. The asterisks and the circles represent the outlier strains.

Fenhexamid

In the sampled vineyards the EC_{50}s of *B. cinerea* strains related to fenhexamid were characterized by low average values, ranging between 0.014 and 0.110 mg/L of active substance. Highly significant differences were found among the EC_{50} median values of the sampled vineyards according to the Kruskal Wallis test (H=54.47; P<0.0001; df=15) (Table 4.2.2). The EC_{50} distributions of the vineyards located in Tirano (SO), Chiuro (SO) and Retorbido (PV) were significantly different from those of the vineyards located in Codevilla (PV), Torrazza Coste (PV) cv Barbera and Monzambano Visconti (MN).

Vineyard	EC_{50}	KW*	Vineyard	EC_{50}	KW*
1 Rovizza di Sirmione (BS)	0.052	bcdef	**9 Retorbido (PV)**	0.110	ef
2 Sirmione (BS)	0.061	cdef	**10 Codevilla (PV)**	0.014	a
3 Adro (BS)	0.055	bcdef	**11 Torrazza Coste (PV) cv Barbera**	0.021	ab
4 Corte Franca (BS)	0.027	abcdef	**12 Torrazza Coste (PV) cv Merlot**	0.028	abcd
5 Tirano (SO)	0.098	def	**13 Monzambano Visconti (MN)**	0.021	abc
6 Chiuro (SO)	0.088	f	**14 Cavriana (MN)**	0.041	abcd
7 S. Anna (SO)	0.039	bcdef	**15 Gonzaga (MN)**	0.086	def
8 Dossi salati (SO)	0.106	bcdef	**16 Mantova (MN)**	0.044	bcdef

Table 4.2.2: Average values of EC_{50} concerning fenhexamid. *Different letters correspond to significant differences among the EC_{50} medians according to Kruskal Wallis test.

In many vineyards, *B. cinerea* strains showed very low EC_{50} values ranging from 0.0001 and 0.05 mg/L. None of the numerous outlier strains showed a Rf greater than 10, suggesting that only sensitive strains were present in the sampled vineyards. The average EC_{50} value reached its maximum value, 0.11 mg/L, in Retorbido (PV), treated with fenhexamid in 2011 and in the previous 4 years.

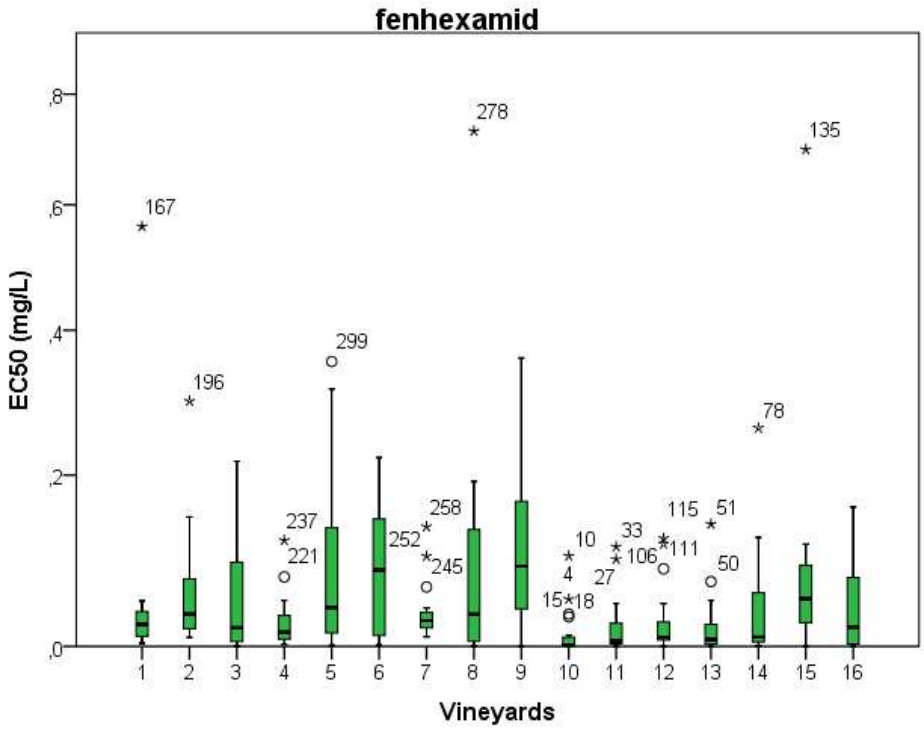


Figure 4.2.3: Ditribution of fenhexamid EC_{50} assessed for the *B. cinerea* strains isolated in 2011 in different vineyards. The asterisks and the circles represent the outlier strains.

No differences were found in the distribution of the EC_{50} of the strains isolated from treated (1 treatment) and untreated vineyards with fenhexamid during the current vegetative season (Figure 4.2.4).

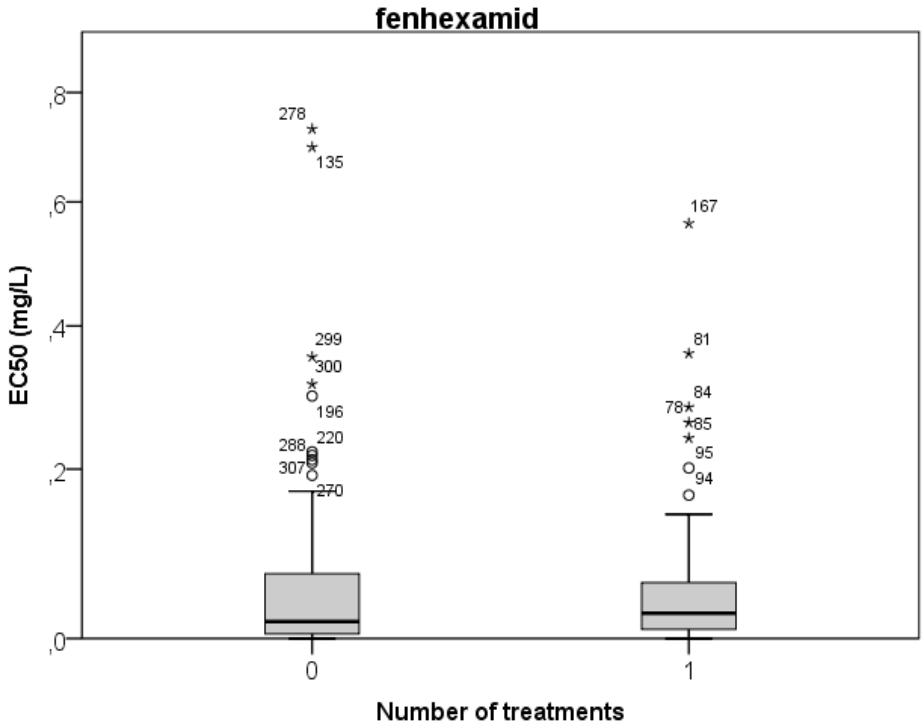


Figure 4.2.4: Distribution of fenhexamid EC_{50} related to the number of treatments carried out in the year 2011 with the same active substance. The asterisks and the circles represent the outlier strains.

Cyprodinil

The average EC_{50} calculated for the sampled vineyards showed values ranging from 0.051 and 0.6 mg/L, with the exception of 5 vineyards (Adro, Codevilla, Torrazza Coste cv Barbera and cv Merlot and Gonzaga) characterized by EC_{50} values higher than 1. No significant differences were found among the median values of EC_{50} of the vineyards according to the Kruskal Wallis test (Table 4.2.3).

Vineyard	EC_{50}	Vineyard	EC_{50}
1 Rovizza di Sirmione (BS)	0.226	**9 Retorbido (PV)**	0.051
2 Sirmione (BS)	0.563	**10 Codevilla (PV)**	42.397
3 Adro (BS)	4.061	**11 Torrazza Coste (PV) cv Barbera**	323.465
4 Corte Franca (BS)	0.107	**12 Torrazza Coste (PV) cv Merlot**	49.849
5 Tirano (SO)	0.617	**13 Monzambano Visconti (MN)**	0.593
6 Chiuro (SO)	0.236	**14 Cavriana (MN)**	0.173
7 S. Anna (SO)	0.064	**15 Gonzaga (MN)**	1.327
8 Dossi salati (SO)	0.894	**16 Mantova (MN)**	0.404

Table 4.2.3: Average values of EC_{50} related to cyprodinil concerning *B. cinerea* strains isolated in the different vineyards

The high EC_{50} average value found in Adro, Codevilla and Gonzaga were due to some outlier *B. cinerea* strains, that were characterized by EC_{50}s placed out of the normal data distribution obtained in the vineyard. The EC_{50}s assessed for the isolated strains varied, in most cases, between 0.003 and 0.5 mg/L, reaching 1.5 only in the two vineyards of Torrazza Coste (PV). Among the numerous outliers, 12 *B. cinerea* strains showed a Rf greater than 10 and were isolated in (Figure 4.2.5):

- Codevilla (PV): strain BC19 (EC_{50}=717 mg/L; Rf=1163) isolated;
- Torrazza Coste cv barbera(PV): strains BC23 (EC_{50}=4831 mg/L; Rf=7836), BC29 (EC_{50}=13.9 mg/L; Rf=22.6), BC102 (EC_{50}=718 mg/L; Rf=1165) and BC110 (EC_{50}=74 mg/L; Rf=120);
- Gonzaga (MN): strains BC123 (EC_{50}=9.3 mg/L; FR=15) and BC130 (EC_{50}=10.5 mg/L; Rf=17);
- Sirmione (BS): strain BC198 (EC_{50}=8.9 mg/L; Rf=14,4);
- Adro (BS): strains BC204 (EC_{50}=12.4 mg/L; Rf=20,2) and BC218 (EC_{50}=51.4 mg/L; Rf=83);
- Dossi Salati (SO): strain BC269 (EC_{50}=12 mg/L; Rf=19.7);
- Tirano (SO): strain BC299 (EC_{50}=7 mG/L; Rf=11).

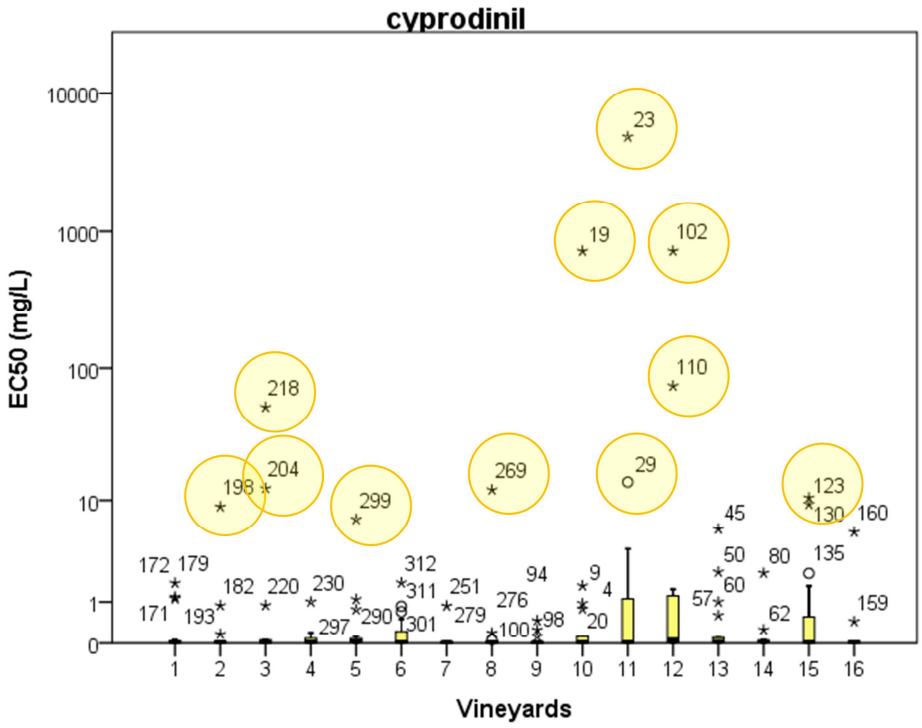


Figure 4.2.5: Distribution of cyprodinil EC_{50} assessed for the *B. cinerea* strains isolated in 2011 in different vineyards. The asterisks and the circles represent the outlier strains. The yellow circles indicate the strains with Rf > 10.

Also for cyprodinil, the number of treatments carried out in vineyard in 2011 using anilinopyrimidines was not associated with a reduced sensitivity. Thus, the distributions of EC_{50}s were similar in untreated vineyards and in those treated two times per year with anilinopyrimidine fungicides (Figure 4.2.6). .

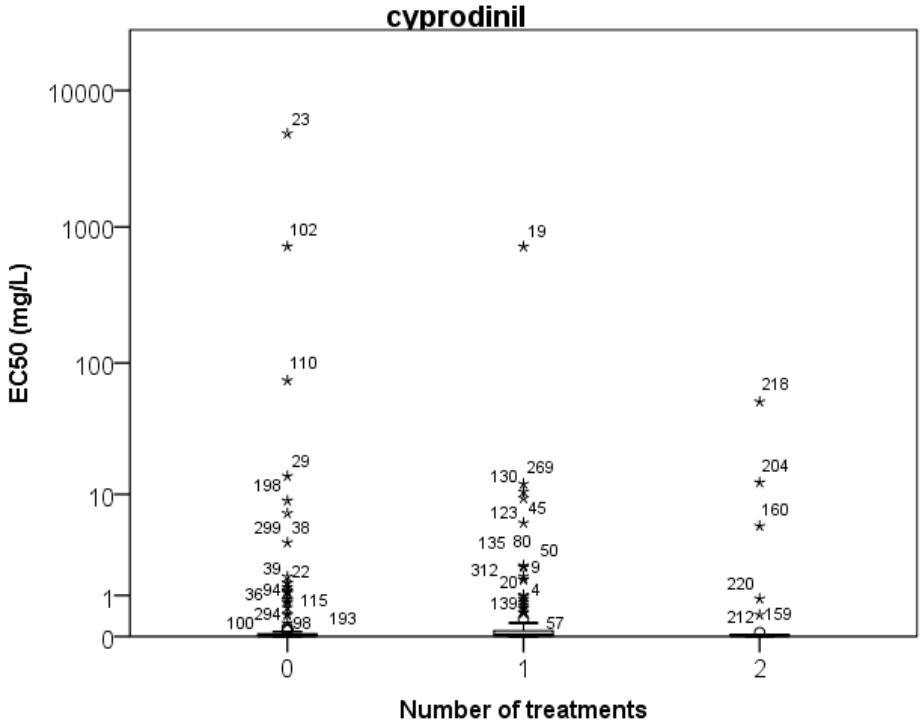


Figure 4.2.6: Distribution of cyprodinil EC_{50} in relation to the number of treatments carried out in the year 2011 with the same active substance. The asterisks and the circles represent the outlier strains.

Fludioxonil

B. cinerea strains isolated in 2011 were characterized by reduced EC_{50} values towards fludioxonil. The average EC_{50} in the selected vineyards ranged from 0.005 to 0.045 mg/L, with the exception of the vineyard located in Torrazza Coste cv Barbera (PV), characterized by an average EC_{50} value of 0.71 mg/L (Table 4.2.4). Such a value was due to the EC_{50} of 4 *B. cinerea* strains showing Rf greater than 10: strain BC21 (EC_{50}=3.46 mg/L; Rf=150), strain BC34 (EC_{50}=1.8 mg/L; Rf=78.5), strain BC37 (EC_{50}=0.97 mg/L, Rf=42) and the strain BC40 (EC_{50}=7 mg/L; Rf=305) (Figure 4.8). Significant differences were found among the median values of EC_{50} obtained in each vineyard according to the Kruskal Wallis test (H=38.1; P<0.001; df=15) (Table 4.2.4). In the majority of the vineyards, the distribution of EC_{50}s showed reduced values, ranging from 0.0001 to 0.05 mg/L. On the contrary, in Chiuro (SO), Dossi Salati (SO), Torrazza Coste (PV) cv Merlot, Gonzaga (MN) and Mantova (MN) vineyards, the EC_{50} upper limit was 0.15 mg/L (Figure 4.2.7).

Vineyard	EC_{50}	KW*	Vineyard	EC_{50}	KW*
1 Rovizza di Sirmione (BS)	0.013	abc	**9 Retorbido (PV)**	0.024	abc
2 Sirmione (BS)	0.013	abc	**10 Codevilla (PV)**	0.017	abc
3 Adro (BS)	0.019	abc	**11 Torrazza Coste (PV) cv Barbera**	0.709	abc
4 Corte Franca (BS)	0.010	a	**12 Torrazza Coste (PV) cv Merlot**	0.024	abc
5 Tirano (SO)	0.023	abc	**13 Monzambano Visconti (MN)**	0.037	ab
6 Chiuro (SO)	0.045	bc	**14 Cavriana (MN)**	0.025	abc
7 S. Anna (SO)	0.005	a	**15 Gonzaga (MN)**	0.019	abc
8 Dossi salati (SO)	0.021	abc	**16 Mantova (MN)**	0.051	c

Table 4.2.4: Average values of EC_{50} concerning fludioxonil, 2011. *Different letters correspond to significant differences among the EC_{50} medians according to Kruskal Wallis test.

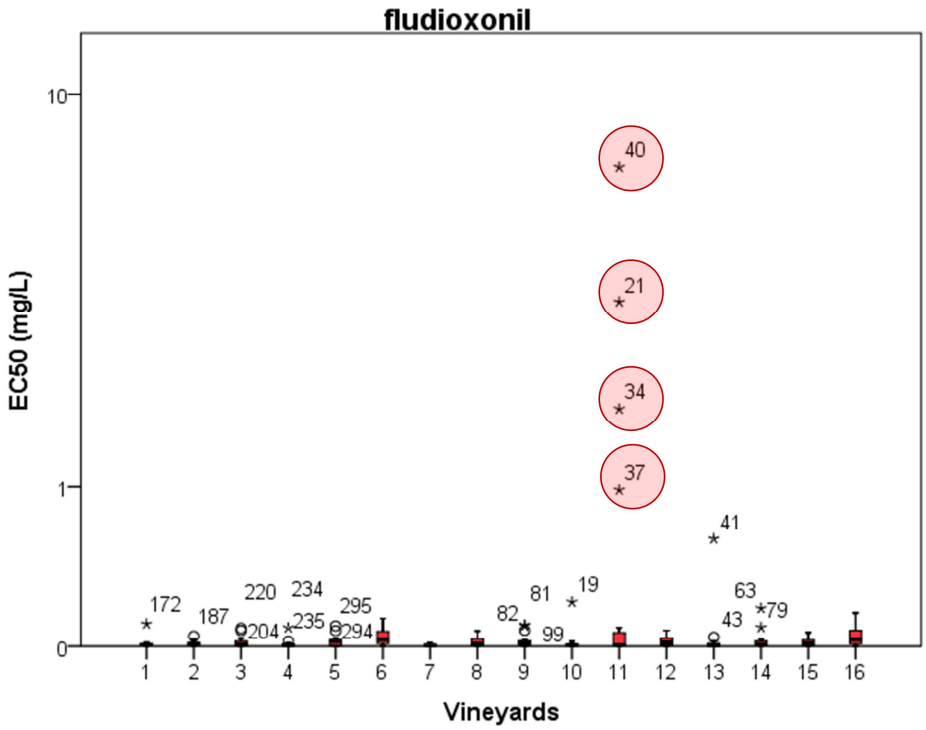


Figure 4.2.7: Distribution of fludioxonl EC_{50} assessed for the *B. cinerea* strains isolated in 2011 in different vineyards. The asterisks and the circles represent the outlier strains. The red circles indicate the strains with Rf > 10.

The distribution of EC_{50}s concerning the *B. cinerea* strains isolated in vineyards treated with fludioxonil in mixture with cyprodinil was wider than those obtained for the strains sampled in untreated vineyards and shows an increasing trend related with the number of treatments carried

out in a single year (Figure 4.2.8). Nevertheless, the four strain with Rf greater than 10 were isolated in an untreated vineyard.

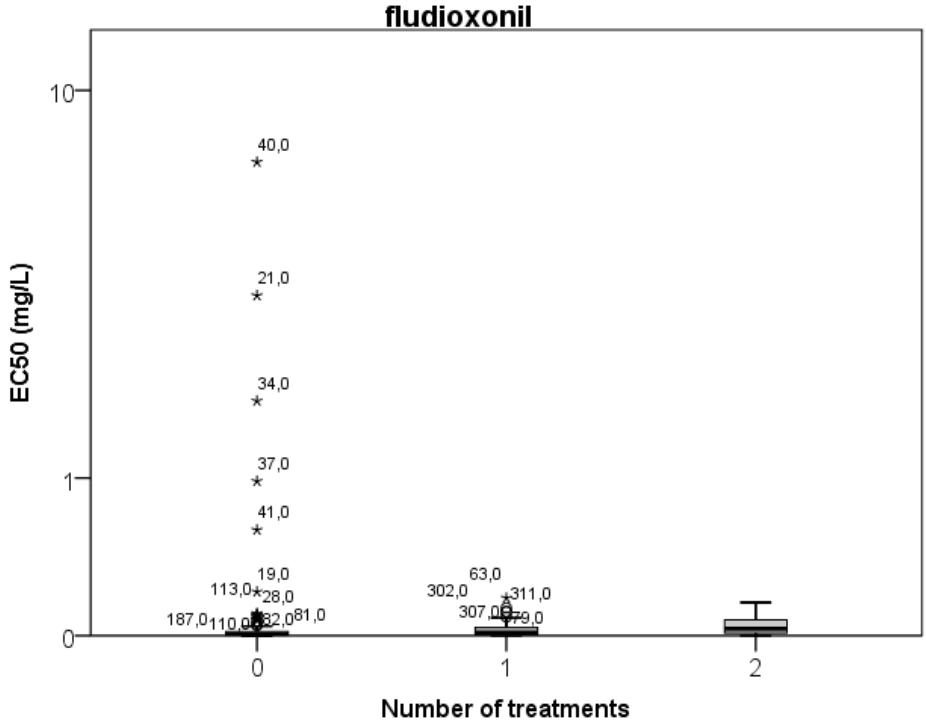


Figure 4.2.8: Distribution of EC_{50} concerning fludioxonil in function of the number of treatments carried out in the year 2011 with the same active substance. The asterisks and the circles represent the outlier strains.

4.2.2 Second year of sampling, 2012

During the second year of sampling, sensitivity assays were carried out on 191 strains for boscalid, 192 for fenhexamid, 185 for cyprodinil and finally 196 for fludioxonil sensitivity.

Boscalid

The EC_{50}s concerning boscalid detected in the 12 vineyards sampled in 2012 were characterized by reduced values and variability (Table 4.2.5, Figure 4.2.9). The average values observed in each vineyard ranged from 0.035 to 1.81 mg/L. No significant differences were found among the EC_{50} values of the vineyards according to the Kruskal Wallis test (H=12.82; P=0.305; df=11).

Vineyard	EC_{50}	Vineyard	EC_{50}
17 Pozzolengo (BS)	1.811	**23 Torrazza Coste (PV)**	0.076
18 Sirmione (BS)	0.047	**24 Torrazza Coste (PV)**	0.097
19 Adro (BS)	0.258	**25 Casteggio (PV)**	0.083
20 Berbenno (SO)	0.042	**26 Gonzaga (MN)**	0.065
21 Berbenno (SO)	0.057	**27 Ponti sul Mincio (MN)**	0.035
22 Chiuro (SO)	0.073	**28 Cavriana (MN)**	0.036

Table 4.2.5: Average values of EC_{50} concerning boscalid, 2012 assessed in each vineyard

The EC_{50} values assessed for the *B. cinerea* strains ranged from a minimum value of 0.00001 to a maximum of 0.63 mg/L of active substance. Only 6 of the numerous strains characterized by an EC_{50} greater than 0.9 mg/L showed a Rf > 10 and were isolated in:

- Pozzolengo (BS): strains BC361 (EC_{50}=5.8 mg/L; Rf=24), BC362 (EC_{50}=7.4 mg/L; Rf=31), BC363 (EC_{50}=4.3 mg/L; Rf=18), BC377 (EC_{50}=6.7 mg/L; Rf=28) and BC379 (EC_{50}=2.4 mg/L; Rf=10);
- Adro (BS): strain BC384 (EC_{50}=2.7 mg/L; Rf=11).

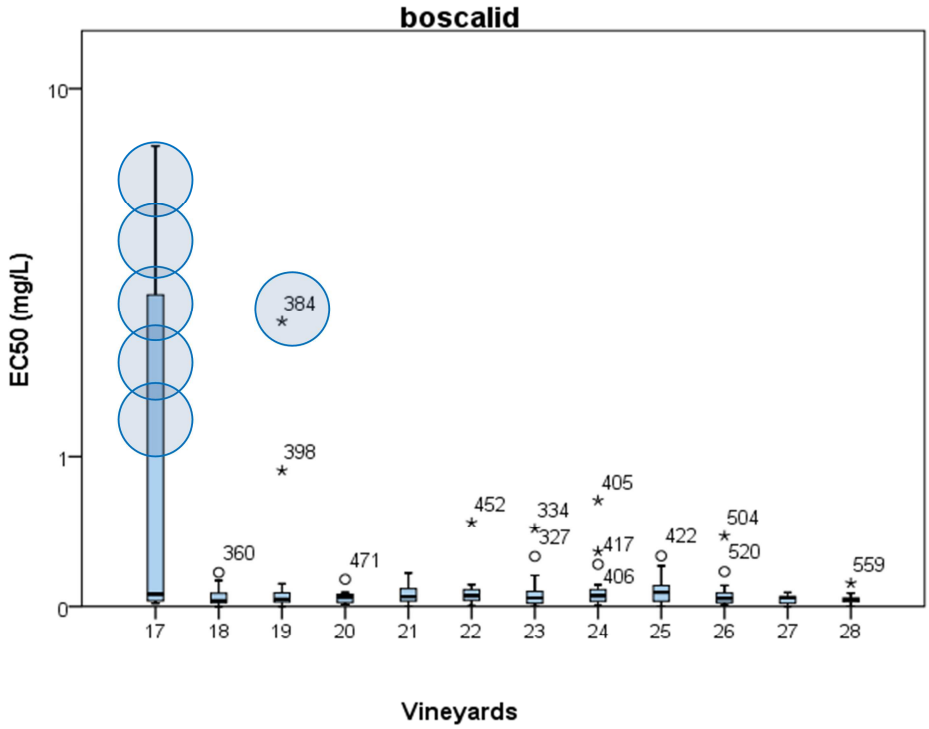


Figure 4.2.9: Distribution of boscalid EC_{50} assessed for the *B. cinerea* strains isolated in 2012 in different vineyards. The asterisks and the circles represent the outlier strains. The blue cirles indicate the strains with Rf> 10.

No difference due to the boscalid application in vineyards during 2012 was detected. However 5 of the 6 strains with Rf higher than 10 were isolated in Pozzolengo vineyard (BS), treated with boscalid for 4 years (Figure 4.2.10).

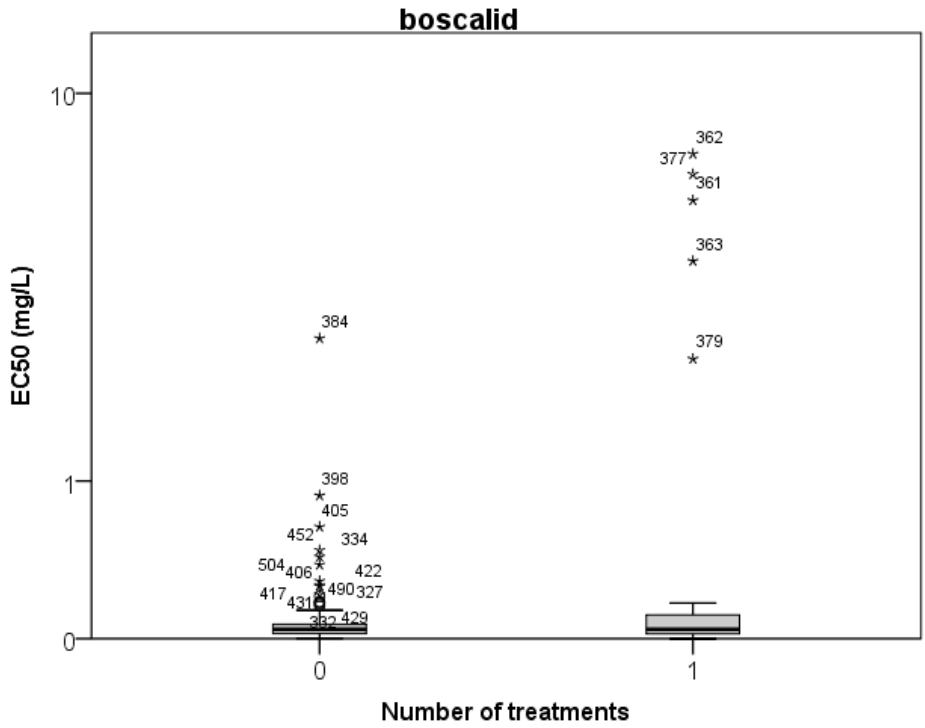


Figure 4.2.10: Distribution of EC_{50} concerning to boscalid in function of the number of treatments carried out in the year 2012 with the same active substance. The asterisks and the circles represent the outlier strains.

Fenhexamid

The averages EC_{50} concerning fenhexamid assessed in the vineyards sampled in 2012 ranged from 0.003 to 0.085 mg/L of active substance. The EC_{50} of the *B. cinerea* strains isolated from the different vineyards varied between very low values: 0.017 and 0.085 mg/L. A *B. cinerea* strain isolated in Adro (BS) showed the highest EC_{50} value and a Rf greater than 10 (EC_{50}=1.1 mg/L; Rf=12) (Figure 4.2.11). The Cavriana vineyard (MN) was the only one treated with fenhexamid in 2012 and the local *B. cinerea* population showed an EC_{50} value of 0.030 mg/L.

Significant differences were found among the median values of EC_{50} obtained for *B. cinerea* strains isolated in each vineyard (H=54.54; P<0.001; df=11) (Table 4.2.6).

Vineyard	EC_{50}	KW*	Vineyard	EC_{50}	KW*
17 Pozzolengo (BS)	0.026	**cd**	**23 Torrazza Coste (PV)**	0.038	d
18 Sirmione (BS)	0.003	**a**	**24 Torrazza Coste (PV)**	0.022	bcd
19 Adro (BS)	0.085	**ab**	**25 Casteggio (PV)**	0.046	d
20 Berbenno (SO)	0.027	**cd**	**26 Gonzaga (MN)**	0.032	cd
21 Berbenno (SO)	0.017	**abc**	**27 Ponti sul Mincio (MN)**	0.023	bcd
22 Chiuro (SO)	0.032	**d**	**28 Cavriana (MN)**	0.030	d

Table 4.2.6: Average values of EC_{50} concerning rfenhexamid, 2012. *Different letters correspond to significant differences among the EC_{50} medians according to Kruskal Wallis test.

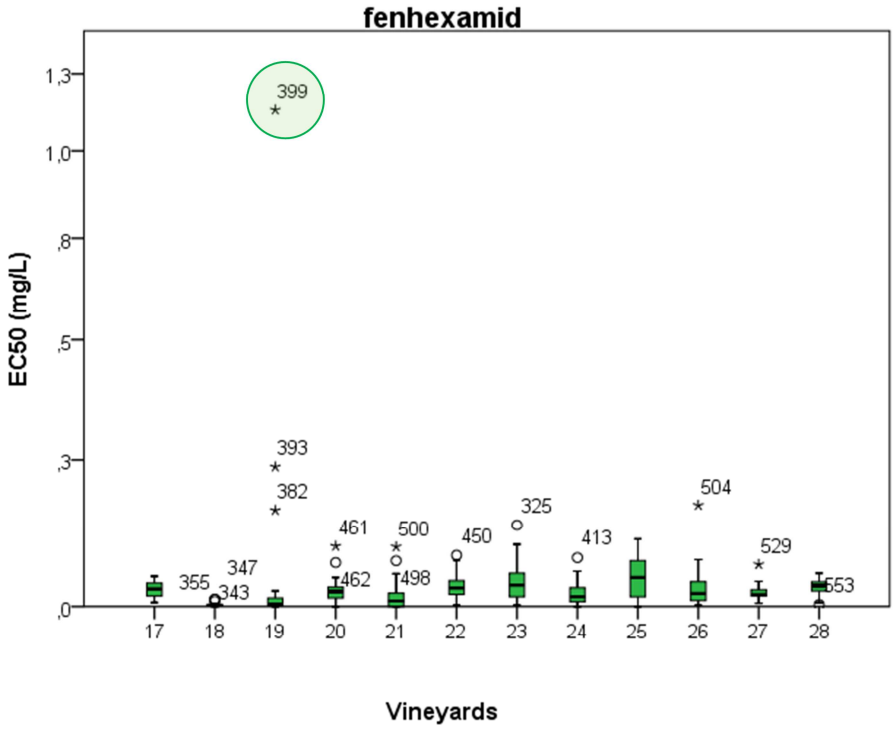


Figure 4.2.11: Distribution of fenhexamid EC_{50} assessed for the *B. cinerea* strains isolated in 2012 in different vineyards. The asterisks and the circles represent the outlier strains. The green circle indicates the strain with RF >10.

Cyprodinil

The EC_{50} concerning cyprodinil obtained for the *B. cinerea* populations isolated in different Lombard vineyards in 2012 were generally characterized by average values lower than 1 mg/L, ranging from 0.010 to 0.972 mg/L. The exceptions were represented by the *B. cinerea* populations isolated in Pozzolengo (BS), Sirmione (BS) and Gonzaga (MN) vineyards, where the average EC_{50} value reached 4.7 mg/L, 25.6 mg /L and 2.6 mg/L respectively (Table 4.2.7; Figure 4.2.12). The high EC_{50} values found in the four above cited vineyards were influenced by the EC_{50}s of the

outlier strains. Significant differences were found between the median values of EC_{50} of the vineyards (*H*=31.82; *P*<0.001; df=11). In total 10 strains were characterized by Rf greater than 10 and were isolated in:

- Pozzolengo (BS): strains BC376 (EC_{50}=40.7 mg/L; Rf=66) and BC366 (EC_{50}=8.9 mg/L; Rf=14.4);
- Sirmione (BS): strain BC351 (EC_{50}=275.5 mg/L; Rf=447);
- Adro (BS): strain BC397 (EC_{50}=10.1 mg/L; Rf=16);
- Torrazza Coste cv Pinot (PV): strains BC418 (EC_{50}=11.8 mg/L; Rf=19.2) and BC420 (EC_{50}=6.3 mg/L; Rf=10.2);
- Berbenno (SO): strain BC473 (EC_{50}=6.6 mg/L; Rf=11);
- Gonzaga (MN): strains BC505 (EC_{50}=20.8 mg/L; Rf=34), BC518 (EC_{50}=11.4 mg/L; Rf=19) and BC519 (EC_{50}=6.2 mg/L; Rf=10).

Vineyard	**EC_{50}**	**KW***	**Vineyard**	**EC_{50}**	**KW***
17 Pozzolengo (BS)	4.677	c	**23 Torrazza Coste (PV)**	0.117	ab
18 Sirmione (BS)	25.579	abc	**24 Torrazza Coste (PV)**	1.017	ab
19 Adro (BS)	0.972	ab	**25 Casteggio (PV)**	0.015	a
20 Berbenno (SO)	0.715	bc	**26 Gonzaga (MN)**	2.634	bc
21 Berbenno (SO)	0.487	abc	**27 Ponti sul Mincio (MN)**	0.058	abc
22 Chiuro (SO)	0.407	abc	**28 Cavriana (MN)**	0.261	abc

Table 4.2.7: Average values of EC_{50} concerning cyprodinil, 2012. *Different letters correspond to significant differences among the EC_{50} medians according to Kruskal Wallis test.

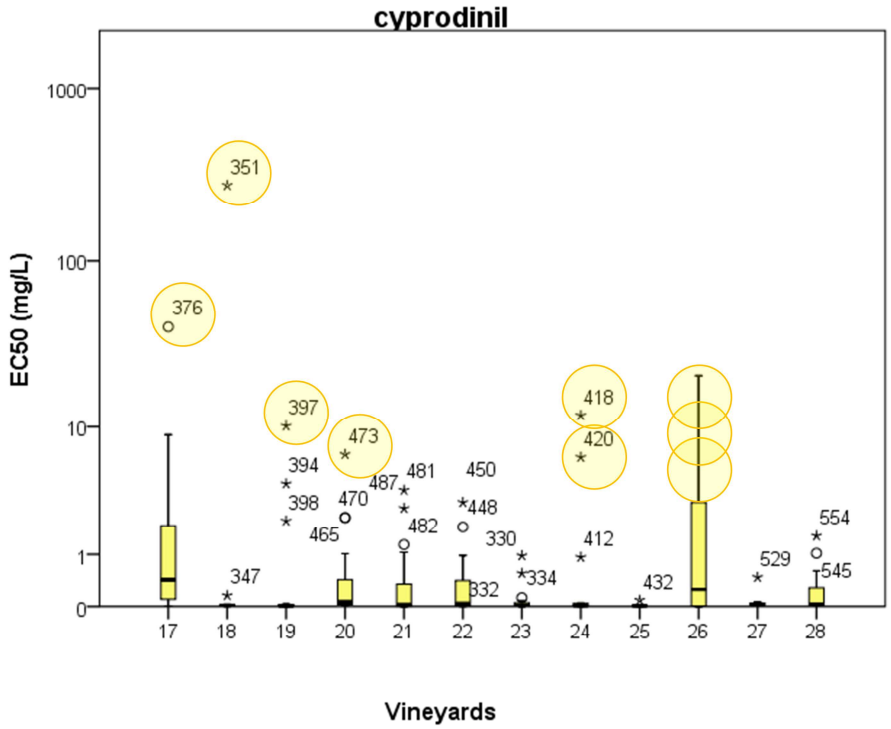


Figure 4.2.12: Distribution of cyprodinil EC_{50} assessed for the *B. cinerea* strains isolated in 2012 in different vineyards. The asterisks and the circles represent the outlier strains. The yellow circles indicate the strains with Rf > 10.

The treatments carried out in 2012 in vineyards using anilinopyrimidine were not associated with a reduction of sensibility of the strains (Figure 4.2.13). The distributions of the EC_{50} in the untreated and treated once or twice per year vineyards were similar. Howeve,r the majority of the outliers *B. cinerea* strains were isolated in the vineyards treated for several consecutive years with anilinopyrimidine.

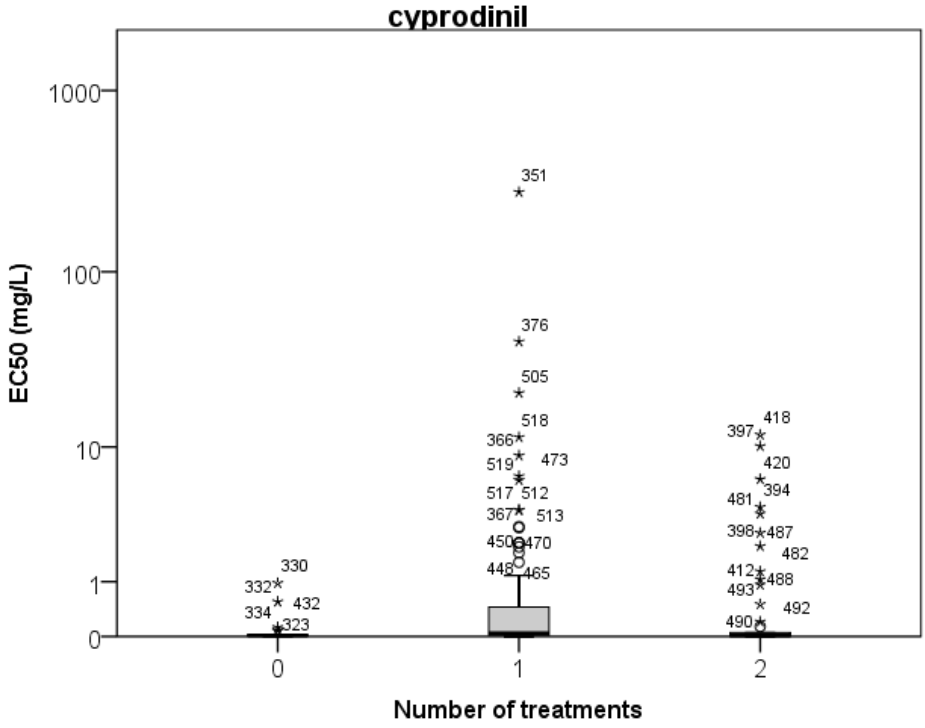


Figure 4.2.13: Distribution of EC_{50} concerning cyprodinil in relation of the number of treatments carried out in the year 2012 with the same active substance. The asterisks and the circles represent the outlier strains.

Fludioxonil

Very low EC_{50} values were found for fludioxonil: the average EC_{50} associated with the sampled vineyards ranged from 0.024 to 0.315 mg/L. The highest EC_{50} values were found in SO and precisely in the three vineyards located in Berbenno and in Chiuro and reached respectively 0.132, 0.308 and 0.315 mg/L (Table 4.2.8; Figure 4.2.14). Significant differences were found between the median values of EC_{50} of the vineyards (*H*F=37.04; *P*<0.0001; df=11).

In 2012, 23 *B. cinerea* strains with Rf > 10 were isolated, 16 of them in SO only. Moreover the distribution of EC_{50} values of Berbenno and Chiuro vineyards were more dispersed. The 23 strains were isolated respectively in:

- Sirmione (BS): strain BC352 (EC_{50}=0.2 mg/L; Rf=10.1);
- Pozzolengo (BS): strain BC372 (EC_{50}=0.2 mg/L; Rf=10.1);
- Adro (BS): strains BC396 (EC_{50}=0.3 mg/L; Rf=11.8) and BC397 (EC_{50}=0.3 mg/L; Rf=12.7);
- Casteggio (PV): strain BC437 (EC_{50}=0.3 mg/L; Rf=11.3);
- Chiuro (SO): strains BC442 (EC_{50}=0.9 mg/L; Rf=37.4), BC443 (EC_{50}=0.3 mg/L; Rf=13.8), BC445 (EC_{50}=0.9 mg/L; Rf=41.6), BC447 (EC_{50}=0.4 mg/L; Rf=19.6), BC448 (EC_{50}=0.9 mg/L; Rf=41),

BC449 (EC_{50}=0.3 mg/L; Rf=14.3), BC452 (EC_{50}=0.5 mg/L; Rf=22.6) and BC454 (EC_{50}=1 mg/L; Rf=41.7);

- Berbenno (SO): strains BC463 (EC_{50}=0.4 mg/L; Rf=16.8), BC473 (EC_{50}=0.4 mg/L; Rf=15.9), BC480 (EC_{50}=1.1 mg/L; Rf=47.8);
- Berbenno (SO): strains BC482 (EC_{50}=0.76 mg/L; Rf=33.3), BC484 (EC_{50}=1.1 mg/L; Rf=50.3), BC487 (EC_{50}=1.3 mg/L; Rf=58.6), BC488 (EC_{50}=0.6 mg/L; Rf=24.3) and BC490 (EC_{50}=1.5 mg/L; Rf=66.4);
- San Benedetto Po (MN): strain BC520 (EC_{50}=1 mg/L; Rf=42.9);
- Ponti sul Mincio (MN): strain BC521 (EC_{50}=0.2 mg/L; Rf=10.7).

Vineyard	EC_{50}	KW*	Vineyard	EC_{50}	KW*
17 Pozzolengo (BS)	0.053	abc	**23 Torrazza Coste (PV)**	0.036	ab
18 Sirmione (BS)	0.069	ab	**24 Torrazza Coste (PV)**	0.023	a
19 Adro (BS)	0.054	ab	**25 Casteggio (PV)**	0.036	ab
20 Berbenno (SO)	0.132	abc	**26 Gonzaga (MN)**	0.084	ab
21 Berbenno (SO)	0.308	bc	**27 Ponti sul Mincio (MN)**	0.049	ab
22 Chiuro (SO)	0.315	c	**28 Cavriana (MN)**	0.024	ab

Table 4.2.8: Average values of EC_{50} concerning fludioxonil, 2012. *Different letters correspond to significant differences among the EC_{50} medians according to Kruskal Wallis test.

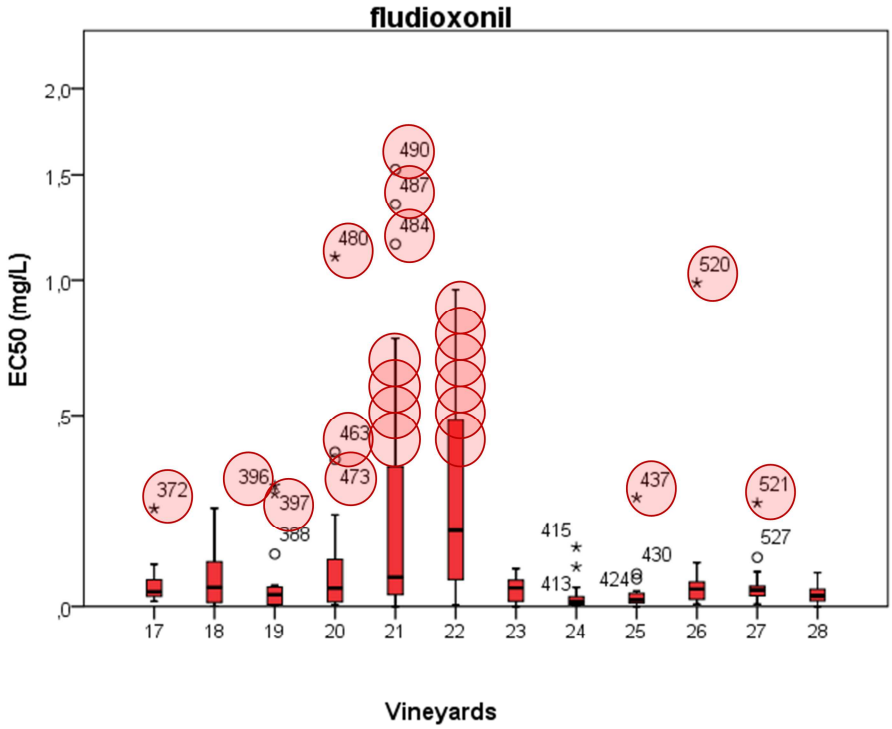


Figure 4.2.14: Distribution of fludioxonil EC_{50} assessed for the *B. cinerea* strains isolated in 2012 in different vineyards. The asterisks and the circles represent the outlier strains. The red circles indicate the strains with Rf >10.

As reported in Figure 4.2.15, the treatments with formulations containing fludioxonil was not correlated with a significant variation in the distribution of the EC_{50} of the *B. cinerea* strains. However the application of fludioxonil was associated with the selection of a greater number of strains characterized by higher EC_{50} values.

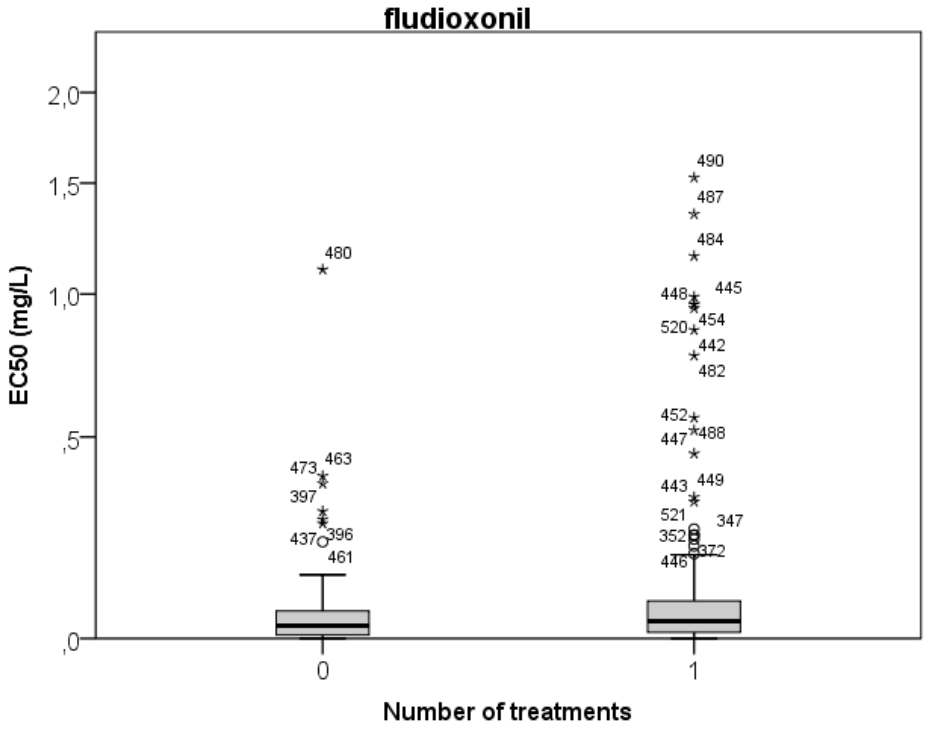


Figure 4.2.15: Distribution of EC_{50} concerning to fludioxonil in relation of the number of treatments carried out in the year 2012 with the same active substance. The asterisks and the circles represent the outlier strains.

4.2.3 Third year of sampling, 2013

In 2013 148 strains were analyzed for boscalid, 146 for fenhexamid, 148 for cyprodinil and 149 for fludioxonil sensitivity.

Boscalid

The EC_{50}s concerning boscalid assessed for the great majority of the isolated *B. cinerea* strains were characterized by low values, ranging from 0.002 to 1.67 mg/L of active substance. The average EC_{50} value in the selected vineyards ranged between 0.074 and 1.725 mg/L (Table 4.2.9; Figure 4.2.16). Significant differences were found between the median values of EC_{50} of the vineyards according to the Kruskal Wallis test (H=18.27; P=0.011; df=7). Among the numerous outliers, 9 strains were characterized by Rf > 10 and were isolated mainly in the province of Brescia, precisely in:

- Cazzago San Martino (BS): strain BC576 (EC_{50}=3.4 mg/L; Rf=14.3);
- Erbusco (BS): strain BC614 (EC_{50}=3.4 mg/L; Rf=14.3);
- Cortefranca (BS): strains BC632 (EC_{50}=4.4 mg/L; Rf=18.5), BC636 (EC_{50}=3.9 mg/L; Rf=16.1) and BC638 (EC_{50}=4.2 mg/L; Rf=17.4);

- Sirmione (BS): strains BC668 (EC_{50}=11.8 mg/L; Rf=49) and BC674 (EC_{50}=2.7mg/L; Rf=11.1);
- Chiuro (SO): strains BC719 (EC_{50}=20.2 mg/L; Rf=83.6) and BC720 (EC_{50}=8.2 mg/L; Rf=34.1).

Vineyard	EC_{50}	KW*	Vineyard	EC_{50}	KW*
29 Cazzago San Martino (BS)	0.243	ab	**33 Adro (BS)**	0.146	a
30 Rodengo Saiano (BS)	0.074	a	**34 Sirmione**	0.883	ab
31 Erbusco (BS)	0.262	a	**35 Torrazza Coste (PV)**	0.091	ab
32 Cortefranca (BS)	0.780	a	**36 Chiuro (SO)**	1.725	b

Table 4.2.9: Average values of EC_{50} concerning boscalid, 2013. *Different letters correspond to significant differences among the EC_{50} medians according to Kruskal Wallis test.

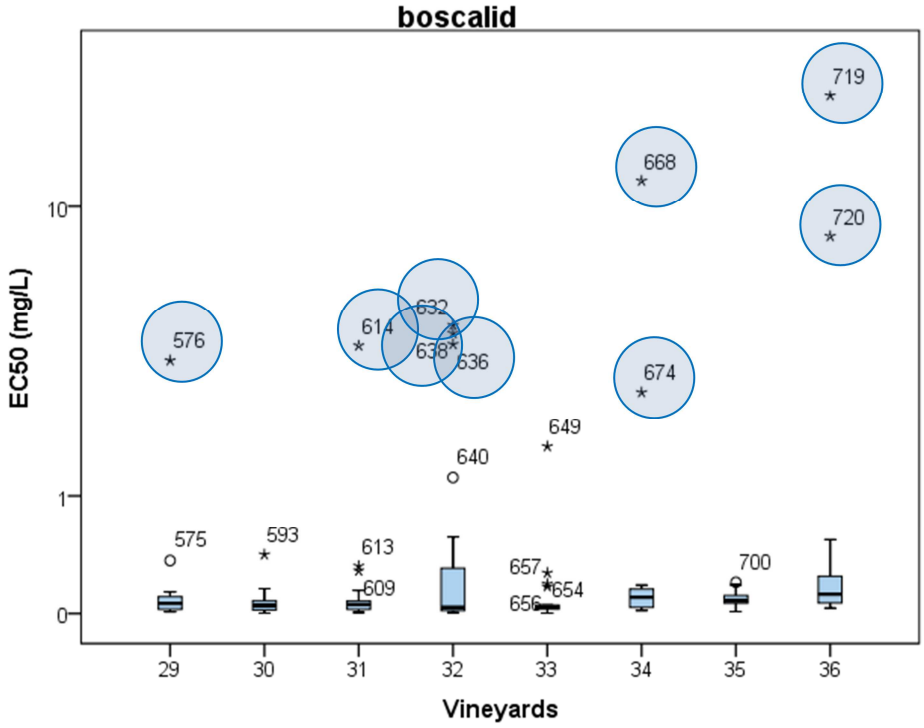


Figure 4.2.16: Distribution of boscalid EC_{50} assessed for the *B. cinerea* strains isolated in 2013 in different vineyards. The asterisks and the circles represent the outlier strains. The blue circles indicate the strains with Rf >10.

No differences due to the use of boscalid in 2013 was detected (Figure 4.2.17).

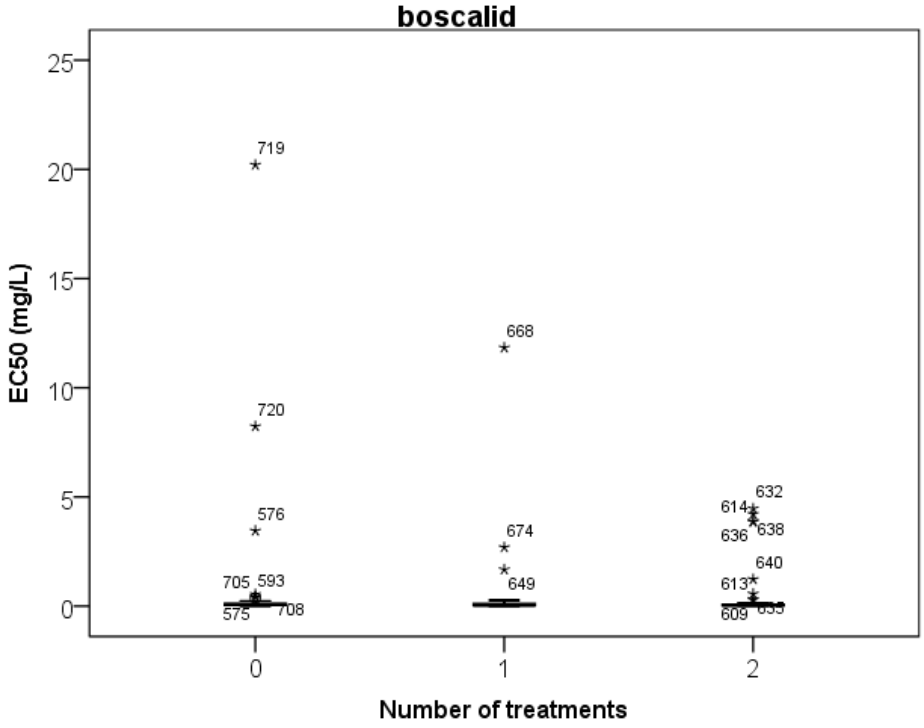


Figure 4.2.17: Distribution of EC_{50} concerning boscalid in relation to the number of treatments carried out in the year 2013 with the same active substance. The asterisks and the circles represent the outlier strains.

Fenhexamid

The EC_{50} concerning fenhexamid for the tested strains were characterized by very low average values ranging from 0.002 to0.220 mg/L of active substance. The median values of EC_{50} associated with the different vineyards (Table 4.2.10; Figure 4.2.18) were not significantly different, according to the Kruskal Wallis test.

Vineyard	EC_{50}	Vineyard	EC_{50}
29 Cazzago San Martino (BS)	0.014	**33 Adro (BS)**	0.022
30 Rodengo Saiano (BS)	0.029	**34 Sirmione**	0.017
31 Erbusco (BS)	0.018	**35 Torrazza Coste (PV)**	0.038
32 Cortefranca (BS)	0.023	**36 Chiuro (SO)**	0.059

Table 4.2.10: Average values of EC_{50} concerning fenhexamid, 2013.

In 2013 no *B. cinerea* strains with Rf greater than 10 were isolated and no treatment with fenhexamid was carried out.

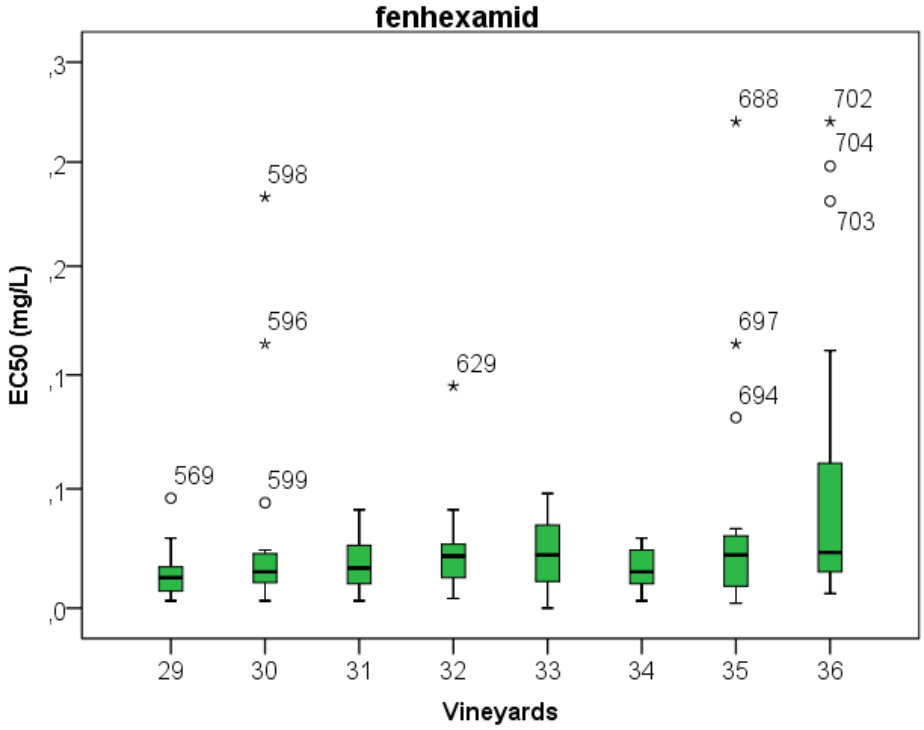


Figure 4.2.18: Distribution of fenhexamid EC_{50} assessed for the *B. cinerea* strains isolated in 2013 in different vineyards. The asterisks and the circles represent the outlier strains.

Cyprodinil

The EC_{50} concerning cyprodinil of the *B. cinerea* strains isolated in Lombardy in 2013 were characterized by values ranging from 0.001 to 3.771 mg/L of active substance. The average EC_{50} values associated with the sampled vineyards varied between 0.032 and 1.584 mg/L (Table 4.2.11; Figure 4.2.19) and significant differences were found among the medians according to the Kruskal Wallis test (*H*=29.98; *P*<0.0001; df=7). 4 *B. cinerea* strains were characterized by Rf greater than 10 and were isolated in:

- Cortefranca (BS): strains BC622 (EC_{50}=9.4 mg/L; Rf=15.3) and BC624 (EC_{50}=18.6 mg/L; Rf=30.1);
- Sirmione (BS): strain BC674 (EC_{50}=26.3 mg/L ; Rf=42.7);
- Chiuro (SO): strain BC711 (EC_{50}=35.3 mg/L; Rf=57.3).

Vineyard	EC_{50}	KW*	Vineyard	EC_{50}	KW*
29 Cazzago San Martino (BS)	0.135	b	**33 Adro (BS)**	0.547	b
30 Rodengo Saiano (BS)	0.103	b	**34 Sirmione**	1.584	ab
31 Erbusco (BS)	0.036	a	**35 Torrazza Coste (PV)**	0.032	ab
32 Cortefranca (BS)	1.551	b	**36 Chiuro (SO)**	2.777	c

Table 4.2.11: Average values of EC_{50} concerning cyprodinil, 2013. *Different letters correspond to significant differences among the EC_{50} medians according to Kruskal Wallis test.

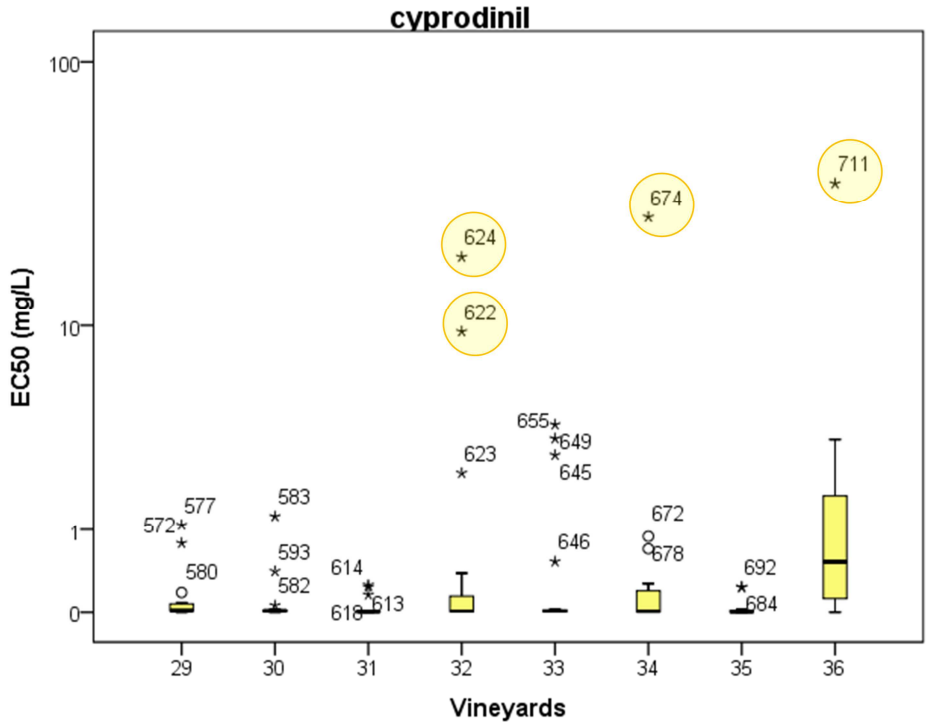


Figure 4.2.19: Distribution of cyprodinil EC_{50} assessed for the *B. cinerea* strains isolated in 2013 in different vineyards. The asterisks and the circles represent the outlier strains. The yellow circles indicate the strains with Rf > 10.

The number of treatments carried out in vineyards in 2013 did not influence the number of *B. cinerea* strains with high Rf (Figure 4.2.20). However the four strains with Rf> 10 were all isolated in vineyards treated at least once with an anilinopyrimidine fungicide.

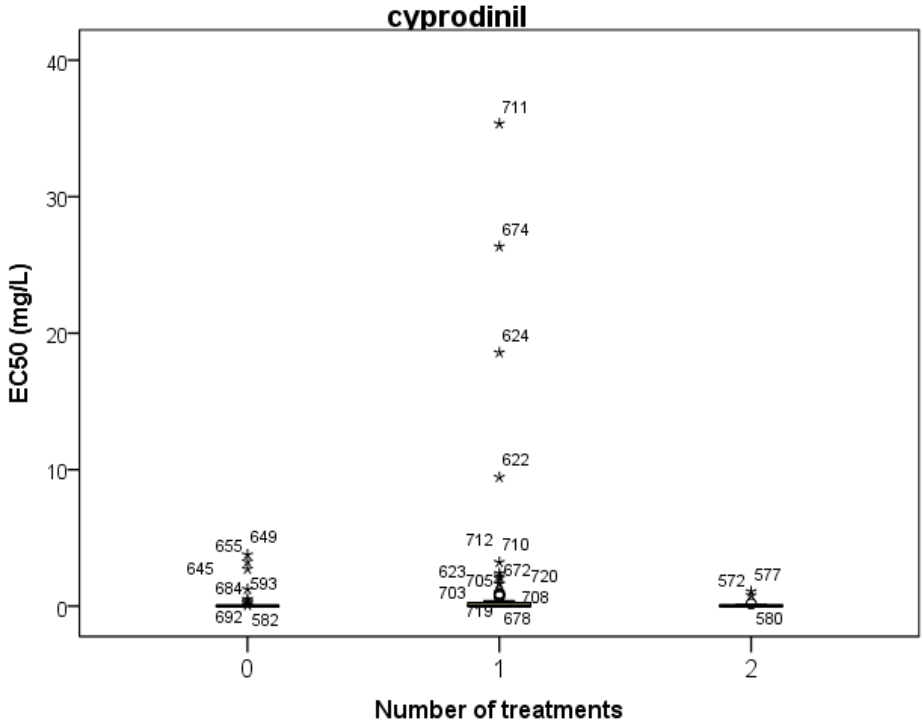


Figure 4.2.20: Distribution of EC_{50} concerning cyprodinil in relation to the number of treatments carried out in the year 2013 with the same active substance. The asterisks and the circles represent the outlier strains.

Fludioxonil

Very low average EC_{50} values ranging from 0.007 to 0.021 mg/L were found for fludioxonil in 2013 in sampled *B. cinerea* populations. The highest EC_{50} value, 0.160 mg/L, was associated with a strain isolated in Torrazza Coste (PV) characterized by a Rf = 7.0 (Table 4.2.12; Figure 4.2.21). No significant differences were found among the median values of EC_{50} of the vineyards, according to the Kruskal Wallis test.

No differences were found between treated and untreated vineyards (Figure 4.2.22).

Vineyard	EC_{50}	Vineyard	EC_{50}
29 Cazzago San Martino (BS)	0.012	**33 Adro (BS)**	0.009
30 Rodengo Saiano (BS)	0.007	**34 Sirmione**	0.008
31 Erbusco (BS)	0.021	**35 Torrazza Coste (PV)**	0.021
32 Cortefranca (BS)	0.009	**36 Chiuro (SO)**	0.020

Table 4.2.12: Average values of EC_{50} concerning fludioxonil, 2013.

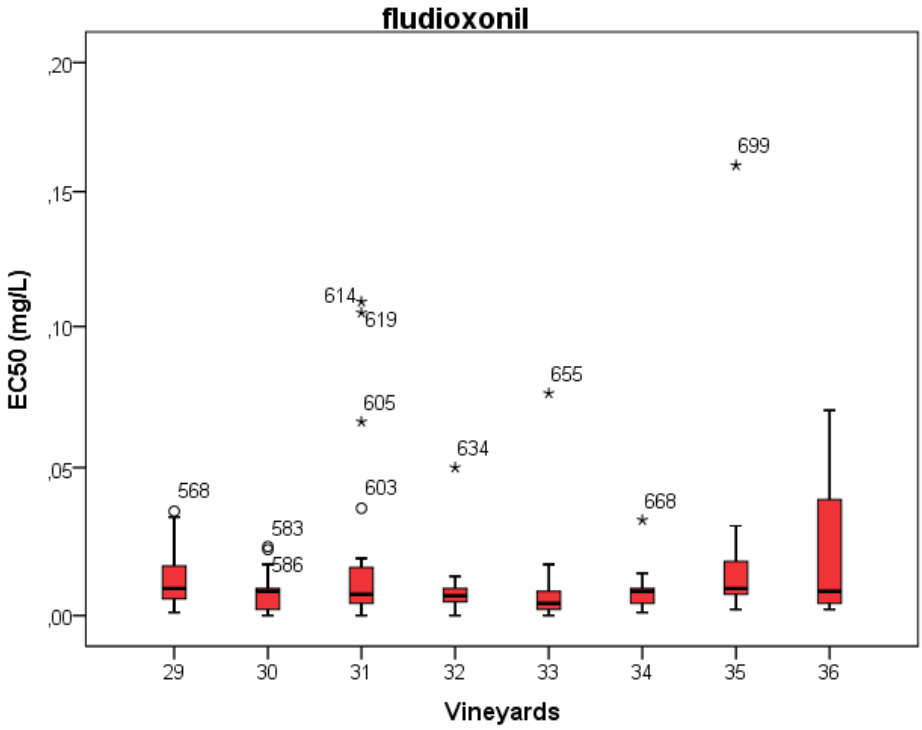


Figure 4.2.21: Distribution of fludioxonil EC_{50} assessed for the *B. cinerea* strains isolated in 2013 in different vineyards. The asterisks and the circles represent the outlier strains. .

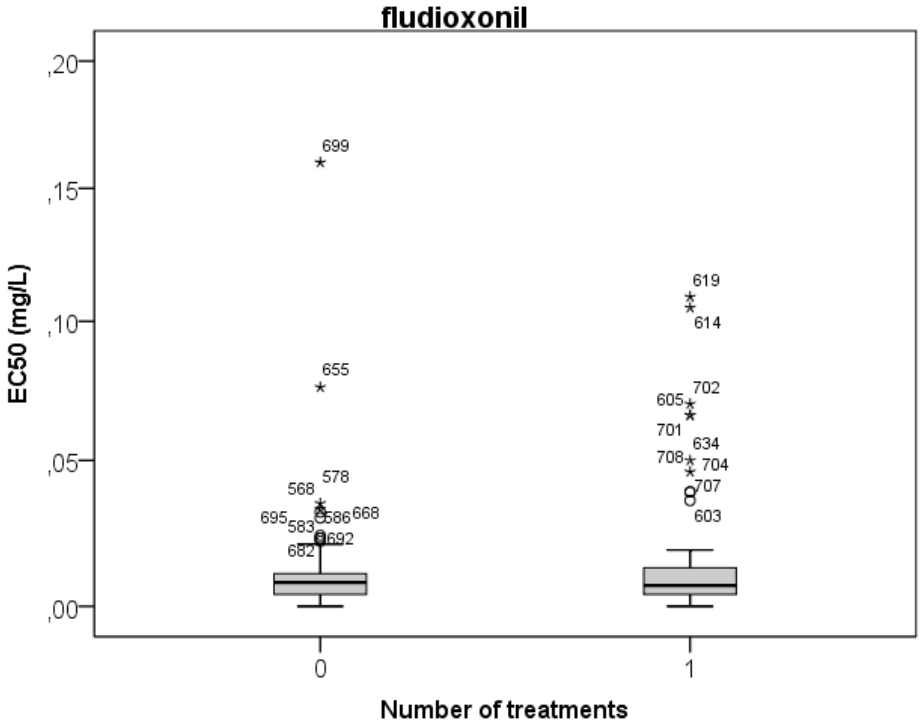


Figure 4.2.22: Distribution of EC_{50} concerning fludioxonil in relation to the number of treatments carried out in the year 2012 with the same active substance. The asterisks and the circles represent the outlier strains.

4.2.4 Temporal variation of *B. cinerea* sensitivity to fungicides

Boscalid

The comparison of the *B. cinerea* populations isolated in the vineyards sampled for a three-year period showed that the distribution of EC_{50} values concerning boscalid did not significantly differ among years. In Sirmione (BS), treated once per year with boscalid, the EC_{50} distribution was similar in 2011 and 2013 and slightly lower in 2012 (*H*=14.7; *P*=0.01; df=2). Two strains with a reduced sensitivity were found only in the third year (Figure 4.2.23; Table 4.2.13).

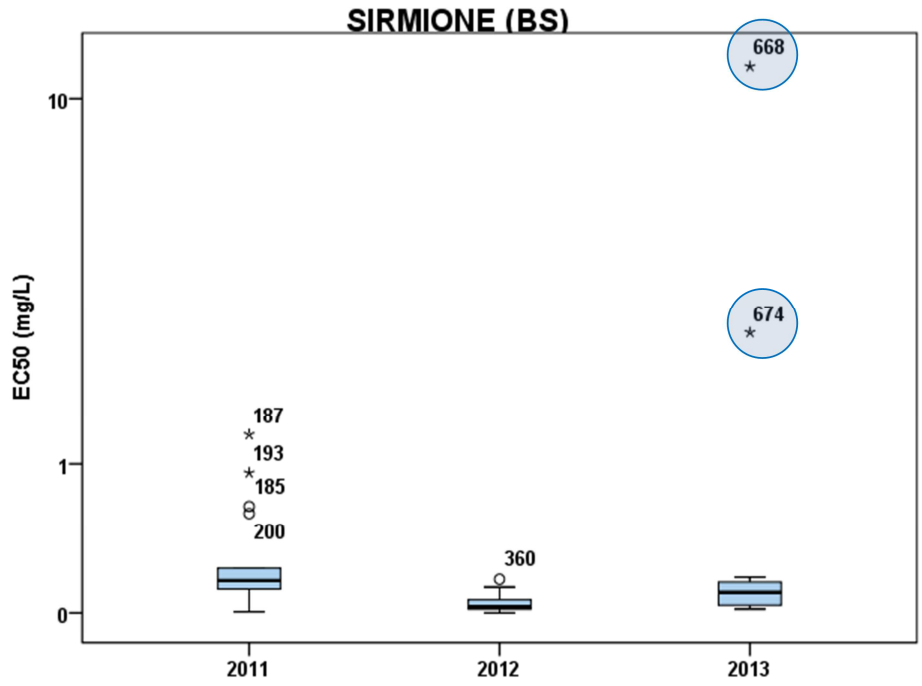


Figure 4.2.23: Distribution of boscalid EC_{50} in 2011-2013 period in Sirmione (BS). The blue circles indicate the strains with a Rf > 10

In Chiuro (SO) vineyard never treated with boscalid, in 2011 and 2013 the EC_{50} distribution was wider than in 2012 (*H*=8.3; *P*=0.016; df=2). Three strains characterized by a reduced sensitivity were isolated from Chiuro and precisely one in 2011 and two in 2013 (Figure 4.2.24; Table 4.2.13).

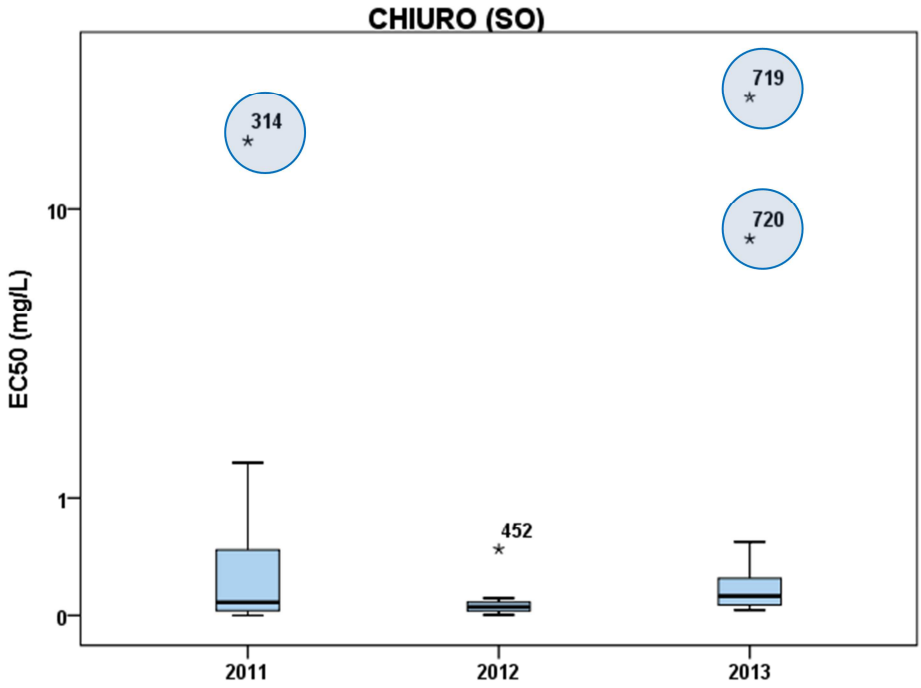


Figure 4.2.24: Distribution of boscalid EC_{50} in 2011-2013 period in Chiuro (SO). The blue circles indicate the strains with a reduction in sensitivity.

The vineyard of Torrazza Coste (PV), treated only once with boscalid in 2013, was characterized by similar EC_{50} distributions in 2011 and 2012, which were significantly different from the wider distribution assessed in 2013 (H=8.9; P=0.011; df=2). No strain with Rf higher than 10 was found in Torrazza Coste (Figure 4.2.25; Table 4.2.13).

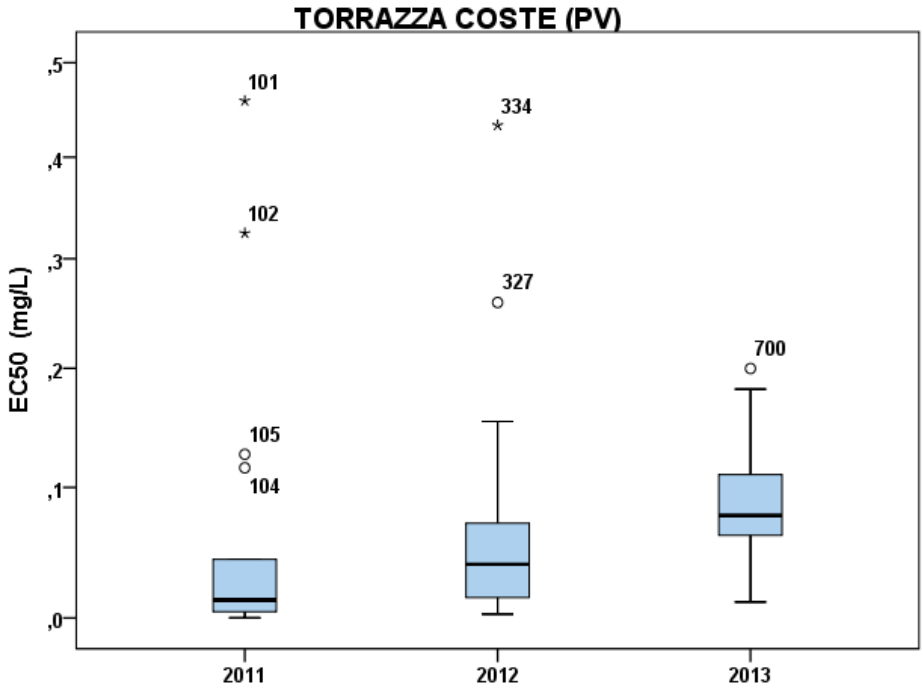


Figure 4.2.25: Distribution of boscalid EC_{50} in the 2011-2013 in Torrazza Coste vineyard (PV).

Vineyard	2011			2012			2013		
	N°	EC_{50}	KW*	N°	EC_{50}	KW*	N°	EC_{50}	KW*
Sirmione (BS)	1	0.29	a	1	0.05	b	1	0.88	a
Chiuro (SO)	-	1.06	ab	-	0.07	a	-	1.72	b
Torraza Coste (PV)	-	0.07	a	-	0.08	a	1	0.09	b

Table 4.2.13: Average values EC_{50} of boscalid assessed in the three vineyards sampled for three years. *Different letters correspond to significant differences among the EC_{50} medians according to Kruskal Wallis test. N°= number of treatments.

Fenhexamid

The selected vineyards were never treated with fenhexamid during the three-year period. In Sirmione (BS) the EC_{50} distribution of *B. cinerea* population was different among years (H=30.4; P<0.0001; df=2). The EC_{50} distribution observed in 2011 was wider than in 2012 which in turn was lower than in 2013 (Figure 4.2.26; Table 4.2.14). No differences were found between the EC_{50} distribution in Chiuro (SO) and Torrazza Coste (PV) vineyards throughout the years (Figure 4.2.27; Figure 4.2.28; Table 4.2.14). No strains with Rf higher than 10 were found in the sampled vineyards.

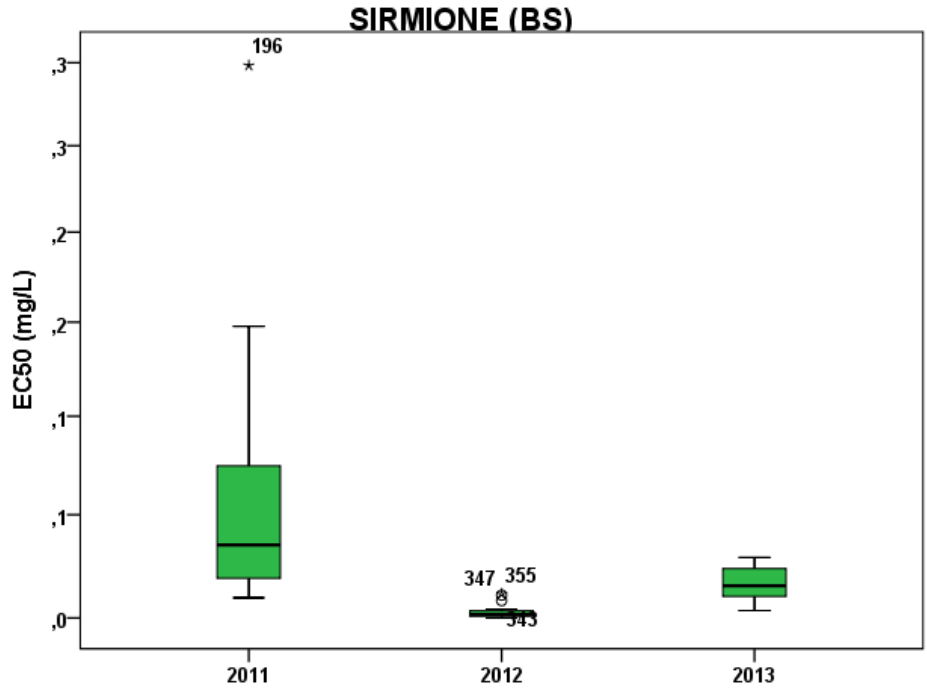


Figure 4.2.26: Distribution of fenhexamid EC_{50} in 2011-2013 in Sirmione vineyard (BS).

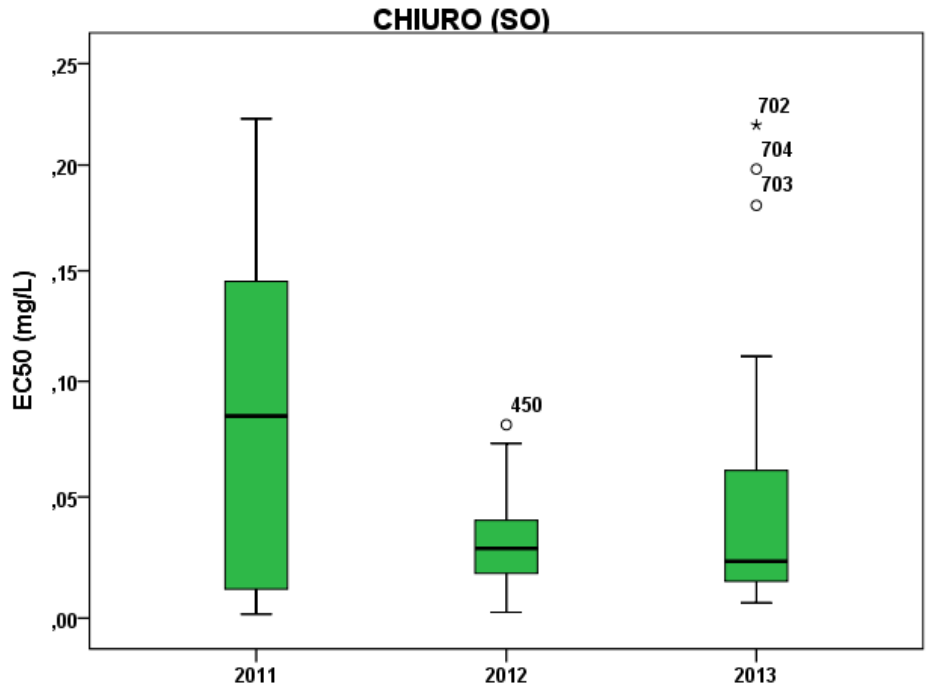


Figure 4.2.27: Distribution of fenhexamid EC_{50} in 2011-2013 in Chiuro vineyard (BS).

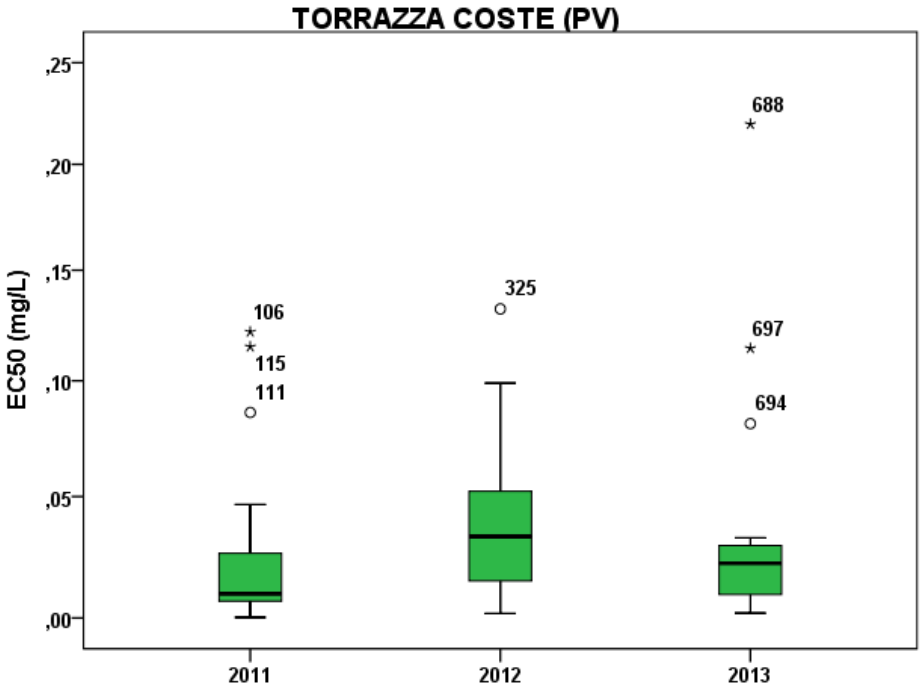


Figure 4.2.28: Distribution of fenhexamid EC_{50} in 2011-2013 in Torrazza Coste vineyard (BS).

Vineyard	2011		2012		2013	
	EC_{50}	KW*	EC_{50}	KW*	EC_{50}	KW*
Sirmione (BS)	0.06	c	0.003	a	0.02	b
Chiuro (SO)	0.09	a	0.03	a	0.06	a
Torraza Coste (PV)	0.03	a	0.04	a	0.04	a

Table 4.2.14: Average values of fenhexamid EC_{50} assessed in the three vineyards sampled for three years. *Different letters correspond to significant differences among the EC_{50} medians according to Kruskal Wallis test.N=number of treatments

Cyprodinil

The EC_{50} distributions concerning cyprodinil assessed for the vineyards of Sirmione (BS) and Chiuro (SO) were similar throughout the years (Figure 4.2.29; Figure 4.2.30; Table 4.2.15). The EC_{50} distributions in Torrazza Coste were similar in 2011 and 2012, during which the anilinopyrimidine were not used, and they were less extended than 2013 (H=12.25; P=0.002; df=2) (Figure 4.2.31, Table 4.2.15).

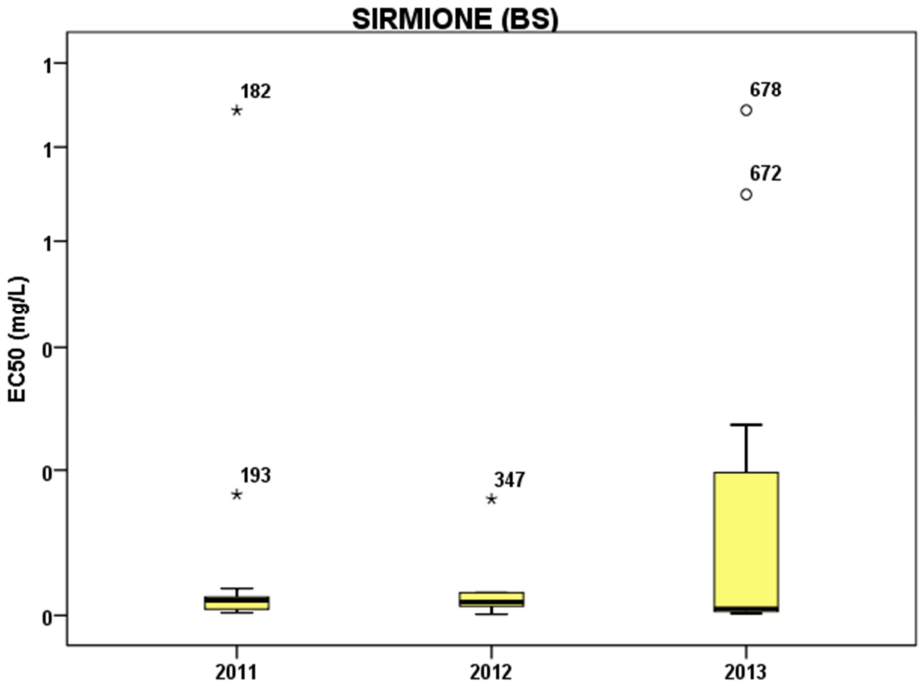


Figure 4.2.29: Distribution of cyprodinil EC_{50} in 2011-2013 in Sirmione vineyard (BS).

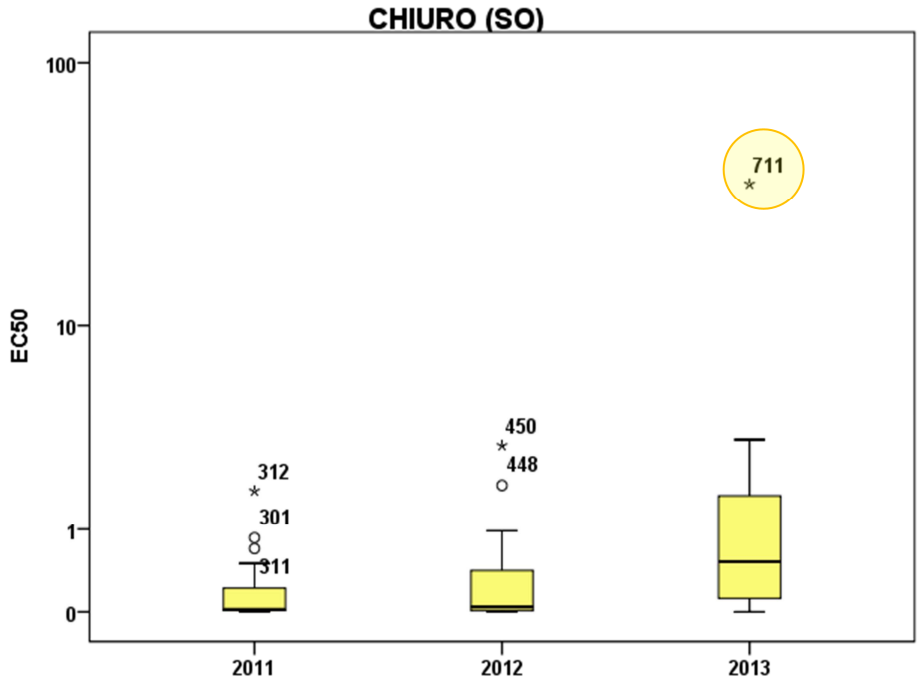


Figure 4.2.30: Distribution of cyprodinil EC_{50} in 2011-2013 in Chiuro vineyard (BS). The yellow circles indicate the strains with a reduction in sensitivity.

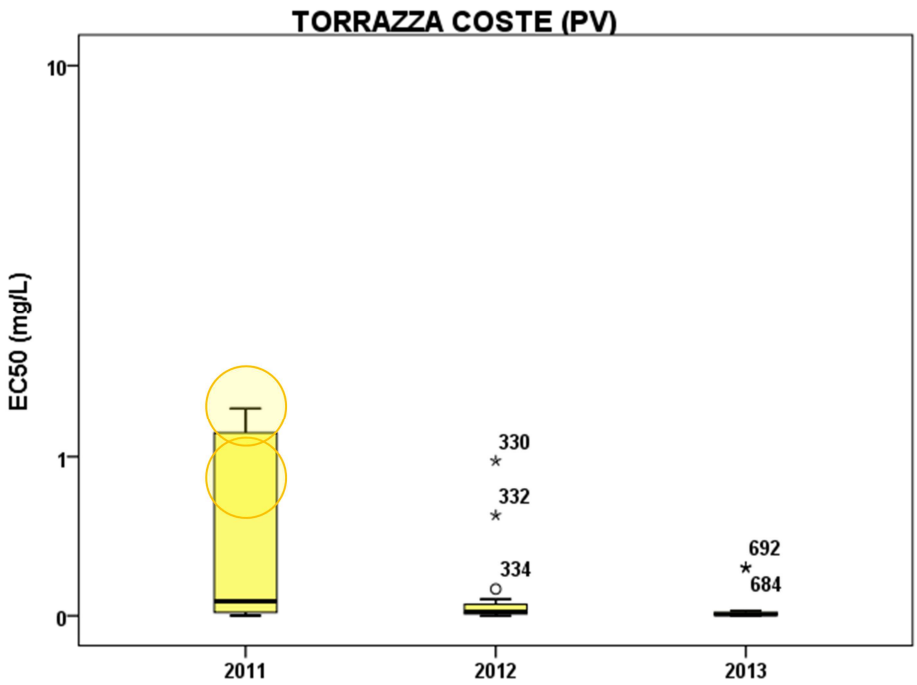


Figure 4.2.31: Distribution of cyprodinil EC_{50} in 2011-2013 in Torrazza Coste vineyard (BS). The yellow circles indicate the strains with Rf>10.

Vineyard	2011			2012			2013		
	N°	EC_{50}	KW*	N°	EC_{50}	KW*	N°	EC_{50}	KW*
Sirmione (BS)	-	0.56	a	1	25.57	a	1	1.58	a
Chiuro (SO)	1	0.24	a	1	0.41	a	1	2.78	a
Torraza Coste (PV)	-	49.85	b	-	0.12	b	1	0.03	a

Table 4.2.15: Average values of cyprodinil EC_{50} assessed in the three vineyards sampled for three years. Averages *Different letters correspond to significant differences among the EC_{50} medians according to Kruskal Wallis test. N= number of treatments.

Fludioxonil

The EC_{50} distributions concerning fludioxonil sensitivity of *B. cinerea* populations were different throughout the years in Sirmione (BS) and Chiuro (SO), and similar in Torrazza Coste (PV) (H=3.7; P=0.160; df= 2) (Figure 4.2.34; Table 4.2.16). In Sirmione and Chiuro the EC_{50} distributions were similar in 2011 and 2013 and wider in 2012 (H=8.7; P=0.013; df= 2 and H=20.1; P<0.001; df=2) (Figure 4.2.32; Figure 4.2.33; Table 4.2.16).

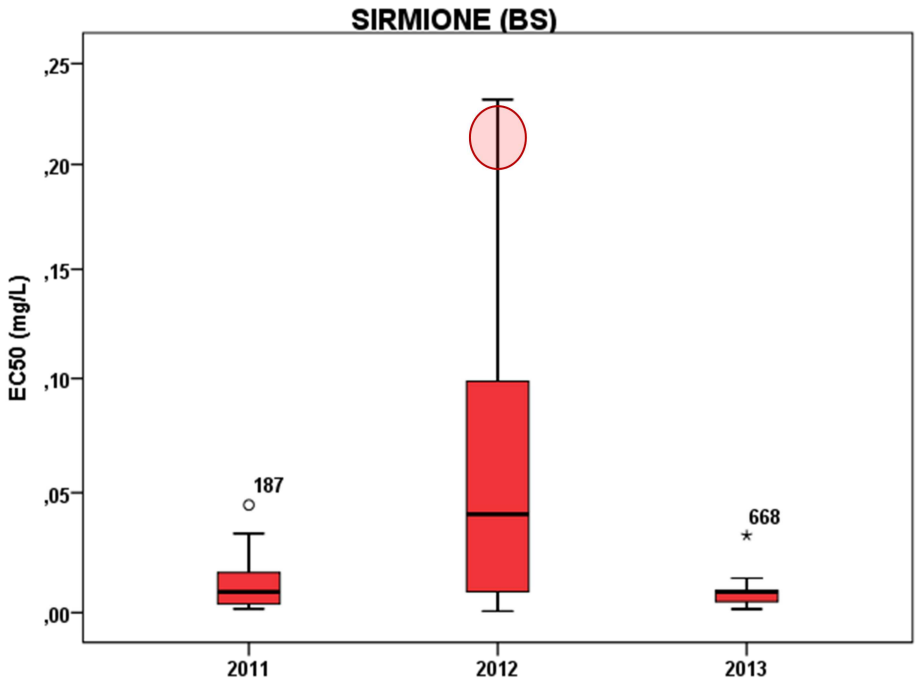


Figure 4.2.32: Distribution of fludioxonil EC_{50} in 2011-2013 in Sirmione vineyard (BS). The red circles indicate the strains with a Rf>10.

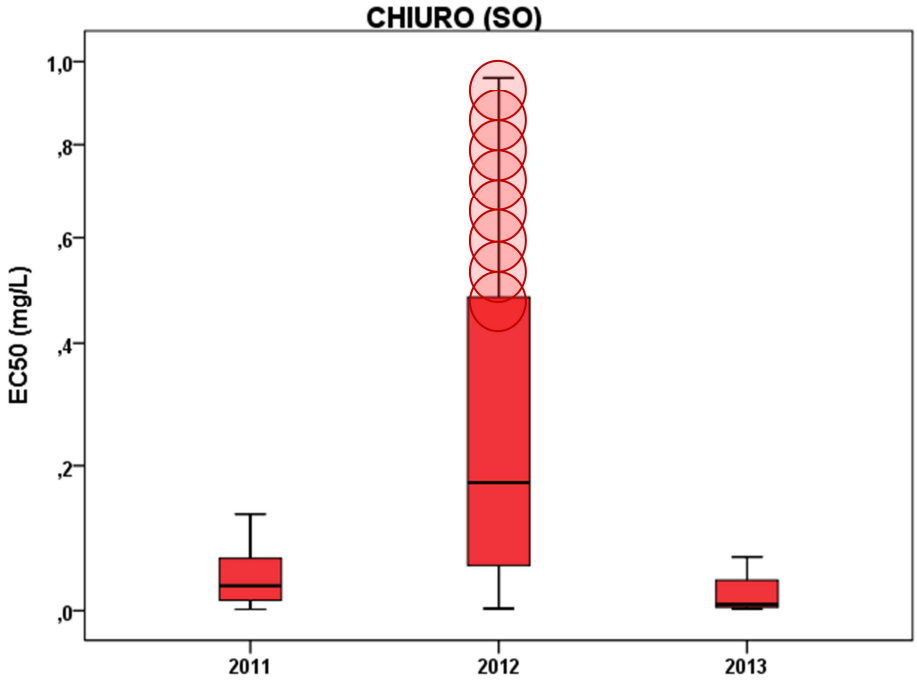


Figure 4.2.33: Distribution of fludioxonil EC_{50} in 2011-2013 in Chiuro vineyard (SO). The red circles indicate the strains with Rf>10.

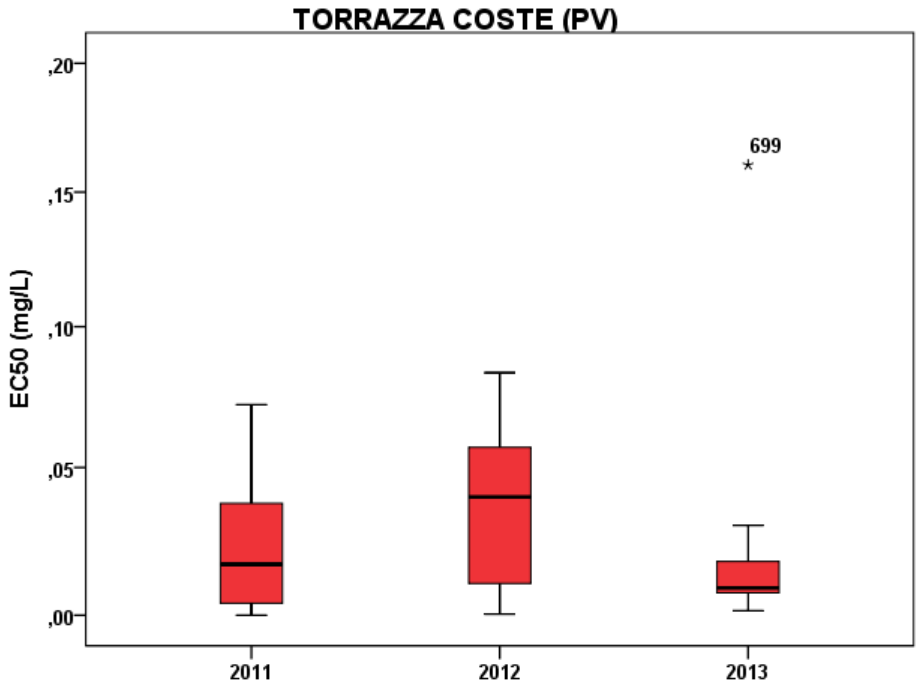


Figure 4.2.34: Distribution of fludioxonil EC_{50} in 2011-2013 in Torrazza Coste vineyard (PV). The red circles indicate the strains with a Rf >10.

Vineyard	2011			2012			2013		
	N°	EC_{50}	KW*	N°	EC_{50}	KW*	N°	EC_{50}	KW*
Sirmione (BS)	1	0.01	a	1	0.07	b	-	0.008	a
Chiuro (SO)	1	0.04	a	1	0.3	b	1	0.02	a
Torraza Coste (PV)	-	0.02	a	-	0.03	a	-	0.02	a

Table 4.2.16: Average values of fludioxonil EC_{50} assessed in the three vineyards sampled for three years. *Different letters correspond to significant differences among the EC_{50} medians according to Kruskal Wallis test.N= number of treatments

4.2.5 Effects of location and year on the EC_{50} distributions

The results of the Friedman test showed the existence of significant differences in the EC_{50} values between year (Y) and location (L), considered at a provincial level (Table 4.2.17).

Source of variation[a]	d.f.[b]	Fr boscalid	Fr fenhexamid	Fr cyprodinil	Fr fludioxonil
L × Y	10	32.32***	23.79**	21.17*	31.45 ***

*, denotes significance at the 0.05 probability level.
**, denotes significance at the 0.01 probability level.
***, denotes significance at the 0.001 probability level.
[a] L: Location (provinces); Y: Year.
[b] d.f.: degrees of freedom.

Table. 4.2.17: Friedman coefficient (Fr) showing the effect of the main factors and their interactions across years on EC_{50} of boscalid, fenhexamid, cyprodinil and fludioxonil.

Considering the EC_{50} distribution of boscalid fenhexamid, cyprodinil and fludioxonil the the interaction of location and years (L x Y) was always significant.

4.2.6 Past treatment effects on the EC_{50} distributions

The relationship between the total number of treatments, carried out in vineyards during the 2006-2013 period, with each active substance and the corresponding EC_{50} distribution was analysed and visualized in box plot graphs (Figure 4.2.35 and 4.2.36).

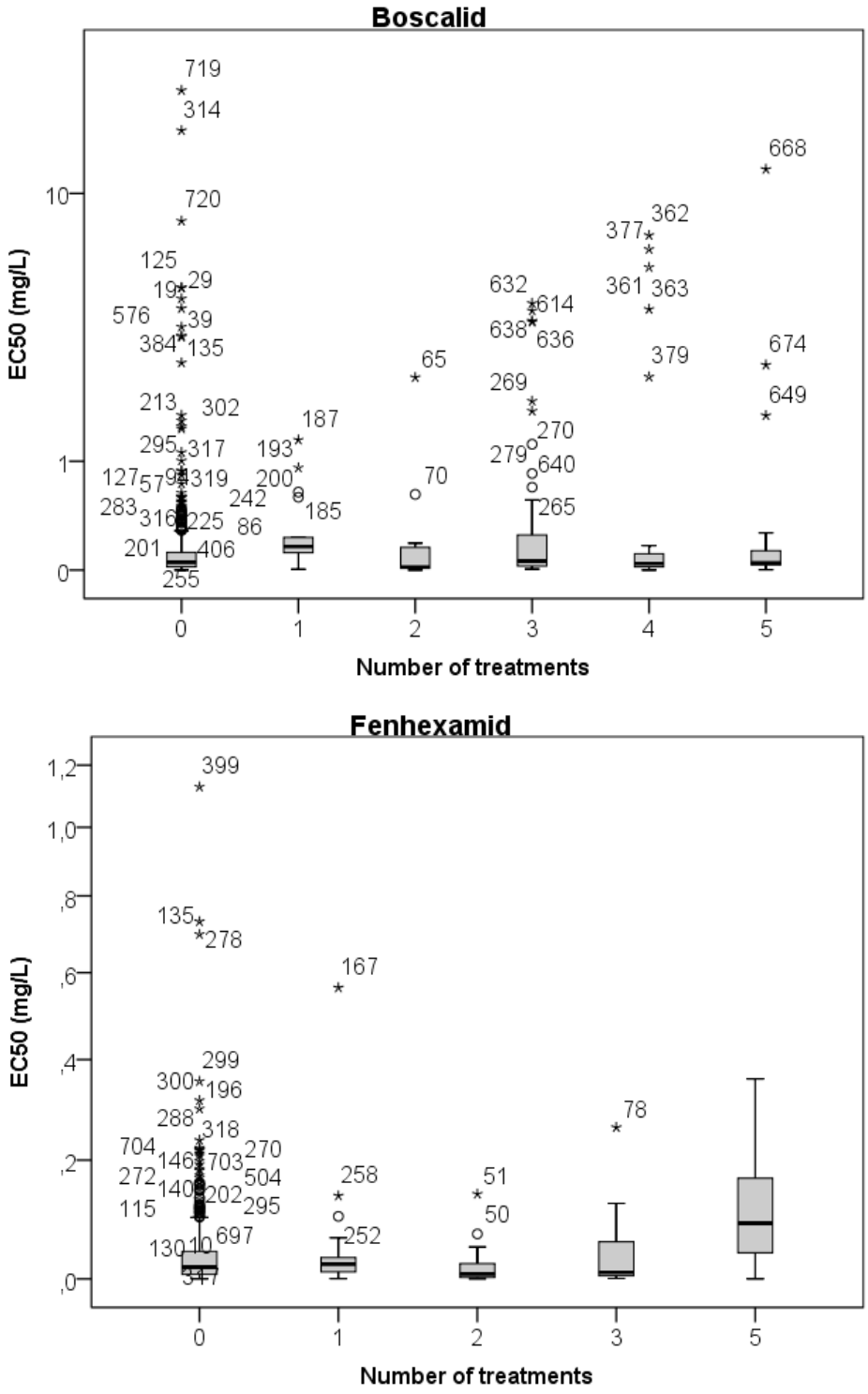


Figure 4.2.35: Distribution of EC_{50} concerning boscalid and fenhexamid related to the total number of treatments carried out from 2006 to 2013. The asterisks and the circles represent the outlier strains.

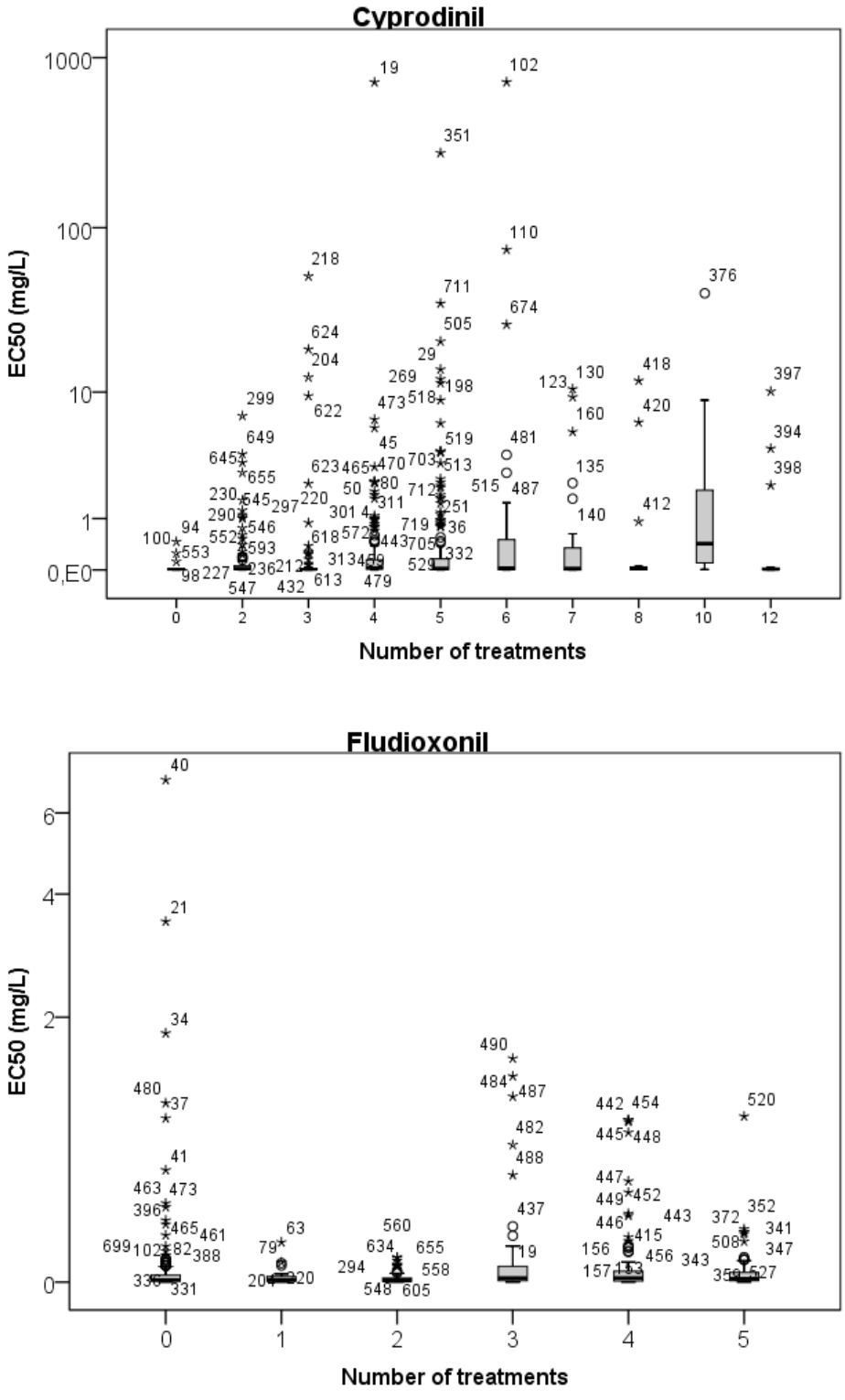


Figure 4.2.36: Distribution of EC_{50} concerning cyprodinil and fludioxonil related to the total number of treatments carried out from 2006 until 2013. The asterisks and the circles represent the outlier strains.

In the majority of the cases, the increase in the number of treatments carried out with all the tested fungicides was associated with a wider distribution of EC_{50}. However, numerous outlier

strains characterized by high EC_{50} and Rf were been isolated in vineyards never treated with the relative active substance.

4.2.7 *B. cinerea* strains showing a reduced sensitivity

In general, the total number of *B. cinerea* strains characterized by a Rf greater than 10 for fenhexamid, cyprodinil and fludioxonil decreased over the years. A slight increase in the frequency of boscalid resistant strains was observed in 2013 (Figure 4.2.37).

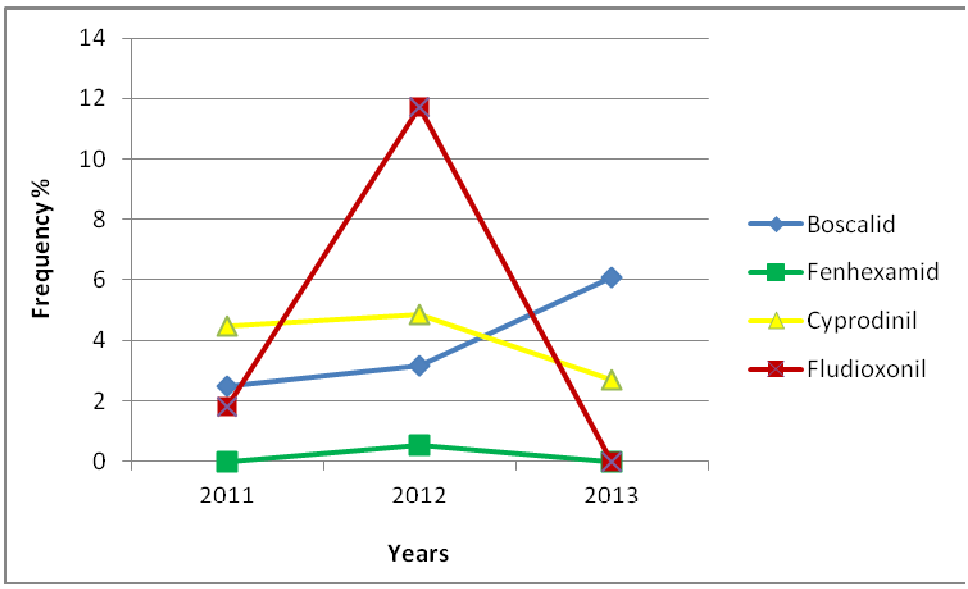


Figure 4.2.37: Frequency of *B. cinerea* strains with Rf greater than 10 found in the three years of study.

The values of EC_{50} for sensitive strains assessed for boscalid, fenhexamid and fludioxonil in the three years of sampling are similar to those calculated by other authors even if, in particular for boscalid and cyprodinil, several strains isolated in Lombardy were characterized by RF > 10. On the contrary, the EC_{50} values concerning cyprodinil are higher than those observed for sensitive strains by other researchers. The annual average EC_{50} concerning cyprodinil are more similar to those calculated for moderately resistant strain by Weber and Hahn (2011).

Excluding fenhexamid, significant differences were detect in the EC_{50} calculated for boscalid, cyprodinil and fludioxonil among years. In particular the EC_{50} mean value for boscalid assessed in 2012 was lower than 2011 and 2013 (H=16.76 ; P>0.001; df=2). In 2011 and 2012 the EC_{50} assessed for cyprodinil were similar and higher from those calculated in 2013 (H=10.03; P=0.007;

df=2). For fludioxonil the EC_{50} assessed in 2011 were similar to those observed in 2013, which show slightly higher values than those observed in 2012 (*H*=69.55; *P*<0.001; df=) (Table 4.2.18).

Authors	Observed effect	Group	EC_{50} (mg/L)			
			Boscalid	Fenhexamid	Cyprodinil	Fludioxonil
Kretschmer *et al*., 2009	Growth at 48 h (A_{600})	S	0.08 ± 0.01	0.05 ± 0.01	0.006 ± 0.001	0.03 ±0.01
Stammler and Speakman, 2006	Growth at 5 days (A_{405})	S	0.01-0.21	-	-	-
Leroux, 2007	Micelium growth	S	0.4	0.01	0.01	0.004
Weber and Hahn, 2011	Length of germ-tubes	S	0.04-0.346	0.06-0.23	0.03-0.48	0.01-0.178
		MR	-	-	0.75-1.44	0.3-0.65
		R	3.43-54.5	>100	1.89-22.7	-
Present study 2011	**Growth at 72 h (A_{492})**	S	**0.18 ± 0.30 b**	**0.05 ± 0.09**	**0.36 ± 0.13 b**	**0.02 ± 0.05 a**
Present study 2012	**Growth at 72 h (A_{492})**	S	**0.06 ± 0.09 a**	**0.03 ± 0.03**	**0.32 ± 0.78 b**	**0.04 ± 0.04 b**
Present study 2013	**Growth at 72 h (A_{492})**	S	**0.10 ± 0.19 b**	**0.03 ±0.03**	**0.24 ± 0.65 a**	**0.01 ± 0.02 a**

Table 4.2.18: EC_{50} values assessed by other authors and calculated in the three years-study for sensitive strains to boscalid, fenhexamid, cyprodinil and fludioxonil. Different letters correspond to significantly different medians according to the Kruskal Wallis test. S=sensitive strain; MR= medium resistant; R=resistant.

Five strains isolated in 2011 showed a simultaneous reduction in sensitivity towards different active substances. Three of them were isolated from samples collected in Oltrepo Pavese and precisely the strains BC19 in Codevilla (PV) and BC29 and BC38 in Torrazza Coste (PV), which were resistant to boscalid and cyprodinil. The strain BC37, isolated from Torrazza Coste (PV), was resistant to fludioxonil and moderately resistant to boscalid whereas the strain BC135, coming from Gonzaga (MN), was resistant to boscalid and moderately resistant to fenhexamid. In 2013, the strain BC674 isolated in Sirmione (BS) was resistant both to boscalid and cyprodinil.

4.2.8 Mycelial growth rate assays and phenotypic characterization of *B. cinerea* strains resistant to boscalid

The mycelial growth rate of the *B. cinerea* strains isolated in 2012 and 2013 and characterized by Rf>10 for boscalid and the mycelial growth of some sensitive strains randomly chosen was assessed at three different temperatures: 15, 20, 25 °C.

No significant differences were found between the growth rate of the two groups of strains at 15, 20 and 25 C° according to the non parametric Mann Whitney U test (U=1415; P=0.052; df=2, U=1493; P=0.173; df=2 and U=1493; P=0.293; df=2 respectively).

No differences were found among the frequency of the four classes of phenotypes (Figure 4.2.38) of the sensitive *B. cinerea* strains according to the Chi square test. On the contrary, significant differences were observed among the four classes for the resistant strains according to the Chi square test (χ^2=9.26; P=0.026; df=3). The majority of resistant strain belonging to the mycelial phenotype were characterized by evident sporulation.

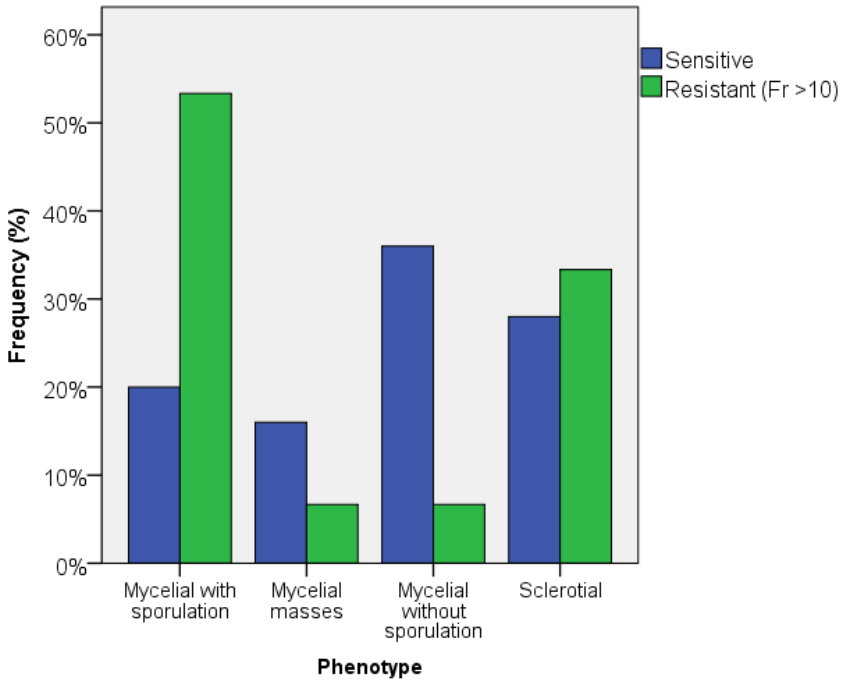


Figure 4.2.38: Frequency of phenotypes in sensitive and resistant *B. cinerea* strains of boscalid.

4.3 Molecular characterization of *B. cinerea* strains

4.3.1 Detection of transposable elements *Boty* and *Flipper* in *B. cinerea* populations

706 *B. cinerea* strains were analyzed in order to detect the presence of the two transposable elements *Boty* and *Flipper*. Highly significant differences were found in the distribution of the four molecular classes (χ^2=535.04; *P*<0.0001; df=3). The *transposa* strains, characterized by the presence of both transposable elements, were the most abundant molecular types (60.2 %), followed by *Boty* (25.1 %), *vacuma* (11.5 %) and *Flipper* (3.3%) (Figure 4.3.1). Only for *transposa* were detected differences in the percentages among years (χ^2=78,36; *P*<0.0001; df=2): a clear decrease of *transposa* strains over the years was reported (Figure 4.3.2).

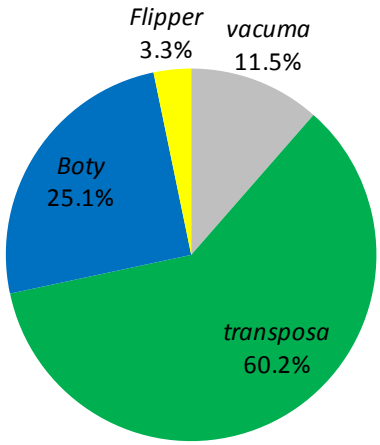
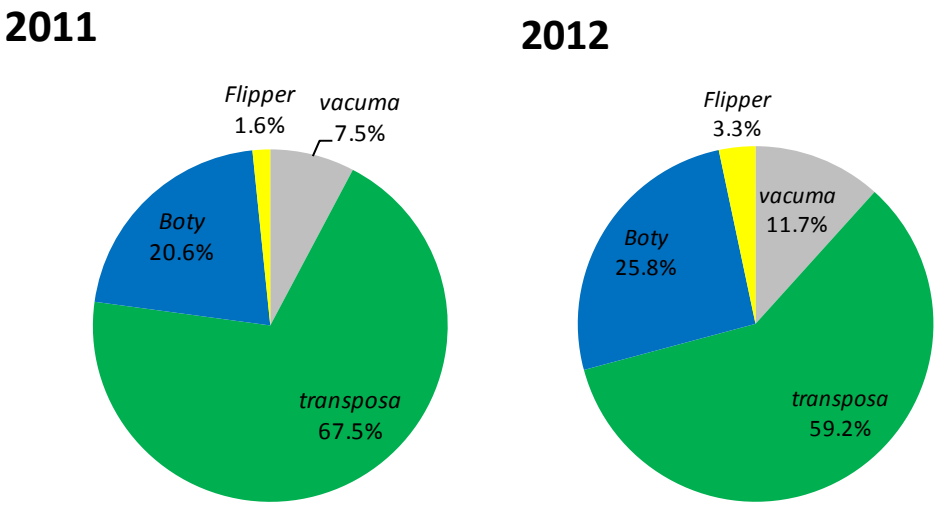


Figure 4.3.1: Occurrence of the four molecular types in the whole *B. cinerea* population.



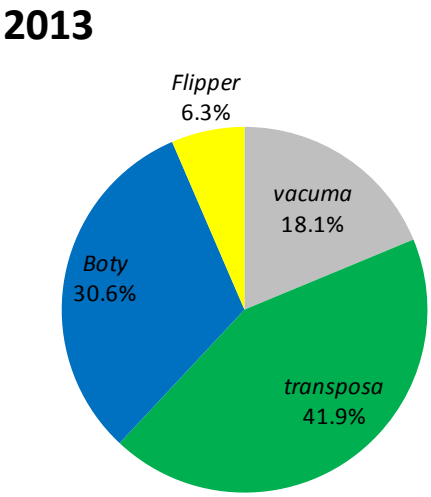


Figure 4.3.2: Frequencies of the four molecular types of *B. cinerea* populations 2011-2013.

The presence or absence of transposable elements was not associated with higher Rfs concerning all the tested fungicides (*H*=0.519 ; *P*=0.670 ; df=3 for boscalid; *H*=0.946; *P*=0.418 ; df=3 for fenhexamid; *H*=0.267 ; *P*=0.849 ; df=3 for cyprodinil; *H*=0.246 ; *P*=0.864 ; df=3 for fludioxonil). However, all the strains with Rf greater than 10 belong to the molecular class of *Boty* or *transposa*, with the only exception of two strains for the fungicide boscalid and two strains for fludioxonil (Figure 4.3.3; Figure 4.3.4).

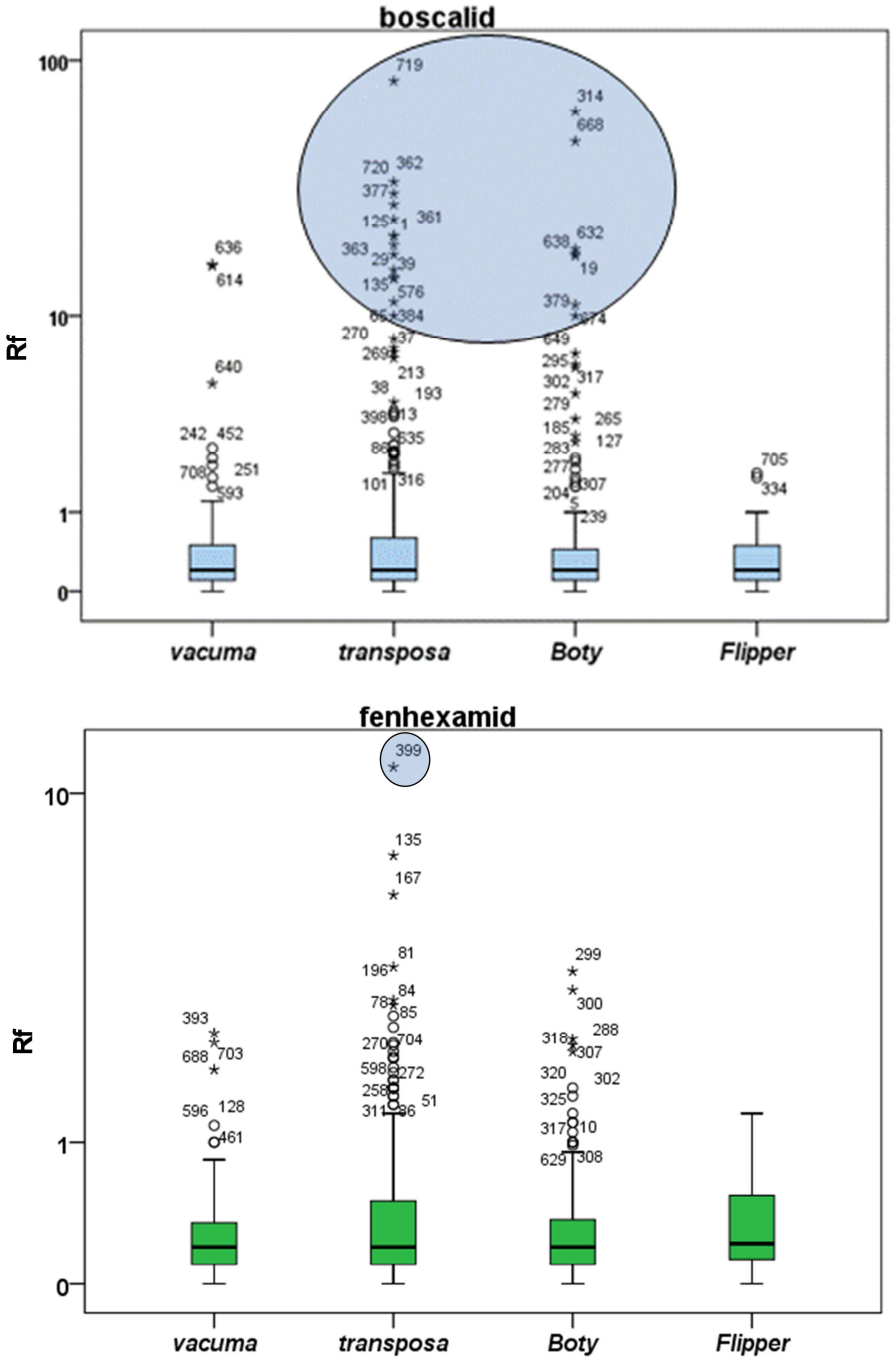


Figure 4.3.3: Distribution of resistant factors (Rf) concerning boscalid and fenhexamid in relation to the molecular types. The strains with Rf greater than 10 are highlighted in blue.

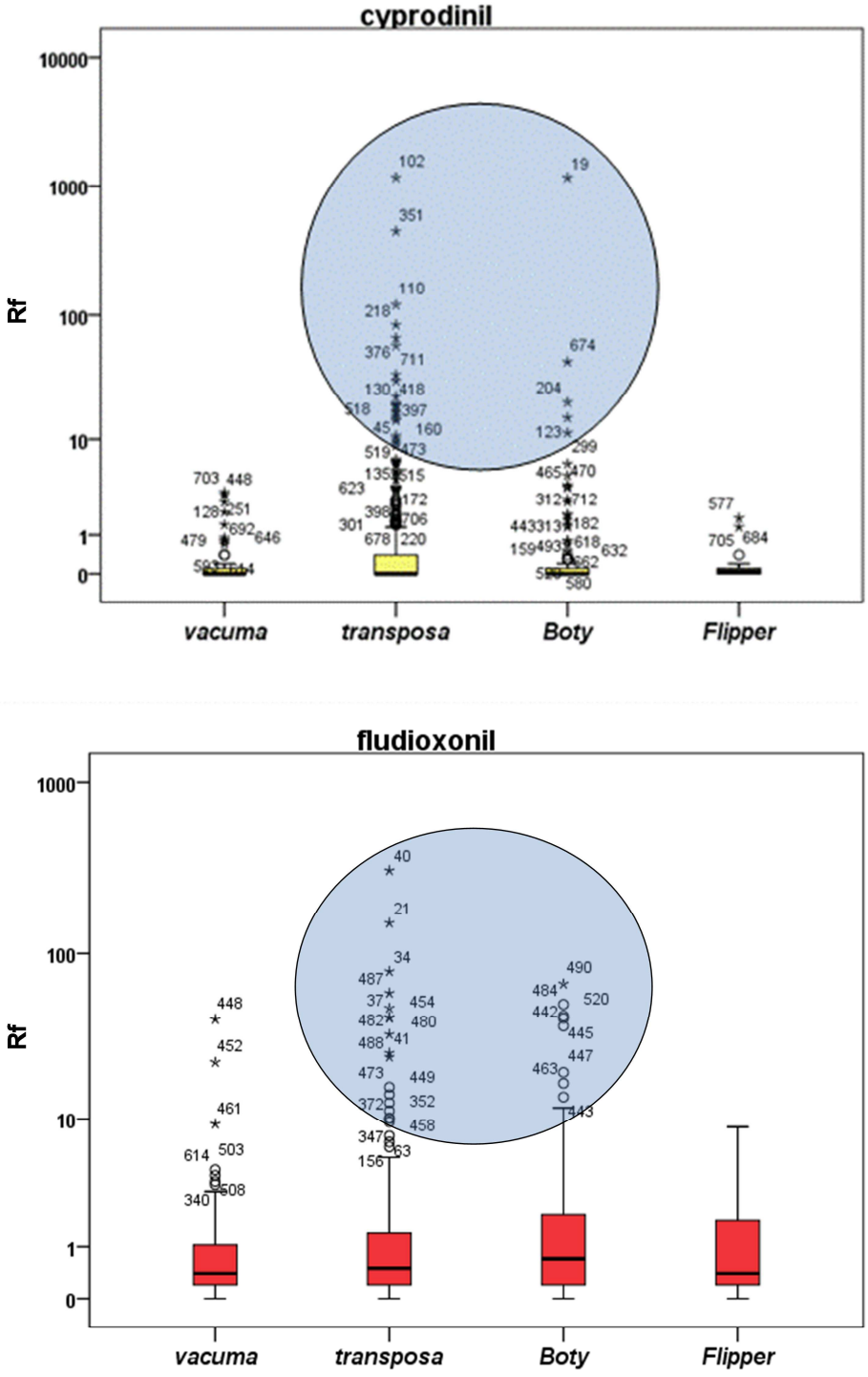


Figure 4.3.4: Distribution of resistant factors (Rf) concerning cyprodinil and fludioxonil in relation to the molecular types. The strains with Rf greater than 10 are highlighted in blue.

4.3.2 Detection of mating types in *B. cinerea* populations

Among the 684 strain screened for the mating types *MAT1-1*, *MAT1-2*, 368 belong to *MAT1-1* (53.8%) and 316 to *MAT1-2* (46.2%): in the sampled population, significant difference was found between the two mating type frequencies (χ^2=3.95; *P*=0.047; df=1). 36 strains gave no amplification product.

In 2011, a population of 309 *B. cinerea* strains was screened and significant difference was found between the *MAT1-1*: *MAT1-2* ratio (χ^2=3.96; *P*=0.046; df=1). In the populations isolated in the provinces of Brescia, Mantova and Sondrio, the *MAT1-1:MAT1-2* ratio was similar to the theorethical 1:1 ratio ratio expected in an idealized random mating population. Only in the *B. cinerea* population isolated in the province of Pavia, the *MAT1-1:MAT1-2* ratio differed from the theoretical 1:1, according to the chi-square test (χ^2=4.81; *P*=0.028; df=1) suggesting that asexual reproduction is more likely in this area. (Figure 4.3.5).

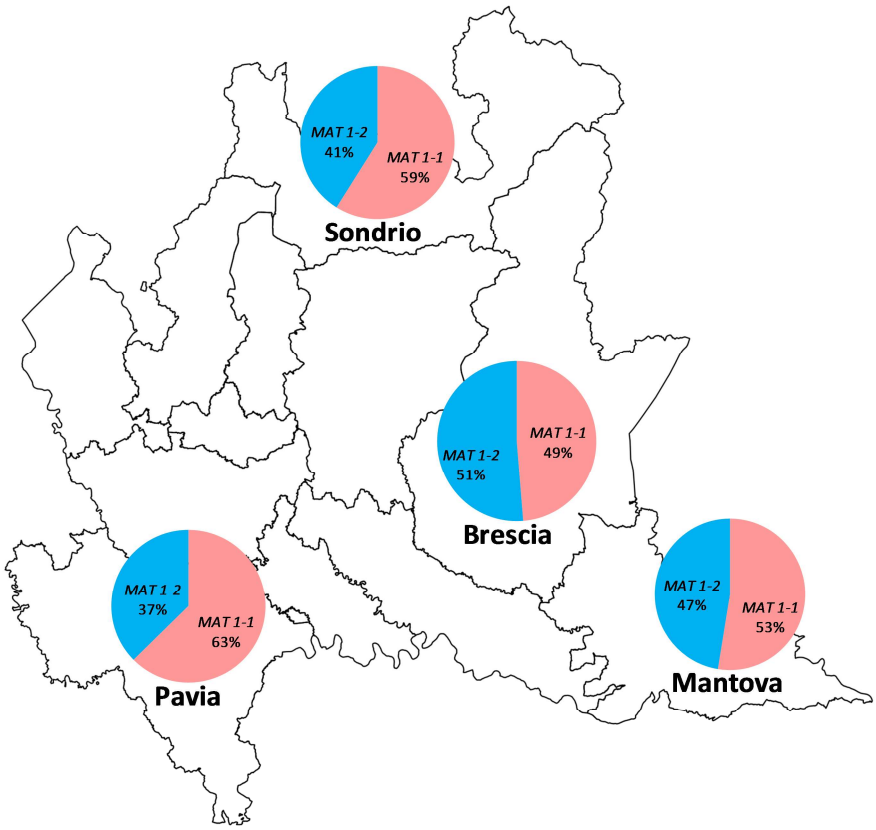


Figure 4.3.5: The *MAT1-1*, *MAT1-2* distribution in the four provinces investigated in 2011.

B. cinerea populations within the vineyards sampled in 2011 apart from Dossi Salati in the province of Sondrio were characterized by *MAT1-1:MAT1-2* ratios similar to the theoretical 1:1 according to the chi-square test (χ^2=4.26; *P*=0.039; df=1) (Figure 4.3.6).

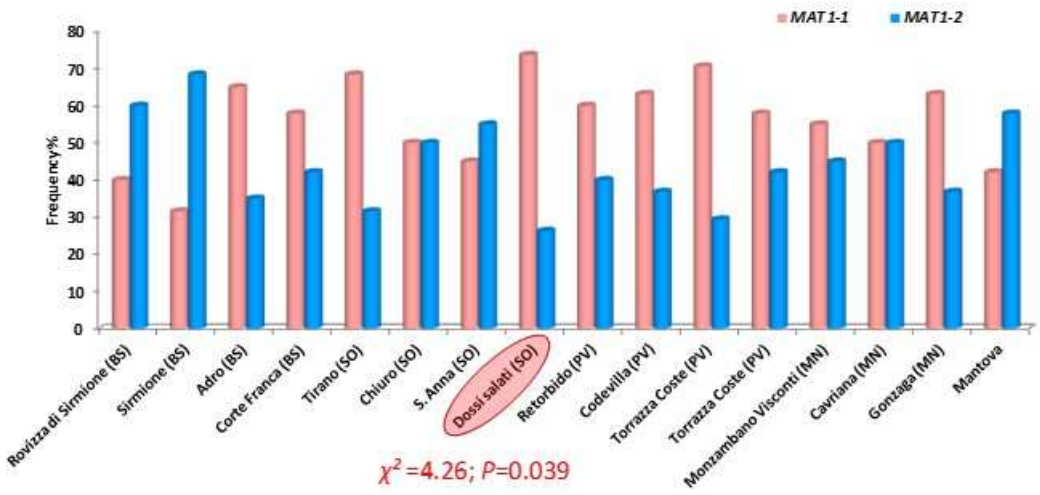


Figure 4.3.6: The *MAT1-1*, *MAT1-2* distribution in the vineyards investigated in 2011.

In 2012, 238 *B. cinerea* strains were analyzed and no difference was detected in the *MAT1-1:MAT1-2* ratio. Concerning the viticultural area level, only in the *B. cinerea* population located in the province of Mantova the *MAT1-1:MAT1-2* ratio differed from the theoretical 1:1 (χ^2=6.12; *P*=0.013; df=1), suggesting a more likely asexual reproduction (Figure 4.3.7).

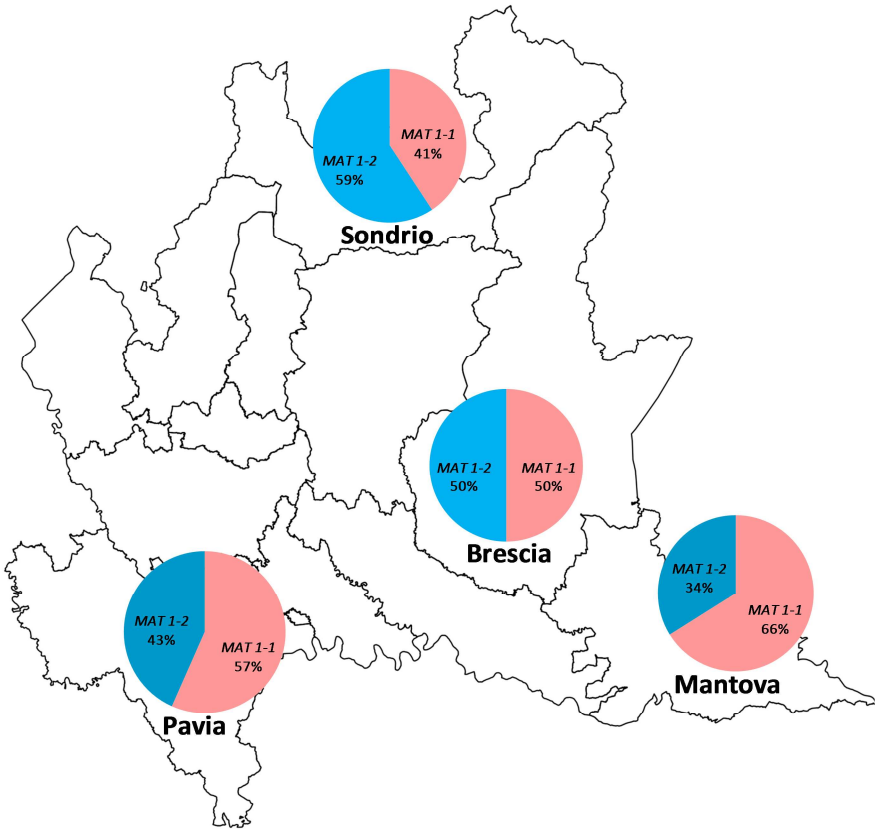


Figure 4.3.7: The *MAT1-1*, *MAT1-2* distribution in the four provinces investigated in 2012.

At a local level, only in the *B. cinerea* populations isolated in Torrazza Coste (PV) and Ponti sul Mincio (MN) the *MAT1-1:MAT1-2* ratios differed from the theoretical 1:1 according to the chi square test (χ^2=5.00; *P*=0.025; df=1 and χ^2=9.80; *P*=0.002; df=1, respectively) (Figure 4.3.8).

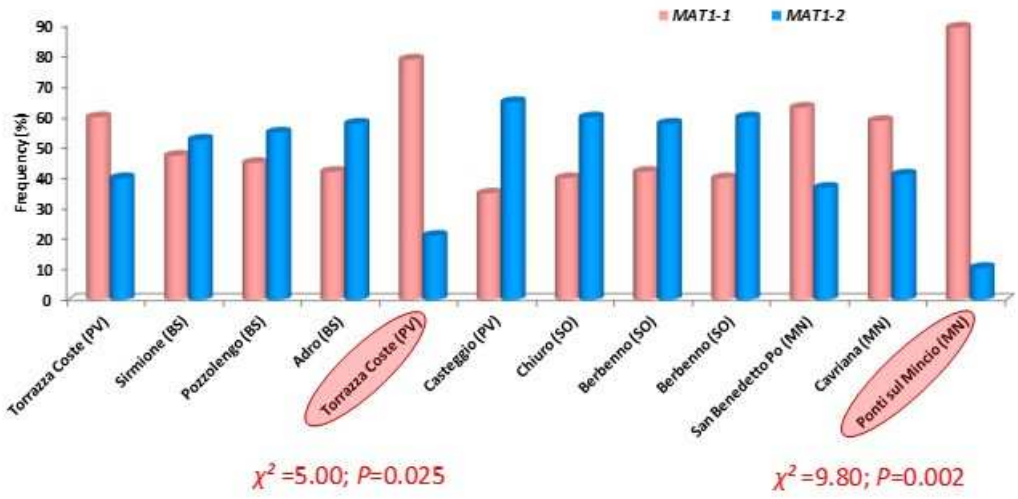


Figure 4.3.8: The *MAT1-1*, *MAT1-2* distribution in the vineyards investigated in 2012.

In 2013 data concerning the 137 *B. cinerea* strains analyzed for the detection of the mating types showed no difference in the regional distribution of the two mating types (χ^2=0.007; *P*=0.932; df=1) (Figura 4.3.9).

In the three investigated provinces, the *MAT1-1:MAT1-2* ratios coincided with the theoretical 1:1 (Figure 4.3.8). The same situation was observed at a local level in all the vineyards sampled (Figure 4.3.10).

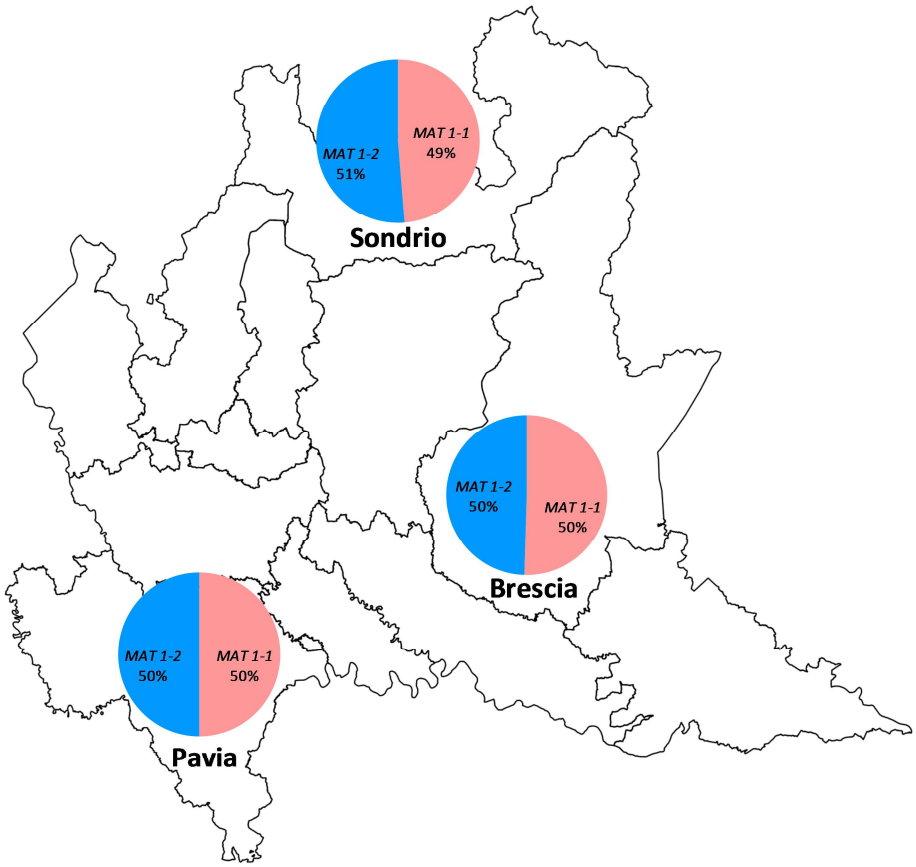


Figure 4.3.9: The *MAT1-1*, *MAT1-2* distribution in the three provinces investigated in 2013.

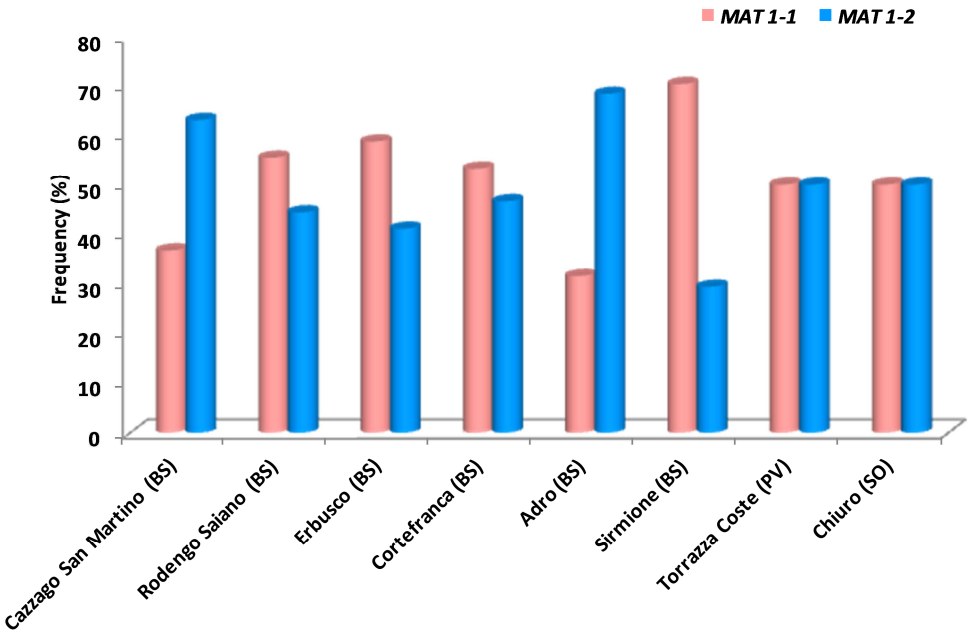


Figure 4.3.10: The *MAT1-1*, *MAT1-2* distribution in the vineyards investigated in 2013.

4.3.3 Detection of the main point mutations in the SdhB subunit for boscalid resistant *B. cinerea* strains

22 *B. cinerea* strains with Rf > 10 for boscalid, (Table 4.3.1) and 7 sensitive strains arbitrarily chosen (Table 4.3.2) were characterized for the two main point mutations linked with resistance to the SDHI boscalid (H272Y or R).

21 of the 22 resistant strains were characterized by the H272Y mutation, resulting in the substitution of histidine by tyrosine. None of the strains had the H272R mutation and the resistant strain BC674 did not show any of the mutations tested.

The point mutation H272Y was found also in 4 of the 7 sensitive tested strains (BC73, BC340, BC380 and BC425).

Strain	Year	Location	EC_{50}	Rf
BC1	2011	BS	5.0	20.9
BC19	2011	BS	4.3	17.8
BC29	2011	BS	4.6	19.2
BC39	2011	BS	3.4	14.1
BC65	2011	MN	2.4	10
BC125	2011	MN	5.0	20.8
BC135	2011	MN	3.7	15.3
BC314	2011	SO	15.4	63.8
BC361	2012	BS	5.9	24.3
BC362	2012	BS	7.4	30.7
BC363	2012	BS	4.3	17.6
BC377	2012	BS	6.7	27.8
BC379	2012	BS	2.4	10
BC384	2012	BS	2.7	11.4
BC576	2013	BS	3.4	14.3
BC614	2013	BS	3.9	15.9
BC632	2013	BS	4.5	18.5
BC636	2013	BS	3.9	16.1
BC638	2013	BS	4.2	17.4
BC668	2013	BS	11.8	49
BC674	2013	BS	2.7	11.1
BC719	2013	SO	20.2	83.6

Table 4.3.1: Resistant *B. cinerea* strains collected in different vineyards in 2011, 2012 and 2013 and their EC_{50} and Rf.

Strain	Year	Location	EC_{50}	Rf
73	2011	MN	0,013	0.1
252	2011	SO	0,029	0,1
340	2012	PV	0,026	0,1
380	2012	BS	0,026	0,1
425	2012	PV	0,016	0,1
617	2013	BS	0,021	0,1
644	2013	BS	0,033	0,1

Table 4.3.2: Sensitive *B. cinerea* strains collected in different vineyards in 2011, 2012 and 2013 and their EC_{50} and Rf.

To verify the presence of the point mutation H272Y in the resistant and sensitive tested *B. cinerea* strains, the PCR products of the strain BC361 were sequenced. The sequence

denominated BC361a.BOSYF originated from primer H272Yfw and BC361a.BOSYR originated from the primer H272Yrev (Table 4.3.3).

Name	Sequence
BC361a.BOSYF	AAGGGAGGTACTTTGTGGAACAGTTATCTTTGTTTGCAGAGTTACAGATGGCTTGCAGATTC CCGTGATCAGAAGAAAGGAAGAACGTAAGGCAGCTTTGGATAACAGCATGAGTTTGTACAG ATGTTACACTATTCTCAACTGCTGGAGATATT
BC361a.BOSYR	AGAGGAGAGAGGTACTTTTTGTGGAACAGTTATCTTTGTTTGCAGAGTTACAGATGGCTTGC AGATTCCCGTGATCAGAAGAAAGGAAGAACGTAAGGCAGCTTTGGATAACAGCATGAGTTT GTACAGATGTTACACTATTCTCAACTGCTGGAGATATT

Table 4.3.3: Results of the sequencing of the two filaments of the strain BC361 amplified with H272Yfw and H272Yrev respectively.

The two sequences were aligned with the AY726618 sequence (*Botryotinia fuckeliana* strain T4 succinate dehydrogenase iron sulphur protein gene, complete coding sequence) present in the Gene Bank of the web site of the NCBI using the software BLAST nucleotide (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The comparison between the two sequences AY726618 and BC361a.BOSYF revealed an identity equal to 94% and an E value of $9x10^{-58}$ and also the presence of the nucleotide substitution leading to the substitution of histidine by tyrosine in the corresponding aminoacid sequence. The length of the sequence was 156 bp (Figure 4.3.11).

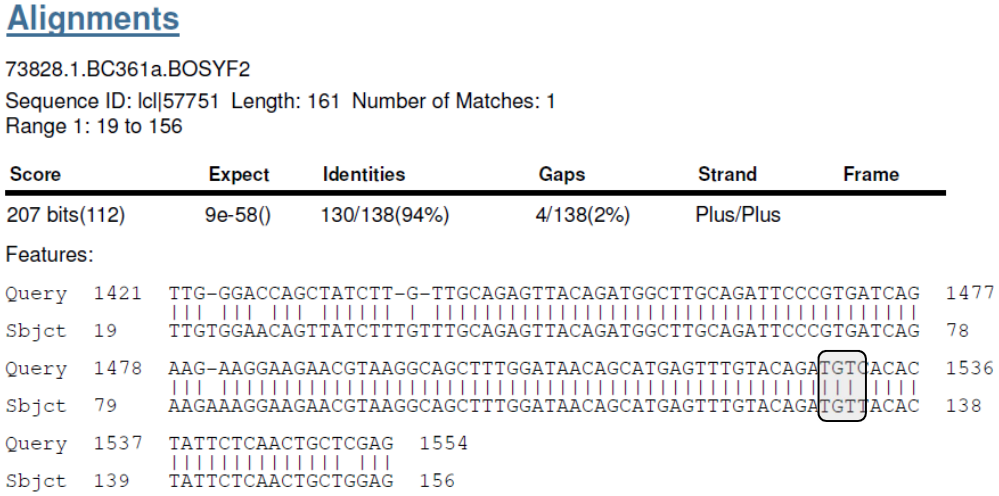


Figure 4.3.11: Alignment of the two sequence AY726618 and BC361a.BOSYF. Shadowed region shows the codon with the nucleotide substitution

The comparison between AY726618 sequence with the BC361b.BOSYR revealed an identity equal to 96% and an E value of $3x10^{-62}$. The length of the aligned sequences was 147 bp (Figure 4.3.12).

Alignments

73828.2.BC361b.BOSYR
Sequence ID: lcl|60251 Length: 156 Number of Matches: 1
Range 1: 9 to 147

Score	Expect	Identities	Gaps	Strand	Frame
222 bits(120)	3e-62()	133/139(96%)	2/139(1%)	Plus/Minus	

Features:

```
Query  1384  ATCTTGCCCCTCCTACTGGTGGAACAGTGAGGAGTACTTGGGACCAGCTATCTTGTTGCA  1443
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  147   ATCTTGCCCCTCCTACTGGTGGAACAGTGAGGAGTACTTGGGACCAGCTATCTTGTTGCA  88

Query  1444  GAGTTACAGATGGCTTGCAGATTCCCGTGATCAGAAGAAGGAA-GAACGTAAGGCAGCTT  1502
             |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  87    GAGTTACAGATGGCTTGCAGATTCCCGTGATCAGAAGAAGGAAAGAACGTAAGGCAGCTT  28

Query  1503  TGGATAACAGC-ATGAGTT  1520
              |   ||||||| || ||||
Sbjct  27    TAATTAACAGCCATAAGTT  9
```

Figure 4.3.12: Alignment of the two sequence AY726618 and BC361b.BOSYR.

4.4 Microsatellite analysis of *B. cinerea* populations

4.4.1 Genotyping and clonal composition of the *B. cinerea* populations

317 *B. cinerea* strains, arbitrarily chosen, were completely genotyped for six microsatellites loci. The six examined microsatellites were all polymorphic and exhibited a number of alleles ranging from 9 to 22. The number of alleles, the allele size range, the effective number of alleles (n^a), the allele frequencies and the unbiased gene diversity are listed in the Table 4.4.1.

Locus	N° alleles	Allele size range (bp)	n^a	h^b
Bc2	22	137-203	4.694	0.787
Bc3	9	209-225	4.128	0.758
Bc5	15	143-175	7.577	0.868
Bc6	12	108-156	1.777	0.437
Bc7	12	105-129	5.181	0.807
Bc10	18	159-197	6.617	0.849

n^a: effective number of alleles.
h^b: unbiased gene diversity.

Table 4.4.1: Number of alleles and size range of the six examined microsatellite in the *B. cinerea* populations.

On the basis of the diagnostic allele (86 bp) at the *Bc6* locus, all individuals were identified as *B. cinerea sensu stricto* (Group II).

B. cinerea genotypes with identical alleles at all loci were assumed to be clones and data were clone-corrected for some analyses to eliminate the effects of asexual reproduction on tests for random mating. The population was subdivided by years, provinces and years/provinces to evaluate the sources of variation both separately and altogether. The number of isolates (N), the number of multilocus genotypes (MLG), the genotypic diversity ($\hat{G}$), the clonal fraction and the number of genotypes represented by two or more isolates in all the considered population are listed in Table 4.4.2.

	Populations	*N*	MLG[a]	$\hat{G}$[b]	Clonal Fraction[c]	g[d]	N° isolates in each repeated genotype
	Whole pop.	317	292	0.999	0.08	19	2,2,4,2,2,2,2,4,3,2, 2,3,2,2,2,2,2,2,2
Years	**2011**	174	156	1.000	0.10	13	2,2,4,2,2,2,3,3,2,2,3,2,2
	2012	99	96	1.000	0.03	3	2,2,2
	2013	44	44	1.000	0.00	0	-
Provinces	**1 BS**	86	84	1.000	0.02	2	2,2
	2 SO	75	72	1.000	0.04	3	2,2,2
	3 PV	83	76	1.000	0.08	7	2,2,2,2,2,2,2
	4 MN	73	72	1.000	0.01	1	2
Years per provinces	**PV2011**	44	39	0.994	0.11	5	2,2,2,2,2
	PV2012	30	29	0.997	0.03	1	2
	PV2013	9	9	1.000	0.00	0	-
	BS2011	34	33	0.998	0.03	1	2
	BS2012	21	21	1.000	0.00	0	-
	BS2013	31	31	1.000	0.00	0	-
	SO2011	51	49	0.998	0.04	2	2,2
	SO2012	20	19	0.994	0.05	1	2
	SO2013	4	4	1.000	0.00	0	-
	MN2011	45	44	0.998	0.02	1	2
	MN2012	28	28	1.000	0.00	0	-

[a] Number of multilocus genotypes.
[b] Genotypic diversity.
[c] Clonal fraction.
[d] Number of genotypes represented by two or more isolates.

Table 4.4.2: Number of multilocus genotypes and clonal composition of *B. cinerea* populations.

Among the 317 analyzed genotypes, 292 distinct MLGs were found. The genotypic diversity ranged from 0.994 to 1.000 and the lowest variability was found among the populations subdivided by years and provinces (PV2011, PV2012 and SO2012). The largest proportion of *B. cinerea* clones was sampled in 2011 in PV. The most frequent genotype was detected 4 times in 2011 and was isolated in the provinces of Pavia and Sondrio.

The estimates of gene flow (N_m) revealed the lowest differentiation (highest N_m) between PV2012 and PV2013, PV2012 and BS2012 and also between BS2011 and PV2013 with N_m varying from 113.9 to 85.3. In the majority of the comparisons, N_m ranged between 42.4 and 10.4. The highest differentiation (lowest N_m) was found between SO2013 and all the other populations, BS2013 with the most of the other populations, BS2012 with SO2011, SO2012 and PV2013 while SO2011 was completely different from SO2012 and BS2012 (Table 4.4.3).

	PV2011	PV2012	PV2013	BS2011	BS2012	BS2013	SO2011	SO2012	SO2013	MN2011
PV2011	-									
PV2012	35.5	-								
PV2013	15.3	113.9	-							
BS2011	10.4	38.8	85.3	-						
BS2012	16.1	92.0	0.0	13.2	-					
BS2013	5.7	6.1	11.0	3.5	19.7	-				
SO2011	15.2	42.4	29.9	9.9	0.0	11.1	-			
SO2012	12.0	38.1	35.0	10.4	0.0	12.1	0.0	-		
SO2013	2.5	3.4	7.9	1.9	7.7	0.0	4.8	7.4	-	
MN2011	0.0	41.9	24.7	13.3	21.4	6.3	21.2	17.3	3.1	-
MN2012	12.4	31.9	25.9	19.7	51.8	5.9	21.3	27.8	3.6	17.6

Table 4.4.3: The estimates of the gene flow (N_m) from six polymorphic loci between *B. cinerea* populations.

4.4.2 Analyses for random mating

Mating-type distributions, index of association I_A and the standardized index of association, $\bar{r}_d$ of uncorrected and clone-corrected samples were tested (Table 4.4.4). Only the uncorrected analysis were carried out for the *B. cinerea* population with no repetition of MLGs.

Considering all *B. cinerea* strains analyzed as an unique population, the multilocus linkage disequilibrium (I_A and r_d) were significantly different from 0, suggesting that panmixia cannot be inferred alone from the data and that some clonality is present.

The mating type ratios did not differ significantly from 1:1 ratio (P>0.05) in the *B. cinerea* populations defined by the year of isolation (2011, 2012 and 2013) but I_A was significantly higher than 0 only for the 2011 and 2012 *B. cinerea* populations and no significant difference was detected in 2013. For these reasons, the hypothesis of panmixia could not be rejected in the *B. cinerea* population isolated in 2013.

Taking into account the populations sampled in different provinces, only the population isolated in the province of Pavia showed a mating type ratios significantly different from 1:1 (P<0.05) and a I_A significantly greater than zero, resulting a mainly clonal population. These data were confirmed by considering the populations divided by year and province of isolation: the mating type ratios differed significantly from 1:1 ratio (P>0.05) in the clone corrected 2011 and 2012 and clone uncorrected 2012 populations isolated in the province of Pavia and I_A were significantly different from 0. On the contrary the *B. cinerea* populations PV2013, BS2011, BS2012, BS2013, SO2013 and MN2012 were characterized by mating type ratios not significantly different

from 1:1 ratio ($P > 0.05$) and I_A indexes not significantly different from 0. Taken together, these data confirmed that *B. cinerea* populations in 2013 were characterized by a high level of recombination and the PV populations were characterized in general by a low level of recombination except in 2013.

Multilocus linkage disequilibrium (LD)			I_A^c	r_d^c	X^{2b}
Whole population[a]		Uncorrected	0.22**	0.045**	4.23*
		Clone-corrected	0.17**	0.036**	7.20**
Years[a]	2011	Uncorrected	0.21**	0.043**	2.14
	2011	Clone-corrected	0.15**	0.031**	3.50
	2012	Uncorrected	0.29**	0.059**	0.65
	2012	Clone-corrected	0.27**	0.055**	0.67
	2013	Uncorrected	0.02	0.003	2.08
	2013	Clone-corrected	0.02	0.003	2.14
Provinces[a]	1 BS	Uncorrected	0.24**	0.049**	0.05
	1 BS	Clone-corrected	0.21**	0.043**	0.20
	2 SO	Uncorrected	0.24**	0.049**	0.86
	2 SO	Clone-corrected	0.21**	0.043**	1.70
	3 PV	Uncorrected	0.27**	0.054**	6.05*
	3 PV	Clone-corrected	0.23**	0.046**	6.04*
	4 MN	Uncorrected	0.17**	0.034**	0.23
	4 MN	Clone-corrected	0.16**	0.032**	0.13
Years per provinces[a]	PV2011	Uncorrected	0.32**	0.065**	1.52
	PV2011	Clone-corrected	0.22**	0.044**	5.07*
	PV2012	Uncorrected	0.36**	0.073**	4.80*
	PV2012	Clone-corrected	0.32**	0.066**	4.17*
	PV2013	Uncorrected	0.31	0.063	0.50
	BS2011	Uncorrected	0.17*	0.034*	0.12
	BS2011	Clone-corrected	0.11	0.021	0.27
	BS2012	Uncorrected	0.09	0.019	0.43
	BS2013	Uncorrected	0.06	0.012	0.33
	SO2011	Uncorrected	0.12*	0.026*	2.88
	SO2011	Clone-corrected	0.11*	0.021*	3.00
	SO2012	Uncorrected	0.51**	0.104**	3.20
	SO2012	Clone-corrected	0.38**	0.079**	2.58
	SO2013	Uncorrected	0.71	0.181	0.21
	MN2011	Uncorrected	0.24**	0.048**	0.38
	MN2011	Clone-corrected	0.22**	0.044**	1.81
	MN2012	Uncorrected	0.04	0.008	1.52

a Clone-corrected samples include a single representative of each multilocus genotype.

b Asterisks (**) indicate that mating-type ratios deviated from 1:1 with *P* = 0.001, asterisk (*) indicates a *P*= 0.01. *P* values for all other samples are > 0.05 (χ^2 < 3.84).

c Index of association (IA) and r_d were calculated with MultiLocus v.1.3b. The majority of the *P* values are <0.001 and were determined by 1,000 randomizations. For those with *P* > of 0.05 (*) or > of 0.01 (**).

Table 4.4.4: Mating type distribution and multilocus linkage disequilibrium for populations of *B. cinerea* considered at regional, provincial, annual and provincial/annual level.

4.4.3 Population structure

Significant genetic differentiations (Φ_{PT}) were detected among *B*. *cinerea* populations sampled in 2011, 2012 and 2013. 2011 and 2012 populations were more similar to each other as measured by Nei's genetic identity. 2013 population was slightly different from both 2011 and 2012 (Table 4.4.5).

***B. cinerea* population**			
2011	**2012**	**2013**	**Population**
-	0.009 (0.001)	0.011 (0.001)	2011
0,963	-	0.007 (0.002)	2012
0,784	0,810	-	2013

Table 4.4.5: Genetic differentiation measured by Φ_{PT} (above the diagonal) and Nei's genetic identity (below the diagonal) between populations of *B. cinerea* sampled in 2011, 2012 and 2013. *P* values for Φ_{PT} are in brackets.

The analysis of molecular variance (AMOVA) showed that 99% of the total variation was distributed within populations and 1% of the variation was explained by differences among populations, with a total Φ_{PT} value of 0.009 (*P*=0.001) (Table 4.4.6).

Source	**d.f.**	**SS**	**MS.**	**%**	Φ_{PT}	***P***
Among Populations	2	10.59	5,29	1%	0.009	0.001
Within Populations	314	901.92	2,87	99%		
Total	316	912.51		100%		

Table 4.4.6: Analysis of molecular variance among and between populations measured by Φ_{PT}

Principal coordinates analysis (PCoA) revealed a considerable overlap among multilocus genotypes belonging to the three populations isolated during the three years of sampling (Figure 4.4.1). The first two coordinates explained 7.5% of the variation. The *B. cinerea* genotypes formed 4 clusters but no evident differences were detected among the clusters taking into account year of isolation.

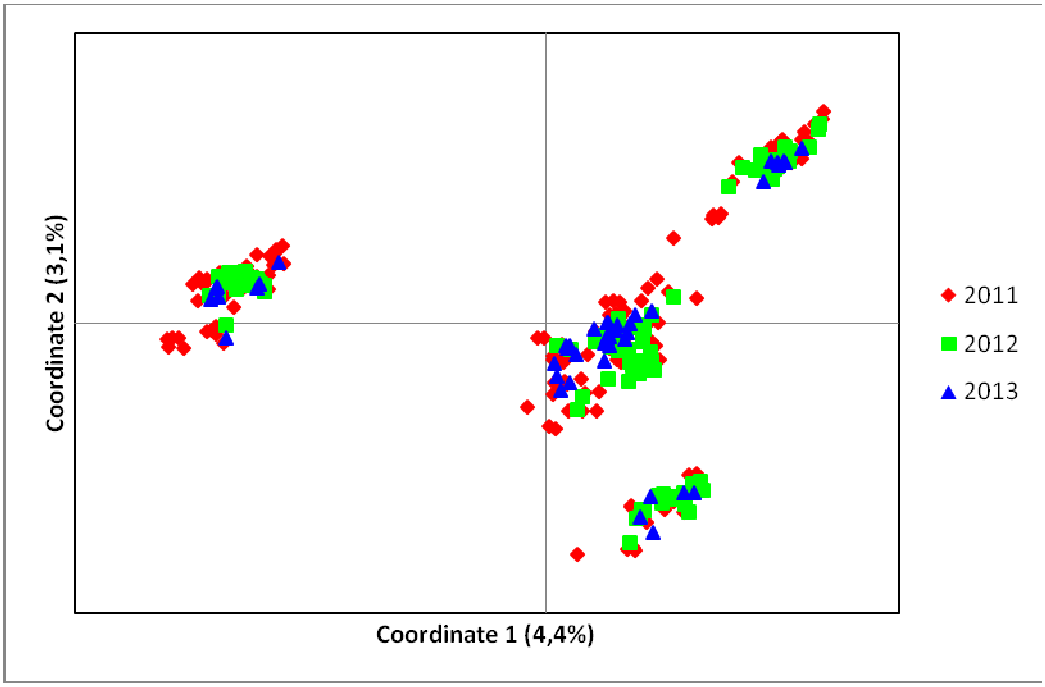


Figure 4.4.1: PCoA plot conducted with Genalex 6.501 on pairwise genetic distances between 317 *B. cinerea* strains from the three annual populations.

Significant genetic differentiations (Φ_{PT}) were detected among the *B. cinerea* populations sampled in the four provinces. The populations isolated in Mantova and Pavia were similar, while those associated with Sondrio and Brescia were different from all the other provinces. Nei's genetic identity showed a similarity between Sondrio and Pavia. (Table 4.4.6).

***B. cinerea* population**				
Brescia	**Sondrio**	**Pavia**	**Mantova**	**Population**
-	0.004 (0.004)	0.004 (0.008)	0.008 (0.001)	**Brescia**
0.939	-	0.007 (0.001)	0.010 (0.001)	**Sondrio**
0.929	0.901	-	0.001 (0.191)	**Pavia**
0.934	0.913	0.966	-	**Mantova**

Table 4.4.7: Genetic differentiation measured by Φ_{PT} (above the diagonal) and Nei's genetic identity (below the diagonal) between populations of *B. cinerea* sampled in four provinces of Lombardy. *P* values for Φ_{PT} are in brackets.

The AMOVA also in this case showed that 99% of the total variation was distributed within populations and 1% of the variation was explained by differences among populations, with a total Φ_{PT} value of 0.006 (P=0.001) (Table 4.4.8).

Source	d.f.	SS	MS	%	Φ_{PT}	P
Among Populations	3	12.603	4.201	1%	0.006	0.001
Within Populations	313	899.911	2.875	99%		
Total	316	912.514		100%		

Table 4.4.8: Analysis of molecular variance among and between populations measured by Φ_{PT}

PCoA revealed considerable overlaps among multilocus genotypes from all the populations (Figure 4.4.2). The first two coordinates explained 7.5% of the variation. The *B. cinerea* genotypes formed 4 separated groups but in general the province, as the year, did not affect the genetic differentiation among MLGs .

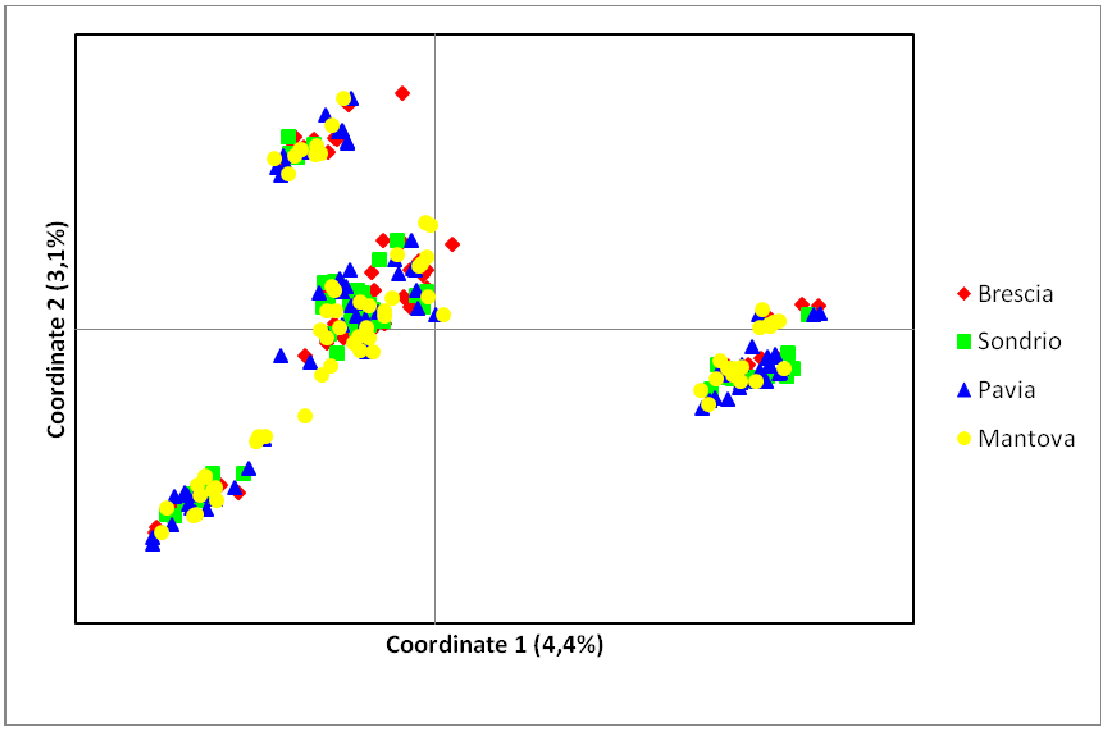


Figure 4.4.2: PCoA plot conducted with Genalex 6.41 on pairwise genetic distances between 317 *B. cinerea* strains from the three provincial populations.

Significant Φ_{PT}s were detected among the populations sampled in 2011, 2012 and 2013 in the four sampled provinces. The PV2011 population was different from all the other populations with the exception of those sampled in the same province but in different years (PV2012 and PV2013) and MN2011. PV2012 population differed only from BS2013 and SO2013 populations. PV2013 populations was similar to all the others with the only exception of the BS2013 population. Both SO2012 and BS2012 populations were different from MN2011, BS2011, PV2011 and BS2013 populations and similar to all the others. BS2011 population was different from all the other

populations but similar to PV2012 and PV2013 populations. BS2013 was different from all the populations with the only exception of SO2013 population. SO2011 was similar to SO2012, BS2012, PV2012 and PV2013 populations but different from the others. SO2013 populations was similar to SO2012, BS2012, BS2013 and PV2013 populations. MN2011 populations was similar to all the three PV populations but different from the others. MN2012 populations was different from SO2012, BS2012, PV2012 and PV2013 populations. The differences assessed for BS2013 and SO2013 were confirmed also by Nei's genetic identity (Table 4.4.9).

PV2011	PV2012	PV2013	BS2011	BS2012	BS2013	SO2011	SO2012	SO2013	MN2011	MN2012	
-	0.014	0.032	0.046**	0.030**	0.080**	0.032**	0.040**	0.165**	0.000	0.039**	**PV2011**
0.902	-	0.004	0.013	0.005	0.076**	0.012	0.013	0.128**	0.012	0.015	**PV2012**
0.785	0.827	-	0.006	0.000	0.043**	0.016	0.014	0.060	0.020	0.019	**PV2013**
0.858	0.917	0.861	-	0.036*	0.126**	0.048**	0.046**	0.205**	0.036**	0.025*	**BS2011**
0.831	0.876	0.826	0.848	-	0.025**	0.000	0.000	0.061	0.023*	0.010	**BS2012**
0.683	0.662	0.680	0.594	0.766	-	0.043**	0.040**	0.000	0.074**	0.078**	**BS2013**
0.866	0.900	0.803	0.848	0.923	0.771	-	0.000	0.094**	0.023**	0.023*	**SO2011**
0.811	0.860	0.773	0.827	0.895	0.738	0.931	-	0.064	0.028*	0.018	**SO2012**
0.310	0.374	0.479	0.306	0.452	0.685	0.438	0.491	-	0.137**	0.122**	**SO2013**
0.965	0.904	0.806	0.878	0.844	0.693	0.885	0.836	0.368	-	0.028**	**MN2011**
0.853	0.891	0.811	0.895	0.884	0.697	0.884	0.864	0.450	0.876	-	**MN2012**

* Significant level P<0.05
** Significant level P<0.01

Table 4.4.9: Genetic differentiation measured by Φ_{PT} (above the diagonal) and Nei's genetic identity (below the diagonal) between populations of *B. cinerea* sampled in four provinces of Lombardy in 2011, 2012 and 2013. *P* values for Φ_{PT} are in brackets.

AMOVA showed that 97% of the total variation was distributed within populations and 3% of the variation was explained by differences among populations, with a total Φ_{PT} value of 0.034 (*P* = 0.001) (Table 4.4.10). In this case, the variance explained within populations was higher compared to those calculated for annual and provincial population.

Source	d.f.	SS	MS	%	Φ_{PT}	*P*
Among Pops	10	43.774	4.377	3%	0.034	0.001
Within Pops	306	670.412	2.191	97%		
Total	316	714.186		100%		

Table 4.4.10: Analysis of molecular variance among and between populations measured by Φ_{PT}

PCoA revealed considerable overlaps among multilocus genotypes from the majority of the populations (Figure 4.4.3). The first two coordinates explained 17.8% of the variation. A relevant pattern could be detected in the distribution of BS2011 population which was more clustered into the upper and lower left quadrant. This difference was confirmed by Φ_{PT} and the Nei's indexes: BS2011 population was significantly different from SO2013 and BS2013 populations. The spatial distributions, in the PCoA plot, of BS2013 and SO2013 were shifted to the right and this differentiation from the other populations was confirmed also by the values of genetic differentiation (Φ_{PT}) and Nei's genetic identity.

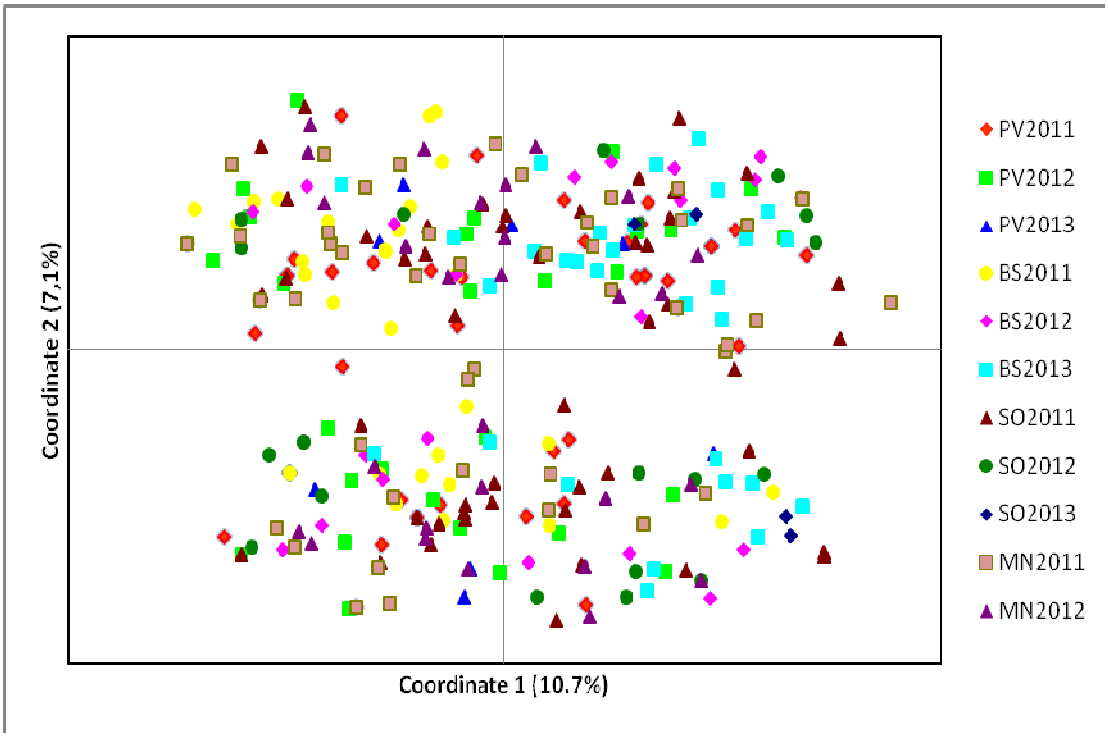


Figure 4.4.3: PCoA plot conducted with Genalex 6.41 on pairwise genetic distances between 317 *B. cinerea* strains of the year per provinces populations.

Significant Φ_{PT}s were detected among populations defined by the four molecular classes of transposons. *Flipper* strains were different to all the others; *Boty* group was different from *Flipper and transposa* but similar to *vacuma* groups. *Vacuma* strains were different from t*ransposa* and

Flipper while was similar to *Boty* strains. *Transposa* group was differentto all the others. The most relevant differentiation measured by Nei's genetic identity was assessed between the *transposa* and *Flipper* populations (Table 4.4.11).

vacuma	***transposa***	***Boty***	***Flipper***	
	0,067 (0,001)	0,001 (0,356)	0,048 (0,01)	***vacuma***
0,838		0.086 (0.001)	0,164 (0,001)	***transposa***
1,002	0,772		0,069 (0,001)	***Boty***
0,778	0,512	0,737		***Flipper***

Table 4.4.11: Genetic differentiation measured by Φ_{PT} (above the diagonal) and Nei's genetic identity (below the diagonal) between populations of *B. cinerea* identified by the four-molecular classes of transposons. *P* values for Φ_{PT} are in brackets.

AMOVA showed that 92 % of the total variation was distributed within populations and 8% of the variation was explained by differences among populations, with a total Φ_{PT} value of 0.076 (*P*=0.001) (Table 4.4.12). It is possible to infer that transposable elements significantly affected the genetic differentiations among *B*. *cinerea* populations belonging to the different molecular classes.

Source	**d.f.**	**SS**	**MS**	**%**	Φ_{PT}	***P***
Among Pops	3	37,719	12,573	8%	0,076	0,001
Within Pops	313	676,467	2,161	92%		
Total	316	714,186		100%		

Table 4.4.12: Analysis of molecular variance among and between populations measured by Φ_{PT}

PCoA revealed considerable overlap among multilocus genotypes between the populations of the molecular class of *vacuma* and *Boty* (Figure 4.4.4). Significant differences were detected in the plot distribution of the *transposa* genotypes which were grouped into the upper and lower right quadrant. As confirmed by Φ_{PT}, the most significant genetic differentiation could be detected between the *transposa* and *vacuma* molecular types: in fact, *vacuma* strains were mainly plotted in the upper and lower left quadrant. The first two coordinates explained 17.8% of the variation.

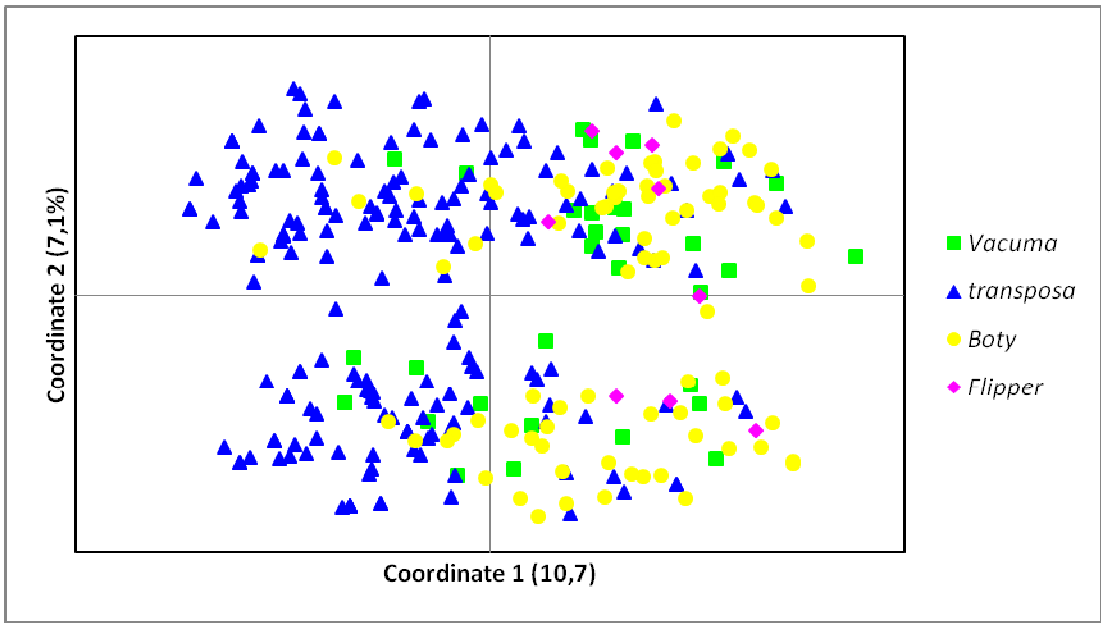


Figure 4.4.4: PCoA plot conducted with Genalex 6.41 on pairwise genetic distances between *B. cinerea* populations defined by the four molecular classes of transposons.

A Bayesian clustering was performed on the 317 *B. cinerea* haplotypes with Structure 2.3.4 software varying *K* from 1 to 10. The highest *Ln P(D)* value was found for *K* = 3 but the computation of the increase of likelihood (*delta(LnPr(X/K*) or *Delta K*) showed that the best gain of explanatory power was for *K* = 4 and then became null or negative (Figure 4.4.5 and Figure 4.4.6).

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	15	-3563.280000	0.376450	—	—	—
2	16	-3385.993750	2.487963	177.286250	140.668971	56.539823
3	17	-3349.376471	2.315850	36.617279	62.147059	26.835527
4	16	-3374.906250	5.675264	-25.529779	624.332721	110.009453
5	16	-4024.768750	93.317821	-649.862500	706.764583	7.573737
6	6	-3967.866667	13.849284	56.902083	—	—

Figure 4.4.5: The highest *Delta K* recorded was for *K*=4. *Delta K* is an ad hoc quantity related to the second order rate of change of the log probability (likelihood) of the data.

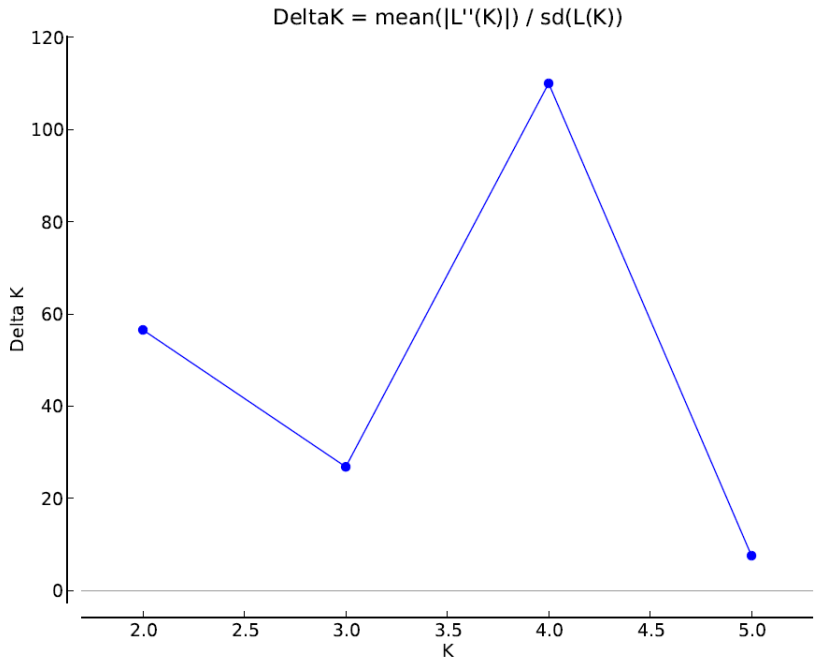


Figure 4.4.6: The magnitude of *ΔK* at each level of *K* used to determine the most probable number of genetic clusters (*K*=4) within *B. cinerea* haplotypes.

The Structure bar plot showed the assignment of haplotypes to clusters to each year of sampling (Figure 4.4.7).

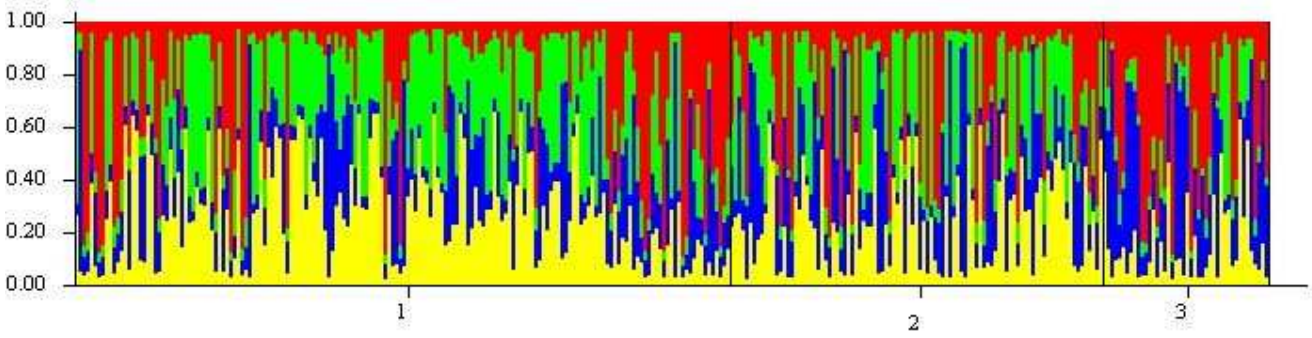


Figure 4.4.7: Bayesian assignment of *B. cinerea* individuals to 3 population cluster (1=2011; 2=2012 and 3 =2013) The structure bar plot showed, with different colours, the assignment of haplotypes at *K*=4.

Bayesian clustering was performed also to verify the differentiation of the populations defined by the four molecular classes of transposons. We have varied *K* from 1 to 10. The highest *Ln P(D)* value was found for *K* = 3 but the computation of the increase of likelihood (*delta(LnPr(X/K)* or *Delta K*) showed that the best gain of explanatory power was still for *K* = 4 and then became null or negative (Figure 4.4.8 and Figure 4.4.9).

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	10	-3527.420000	0.308401	—	—	—
2	10	-3355.000000	2.773686	172.420000	137.870000	49.706422
3	10	-3320.450000	2.045727	34.550000	66.080000	32.301471
4	10	-3351.980000	6.687767	-31.530000	601.527500	89.944452
5	8	-3985.037500	95.328304	-633.057500	—	—

Figure 4.4.8: The highest *Delta K* recorded was for *K*=4. *Delta K* is an ad hoc quantity related to the second order rate of change of the log probability (likelihood) of the data.

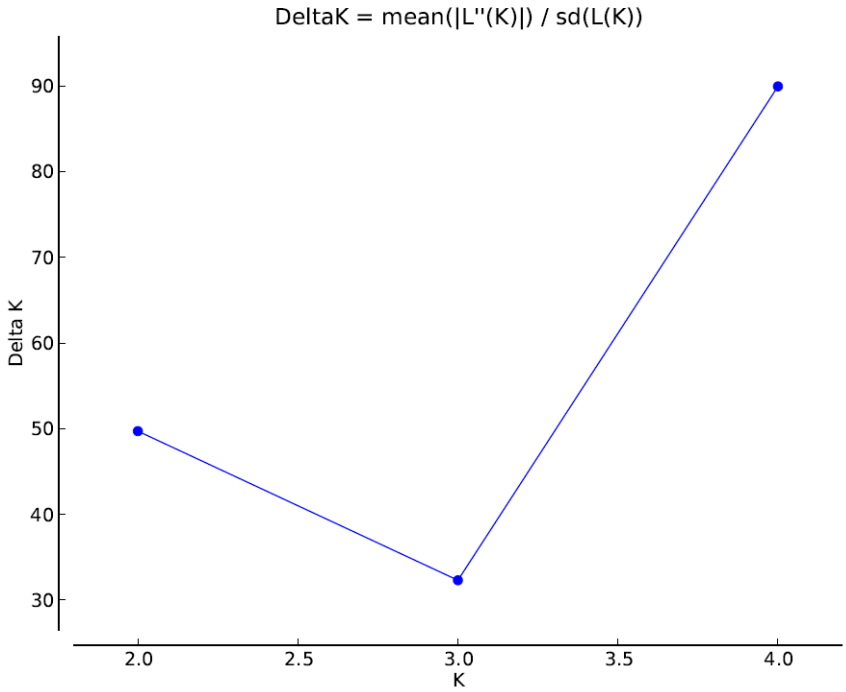


Figure 4.4.9 The magnitude of *ΔK* at each level of *K* used to determine the most probable number of genetic clusters (*K*=4) within *B. cinerea* haplotypes.

The Structure bar plot showed the assignment of haplotypes to cluster to each class of transposons (Figure 4.4.10).

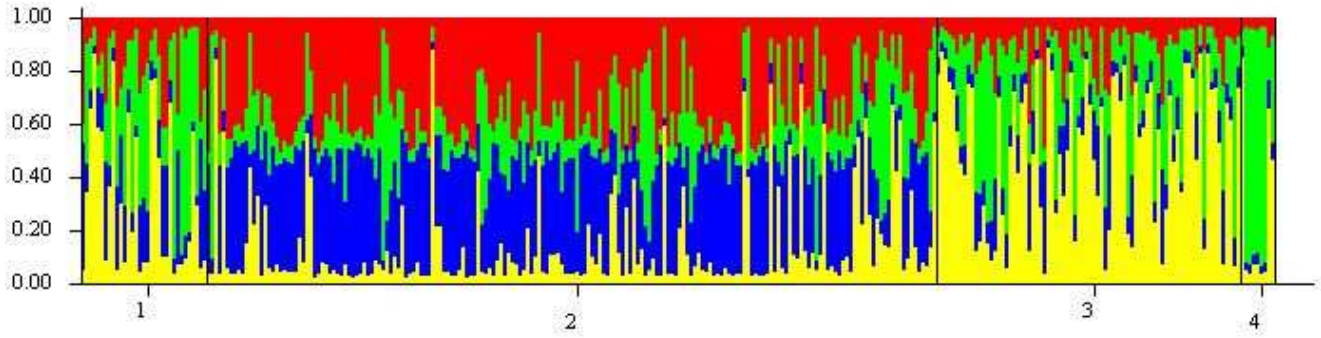


Figure 4.4.10: Bayesian assignment of *B. cinerea* individuals to 3 population cluster (1=*vacuma*; 2=*transposa*, 3 =*Boty* and 4=*Flipper*) The structure bar plot showed the assignment of haplotypes at *K*=4.

A dendrogram, using Mega4 under the rule of the Unweighted Pair Group Method with Arithmetic mean (UPGMA) clustering algorithm, was drawn taking into account the 317 *B. cinerea* molecular fingerprints. Also in this case four main clusters could be distinguished. In general, the haplotypes did not cluster according to the isolation province although there were some sub-clusters with a prevalence of strains coming from a specific site (Figure 4.4.11).

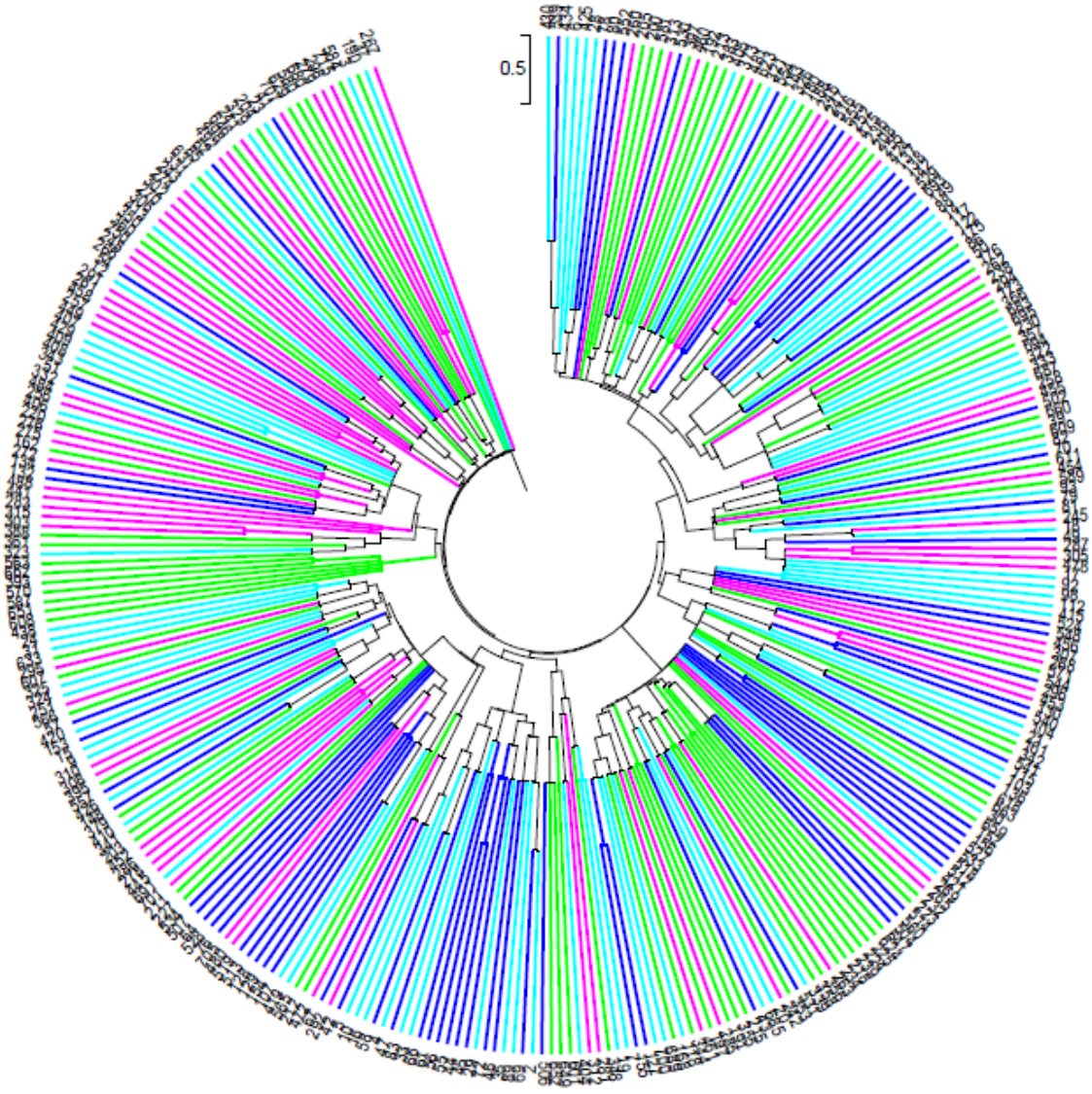


Figura 4.4.11: UPGMA dendrogram of microsatellite obtained for *B. cinerea* population of the province of Mantova (blue), Sondrio (pink), Brescia (green) and Pavia (sky-blue).

Moreover, the UPGMA was computed taking into account the presence/absence of transposons for each *B. cinerea* haplotype. The dendrogram (Figure 4.4.12) showed that *Flipper*

isolates (yellow) mainly belonged to a separated cluster while the other molecular classes of transposons form homogenous but not exclusive groups.

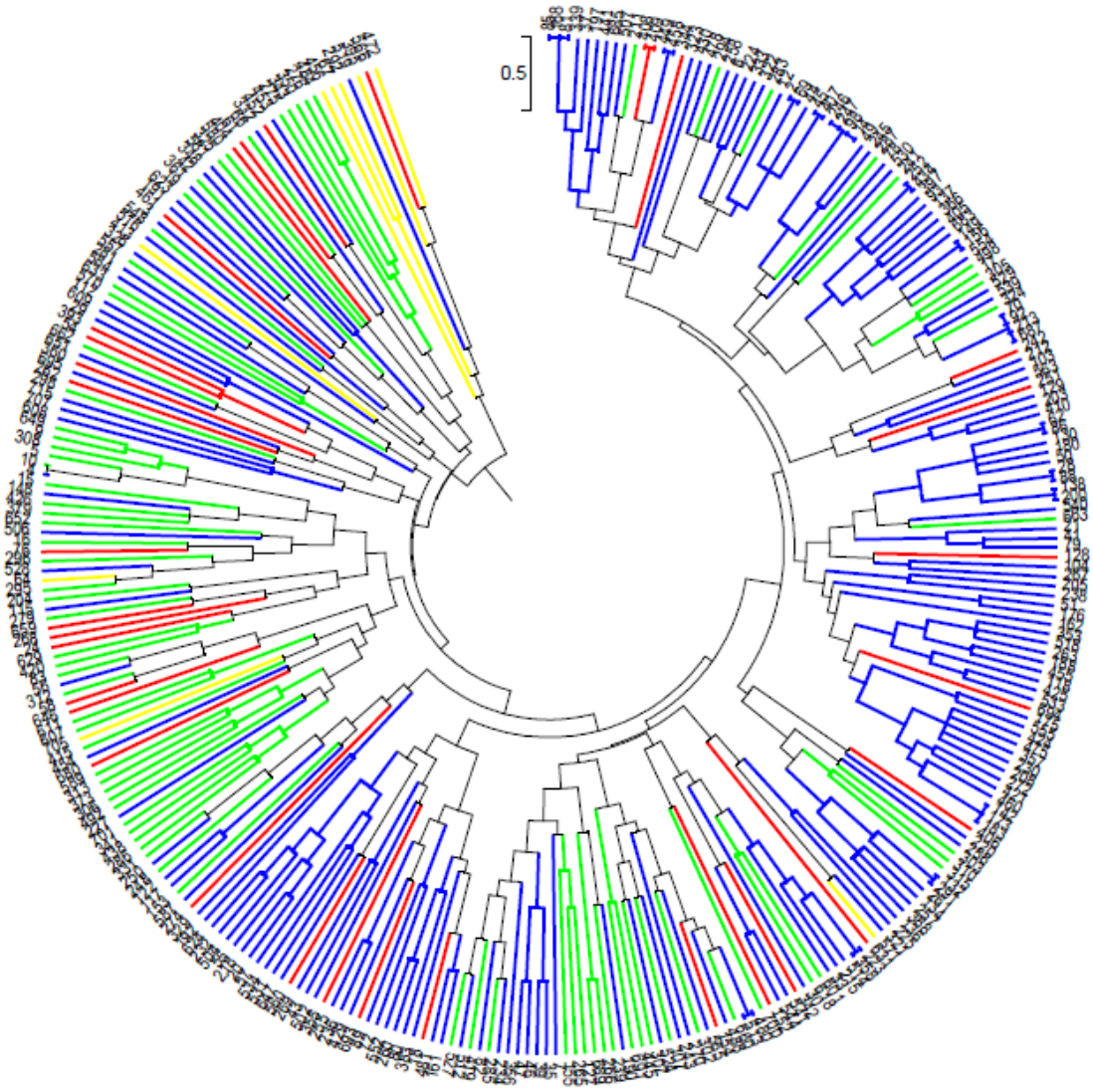


Figura 4.4.12: UPGMA dendrogram of microsatellite obtained for *B. cinerea* population defined by transposable elements: *vacuma* (red), *transposa* (blue), *Boty* (green) and *Flipper* (yellow).

5 DISCUSSION

The present study is the first report on the effect of fungicide treatments, the presence and distribution of transposons and the genetic structure in *B. cinerea* populations isolated in northern Italian vineyards (Lombardy region).

A total number of 36 populations were investigated for their sensitivity to the 4 most utilized active substances in northern Italian vineyards and with a specific single-site mode of action: cyprodinil (anilinopyrimidines), fenhexamid (hydroxyanilides), fludioxonil (phenylpyrroles) and boscalid (pyridine carboxamides).

The EC_{50} values for sensitive *B. cinerea* strains assessed for boscalid, fenhexamid and fludioxonil during the three-years sampling are similar to those calculated by other authors. Significant differences were found between years for boscalid, cyprodinil and fludioxonil but they were of small entity. The EC_{50} values of the strains sensitive to boscalid fell within the range reported by Weber and Hahn (2011) and Leroux (2007), while the very low EC_{50}s found for fenhexamid and fludioxonil were similar to those calculated by Weber and Hahn (2011) and Kretschmer and collaborators (2009). On the contrary, the EC_{50} values concerning cyprodinil are higher than those observed for sensitive strains by other researchers (Kretschmer *et al*., 2009; Leroux *et al*., 2007). The EC_{50}s values of cyprodinil assessed in this study for sensitive strains were close to those calculated by Weber and Hahn in 2011 for medium-resistant strains. The whole EC_{50} distribution of cyprodinil was shifted towards values that denote a reduction in sensitivity and a loss of efficacy of these class of fungicides. This can be due to the fact that the anilinopyrimidines, which include cyprodinil, mepanipyrim and pyrimethanil, have been the most utilized active substances in vineyards over the last decade and therefore they have exerted the highest selection pressure. In Lombardy the anilinopyrimidines have been used one to two times for vegetative season (alone or in mixture with fludioxonil) in the great majority of the vineyards. Their repeated use and the lack of alternation with active substances characterized by a different mode of action, probably selected a population with a reduction in sensitivity. Populations of *B. cinerea* resistant to cyprodinil have already been reported on table grape and strawberry in southern Italy (De Miccolis Angelini *et al*., 2014a), in French vineyards (Leroux *et al*., 1999), in vegetable fields (Myresiotis *et al*., 2007) and in lettuce crops in Greece (Chatzidimopoulos *et al*., 2013), in south west German vineyards with increasing frequency from 2006 to 2008 (Leroch *et al*., 2011), in Swiss experimental plots intensively sprayed with anilinopyrimidine (Forster and Staub, 1996), from strawberry fields in Florida (Amiri *et al*., 2013) and from strawberry in North and South Carolina (Ortuno *et al.,* 2013).

Furthermore, in the present study, 25 *B. cinerea* strains resistant to cyprodinil were found in different vineyards and years. No mutation was never found in *BcmetC* (Fritz *et al.,* 2003), and the mutations detectedin *BcmetB* (S24P and 164V) were also found in susceptible strains (Sierotzki*et al*., 2001). Further studies are therefore required to identify the primary target of anilinopyrimidines and the resistance mechanism. It has been suggested that they could affect protein secretion pathways involving the Golgi complex (Leroux *et al*., 2002).

In recent studies exploring the mechanisms of resistance in boscalid-resistant strains, seven different amino acid changes in the *SDHB* subunit were found and correlated with resistance to boscalid. The most frequent mutation is the replacement of an histidine (H) with a tyrosine (Y) at codon 272, followed by other amino acids substitutions such as arginine (R), leucine (L) or valine (V). At codon 230 was found a nucleotide change resulting in an asparagine to isoleucine (N230I) substitution while at codon 225 was found a SNP leading to the presence of phenylalanine (P225F) or leucine (P225L) instead of proline in the amino acid sequence (Sierotzki and Scalliet, 2013; De Miccolis Angelini *et al.,* 2014a). Three different mutations at codon 272 were found in *B. cinerea* strains resistant to boscalid isolated from grape and strawberry in southern Italy (De Miccolis Angelini *et al.*, 2014a). Three amino acid replacements, N230I and H272Y/R, were already known, whereas the H272V mutation was detected for the first time. In Greece, H272R was predominant in strains of *B. cinerea* isolated from strawberry. In the same study, H272Y was present at lower frequencies and the H272L was found only in one strain: the two point mutations were associated with moderate levels of resistance to boscalid (Veloukas *et al*., 2011). In Florida, strains isolated from strawberry showed at high frequency the mutation H272R, followed by the H272Y and H272L, and the H272Y mutation was associated with high to very high levels of resistance to boscalid (Amiri *et al*., 2014). The predominance of H272R mutation followed by H272Y was also found on *B. cinerea* strains isolated from strawberry and blackberry (Li *et al.,* 2014) and from apple (Yin *et al.,* 2011). In the latter case, the level of resistance ranged from low to very high within isolates carrying the H272R and H272Y mutations respectively. Indeed, De Miccolis *et al*, 2010 found that *B. cinerea* laboratory mutants, possessing the H272Y mutation, showed a low level of resistance. Leroux *et al*., 2010 found that the H272Y substitution is the most frequent genotype in French and German vineyards and was associated with low level of resistance.

The 22 *B. cinerea* strains resistant to boscalid in the present study were screened for the presence of the two main point mutations detected in *B. cinerea* strains isolated from grapevine and linked with resistance to the SDHI boscalid (H272Y or R) (Yin *et al*., 2011 and Amiri*et al*.,

2014). 21 strains were characterized by the H272Y mutation, resulting in the substitution of histidine by tyrosine. None of the strains had the H272R mutation. The point mutation H272Y was also found in 4 of the 7 sensitive tested strains. Therefore in northern Italian vineyards the H272Y mutation, found at high frequency, is not always associated to high resistance levels.

As in the present study, relevant variation in sensitivity have been observed across isolates of the same species carrying the same mutation in the SDH enzyme. H272Y was found in sensitive *B. cinerea* strains isolated from apple in 2009 in the United States (Yin *et al*., 2011) and in studies using artificially generated mutants (De Miccolis Angelini *et al*., 2010). Moreover, in a recent study De Miccolis Angelini and collaborators (2014) found that substitutions at position H272, responsible for a high level of resistance to boscalid, confer sensitivity (H272R), hypersensitivity (H272Y) or moderate resistance (H272V) to another SDHI, fluopyram. Taken together, these results indicate that other factors besides the target sequence are involved in the sensitivity to SDHIs (Scalliet *et al*., 2012) and that only certain mutations are suspected to reduce the efficacy of the newest generation of SDHI fungicides (Sierotzki and Scalliet 2013).

Despite of this, in a recent study (Laleve *et al*., 2014) was found that the successful establishment of the sdhBH272Y mutant is not surprising, because the H272Y substitution was the only substitution that did not decrease respiration and SDH activity. Moreover, the sdhBH272Y mutant was only weakly affected in terms of the fitness parameters tested.

During the present study *B. cinerea* isolates generally showed a good sensitivity towards fenhexamid and only one individual characterized by a high resistance factor (Rf>10) was detected. The limited use of fenhexamid in Lombard vineyards results in a good sensitivity of the populations to this active substance. Strains belonging to the minor species *B. pseudocinerea* and naturally resistant to fenhexamid were present in wild populations before the introduction of this fungicide on the market (Walker *et al*., 2013). Resistance to fenhexamid in *B. cinerea* has been reported in vineyards located in southern and northern Italy, France, Israel, Germany and Chile (Albertini and Leroux, 2004; Esterio *et al*., 2007; Bertetti *et al*., 2008; Fillinger *et al*., 2008; Korolev *et a*l. 2011, Leroch *et al.*, 2011; De Miccolis Angelini *et al*., 2014); in strawberry fields in Florida and in north and south Carolina (Amiri *et al*., 2013; Grabke *et al*., 2013); in lettuce in Greece (Chatzidimopoulos *et al*., 2013); and in a single case from different vegetables in Greece (Myresiotis *et al*., 2007).

Limited level of resistance was found also for fludioxonil: very low EC_{50} were assessed in all the populations sampled, even if 28 resistant strains (Rf>10), for the great majority isolated in 2012 in the province of Sondrio, could be found. Resistance to the phenylpyrrole fludioxonil and always

combined with anilinopyrimidine resistance was found at low frequencies in grapevine isolates in southern Italy (De Miccolis Angelini *et al*., 2014a), Israel (Korolev*et al.*, 2011), and South West Germany (Leroch *et al*., 2011). Probably the climatic condition or other elements related to the location could have favoured the presence of *B. cinerea* strains with a reduction in sensitivity.

Five strains isolated in 2011 showed a simultaneous reduction in sensitivity towards different active substances (multidrug resistance, MDR). Strains exhibiting patterns of multiple resistance are often found in vineyards subjected to intense selective pressure (Leroux *et al*., 1999; De Miccolis Angelini *et al*., 2014a) and result from the accumulation, in a single cell, of different mutations conferring resistances to distinct fungicides. Strains with different MDR phenotypes were previously described (Chapeland *et al.,* 1999): MDR1 strains show considerable resistance levels mainly towards fludioxonil, cyprodinil and tolnaftate; MDR2 strains are characterized by increased resistance to fenhexamid, tolnaftate, cycloheximide and cyprodinil; and MDR3 strains show the highest levels and broadest spectrum of resistance against most fungicides tested. The few MDRs found in the present study seems not to belong to these three MDR phenotypes. In fact, all the strains were resistant or moderately resistant to boscalid and, at the same time, resistant to another classes of fungicide. Three of them were isolated from samples collected in Oltrepo Pavese and were resistant to boscalid and cyprodinil. A strain isolated from Torrazza Coste vineyard (PV), was resistant to fludioxonil and moderately resistant to boscalid, whereas another one, coming from Gonzaga (MN), was resistant to boscalid and moderately resistant to fenhexamid. Finally, a strain isolated in Sirmione (BS) during 2013 was resistant both to boscalid and cyprodinil.

The comparison of the *B. cinerea* populations isolated in the vineyards of Chiuro (SO), Torrazza Coste (PV) and Sirmione (BS), sampled for a three-year period, did not always show a clear relationship between the treatments carried out and the distribution of EC_{50}. Only in Torrazza Coste vineyard, the application of boscalid during 2013 grapevine growing season was associated with a wider EC_{50} distribution. The three vineyards were never treated with fenhexamid during the three-year period: as a consequence, the EC_{50}s were characterized by low values and their distribution showed only weak differences over the three years of monitoring activity. For cyprodinil, the relationship between EC_{50} distributions and the treatment carried out in a single year was not clear. But if we considered the treatments carried out in previous years we can supposed that previous treatments in past years could have selected resistant phenotypes which persist in vineyard also in the absence of selection pressure. Also for fludioxonil the relationship between treatments and EC_{50} distribution over the three years was unclear: in general in the

three vineyards a wider distributions was observed in 2012, probably caused by particular climatic condition.

Furthermore, the relationship between the total number of treatments, carried out in vineyards during the 2006-2013 period with each active substance, and the corresponding EC_{50} distribution was analysed. Generally, the repeated use for several years of the same mode of action resulted in the selection of strains with a reduction in sensitivity and a loss of efficacy of the fungicide: this is evident especially for the anilinopyrimidines, even if wider distribution associated to the repeated use were found also for the other fungicides. However, several strains were isolated in vineyards never treated with the relative active substance, so we can assume the natural presence in the populations of this resistant genotypes or migration from neighbouring vineyards.

On the basis of the diagnostic allele at the *Bc6* locus (Walker *et al*., 2011), all individuals sampled in Lombardy were identified as *B. cinerea sensu stricto* (Group II), confirming a similar previous study of Vercesi *et al*. (2014) performed in northern Italy, on the Oltrepo Pavese hills. The same results were obtained in Tunisia (Ahmed and Hamada, 2005; Karchani-Balma *et al*., 2008), Greece (Samuel et al., 2012), Chile (Esterio *et al*., 2011), southern Asia and Australia (Isenegger *et al*., 2008). *B. cinerea* Group I was found, only at low frequencies, in French vineyards (Giraud *et al*. 1997; Martinez *et al*., 2003; Martinez *et al*., 2005; Walker *et al*. 2011), on strawberry and rape plant in Hungary (Fekete *et al*., 2012). Two strains were found in a German vineyard (Kretschmer and Hahn, 2008) and a single strain on rooibos in the Western Cape of South Africa (Wessels *et al*., 2013). Since no Group I strains were isolated during the present study, it is possible to conclude that *B. pseudocinerea* is epidemiologically irrelevant in northern Italian vineyards.

The causes of the genotypic variation in *B. cinerea* are thought to be sexual reproduction, heterokaryosis, aneuploidy and extrachromosomal elements deriving from mitochondria, viruses and plasmids (Beever and Weeds, 2007). Beside all the above cited sources of genetic variation, it has also been postulated that variability might be due to the activity of transposable elements (Levis *et al*., 1997; Mac Donald, 1993; Smith and Corces, 1991). Transposable elements (TEs) are mobile, repetitive DNA sequences of genomes that can alter the sequence, expression and chromosome rearrangements of genes. These changes have the potential to cause wide-ranging genetic variation, giving populations the flexibility to adapt successfully to environmental conditions, particularly in species that do not have a sexual phase (de Lima Fávaro *et al*. 2005). *B. cinerea* populations belonging to Group II show four molecular classes based on transposons:

transposa have both *Boty* and *Flipper* TEs; *vacuma*, with no TEs; and strains containing *Boty* or *Flipper* TEs only.

The four molecular classes of *B. cinerea* strains isolated at harvest in the present study showed significant differences in their frequencies. *Transposa* strains, characterized by the presence of both transposable elements, were the most abundant molecular type (60.2 % of the total population), followed by *Boty* (25.1 %), *vacuma* (11.5 %) and *Flipper* (3.3%). In all the three years of sampling, the frequencies remained almost the same and differences in the percentages among years were detected only for the molecular class of *transposa*: a clear decrease of *transposa* strains over the years was reported, followed by a not significant increase of the three remaining classes. The predominance of *transposa* strains was reported also by other authors (Muñoz *et al*., 2002; Ma and Michailides, 2005; Kretschmer and Hahn, 2008; Vaczy *et al*., 2008; Esterio *et al*., 2011; Samuel *et al.*, 2012), particularly at harvest (Giraud *et al*., 1997; Martinez *et al*., 2005; Vercesi *et al*., 2014), while *vacuma* strains are detected mainly on floral residues and strongly decrease during summer.

Vacuma strains, frequently isolated from senescent tissues, show marked saprophytic capability linked to rapid growth on highly nutritive media and reduced pathogenicity (Martinez *et al.*, 2005). *Transposa* isolates are characterized by greater virulence on grape berries, and are more frequently resistant to the fungicides vinclozolin and carbendazim (Giraud *et al.*, 1997; Martinez *et al.,* 2003).The presence or absence of transposable elements was not associated with higher Rfs concerning all the tested fungicides in this study. With the only exception of two strains for the fungicide boscalid and two strains for fludioxonil which appertain to the class of *vacuma*, the great majority of the strains with Rf>10 found in Lombardy belongs to the molecular class of *Boty* and *transposa,* therefore by the presence of a single or both TEs. The presence of these elements could probably induce mutation or rearrangements of the genes involved in resistance to fungicides. The relationship between the presence of TEs and a reduction in sensitivity through fungicides should be more deeply investigated.

Contrasting results were obtained in *B. cinerea* populations on differentiation among transposon genotypes: weak correlations were found between transposon frequency and the geographic origin of the isolate (Munoz *et al*., 2002; Vaczy*et al*., 2008), the year of isolation (Váczy*et al*. 2008), or different hosts (Ma and Michailides 2005; Asadollhai *et al*., 2013). Results were also contradictory regarding temporal distribution (Martinez *et al.,* 2005, 2008). Phylogenetic studies (Albertini *et al*., 2002; Fournier *et al*., 2003, 2005; Iseneggeret *al*., 2008) could not provide sound evidence for genetic differentiation between transposon genotypes.

These contrasting results indicated that transposon alone could not clearly define population structures of *B. cinerea* populations (Fekete *et al*., 2012). For the same reason Asadollahi and collaborators (2013) concluded that microsatellites are the most informative markers, while transposon are not informative for differentiation.

An asexual reproductive system is characterized by low genotypic diversity and many clones (Mc Donald, 2004), whereas sexual reproduction involves a high genotypic diversity and no discontinue clones. In a mixed model we can expected intermediate genotype diversity, many genotypes and some clones. A mixed reproduction system has the best advantages of both sexual and asexual system. The clone of an asexual population will have a limited potential to adapt to a changing environment and its evolutionary potential is limited to the random mutations that occur in a clonal lineage. But in a stable environment this genotype possess a co-adapted combination of genes that were fit to these conditions. On the other hand, in a sexual reproductive system the independent assortment of genes produce a population that is able to adapt quickly to a changing environment. But these organism are less likely to form coadapted genes complex that can optimize fitness in a stable environment.

Apothecia of the perfect stage of *B. cinerea*, have been rarely found in nature (Polach and Abawi, 1975) but can be obtained in laboratory (Faretra and Antoniacci, 1987), therefore the occurrence of recombination through sexual reproduction in the pathogen populations has been for a long time matter of debate. The reproductive systems of *B. cinerea* is surely asexual but could be also sexual or a mixture of the two.

Based on the mating type screening, in *B. cinerea* populations of Lombardy a combination of asexual and sexual reproductive systems seems likely to occur. The mating type ratio, as a first indication of recombination, was similar to the theoretical 1:1 in the majority of the populations, with the only exception of the vineyard of Dossi Salati (SO) in 2011 and Torrazza Coste (PV) and Ponti sul Mincio (MN) in 2012. As in other analysed populations, the frequency of the strains with the allele *MAT1-1* was in general slightly higher than that with allele *MAT1-2* (Beever and Parkes, 1993; Faretra and Pollastro 1993; Van der Vlugt-Bergmans *et al*., 1993).

The multilocus structure of the Lombard *B. cinerea* populations revealed that some populations, such as those located in the provinces of Pavia, Brescia and Sondrio isolated in 2013 and Brescia in 2011 and 2012, seemed to carried out recombination mainly by sexual reproduction, while in all the other populations panmixia alone could not be inferred and some clonalty must be present. Furthermore, four haplotypes were found in successive vegetative seasons suggesting persistence and survival among growing seasons, probably as mycelium or

sclerotia during the winter. Some haplotypes were isolated in the province of Pavia, Mantova and Brescia in 2011 and the same in the province of Pavia in 2013, two vegetative seasons later. Other eight haplotypes were isolated in different provinces, suggesting a high gene flow and low differentiation between populations. The measure of the Gene Flow (N_m) revealed very low differentiation between the populations of the province of Pavia sampled in the three years of study, with a slight differentiation only between the population sampled in 2011 and 2013. The populations of Pavia were more similar to those of Brescia: and precisely the populations sampled in 2012, in Pavia and Brescia, showed the lowest differentiation followed by the population of Pavia 2013 and Brescia 2011 and the populations of Pavia 2012 and Brescia 2011. Some similarity were found also between the province of Pavia and Sondrio (PV2012 and 2013 with SO 2011 and 2012). On the contrary the highest differentiation and therefore the lowest gene flow was assessed between all the populations of the provinces of Sondrio against all populations of the provinces of Brescia and between the provinces of Mantova and Pavia sampled in 2011.

The evidence of the presence of a certain quote of genotype flow was confirmed also by AMOVA using as sources of variation years and provinces: in the two cases the most of genetic diversity (99%) was present within subpopulations, indicating high similarity among populations both between years and provinces. A slightly higher variation among populations was reached when the two sources of variation were analysed together: 97% of genotypic variation was found within populations and only 3% among them. Similar results were found in Spain in *B. cinerea* populations isolated on vegetable crops in greenhouses (Alfonso *et al*., 2000) and in the South Africa on rooibos seedlings (Wessels*et al*., 2013). Slightly higher values were found within *B. cinerea* populations (90%) sampled from chickpea plants in Bangladesh (Isenegger *et al*., 2008), from different hosts and regions in Tunisian populations (89%) (Karchani-Balma*et al*., 2008) and from grapevine and bramble in French regions (91-93%) (Fournier and Giraud, 2007)

The computed PCoA on the years and those assessed for the provinces revealed considerable overlaps among multilocus genotypes but, in contrast, some differences between populations were visualized in the PCoA assessed for the years and provinces: as confirmed by the low value of the Gene Flow, by Φ_{PT} and Nei's genetic identity, clear differences in the spatial distribution of the *B. cinerea* population of Brescia sampled in 2011 and the populations of Sondrio and Brescia of 2013 were found.

To investigate the possible genotypic variability of the four molecular class of transposons we performed an AMOVA on them. Also in this case the greatest part of the observed variance came from the variation within each population but a significant and higher genetic diversity (8%) was

found among the population defined by the four molecular classes of transposon. Such a result is clearly higher than those assessed previously for the font of variation year, provinces and the combination of the two, suggesting that transposable elements significantly affected the genetic differentiations among *B*. *cinerea* populations belonging to the different molecular classes. This results and the genetic differences between molecular classes was supported also by the PCoA, the Bayesian clusteringand the dendrogram: the Bayesian clustering showed that the best assignment was found for K=4, that is the number of transposable elements, and differences, visualized in the Structure bar plot and in the dendrogram, were evident. As confirmed by Φ_{PT} the class of *Flipper* was different from the others and the only two similar classes were *Boty* and *vacuma*. The highest differentiation measured by Nei's genetic identity was found between *Flipper* and *vacuma* molecular classes. A cluster containing the majority of *Flipper* and separated from all the other was evident in the dendrogram. In the dendrogram, the other molecular classes of transposons form homogenous but not exclusive groups.

Taking into account the reduced number of *Flipper* strains, it seems that *Flipper* genotypes group in a separate class, genetically different from the others. Differences were also found in all the other classes with the only exception of an overlap of the molecular classes of *Boty* and *vacuma*.

In conclusion, the status of the *B. cinerea* populations in Lombardy assessed in the present study denoted a good sensitivity to the four active substances tested. Particular attention must be taken for the use of anilinopyrimidine with the implementation of disease control strategies which may include a single treatment per season with this class of fungicide and an alternation in the season or between years of the different available fungicide modes of action. Many strains with a reduced sensitivity to boscalid, cyprodinil and fludioxonil were found in *B. cinerea* populations and it could be interesting to carry out further analysis on the fitness of these strains to evaluate their ability to grow and reproduce in the absence of fungicide.

In the present study, the mutation H272Y was associated to high resistance levels but also to sensitivity, so it is possible to conclude that molecular methods able to discriminate strains resistant to boscalid can not be based simply on the detection of H272Y point mutation.

Genotypic differentiation was detected among the haplotypes and the presence/absence of transposable elements: the *Flipper* molecular type seems to be completely different from all the others. Indeed, very weak differentiation was found between geographic origin and years of sampling suggesting that Lombard *B. cinerea* population were similar to each other.

Application of the index of association, the chi-square test, and the phi test consistently indicated that the population of northern Italian isolates of *B.cinerea* undergoes sexual reproduction and therefore a mixture of a sexual and asexual reproduction can be assumed. This finding is in accord with the successful adaptability of *B. cinerea*.

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