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Phenotype and genotype characterization of *Monilinia* spp. isolates and preformed antifungal compounds in peach peel fruit at different developmental stages

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Abstract

The brown rot fungi belong to a group of fungal pathogens that causes considerable damage to cultivated fruits trees, particularly stone fruits and apples in the temperate regions of the World and during the postharvest with an important economic impact. In particular in Italy, it is important to monitor the *Monilinia* population to control economic losses associated to the peach and nectarine market.

This motivates the research steps presented in this dissertation on *Monilinia* Italian isolates. The *Monilinia* species collected from stone fruits have been identified using molecular analysis based on specific primers. The relevant role of *M. fructicola* was confirmed and, for the first time, it was found also on apple fruits.

To avoid the development of resistant strains and implement valid treatment strategies, the understanding of the fruit natural resistance during different developmental stages and the assessment of the *Monilinia* sensitivity/resistance to fungicides are required.

The relationship between the inhibition spots and the phenolic compounds in peach fruit peel was highlighted in this research.

Three methods were used to assess isolate resistance/sensitivity, the amended medium, the Spiral Gradient Endpoint Method (SGD) and the Alamar Blue method.

The PCR was used to find possible mutation points in the b-tubulin gene that is responsible for fungicide resistance. Interestingly, no mutation points were observed in resistant *M. laxa* isolates, suggesting that the resistance could be stimulated by environmental factors.

This lead to the study of the effect of the temperature on the resistance and the preliminary results of in vitro tests showed that maximum inhibition was observed at 30°C.

Chapter 1

Introduction

A diet rich in fruit and vegetables plays a significant role in improving the health of people around the world. An increase of fruit consumption and its promotion as alternative to “junk food” are encouraged by the European Community and USA. In the past few decades, the fruit industry has changed considerably in order to adapt itself to the fast evolution of the market and to the requirements of consumers increasingly concern about food healthiness and environmental issues. In addition fruits and vegetables contribute significantly to the economy of the European countries in the Mediterranean area, among the leading exporters of fruit in the world (79). In this context, world peach production increased during the first decade of 21st century and reached 20 Mt. During the same period the production in Europe, over 95% of which is from EU countries, slightly decreased.

The top 15 producers of stone fruits are: China, Italy, USA, Spain, Greece, Turkey, Iran, Egypt, Chile, France, Mexico, Brazil, Argentina, South Africa and South Korea. China produces 50% of the World production, followed by the Mediterranean countries (with 6 countries in the top 15), North America, and South America. Iran, South Korea and South Africa stand among the top 15 producers.The Italian production remained stable with 1.6 Mt.

Peach and nectarine export is growing at an higher rate than the production. European import/export market is predominant, with 75% and 82% of the overall imports and exports, respectively. Among the

top 15 exporting countries, 11 are European, with Spain and Italy at the top positions. Russia, Poland, and Ukraine are increasing their exports. The first non-European exporting countries are the US and Canada. Italy and France, that were mainly exporters, have become importers (34, 103).

Transport by road, rail, sea or air allows fruit (and vegetables too) to reach large areas of the World; however, to be distributed overtime and space, these products need to be subjected to a short or long retention period, in relation to product characteristics and market demands.

Peaches, nectarines, plums and apricots (climacteric fruits), able to ripen on or off the tree, are usually harvested mature but not ripe. The maturity indices currently recommended for their harvest are based on the compromise between those parameters that would ensure the best eating quality to the consumer and those that provide minor losses during handling, storage and marketing (59, 90). The use of appropriate postharvest technology procedures in the last few decades has greatly reduced product losses from production, the application of low temperature storage delays fungal growth in general and fruit ripening, which translates into reduced development of disease (73).

When the stone fruits are transferred to higher temperature than that used in storage to develop their typical flavor and texture and reach optimal eating quality, the pathogens resume growth and develop rapidly into decay lesions (21, 46, 90, 91, 103, 104). In addition, excessive handling in self-service display or suboptimal disposal of fruits at retail store often cause mechanical injuries (punctures, cuts or bruises), that create infection opportunities for pathogens that can development diseases after purchase (114, 122, 124). Losses occurring at the consumer level are generally not taken in consideration, but can

negatively affect consumer satisfaction and propensity to consumption (78).

Stone fruits are characterized by a relatively short postharvest life, passing quickly from ideal ripeness to over-mature fruits, depending in part of temperature and handling practices (58, 78). Protection from heating after harvest and rapid movement to cooling are thus important to avoid excessive flesh softening but especially to reduce postharvest decay. Brown rot cauded by *Monilinia laxa* (Aderhol&Ruhland), *Monilinia fructigena* (Aderhol&Ruhland) and *Monilinia fructicola* (G. Winter) is the major postharvest decay of stone fruits, follow by gray mould caused by *Botrytis cinera* (Pers.:Fr) and Rhizopus rot caused by *Rhizopus stolonifer* (Ehrenb.:Fr) (21)

1.1 Brown rot of stone fruit

The brown rot fungi belong to a group of fungal pathogens that causes considerable damage to cultivated fruits trees, particularly stone fruits and apples in the temperate regions of the World.

Monilinia spp. can cause severe losses in peaches (*Prunus persica* (L.) Batsch) worldwide, with an important economic impact. Direct yield losses result from infection of flowers, blossom and twig blight, and from fruit rot at harvest and postharvest (80). Losses depend on weather conditions and are especially severe if high humidity, warm temperatures, and abundant rainfall prevail prior to harvest (15, 104). Indirect losses are due to the cost of fungicide application during the bloom and during the pre-and postharvest period (118). Marketing of decay-free fruits, especially to long-distance markets, can consequently be problematic overall for European countries, where postharvest fungicide treatments are not allowed and brown rot is

controlled only by fungicide spray programs in the field. Preharvest treatments can be fundamental as a consequence of disease epidemiology and the importance of controlling latent infections (66).

The plant pathogen is primarily a wound pathogen and invades the fruit through wounds and injuries that occur before and, most importantly, during and after harvest. The conidia can also penetrate through micro lesions, stomata and lenticels of fruit skin; in some cases, infections occurring in the field remain quiescent until the fruit reaches ripening, allowing *Monilinia* to overcome host defenses (30). Latent infections by *M. fructicola* or the closely related *M. laxa* have been documented in peach for the first time in 1971 (57), later the latent infections by *M. fructicola* on peach fruits were described by Schlogbawer (111) and by Michailidis in California (84). Other authors reported also latent infection in apricot (111),plum (82, 94, 111), prune (83) and cherry (131, 36). A better understanding of the importance of latent infection in the epidemiology of brown rot could facilitate the early detection of fruit rot risk before harvest and consequently increase lead time for disease management decision (1, 2, 36, 82, 83, 84).

1.1.1 History

The brown rot diseases of fruits have been extensively studied in Europe for over 150 years, in North America for about 100 years, and more recently in other parts of the World, as Australia, New Zealand, China and Japan.

In Europe there are references on fruit diseases in early literature that could be attributed to the brown rot fungi. The first authentic description of brown rot fungus on fruit was published in 1796 by Persoon. During the 19th Century there was a greater awareness of

fungal plants diseases in Europe and the number of publications increased, including those concerned with the brown rot fungi on fruits. By the end of the 19th Century it had been established that there are two brown rot species in Europe: *M. fructigena* and *M.laxa* (18).

M. fructicola causes brown rot of stone fruit in India, Japan, the Republic of Korea, Oceania, and North and South America. It was considered a quarantine pathogen by EPPO (A1 list) until 2001 when it was detected in peach orchards in France (64), later in the Czech Republic (28) and in 2006 in mummified peach fruit and trees in an orchard located in Lleida (Spain) (25). Stored nectarines from two orchards in Lagnasco (Italy) were found infected in 2008 (100). Following these communications *M. fructicola* was inserted in A2 EPPO list (29, 31, 32, 96). *M. laxa* is a quarantine pathogen in China (though there is no finding) while in some States of the North America it was detected for the first time in 2008 on "Surefire" cherries in Niagara, in 2009 on cherries from Wayne, and in 2011 on sweet cherries and plums in New York, Rhode Island and Massachusetts by Cox et al. (23, 125).

1.1.2 Symptoms

The pathogen infects aerial parts of host plants with a variety of symptoms, including blighting of blossoms, cankers on woody tissues and rotting of fruits.

Blossom blight is the first symptom in the spring, it appears on the blossoms, and develops when spores (conidia) land on and penetrate on flowers stem of susceptible plants. In humid weather (moist and moderately warm) blighting of flowers results in a reduction in the set of fruit and also in the infections. Any part of the flowers, the stigma, stamens, petals or sepals can be sites of infection. The infected tissue

turns dark brown and is covered with the grayish-brown conidia of the fungus and later shrivels and dries up, with the rotting mass clinging to the twigs for some time. Some infected blossoms fall to the ground but others can remain attached to the tree for long periods, and during moist weather the pathogen within the withered flowers produces tufts of spores over the infected tissues. The chains of conidia provide the inoculum for other parts of the same or neighboring trees.

At the base of infected flowers, small, sunken cankers develop on twigs around the flower stem, which sometimes they encircle and cause twig blight. The fungus grows from the floral parts through the peduncle in the twigs where the infected tissues appear brown, as collapsed area. These symptoms gradually extend up down the twig. Gum may exude from the infected shoots and gray to brown tufts of spores produced in sporodochia often are found on infected tissues. Gray tan-colored tufts of spores emerge on blighted twigs andinfected blossoms. These parts (such as shucks) may cling to enlarging fruit, and with moisture, they tend to produce spores resulting as sources of inoculum for infection of the developing green fruit.

Stem cankers develop usually from blighted twigs or fruit spurs by growth of mycelium into larger limbs of the tree. In the early stages of canker formation the bark in the infected area dies, the tissue beneath the bark becomes sunken and discolored and finally an open wound develops. Gum is often exuded in the diseased area. Usually callus tissue develops around the canker and restricts further growth, so that the cankers heal up. If environmental conditions are suitable for sporulation, active cankers sometimes produce conidia and thereby provide a source of inoculum. Occasionally the wounds continue to develop for several years and extensive damage of even death of the

tree may be caused, but this is often the result of invasion by secondary organisms (5, 18).

Infections of green fruit are not frequent, they show soft water-soaked, dark areas, rot first develops in clustered fruit, in fruit contact spots, and insect- or wind-damaged fruit, since clustered fruit are more favorable for disease development. For instance, fruit-to-fruit contact surfaces have microcracks, thinner cuticles, and micro-environmental conditions predisposing fruit infections (85). When the fruit approaches maturity, symptoms appears as small, circular, brown spots that spread rapidly in all directions. One large or several small rotten areas may be present on the fruit, which finally becomes completely rotted or dries up. The mummified fruits can hang from tree or, alternatively, fall to the ground where they remain through the winter months, partly or completely buried in soil or leaf litter (20, 52, 83, 86, 123)

Under moist conditions and on soft, ripe fruit, almost all the surface is covered with conidial tufts or vegetative mycelium, but when the relative humidity is low and/or the fruits are not ripe, no mycelium and little or no conidial tuft develop (85). Green fruits are more resistant to the brown rot fungi than those approaching the ripening stage (77, 83, 93).

Disease development in orchard depends on the pathogen’s inoculum potential, microclimatic conditions, and cultural practices (42, 43, 79).

Figure 1: Mummy of peach fruits

Figure 2: *Monilinia* brown rot on a peach blossom, showing gumming, and wilted blossoms through which infection occurred

Figure 3: *Monilinia* brown rot on abort peach fruit

Figure 4: *Monilinia* brown rot on the peach fruit

Figure 5: Postharvest rotting of fruits

1.1.3 Hosts

M. laxa, *fructicola* and *fructigena* could infect all drupaceous species and also many other members of the *Rosaceae* may serve as hosts to these fungi. The main commercial crops that are infect by *M. laxa*, *M. fructicola* and *M. fructigena* include *Prunus* species peach and nectarine (*P. persica*(L.) Batsch), apricot (*P. armeniaca*L.), plum (*P. domestica*L.), sweet cherry (*P. avium*L.), sour cherry (*P. cerasus*L.) and almond (*P. amygdalus*Batsch)andapple (*Maluspumila*Mill.), pear (*Pyruscommunis*L.) (5).

Figure 6: *Monilinia laxa* on peach tree

Figure 7: *Monilinia laxa* on cherry tree

1.1.4 The Pathogen

The mycelium produces chains of elliptical *Monilia*-type conidia on hyphal branches arranged in tufts (sporodochia). The old colonies of *M. fructicola* natural substrate or in artificial substrate like potato dextrose agar (PDA) or agar technical charge with 1/3 of V8 Juice (V8) can also produce microconidia (spermatia) in chains on bottle-shaped conidiophores. The spermatia do not germinate, but seem to be involved in fertilization of the fungus (18). The apothecium (only *M. fructicola*has the sexual stage) originates from pseudosclerotia formed in mummified fruit buried partly or wholly in the soil or debris, and usually more than 20 apothecia may form on one mummy (42, 43,

86). The inside or upper surface of the apothecium is lined with thousands with sterile hyphae (paraphyses). Each ascus contains eight single-celled ascospores (5).

1.1.5 Life cycle

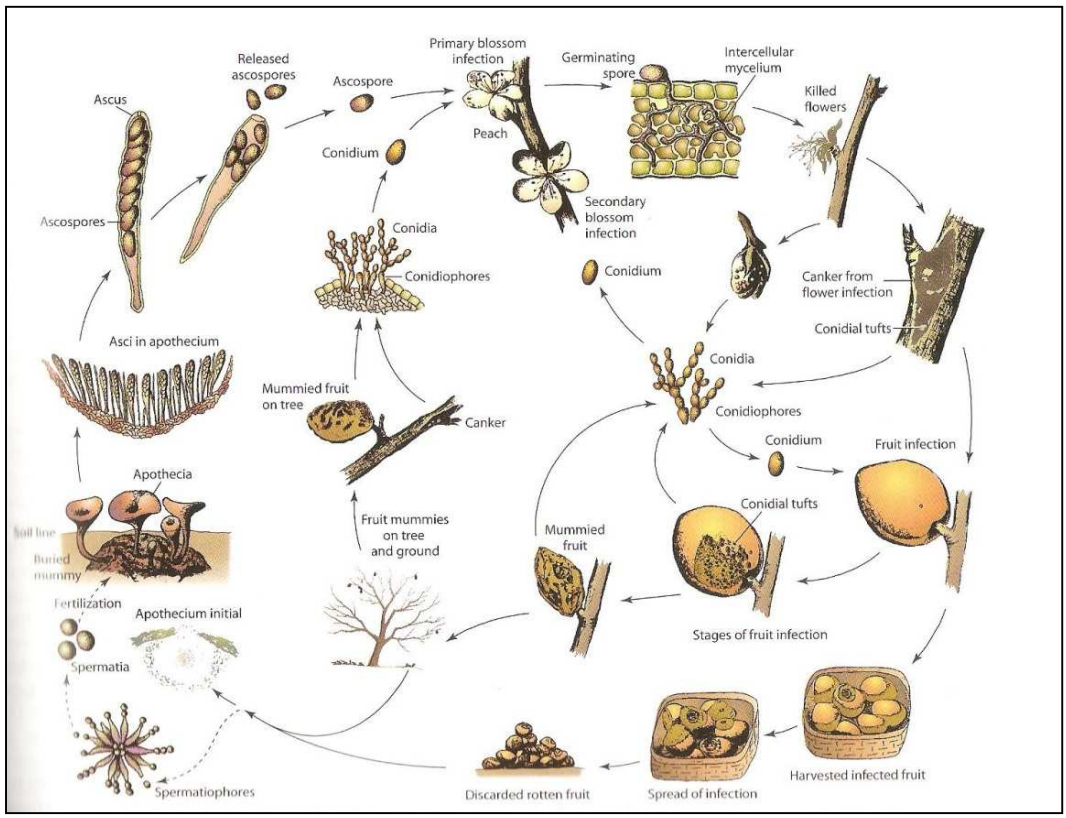


Figure 8: The disease cycle of brown rot of stone fruits (From Georfe N. Agrios: Plant Pathology. Fiveth Edition) (5)

The pathogen overwinters as mycelium in mummified fruit on the tree and in twig cankers or as pseudosclerotia in mummies on the ground. The new conidia are produced by mycelium mummified fruit on the tree and in twig cankers, whereas the pseodiosclerotia in mummified fruit buried in the ground produce apothecia, which form asci and ascospores. *M. laxa* conidia in mummified fruits or twig cankers and *M. fructicola*ascopores in mummies in the ground can cause the first infection: blossom infections.

The conidia are windblown or carried on floral parts by rainwater splash or insects. The ascospores are discharged by the ascus, forming a whitish cloud over the apothecium. Air currents then carry the ascospores to the flowers. Temperature and wetness duration play critical roles in the number of the infected flowers (86).

In humid weather the mycelium produces numerous conidial tufts on the rotten, shriveled floral parts from which new masses of conidia are released. The mycelium advances rapidly into the blossom petioles and into the fruit spurs and the twigs, where a depressed, reddish-brown, shield-shaped canker forms. The surface of the canker is soon covered with conidial tufts, these conidia are the inoculum for fruit infection later in the season when the fruit begins to ripen. The conidia can penetrate the fruits from wounds and lesions, and in some cases they also gain access through stomata (30). The fungus grows intercellularly at first and secretes enzymes that cause maceration and browning of the infected tissues, it invades the fruit quite rapidly while it also produces conidial tufts on the already rotted area. The new conidia can infect more fruits. The entire fruit become completely rotten and mummified. The mummified fruits can remain on the tree or fall on the ground where the mycelia can survive long periods of adverse environmental conditions, particularly those of winter (5, 52, 54).

1.2 *Monilinia* species: morphology, culture characteristics, and molecular analysis

Identification of *Monilinia* species used to be based on the symptoms, host, morphology, and culture characteristics of the fungus (9, 10, 18).

M. laxa shows a lobed type of mycelial growth. Colonies of *M.laxa* are grayish-brown or hazel, the margins are serrulate, the colonies form rosettes with black arcs on Potato Dextrose Agar (PDA), and sporulation is sparse. *M. laxa* has lower growth rates, about half of that of *M. fructicola* (31). Conidia are sparsely produced on PDA, while more abundantly when they are cultivated on the media V8juice or Tomato (based on V8 juice or tomato puree respectively)rich in carbon and nitrogen sources. The stromata of *M.laxa* (in mycological terms a stroma consist of a compact, mattress-like mass or matrix of vegetative hyphae, on which fructifications are often formed) are grayish or hazel in color (Figure 9) .

M. fructicola grown on PDA at 25°C in the dark, forms colonies with active growing entire margins and fluffy. The bands are usually light buff in color. Large numbers of conidia are formed on V8 juice or Tomato media, in old cultures, the circles take on a dark buff color. Black stromatal crusts develop on the surface or in the medium but usually only after the mycelium has covered the Petri dish. There is considerable variation among isolates of *M. fructicola*, particularly in the amount of stromatal tissues on media like. The stromata of *M. fructicola* are grayish or hazel in color. Abundant microconidia may be apparent macroscopically as black raised areas, particularly at the edge of the Petri dish (Figure 9).

M. fructigena colonies grown on PDA have entire margins, the mycelium enlarges uniformly to the edge of the plate and it is yellowish or buff-colored. On alternating periods of light and dark, some isolates produce distinct concentric rings while others either do not sporulate or from only indistinct sporangeus bands (18).

Figure 9: Cultures of *Moniliniafructicola* (right plate) contrasted with *M. laxa* (left plate) on potato dextrose agar after 10 days at 25°C

Identifications is possible by combining culture characteristics, such as growth rate, growth pattern and color with morphological data such as the conidial dimensions and the length of the germ tube. Conidia are useful for *Monilinia* identification: conidia of *M. fructigena* are uniformly larger than those of *M. fructicola* and *M. laxa* (mostly 17-21 x 10-13 µm), and often form two germs tubes per conidia (24, 123) *M. laxa* produces conidia similar in size to that of *M. fructicola*, germ tubes are single but short (150-350 µm) and twisted (32). However, conidial size is very variable and influenced by environmental factors and host species (31, 123). Most of these characteristic are quantitative and overlapping, and atypical isolates of *M. fructicola* may be misidentified as *M. laxa* and vice versa (123)

Consequently, classical methods alone are not adequate for phytosanitary diagnosis of *M. laxa* and *M. fructicola*.

The diagnostic protocol recommends isolation of *Monilinia* spp. from the host, followed by species-specific PCR. When mycelium is present on the examined sample, direct PCR is also possible (56).

To develop a more reliable method, Ioos and Frey (55) designed specific primers pair for the ribosomal internal transcribed spacer 1

(ITS1) region, the 5.8S rRNA gene, and the ITS2 region between the 18S and 28S rRNA genes. These primer pairs were specific for *M. laxa*, *M. fructicola*, and *M. fructigena* and can be used on conventional PCR assays, included a manual DNA isolation or on the TaqMan assays included automatic DNA isolation.

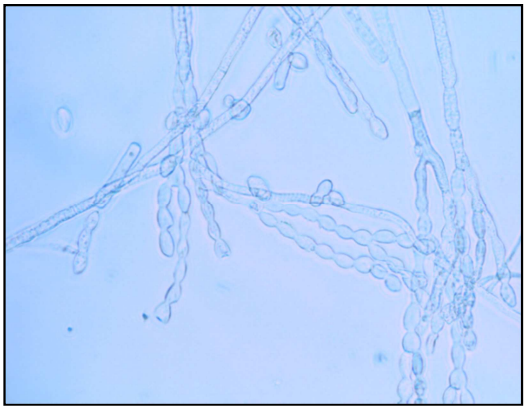


Figure 10: Typical conidial chains of *Monilinia laxa* (200x)

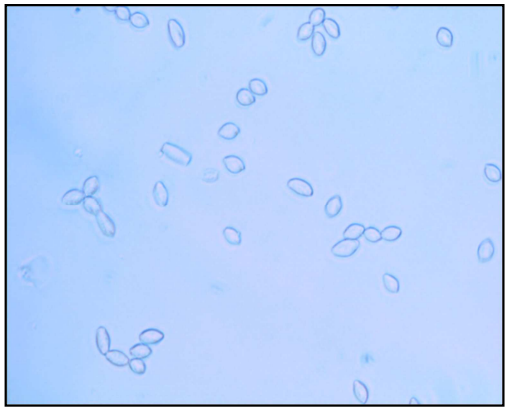


Figure 11: Typical conidia of *Monilinia laxa* (200x)

Figure 12: *Monilinia fructicola* microconidia may be apparent macroscopically as black raised areas, particularly at the edge of the Petri dish (200x)

1.3 Latent infection

Postharvest disease is often classified as “quiescent” or “latent” infection according to how infection is initiated.

The latent, dormant or quiescent infection is a condition in which the pathogen spends a certain period of time during the host’s life in a quiescent stage until it becomes active (124). Jan der Plank (122) reported that a latent infection may occur in the period between spore landing and the production of new spores by the parasitized tissue. Swinburne (114), to avoid confusion, proposed that the term “period of quiescence” was to differentiate the quiescent parasitic relationship from the latent period. Quiescence can be enforced during the various processes of fungal attack, which suggests that the quiescence may occur during any of the processes leading from fungal germination to colonization.

There are different mechanisms of pathogen quiescence and several studies reported that brown rot incidence during fruit development highly varies: green fruits appear more resistant to the pathogen than later ripe fruits (5). *Monilinia* spp. use the ungerminated and germinated spores correlated with cuticle and cell-wall thickness (2). The quiescent period lengthened as the cuticle and cell-wall thickness of peaches increased (1, 2, 81). Michailidis and Johnson reported that in nectarines, the development of quiescent infections in *M. fructicola* was increased with the decreasing of the fruit cuticle thickness (81). Mechanical barriers set up by the host may therefore be sufficient to arrest infection, delay or completely prevent the disease (1, 2, 112). However, in apricots when the fruit ripened, viable hyphae of *M. fructicola* present in the tissue as latent infections escaped from the arrested lesions and caused fruit decay (127). Different hypotheses

have been proposed to explain the association between the decrease in *Monilinia* susceptibility and the green fruit stage: i) the higher mechanical resistance, ii) a different biochemical response between unripe and ripe fruits, iii) the inhibitory substances abundant in the tissue of fruits at this stage. More recently, susceptibility of peach fruit to *M. laxa* has been deeper characterized by monitoring it during the entire fruit growth. It was found that during the period corresponding to the pit hardening stage fruit susceptibility drastically decreases (38, 77, 93). No difference in the rot incidence was found between wound and un-wound fruit, suggesting that the resistance is probably associated to a specific biochemical response of the fruit, rather than to a higher mechanical resistance (77). The cultivar Bolinha, a South American peach, is known to have a high degree of resistance to brown rot (16). This cultivar differs from more susceptible ones for different features, including a higher concentration of phenolic compounds. Similarly, an inverse relation between the severity of brown rot and the polyphenolic content was already found in apples (128). Some of these compounds, such as chlorogenic acid, caffeic acid, the catechin and epicatechin, were used in *in vitro* assays to evaluate their antifungal potential as inhibitors of spore germination and fungal growth. The antimicrobial activity of several phenolic compounds, such as anthocyanins and chlorogenic acid (3-caffeyl-quinic) is well established (12, 128). Consistently, an increase in the total phenolic compounds in peach skin during the pit hardening stage was already shown; in particular, catechin and chlorogenic acid were found as the major phenolic compounds (8, 16, 44).

However, the molecular basis of this phenomenon is still not well understood, Zubini (137) associated the presence of inhibitory compounds in the peel of peach fruits during the pit hardening to the

variation in the gene expression of key genes of the phenylpropanoid pathway; transcript levels of these genes were determined in susceptible and resistant fruits. These data suggest a critical change in the expression level of the phenylpropanoid pathway from 7^{th} to 8^{th} week after full bloom; such change could be directly associated to the peach growth and could indirectly determine the decrease of susceptibility of peach fruit to *Monilinia* rot during the subsequent weeks.

1.4 Thin layer Chromatography

Chromatography is a technique for separating mixtures of molecules, or for characterization of individual molecules, and is the method of choice for secondary metabolites as a whole (13). A mobile phase (can be of a wide range of different solvents) containing the components to be separated, is passed over a stationary phase (can also be of widely different types) to elicit separations. There is an interplay between the three main elements: a) stationary phase (from active to inactive), b) mixture to be separated (from nonpolar (lipophilic) to polar (hydrophilic), and c) mobile phase (from nonpolar to polar). Physical and chemical forces act between the solutes and the two phases to cause separations.

In TLC (Thin-layer chromatography) the stationary phase is layered over a support plate which can be glass, aluminum, or plastic. The basic procedure for classical TLC consists of the following steps: a) the sample solution is applied to the plate origin as a spot or zone, b) the sample solvent is allowed to evaporate from the plate, c) the plate is placed in a closed chamber containing a shallow pool of mobile phase on the bottom, d) the mobile phase rises by capillary through

the applied spot, e) development is continued until the solvent front is about 10 to 15 cm beyond the origin, f) the plate is removed from the chamber and the solvent front is marked, g) mobile phase is removed from the plate by drying in air or applying heat, h) if the compounds are not naturally colored or fluorescent, a detection reagent is applied to visualize the zones and, i) the positions of the zones are used for qualitative identification of compounds (40, 99). The metabolite identity can determine by compering colors and Retardation factor (Rf) values to those on database or in the literature. Rf is defined as the ratio of the distance moved by the center of a spot to the distance traveled by the solvent front (33).

TLC can be used both as an analytical and a preparative technique, and it has been regarded traditionally as a simple, rapid, and inexpensive method for the separation, tentative identification, and visual semi-quantification of a wide variety of substances. Despite the widespread use of TLC, the technique usually has not been considered to be highly efficient or quantitative (40).

The TLC technique is previously used in other studies as to make a preliminary investigation of the antifungal compounds in mango, avocado, strawberry fruits, geraldton waxflower, and soybeans (4,27, 60, 115, 116).

1.5 Brown rot control

The main principles of integrated brown rot management consist of minimizing inoculum potential in orchards, reducing risk of blossom and fruit infections, and making correct decisions on disease management strategies.

Conidia produced in overwintered fruit mummies, fruit stalks, scars and buds, and in cankerous lesion act as primary inoculum sources and cause blossom bight.

Several orchard cultural practices are related to changes in inoculum potential. Proper coordination of fruit thinning and irrigation can significantly reduce the inoculum potential. An empirical recommendation is to time the orchard irrigation in such a way so that the thinned fruit on the ground remains for at least 2 weeks without becoming wet (18).

In some cases, infections occurring in the field remain quiescent until favorable conditions allow the pathogen to overcome host defenses, such that disease symptoms frequently develop during the postharvest phase. To prevent infections at harvest and during storage and transit, fruit should be picked and handled with the greatest care to avoid punctures and skin abrasions on the fruit, which enable the brown rot fungus to gain entrance more easily. All fruit with brown rot spots should be discarded. For this reason it is very important not only to use the fungicides but also monitoring that allows the discovery potential resistant strains (20, 26).

Fungicide sprays are usually used to control brown rot in commercial peach orchards. The number of sprays per season may differ depending on weather conditions and location. A grower may apply three fungicide sprays during bloom and three more before harvest in Brazil (72). The official protocol of fungicides sprays of the U.S. recommends a treatment with effective fungicide 3-4 weeks before harvest, when the fruit changes color, and an application immediately before harvest (from 1 to 3 days). This application is needed to provide fruit with an adequate shelf life. If the orchard has a history of brown rot problems a grower needs to apply fungicide coverage of

green fruit, particularly if weather is wet (6). In China the most prevalent method of controlling brown rot postharvest decay is to combine postharvest treatments with dichloran and preharvest spraying with fungicides such as benomyl, captan or iprodione (65).

In Europe brown rot is controlled by fungicide spray programs in the field and postharvest fungicide applications are not allowed (120).

Postharvest losses are estimated to be 5-10% when postharvest fungicides are used, in countries where these treatments are not authorized, losses may reach 50% or more when favorable conditions are present (88).

In Greece, the benzimidazole fungicides thiophanate methyl and carbendazim are widely used for controlling brown rot of peaches. During the bloom a good control of *M. laxa* in peach flowers is carbendazim (76, 117). The synthetic pesticides mostly used in Italy belong to four different groups according to the mechanism of action and to seven different chemical groups.

Italian integrated brown rot management consists of two or three chemical treatments, one during the full bloom and one seven or ten days before the harvest with demethylation inhibitor fungicides (DMI) or quinone outside Inhibitors (QoI), used by themself or in combination with different mechanism of action fungicides such as Fludioxonil and Cyprodinil or Pyraclostrobin and Boscalid to avoid developing the resistant strains (Appendix 4) (120).

1.5.1 Sensitivity of *Monilinia* spp. to tebuconazole and thiophanate methyl

The assessment of the resistance to fungicides is an important tool to determine their efficacy and the useful lifetime.

Demethylation inhibitor fungicides (DMI) appeared in 1980s are widely used against brown rot and have effectively replaced methyl benzimidazole carbammates (MBC), since its repetitive use has selected fungicide-resistant populations (26, 113, 133, 135).

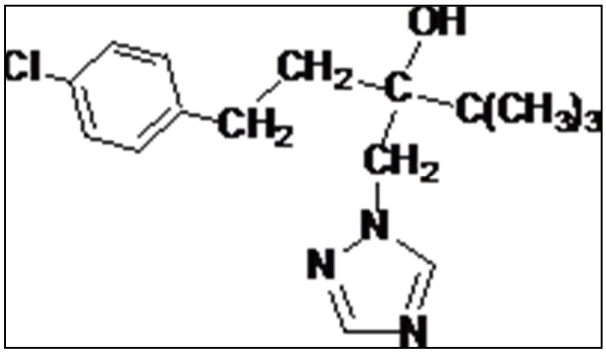


Figure 13: Tebuconazole (from The Pesticide Manual) (119)

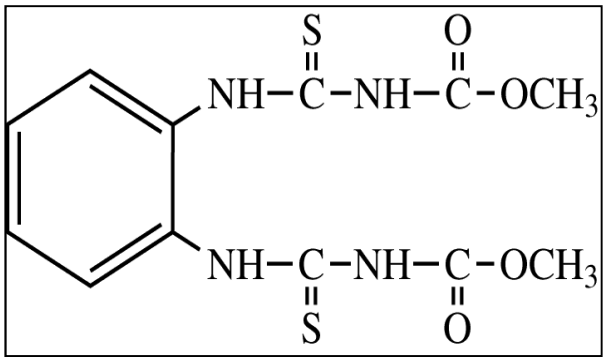


Figure 14: Thiophanate methyl molecule (from The Pesticide Manual) (119)

Therefore, DMI fungicides have become essential to growers to control many plant diseases. The class counts more than 20 fungicides with the same mode of action (39, 107), and many of them are used widely in crop protection. They are subject to resistance problems because they are used almost exclusively for *Monilinia laxa, M. fructicola* and M. *fructigena* control during flowering and preharvest period (20, 106). DMI and to a lesser degree MBC fungicides are used in many European orchards to prevent the *Monilinia* rot (32, 87). Tebuconazole, propiconazole and fenbuconazole have become the leading DMIs used in Italy.

In the past Italian *M. laxa* and *M. fructigena* were controlled by chemical treatments based on MBCs, but three decades ago DMIs were introduced in substitution of MBCs because resistant strains were developed; however, thiophanate methyl is still used against cancer on peach tree branches (20, 26).

The degree of toxicity of a fungicide against the target organism is generally calculated by *in vitro* trials and expressed as the 50%

effective concentration (EC_{50}). It represents the fungicide concentration at which mycelia growth or spore germination of the target pathogen is reduced of the 50%. In fungicide resistance management, baseline sensitivities have become critical parameters that define the distribution of EC_{50} values in a fungal population.

Methods to assessed the sensitivity to fungicides are:

a) Amended medium. Traditionally, EC_{50} values are determined by serial dilution of a fungicide in agar medium, this method compares the growth of isolates on fungicide amended medium relative to the growth on non-amended medium (107). The agar dilution method requires the preparation of large amounts of agar media, several fungicide stock solutions and at least three plates for each fungicide concentration, then the plates inoculums, and the measuring of fungal growth after incubation. The result cannot be quickly determined and may take several weeks for recalcitrant fungi.

b) Spiral Gradient Endpoint Method (SGD).For determining minimal inhibitory concentrations of antibacterial agents in 1990 the SGD method was introduced and has been extensively used in clinical studies of bacteria (98). Other researchers have used the SGD method with a wide range of bacteria (47, 129, 130) and Forster et al. applied the method to fungi (37). In this method a precise volume of stock solution of attest substance is plated in an Archimedes spiral on a rotating agar plate, from the center to the perimeter, forming a 2.5-log dilution in a radial concentration gradient In this way fungicide stock concentrations are applied to the agar plate. Fungicide stock solution can be determine by the Spiral Gradient Endpoint (SGE) software (Spiral Biotech, Nerwood, MA) if information on the toxicity of the fungicide is available. Test fungus, previously grown on paper, cellophane or agar strip, is applied along radial lines. Plates are

incubated for days at suitable temperature and the radial location of the transition from growth to reduced growth is measured. To determine most accurately local fungicide concentrations in the agar, a stock concentration should result in 50% inhibition at locations in the midrange of the radial distance between the center and the periphery of the plate. Local fungicide concentrations along the concentration gradient are computed from several key parameters entered into SGE program. These parameters are: molecular weight of the antimicrobial agent and the time after application of antimicrobial agent. In fact, after application of antimicrobial agents, plates should be incubated for 1-4 h to allow a gradient to form before the test organism is applied. After this period the gradient changes very slowly. A time factor for diffusion is included in the SGE software, ranging from 1 to 2 days of incubation option selected for determining EC_{50} values. For mycelial growth assays the optimal settings is 2-day option. The minimum inhibitory concentration is computed from the measured radial location by the SGE.

The SGD method takes much less time than traditional method, it is cost-effective, accurate, and reproducible while requiring fewer supplies. The computer software for calculating fungicide concentrations is user-friendly and helpful in creating toxicity databases.

The fungal colony shapes can morph in different fungus-fungicide combinations, in fact the shape has to be determined primarily by the fungicide but also by fungus. In general, fungal growth responded gradually to changes in fungicides concentrations. In some interaction the response is more abrupt and it could be very difficult to determine the right values (37).

c) Alamar Blue method. The third way to determine the EC_{50} is based on Alamar blue (AB). AB is an oxidation-reduction indicator dye, commonly used as indicator of microbial respiration, it can provide results within 24 hours, depending on germination velocity of the conidia . In the presence of actively growing cells, the AB changes from an oxidized, nonfluorenscent blue form to a reduced, fluorescent pink form. Inhibition of growth maintains an oxidized environment, leaving the indicator blue. AB is nontoxic and stable, allowing long incubator periods without distorting results (95, 99). As a colorimetric test, the results can easily be discerned with the naked eye, and more rapid and exact measurements can be taken with spectrophotometer. The microtiter plates potentially allow for a high-throughput screening of multiple isolates, multiple fungicides, and multiple fungicides concentrations. Collection of results was less time consuming because 96-well plate reader took absorbance readings in a matter of seconds (21). Several studies in medical microbiology have successfully used AB to quantify the fungal resistance to drugs (22, 97, 101). However, quantification and optimization of inoculum is needed, especially the quantity of inoculum, since greater efficacy is observed when smaller amounts of inoculum are used (95, 102). Unfortunately there are few disadvantages of this method. The first it is the reliance on quantifiable reproductive propagules such us spores, and it may not be useful for assaying fungicide sensitivity in pathogens that do not readily produce spore in culture. The second is the presence of possible contaminant fungi, yeast or bacteria on the surface of fungal hyphae or infected plant tissue that can interfere with the results and prevent the assay directly on field samples. The third is that only pure cultures of sporulating fungal pathogens would be amendable to this technique (22).

Like the mycelial growth assay and the Spiral Gradient Endpoint Method, the AB assay can be used to quantify fungicides sensitivity phenotypes but is unable to identify the genotype and does not provide an alternative to polymerase chain reaction-based studies to identify genes that confer fungicide resistance.

1.5.2 Characterization of methyl benzimidazole carbammates resistance in *M. laxa* and *M. fructicola*

The MBCs were used to control almost exclusively the brown rot in stone fruit orchards during the 1980s in Italy. Genotype resistance to MBC fungicides has been detected in many fungal species. In most cases, resistance is associated with point mutations in the β-tubulin gene which result in altered amino-acid sequences at the benzimidazole binding site (61).

Previous studies by Ma et al. (74, 75), showed that in the phenotype of *M. laxa* field isolations, low resistance to thiophanate methyl is determined by a change in the codon position 240 converted from CCT (leucine) to TTC (phenylalanine) (75). Other *M. fructicola* strains that showed phenotype high resistance to thiphanate methyl have the sequence CAT (histidine) at codon position 6 in the β-tubulin gene while thiophanate methyl low resistant *M. fructicola* strains have codon 6 converted from CAT (histidine) to TAT (tyrosine). *M. fructicola* strains that are sensitive or low resistant to thiophanate methyl have the sequence GAA (glutaminic acid) at codon 198 in the β-tubulin gene, while the thiophanate methyl high resistant *M. fructicola* strains have a punctual allelic change ad codon 198, GCA (alanine) instead of GAA (glutaminic acid) (74) (Table 1).

Codone	Alteredaminoacidic	Pathogen	Bibliography
6	His →Tyr	***Moniliniafructicola***	Ma *et al.*, 2003
50	Tyr →Cys	*Cladobotryumdendroides*	McKay*et al.*, 1998
167	Phe →Tyr	*Cochliobolusheterostrophus* *Penicilliumexpansum*	Gafur*et al.*, 1998 Baraldi*et al.*, 2003
198	Glu →Ala	*Botryotiniafuckeliana* *Helminthosporium solani* ***M. fructicola*** *Penicilliumaurantiogriseum* *Penicilliumexpansum* *Tapesiayallundae* *Tapesiaacuformis* *Venturiainaequalis* *Venturiapirina*	Luck e Gillings, 1995 McKay e Cooke, 1997 Cunha e Rizzo, 2003 Ma *et al.*, 2003b Koenraadt*et al.*, 1992 Baraldi*et al.*, 2003 Koenraadt*et al.*, 1992 Albertini *et al.*,1999 Koenraadt*et al.*, 1992
	Glu →Gln	*T. acuformis* *T. yallundae* *H. Solani*	Albertini *et al.*, 1999 McKay e Cooke, 1997
	Glu →Gly	*T. acuformis* *T. yallundae* *V. Inaequalis*	Albertini *et al.*, 1999 Koenraadt*et al.*, 1992
	Glu →Lys	***M. fructicola*** *P. aurantiogriseum* *P. digitatum* *Sclerotiniahomoeocarpa* *V. inaequalis* *P. expansum* *T. yallundae* *T. acuformis*	Koenraadt*et al.*, 1992 Albertini *et al.*, 1999 Baraldi*et al.*, 2003 Albertini *et al.*, 1999
	Glu →Val	*P. expansum*	Albertini *et al.*, 1999
200	Phe →Tyr	*P. aurantiogriseum* *P. italicum* *V. inaequalis* *V. pirina* *T. yallundae*	Koenraadt*et al.*, 1992 Albertini *et al.*, 1999
240	Leu →Phe	***Monilinialaxa*** *T. yallundae*	Ma *et al.*, 2005 Albertini *et al.*, 1999

Tab. 1: Mutation points in β-tubulin gene reported in field isolates (47)

1.6 Influence of temperature on mycelium growth, spore production, germination and resistance to fungicide in *M. laxa* and M. *fructicola*

The pathogens require certain minimum temperatures to grow and carry out their activities. In temperate regions, the low temperatures of late winter, and early spring (0-5°C) are below the minimum required by most pathogens. With the advent of higher temperatures (15-30°C) the *Monilinia* spp. become active and can infect the plant if other environment conditions like water (moisture) and light are favorable, causing the disease (5, 19, 69).

The optimal temperature range for germination of *M. laxa* conidia is 15-30°C. The species is even able to germinate at suboptimal temperatures (0 and 35°C), but at 38°C no germination was observed (19).

M. fructicola also has a wide optimum temperature range for germination, from 15 to 30°C, and can germinate over 35°C (though no germination was observed at 38°C) and less than 0°C. The effect of temperature on the ability of *M. fructicola* conidia to germinate was previously reported by Casals et al. (19, 69) and Luo et al. (70, 71).

The optimum germination range of *M. fructigena* is 15-30°C (19).

In a previous study on the sensitivity of *M. fructicola* to benzimidazoles, Ma et al. observed that benzimidazole LR isolates showed sensitivity to low temperature, whereas HR isolates exhibited sensitivity to high temperature (74, 75).

The temperature can influence the resistance to DMI of *M. fructicola* strains. Several mechanisms of resistance to DMI fungicides have been described in plant pathogenic fungi. The most common of those mechanisms found in field isolates of plant pathogens include

mutations and overexpression of the cytochrome P450 14a-demethylase (CYP51). This overexpression was associated with a repetitive genetics called “Mona” located up-stream of the MfCYP51 gene. The Mona element contains a predicted promoter sequence, which likely triggers the elevated expression of MfCYP51 (67). The Mona element contains a putative sequences and has been found in DMI-resistant isolates of several eastern states of U.S. (67, 126). The methylation status of the Mona element and a portion of the MfCYP51 gene before and after storage at 4°C was investigated because the resistant strains during the conservation lost this characteristic, but the experiment indicated that neither a deletion of Mona element nor methylation of Mona or the MfCYP51 gene was associated with the resistance decline. Some studies suggest that other genes or other parts of the genome were methylated and thus were involved in propiconazole resistant instability (67, 138).

The knowledge of the temperature sensitivity of resistant field isolates can have a direct impact on the strategies for brown rot management.

However, the influence of temperature in DMI resistant strains in orchard during the season has not been investigated and reported in literature.

Chapter 2

2.1 Aim

Fruits contain secondary metabolites with antifungal properties, that are mostly concentrated in the peel at immature stage and decline during ripening in coincidence with fungal rot development (4). Some authors have attributed the variation in natural disease resistance in peach fruits to skin strength (16, 70) and the change in the expression level of the phenylpropanoid pathway on pit hardening period (137). The concentration in peach peel of chlorogenic acid, caffeic acid and caffeolic acid during peach fruit developmental stages canges (8, 45, 63), but more comprehensive studies of peach fruit antifungal systems at different phenological stages are not present in the literature. This type of investigation, that has been conducted on avocado, mango, and strawberry (4, 116) but not on peach, would provide a better understanding of peach fruit susceptibility at different stages, and consequently improve the pathogen control and reduce production costs and environmental risks.

In this spirit, the contribution presented in this dissertation is on the study of *Monilinia* populations in Italy, with the focus DMI and MBC fungicide–resistant populations. This research activity finds its justification in the previous studies conducted in China (65), California (82, 84, 86, 132), New York (126), Georgia (6, 7, 113), South Carolina (7, 51, 62, 68, 136), Brazil (72), Greece (118) and Spain (87) that reported different grades of resistance to the benzimidazole and dimethylation inhibitors in isolate of *M. laxa* and *M. fructicola* collected from stone fruits orchards. The Greek researches recently have reported low or none benzimidazole and

dimethylation inhibitors resistance strains from different local orchards (76, 117).

More in detail, the objectives of this dissertation were:

1) investigate to *M. laxa*, *M. fructicola* and *M. fructigena* Italian population;

2) study of peach fruit susceptibility at different phenological stages by *in vivo* trials and TLC assays;

3) determine the sensitivity to two fungicides, thiophante methyl and tebuconazole by multiply assays;

4) explore the molecular mechanism of benzimidazole resistance;

5) consider the temperature influence on fungicide sensitive and resistant strains.

Chapter 3

Materials and methods

3.1 Population of *Monilinia* spp. in Italy

Peach and nectarine are two of the most important crops of fruit trees in Italy. The largest production of peaches and nectarines per hectare was recorded in the Northern regions (44,232 ha, 196 peach and nectarine productions per hectare), followed by the Southern (36,656 ha, a 179 peach and nectarine productions per hectare) (34). Emilia Romagna, and in particularly Romagna, is the first Italian region that has received the Igp high quality brand for its peaches and nectarines (130); followed by Veneto, Campania, Basilicata, Calabria, Sicily, and Sardinia (34). These areas are subjected to high humidity that favors fungal infection of stone fruit causing pre-harvest and postharvest decays. Serious losses of fruit are caused by pre-harvest brown rot, particularly when weather conditions are wet. Fungi of the genus *Monilinia* are the primarily responsible for the occurrence of brown rot on fruits (20).

3.1.1 Isolation and identification of the pathogen

During two years (2010 and 2011), fruit samples were collected from different Italian growing areas. In particularly, in 2010 over two hundred rotten stone fruits (peaches, nectarines, plums, cherries, apricots), apples and pears were sampled randomly at harvest from different orchards located in Emilia Romagna and from the packaging

house (Apofruit, Cesena). In 2011 over three hundred rotten fruits were collected from different peach and apple orchards located in Emilia Romagna, Veneto, Lombardia and Sardinia, and from packaging house Apofruit.

To isolate the pathogen, small pieces of fruits, approximately 2 mm in diameter, were taken on the margin of rotten tissues and placed on PDA (see Appendix 1) with antibiotics (0.1%) (neomicyn and streptomycin) (see Appendix 2) in Petri dishes (90 mm in diameter) and incubated at 25°C for a week. Single-spore cultures were prepared from colonies growing on PDA. Conidia were harvested by adding 5 ml of sterile water with Tween 80 (0.05%) and rubbing the sporulating mycelial mat with a sterile plastic loop. The conidial suspension was filtered through two layers of cheesecloth and then 100 µl were spread on MEA (see Appendix 1), with antibiotics as previously described.

When on the surface of rotten fruits there was an evident abundant spore production, conidia were scraped from each fruit with a sterile toothpick and transferred to 1 ml of sterile distilled water and Tween 80 (0.05%). Subsequently, an aliquot of 100 µl was evenly distributed on MEA with antibiotic. Petri dishes were incubated at 25° C (104). After two days, three different single spores were selected and placed on PDA plates and incubated at 25°C for 5 to 7 days and isolated pathogens were stored in growth chamber until use. Morphological identification of various isolates was done using various taxonomic keys (31, 32, 123). The conidial dimension, germ tube, the sporulation and colony shape after seven days in the dark were evaluated on water, V8 agar and PDA (see Appendix 1). Subsequently, the sequencing of ribosomal DNA ITS regions was used to confirm the diagnosis.

PCR reactions were performed using specific primers ITS1Mfc1 and ITS4Mfc1 for *M. fructicola*, ITS1Mfgn1 and ITS4Mfgn1 for *M. fructigena* and ITS1Mlx1 and ITS4Mlx1 for *M. laxa* (Table 2) (55, 121) using mycelia from pure cultures, following protocols described by Iotti and Zambonelli (56).

A small portion approximately 0,01-0,02 mm^2 wide of mycelium was removed from young colony (5 days) and transferred directly into the PCR tube containing 100 µl of sterile water. In order to break mycelium cells, the tubes were placed in a T gradient thermal cycler (Biometra, Gottingen) for 15 minutes at 99,9°C and immediately transferred and placed on ice for 15 minutes. Ten microliters of this solution were used as template in a 50 µl of PCR reaction containing 10 mM Tris/HCl (pH 8.3), 50 mMKCl, 1.5 mM MgCl2, 150 µM for eachdNTP, 300 µM for each primer, and 1.5 U of TaKaRaTaq DNA polymerase (Takara, Otsu, Japan). Twenty micrograms of Bovine Serum Albumin (BSA) (Fermentas, Vilnius, Lithuania) were added. The amplification reactions were carried out in a T gradient thermal cycler with an initial denaturation at 95°C for 6 min followed by 30 cycles at 94°C for 30 s, 62,5°C for 1 min and 72°C for 1 min and a final extension step of 72°C for 10 min. PCR products were run on 1.5% agarose gels, according to the standard methods (109) with a 1kb bp DNA ladder (GeneRule 1 Kb bp, DNA Ladder, Fermentas GmbH) to size fragments. Gel was stained with ethidium bromide, visualized and photographed under UV light (121).

In order to store the pathogen isolates, all isolates in this study were grown on filter paper disks placed on PDA and, after 7 days, disks with mycelium were removed, desiccated, and stored at -18°C (68).

Table 2: Oligonucleotides used in this study (55)

Primer	Sequence 5'-3'	Target
ITS1 Mlx1 fw	TATGCTCGCCAGAGAATAATC	*M. laxa*
ITS4 Mlx1 rw	TGGGTTTTGGCAGAAGCACACC	*M. laxa*
ITS1 Mfc1 fw	TATGCTCGCCAGAGGATAATT	*M. fructicola*
ITS4 Mfc1 rw	TGGGTTTTGGCAGAAGCACAC	*M. fructicola*
ITS1 Mfgn1 fw	CACGCTCGCCAGAGAATAACC	*M. fructigena*
ITS4 Mfgn1 rw	GGTGTTTTGCCAGAAGCACACT	*M. fructigena*

3.2 Susceptibility of peach fruit to *M. laxa, M. fructicola* and *M. fructigena* during phenological stages

3.2.1 Fruit

For these experiments 'Red Heaven' peach from five-year-old fungicide-free orchard located in Cadriano, Bologna (Italy) (with coordinates 44.559592°-11.410246°) were used. The orchard system was open vase with P.S. A6 rootstock (*Prunus persica*, DCDSL, Pisa, Italy).

In 2010 and 2012 fruits were weekly harvested during a 12-week-long period, starting from the fourth week after full bloom until full maturity.

A sample of 260 fruits were used at each sampling date. Before each inoculum, fruit were washed with sodium hypochlorite (1% active chlorine), rinsed in distilled water and left to dry at room temperature for 1 hour. At each sampling date, 20 fruits were assessed for fruit growth, measuring the diameter [mm], weight [g], percentage of soluble solids and stage of pit hardening. To individuate the pit

hardening stage, the equatorial zone of fruit was cut with a knife; the pit was considered hardened when the knife could not penetrate. Two hundred fruit were divided in two equal lots. Each fruit of the first lot was wounded with a sterile nail (one wound per fruit in the equatorial zone) while the fruits on the second lot were left unwounded.

3.2.2 Fungal pathogens

M. laxa (isolate ML4), *M. fructicola* (isolate MCL2) and *M. fructigena* (isolate MCG1) were grown on V8 agar at 25°C for 7 days, conidial suspensions were obtained as described before. The conidia were eluted in water with Tween 80 (0,05%) previously sterilized, and the conidia quantified with a haematocytometer; the appropriate concentration was adjusted with sterile distilled water.

At each sampling time, fruits were dipped for 1 min in the ML4 or MCL2 or MCG1 conidial suspension (10^5 conidia ml^{-1}). The negative control was dipped in distilled water. After inoculation, fruits (wounded and unwounded) were kept at 20°C and 95% RH for 7 days and the incidence of rot was determined as the percentage of infected fruits. The sample unit was represented by 4 replicates of 10 fruit each.

Isolates ML4, MCL2 and MCG1 were maintained on PDA and passed on fruits annually to verify pathogenicity (75, 135).

3.3 Preformed antifungal compounds in peach fruits

3.3.1 Crude extracts from peach peel

In 2012, a sample of ten fruits of peach ‘Red Heaven’ from orchard located in Cadriano, (Bologna), ‘Tardibelle’ and ‘Maycrest’ from orchards located in Cesena were weekly harvest starting from four

week from full bloom until full maturity. Immediately after harvest, the fruits were peeled and the peels immediately snap-frozen in liquid nitrogen and stored at -80°C until use. Each sample extract was prepared according to the procedure of Terry et al. (116) with some modifications. Two grams of peach peel were ground in a liquid nitrogen and homogenized in ethanol (99% v/v) at -18°C (3 mL g^{-1}) (HK 3300, FALC) for 15 minute g^{-1}. The homogenate was filtered under vacuum through Whatman No. 3 filter paper by a 5.5 cm-diameter Buchner funnel. The filtrate was concentrated in a rotary evaporator (Buchi Rotovapor, Buchi Labor technik AG) under vacuum (Vacuum pomp 707, Asal, Milan) of ca. 0.6 kPa at 40°C to approximately one third of the original volume. Dichloromethane 99% was added to one third of the volume of concentrated using a separating funnel. The lowest dichloromethane layer was pooled and dried by adding 0.5 g of anhydrous $MgSO_4$, filtered through Whatman No. 2 filter paper and evaporated to dryness in the rotary evaporator at 40°C. The material was re-suspended in ethanol (0,2 mL g^{-1}) (27) and stored at -18°C until used for 1D Thin Layer Chromatography (TLC), fungal bioassay or TLC detection procedures.

The crude extraction was repeated twice at all stages of peach fruit maturity, on fruit without symptoms of fungal infection.

3.3.2 Thin Layer Chromatography

Glass TLC plates (20 cm x 20 cm) coated with silica gel 60 with concentration zone and fluorescent indicator 254 nm (Fluka, St. Louis, MO) were used. The plates were pre-conditioned in an oven at 110°C for one hour. The plates were spotted using a micro-pipette with 5, 10, 20, 30, 40 and 100 µl of re-suspended crude peel extracts. The extracting solvents (99% (v/v)) were used as negative controls. TLC

plates were developed in one dimension (1D) at approximately 22°C in a TLC tank (20 cm x 20 cm x 10 cm) and closed with a lid to create a solvent saturated atmosphere. 1D TLCs were developed in 100 ml of running solvents comprised of hexane:ethylacetate:methanol in 4 different elution systems a) 60:40:1, b) 60:40:10, c) 60:40:20 and d) 60:40:30 (114). The TLC 1D plate chromatogram was air-dried and then either used for antifungal bioassays or sprayed with chemical reagents.

3.3.3 TLC bioassay

In order to carry out the TLC bioassay, a single-spore isolate of *M. laxa* (ML4) obtained from a naturally infected peach was cultured in V8 agar (66) at 25°C for seven days. Than a conidia suspension was prepared as previously described at a concentration of 10^7 conidia ml^{-1}. Developed chromatograms were covered with a thin layer (2 mm) of PDA at 45°C amended with antibiotics (0.1%) and with conidia suspension of *M. laxa* at final concentration of 10^5 conidia ml^{-1}. TLC plates were incubated at 4°C for three days in sealed plastic containers and then stored at 25°C for other three days. Spots of fungal inhibition, i.e., where mycelial growth was absent, indicated the presence of antifungal activity (60). Weak antifungal activity was defined as areas with less dense, lighter colored mycelium. The retention factor (R_f) and areas of inhibition were measured and recorded. For each week-crude extract two TLC plates were used.

3.3.4 Preliminary identification of antifungal compounds

Detection procedures for compounds on 1D TLC included inspection under visible and ultraviolet (254 nm, Koninklijke Philips N.W.,

Amsterdam) light using fluorescent TLC plates. Phenolics were detected by spraying chromatograms with either 1.89 M sodium carbonate followed by Folin Ciocalteu (Folin-Ciocalteu; diazotized sulfanilic acid, Fluka, St. Louis, MO) reagent (1:3 v/v water) and fumigation with ammonia (128). After examination, ammonia was removed from the plates by air drying for 1h at room temperature. The R_f and colour of detected spots were recorded and compared to zone of inhibition on duplicate bioassay chromatograms (115, 116).

3.4 Sensitivity of *M. laxa*, *M. fructicola* and *M. fructigena* to fungicides

Fungicides are currently used in an integrated scheduled program to control brown rot and avoid economic losses worldwide; in Italy only two or three treatments in the field are permitted: during bloom and just before harvest. The third treatment is reserved for late cultivars or under weather conditions favorable for brown rot. Thus, the assessment of the resistance to fungicides is an important tool to determine their efficacy and the useful lifetime.

3.4.1 Amended medium

The sensitivity to fungicides was evaluated on 32 samples (see Appendix 6) of *M. fructicola* (27 samples) and *M. fructigena* (5 samples) obtained as described in Section 3.1.1.

In order to evaluate the sensitivity to fungicides in *M. fructicola* and *M. fructigena* strains, dishes of 25 ml of PDA were amended with thiophanate methyl (EnovitMetil FL, Sipcam) or tebuconazole (Folicur SE, BayerCropScience) (see Appendix 5) at concentrations of 0.1, 0.3, 1, 3, 10, 30, 60, 120, 240 μg ml^{-1} and 0.01, 0.03, 0.1, 0.3, 1, 3,

10, 30 μg ml^{-1}, respectively. PDA was autoclaved and fungicides were incorporated after cooling (50°C) (37). A mycelium plug (6 mm in diameter), taken from the margin of 5-day-old actively growing colonies on PDA of each isolates, was transferred to each amended PDA dish. They were incubated at 25°C in the dark and the diameter was recorded after 6 days. Four replicates were used for each isolate at different fungicide concentrations, while four un-amended PDA dishes for each isolate were used as control. The experiment was repeated twice.

3.4.2 Spiral Gradient Endpoint Method (SGD)

The experiments relative to SGD were conducted at Clemson University (School of Agricultural, Forest & Environmental Sciences, Clemson, SC).

Sixty isolates of *M. laxa* listed in Appendix 7 were used. Fifty six isolates were isolated from pathogens from peaches, nectarines, cherries, plums, and apricots cultivated in Emilia Romagna orchards. Nineteen samples were kindly provided by Emilia Romagna Regional Plant Protection Centre and four isolates by Dr. K. D. Cox (Department of Plant Pathology and Plant-Microbe Biology, New York State Agricultural Experiment Station, Cornell University, Geneva, NY).

The active ingredients were the same used for amended medium trial while the commercial products were Topsin-M 70WP (thiophante methyl) and Elite (tebuconazole) (see Appendix 5).

For the mycelial growth assay the fungi were grown on PDA. A single 5 mm fungal plug was taken from the periphery of a *M. laxa* colony grown for 4 days on V8 agar, and shacked on vortex in a sterile tube containing 4 ml of sterile water and 2 g of 5-mm sterile glass beads

until the plug was completely destroyed (89). To produce mycelial inoculum for the spiral gradient dilution method an aliquot of 2 ml of mycelial suspension was sprayed on the surface (5.3x0.6 cm) of filter paper strips (P8-Fisher Braund), previously autoclaved and then placed in petri dishes containing V8 Juice agar. The plates were incubated for 3 days at approximately 25°C under illumination without Parafilm.

According to the protocol provided by Forster (37), the fungicide was added on the surface of 15 cm-diameter petri dishes (Fig. 15). The addition of V8 juice (1%) to PDA is required to improve the mycelial growth in less time.

Petri dishes were placed on a flat surface, and 50 ml of modified PDA was poured into each dish to form a 3.3 mm-thick layer of agar under aseptic conditions as suggested by the SGE manual. The plates were prepared at least 24 h before applying aqueous stock concentrations of fungicide to the agar. Appropriate stock tebuconazole and thiophanate methyl concentrations of 400 and 50 μg ml^{-1}, respectively, were determined by SGE software using the exponential mode calculation option. A total of 50 μl of fungicide solution was applied to each plate with a spiral plater (Spiral System Model D, Spiral Biotech, Nordwood, MA) using the exponential mode of application, resulting in a radial gradient of approximately 1:300 in a 15 cm dish. The starting point of the spiral is marked at the edge of the petri dish. The center of the spiral was marked on the agar surface. The plates were incubated at room temperature for 3 h to allow the fungicide to diffuse and form a radial concentration gradient over the plate. A sterilized cork borer (12 mm diameter) was used to remove the agar disk from the center of the plate to create a fungicide-free region of substrate. The application of fungicide by the stylet of the spiral plate starts off-

center and the removal of this agar disk prevented the growth of the fungus across the center on the plate into neighboring inoculated areas.

According to the scheme provided by SGE software, the mycelium-covered paper strips were radially applied across the fungicide concentration gradient on the spiral gradient dilution plate. Forceps were used to carefully place paper strips over the plate position lines, with the mycelium facing down.

For each isolate, two replicates (i.e, two plates) were prepared for each fungicide, and performed three. The control consisted of PDA plates sprayed only with water without fungicides. The plates were incubated at 25°C for three days.

After incubation, the radial growth of the fungus in each of the replicates was measured, and the values were averaged. In mycelial growth assays, the location where fungal growth was inhibited by 50% with respect to the growth on the control was determined as illustrated in Figure 16. The radial distance between this location and the center of the plate was measured. In the SGE software, this distance is a key parameter to determine the local fungicide concentration in the agar at the location of 50% inhibition.

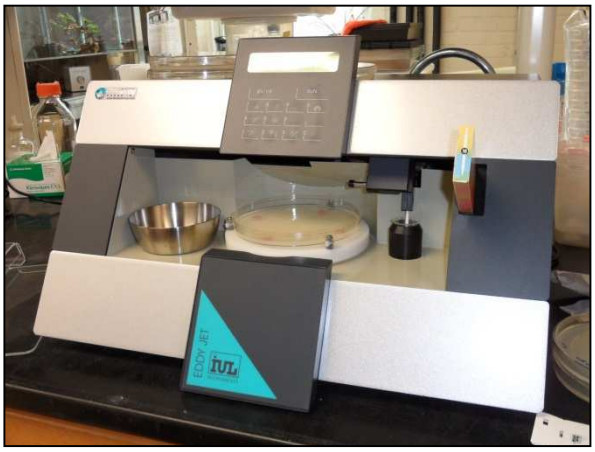


Figure 15: Spiral plate machine used in the fungicide experiment

Figure 16: Isolates of *M. laxa* grown on PDA amended with tebuconazole (stock concentration 50 mg ml^{-1}) applied in a spiral gradient dilution by means of spiral plater in a 15 cm Petri dish (right). The strips were placed along radial lines across the fungicide concentration gradient. Paper strips covered with mycelium of *M. laxa* on the control (left). After 3 days of incubation, three measurements were taken: radial growth of the controls (a), the location on the spiral gradient dilution plate where fungal growth is inhibited by 50% (b), and the distance between the center of the plate and the 50% growth point (c)

3.4.3 Alamar Blue method

Before applying the rapid method AB test, it was necessary to quantify and optimize three parameters, the incubation time, the substrate and the pathogen concentration (22). The best parameters used for AB assay with *M. laxa* and *M. fructicola* and the fungicides tebuconazole and thiophante methyl are listed in table 3.

Table 3: Best parameters for AB assay with *M. laxa* and *M. fructicola*

Conidial concentration[a]	10^5 on total volume of 200 µl in each well
Substrate	PDB half dose (12 gL^{-1})
Alamar Blue[a]	20 µl in each 200 µl well
Incubation time	12 h

[a]The conidial concentration and AB quantity for each well were the same as reported by Cox (21)

Four *M. laxa* and eight *M. fructicola* isolates from single conidia were chosen for comparison among traditional amended agar and AB assay

with tebuconazole, and eight *M. laxa* and eight *M. fructicola* isolates were treated with thiophanate methyl and the EC_{50} was compared (Appendix 8).

MEA was amended with fungicides as described above. Final concentrations were adjusted such that the inhibition values of conidial germination were well distributed over a range of concentrations: tebuconazole 0.001, 0.003, 0.01, 0,03, 0.1, 0.3, 1, 3, 10, 30, 100 μg ml^{-1} and thiophanate methyl 0.1, 0.3, 1, 10, 30, 60, 120, 240 μg ml^{-1}. The control was represented by medium without fungicide. The experiments were conducted in the same conditions as described above. The experiment with the amended medium was repeated two times with three replicates for each concentration. Averaged data obtained by the agar dilution method were used for computing the inhibition percentage of conidia germination. EC_{50} values were determined. Sixty-four isolates of *M. fructicola* and twenty-eight isolates of *M. laxa* previously tested with tebuconazole and thiophanate methyl inhibitory doses representing a range of different sensitivities were selected for AB test (Appendixes 9 and 10). All assays were performed in 96 rounded-bottom polystyrene microtiter plates (Corning, Corning UK) as described by Cox (22) with some changes. Each well contained 80 μl of half PDB dose with a conidial suspension (10^5) of *M. laxa* or *M. fricticola*. An additional 100 μl of PDB amended with the final concentration of tebuconazole 0.001, 0.01, 0.1, 1, 10, 100 μg ml^{-1} or thiophanate methyl 0.3, 1, 10, 30, 60, 120, 240 μg ml^{-1} was added to each well, followed 20 μl of AB dye (Sigma-Aldrich) as an indicator of cellular respiration. The total volume was 200 μl. All microtiter plates included internal standards of half PDB dose and AB without *M. laxa* and *M. fructicola* spores, at each concentration of tebuconazole or thiophanate methyl. Fungal

respiration was then determined by measuring the absorbance of the dye at 570 nm (λ1 red light reflectance, blue light absorbance) and 600 nm (λ2 blue light reflectance, red light absorbance) on a tunable microplate reader (Microplate Absorbance Reader Infinite F50, Tecan, Salzburg, Austria). Percentage reduction in AB was calculated by the manufacturer's equation (3):

a) Equation for calculating the reduction percentage of AB using absorbance

Reduction percentage of AB =

$$\{[(O2 \times A1)-(O1 \times A2)]/[(R1 \times N2)-(R2 \times N1)]\}x10$$

Where:

O1=molar extinction coefficient (E) of oxidized AB at 570 nm

O2=E of oxidized AB at 600 nm

R1=E of reduced AB (Red) at 570 nm

R2=E of reduced AB at 600 nm

A1=absorbance of test wells at 570 nm

A2=absorbance of test wells at 600 nm

N1=absorbance of negative control well (media plus AB but no conidia) at 570 nm

N2=absorbance of negative control well (media plus AB but no conidia) at 600 nm

In order to determine the resistance to fungicide on fungal pathogen, the Alamar Blue relative percentage reduction in the control was preliminary evaluated. As described by Cox (22) there is a relationship between the relative percentage reduction of Alamar blue in control (30%) and the possibility that the isolate is resistant.

b) Equation for calculating the percent difference in reduction between treated and control cells

Percent difference between treated and control conidia=

$$\{[(O2 \times A1)-(O1 \times A2)]/[(O2 \times P1)-(O1 \times P2)]\}x100$$

Where:

O1 = molar extinction coefficient (E) of oxidized AB at 570 nm

O2 = E of oxidized AB at 600 nm

A1 = absorbance of test wells at 570 nm

A2 = absorbance of test wells at 600 nm

P1 = absorbance of positive growth control well (conidia plus AB but not test agent) at 570 nm

P2 = absorbance of positive growth control well (conidia plus AB but not test agent) at 600 nm

Table 4: molar extinction coefficients for AB at different wavelenghts

Wavelength	Reduced (R)	Oxidized (O)
570 nm	155677	80586
600 nm	14652	117216

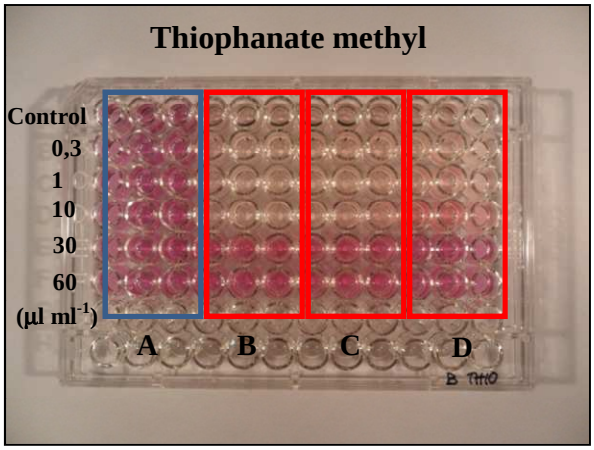


Figure 17: The AB is a colorimetric test. A it is the internal standard (of PBD half dose and AB without *Monilinia* spores); B, C and D are different *Monilinia* samples. In the Control row, spores were added, AB and PDB half dose, but no fungicide. 0.3, 1, 10, 30 and 60 µl ml^{-1} were different concentrations of thiophanate methyl used in the AB test

EC_{50} values (μg ml^{-1}) were calculated by regressing the percentages of the relative difference between treated and control conidia against the logarithm of inhibitor concentrations. The reported data were the average of two experiments, with three replicate (micro-wells) per experiment. The relationship between the EC_{50} derived from AB test and amended medium test was determined by linear regression of data.

3.4.4 *In vivo* assays

M. laxa and *M. fructicola* isolates with different EC_{50} values were selected to determine their sensitivity to tebuconazole and thiophanate methyl (Tables 5 and 6) in *in vivo* trials. In order to assay the sensitivity to fungicides (tebuconazole and thiophanate methyl) *in vivo* trials, 'Red Heaven' peach harvested at commercial maturity from the orchards previously cited, stored at 0°C and used within 2 weeks from harvest were used. For each experiment, the fruits were washed for one minute in hypochlorite solution (1%) then were gently rinsed for one minute in water and left to dry at room temperature for 1 hr. Peaches were inoculated as described in section 3.2; each peach was wounded in three different points on the upper surface with a sterile nail and a 10 μl of spore suspension (10^3 conidia ml^{-1}) of *M. laxa* or *M. fructicola* was immediately placed in the wound. For each isolate, 30 peaches (three replicates of 10 fruit each) were inoculated. The inoculated fruits were closed in a plastic bag to maintain the humidity (> 95%). Holes was drilled in the plastic bags to permit gas exchanges. Each fungicide was applied 6 h before inoculum (protective treatment) at half the label rate (36 ml/10 l and 92.5 ml/100 l, respectively) with a hand sprayer to runoff. Peaches were

maintained at 25°C, and the disease incidence (percentage of diseased fruits) was evaluated 5 days after inoculation. The control was represented by fruit inoculated with the pathogens and sprayed only with tap water. The experiment was repeated three times.

Table 5: Efficacy of thiophanate methyl (EnovitMetil FL, Sipcam) treatment against some *M. laxa* and *M. fructicola* isolates in *in vivo*

Fungus	Isolate no.	EC_{50} (µg ml^{-1})	S/R
M. laxa	M7454	0.029[a]	S
M. laxa	ML12BO	0.084[a]	S
M. laxa	M20FC	0.210[a]	S
M. laxa	M7784	0.412[a]	S
M. laxa	ML7	0.544[a]	S
M. laxa	ML13	0.781[a]	S
M. laxa	ML11	0.876[a]	S
M. laxa	M15FC	1.357[a]	R
M. laxa	ML5	2.231[a]	R
M. laxa	ML8B	2.270[a]	R
M. laxa	M23	3.12[a]	R
M. fructicola	MCL4	0.51[b]	S
M. fructicola	MCL19	3.69[b]	R
M. fructicola	MCL9311	10.05[b]	R
M. fructicola	MCL13	22.74[b]	R
M. fructicola	MCL10	34.08[b]	R
M. fructicola	MCL12	37.05[b]	R

[a] Values determined by Spiral Gradient Endpoint software with 2-days - incubation option. For each fungal isolate, two replicate plates were prepared for fungicide and the experiment was conducted two times. Data for each fungus-fungicide combination were averaged over the replicate petri dishes and the repeated experiments.

[b] Values determined by amended medium where EC_{50} is the effective concentration of the fungicide at which mycelial growth was inhibited by 50%. All values are averaged over two experiments, with four replicate petri dishes per experiment

Table 6: Efficacy of tebuconazole (Folicur SE, Bayer CropScience) treatment against some *M. laxa* and *M. fructigena* isolates in *in vivo* assay

Fungus	Isolate no.	EC_{50} (µg ml^{-1})	S/R
M. laxa	ML7	0.008 [a]	S
M. laxa	ML10B	0.010 [a]	S
M. laxa	ML12BO	0.019 [a]	S
M. laxa	M20FC	0.026 [a]	S
M. laxa	M7454	0.125 [a]	R
M. laxa	M27FC	0.163 [a]	R
M. laxa	M23	0.180 [a]	R
M. laxa	ML8B	0.320 [a]	R
M. fructigena	MCG1	0.118 [b]	R
M. fructigena	MCG3	0.124 [b]	R
M. fructigena	MCG4	0.132 [b]	R
M. fructigena	MCG5	0.155 [b]	R
M. fructigena	MCG2	0.172 [b]	R

[a] Values determined by Spiral Gradient Endpoint software with 2-days incubation option. For each fungal isolate, two replicate plates were prepared for fungicide and the experiment was conducted two times. Data for each fungus-fungicide combination were averaged over the replicate petri dishes and the repeated experiments.

[b] Values determined by amended medium where EC_{50} is the effective concentration of the fungicide at which mycelial growth was inhibited by 50%. All values are averaged over two experiments, with four replicate petri dishes per experiment.

3.4.5 Isolation of the β-tubulin gene from *M. laxa* and *M. fructicola* isolates

Ten isolates of *M. laxa* (six sensitive (S) to thiophanate methyl: ML7, ML12BO, ML13, M20FC, M7454 and M7784; four low resistant (LR): ML5, ML8B, M15FC and M23) and four *M. fructicola* isolates (with different resistance grades to thiophanate methyl: MCL4 (S), MCL10, MCL13 and MCL19 (R)) were used for the analysis of the DNA sequence of the β-tubulin gene.

To extract the DNA, the mthod proposed by Chi (92) was followed. Each isolate was grown on PDA at 23°C for 7 days and ten to twenty mg of mycelium were harvested and placed into a 0.5 ml extraction buffer (1M KCl, 100 mMTris-HCl, 10 mM EDTA). The fungal mass was completely pulverized with the electric grinder for 30 s. Cell lysates were centrifuged at 10,000 rpm for 10 min. The supernatant was decanted into the tubes containing 0.3 ml of 2-propanol. The lysate and 2-propanol were mixed by inverting the tube ten times and the tube was centrifuged at 13,000 rpm for 15 min. The supernatant was discarded and the remaining 2-propanol was evaporated at 37°C for 15 min. Fifty microliters of sterile water for PCR were added and the DNA pellet was re-suspended.

The PCR primer pair TubA (AAATGCGTGAGATTGTA) and TubR1 (TGTACCAATGCAAGAAAGCCTT) was used to amplify the β-tubulin gene fragment from *M. laxa* (75). According to Ma et al. (75) the PCR reaction was performed in 50 µl volume containing 2.5 µl of DNA solution, 0.2 µM of each primer, 10 mMTris/HCl (pH 8.3), 50 mMKCl, 1.5 mM MgCl2, 150 µM for each dNTP, 200 µM for each primer, and 1.5 U of TaKaRaTaq DNA polymerase (Takara, Otsu, Japan). The PCR amplifications were performed using the following parameters: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 94°C for 40 s, annealing at 50°C for 40 s, extension at 72°C for 1 m and 30 s, and final extension at 72°C for 10 min. PCR products were examined by electrophoresis in a 1.5% agarose gel in 1x Tris-acetate (TAE) buffer. The PCR product (approximately 1.6-kb) from each isolate was purified using ExoSAP-IT (Affymetrix, Inc., US). The purified fragment from each isolate was cloned in TOPO TA Cloning (Invitrogen, Carlsbad, CA) following the manufacture instructions, and plasmids were used to

chemically transform DH5α *E. coli* cells. Transformant cells were grown overnight at 37°C in LB (see Appendix 1) solid media with ampicillin to a final concentration of 100 μg ml^{-1}. Liquid cultures were inoculated with a single colony and grown overnight in agitation at 37°C. Plasmids were purified from liquid culture using the PureLink Quick Plasmid Miniprep kit (Invitrogen, Carlsbad, CA) following the manufacturer instructions and sequenced by CUGI (Clemson University Genome Institute, Clemson University, SC) using generic primers M13f and M13R and internal primers MfTF (TCGACAATACAAAATCACTCCGC) (75) and MfTR3 (GACAGGTGGTAACACCGGACAT) (74). The chromatograms were analyzed with Chromas lite software, and the obtained sequences were translated into amino-acid sequences using Expasy free program (www.web.expasy.org). The sequence of deduced amino acids was aligned using the computer program Clustal X (www.ebi.ac.uk/clustax European Bioinformatics Institute, Cambridge, UK). The DNA sequence and deduced amino-acid sequence from S isolates of *M. laxa* were also compared with those from other phytopathogenic fungal species using BLAST of NCBI/GeneBank available on line at www.ncbi.nih.gov/BLAST.

3.5 Influence of the temperature on *Monilinia* spp.

3.5.1 Effect of the temperature on *in vitro* mycelial growth, germination and spore density

The effect of temperature on the mycelium growth, spore production and germination of *M. laxa*, *M. fructicola* and *M. fructigena* was investigated. Three different isolates of *M. laxa* (ML4, ML2, ML9),

M. fructicola (MCL2, MCL3, MCL17) and *M. fructicola* (MCG1. MCG2 and MCG3) were chosen randomly from the isolates of our collection and incubated at -1, 0, 2, 3, 4, 4.5, 5, 10, 15, 20, 25, 30, 40°C for 15 days. The effect of temperature was also evaluated storing the isolates for 30 days at 0, 2, 3, 4, 4.5, 5°C. The effect of the temperature on the mycelium growth of pathogen was determined *in vitro* by transferring mycelial plugs (6 mm in diameter) derived from the edge of 7-day-old colonies in the center of Petri dish containing PDA. The dish was sealed with Parafilm and incubated at the temperatures above mentioned in darkness. The temperatures were automatically recorded by a data logger (Siemens Multireg C1732, Germany). The colony diameter was measured daily or weekly depending on the temperature of incubation (51). The sample unit was represented by three dishes for each isolate and temperature

To determine the influence of temperature on the spore production at 0. 2, 3, 4, 4.5 and 5°C after 30 days, the isolates from *M. laxa*, *M. fructicola* and *M. fructigena* were cultured on V8 agar in the darkness at 25°C for one week at the temperatures described above. Subsequently the surface of colony was washed with 5 ml of sterile distilled water and Tween-80 (0.5%). The conidial concentration was measured by the haemocytometer. Experiments were conducted with three Petri dishes (i.e., three replicates) per temperature and conidia count was averaged over the three replicates (19).

The influence of temperature on conidia germination was determined at 0, 2, 3, 4, 4.5 and 5 °C after 30 days on the three isolates of *M. laxa*, *M. fructicola* and *M. fructigena* listed above. The fungi were inoculated on V8 agar and incubated in the darkness at 25°C for one week, than the colonies were washed by adding 5 ml of sterile water and Tween 80 (0,05%) and the sporulating mycelial mat rubbed with a

sterile plastic loop. The conidial suspension was filtered through a layer of cheesecloth and the concentration adjusted to 10^3 conidia per ml. An aliquot of 100 µl of conidial suspension of each isolate were spread on MEA amended with 0.1% of antibiotics. The dishes were incubated at the temperatures mentioned above for 30 days. Subsequently, the colonies forming the units were counted and the results were expressed as a percentage of culturable conidia (87).

3.5.2 Influence of the temperature on the resistance and sensitivity of *M. laxa, M. fructicola* and *M. fructigena* isolates to tebuconazole and thiophanate methyl. *In vitro* trials

To determine the influence of the temperature on fungicide treatments sensitive and resistant isolate of *M. laxa*, *M. fructicola* and M. *fructicgena* listed in table 7 were used. PDA plates amended with tebuconazole or thiophante methyl at a concentration corresponding to EC_{50} value for each isolate were inoculated with a 6 mm mycelial plug taken from the edge of a 7-day-old colony of each isolate. Plates were incubated at 15, 20, 25, 30°C for seven days in the dark, and mycelial growth was determined measuring the radial growth with a ruler (74, 75). PDA plates without fungicides represented the control. The sample unit was represented by three dishes for each isolate-fungicide concentration and the experiment was performed two times.

3.5.3 Influence of the temperature on the resistance and sensitivity of *M. laxa* and *M. fructicola* isolates to tebuconazole and thiophanate methyl. *In vivo* trials

M. laxa and *M. fructicola* isolates with different EC_{50} values were selected to determine their sensitivity to tebuconazole (Folicur SE,

Bayer CropScience) and thiophanate methyl (EnovitMetil FL, Sipcam) (Table 7) in *in vivo* trials on peaches inoculated artificially.

'Red Heaven' peaches harvested two weeks before the commercial harvest from experimental orchards previously cited were used. The methodology of inoculation was the same as described in Section (3.2). Each fungicide, i.e., thiophanate methyl (EnovitMetil) and tebuconazole (Folicur) was applied 6 h before inoculum at a concentration of half the label rate (92.5 ml/100 l and 36 ml/10 l respectively) with a hand sprayer to runoff. Fruits were maintained at 15, 20, 25 and 30°C; the disease incidence (percentage of diseased fruits) and the lesion diameter were evaluated 7 days after inoculation. The control was inoculated as described and treated only with tap water. The sample unit was represented by 30 fruit (three replicates of 10 fruit each) for each combination of fungicide and temperature. The experiment was repeated three times.

Table 7: *M. laxa*, *M. fructicola* and *M. fructigena* isolates used *in vitro* and *in vivo* temperature assay. In the *in vitro* assay PDA plates were amended with the EC_{50} value determined for each isolate. Plates were incubated at 15, 20, 25, 30°C for seven days in the dark, and mycelial growth was recorded. In the *in vivo* assay peaches were wounded and inoculated with a spore suspension (10^3conida ml^{-1}) of *M. laxa* or *M. fructicola*. Six hour before the inoculum the fungicide at a concentration of half (92.5 ml/100 l and 36 ml/10 l respectively) label rate was applied with a hand sprayer. Peaches were stored at 15, 20, 25 and 30°C for 7 days.

Isolate	Fungus	Fungicide	EC_{50} (µg ml^{-1})	*In vitro* assay	*In vivo* assay
ML5	*M. laxa*	tebuconazole	0.03	n.d.[a]	d.[b]
		thiophanate m	2.231	d.	d.
ML8B	*M. laxa*	tebuconazole	0.32	d.	d.
		thiophanate m	2.27	d.	d.
ML9B	*M. laxa*	tebuconazole	0.021	d.	n.d.
		thiophanate m	0,061	d.	n.d.
ML11	*M. laxa*	tebuconazole	0.017	n.d.	n.d.
		thiophanate m	0.87	d.	d.
ML13	*M. laxa*	tebuconazole	0.026	n.d.	d.
		thiophanate m	0.781	d.	n.d.
ML21	*M. laxa*	tebuconazole	0.027	d.	n.d.
		thiophanate m	0.331	d.	n.d.
ML34	*M. laxa*	tebuconazole	0.020	d.	n.d.
		thiophanate m	0.211	d.	n.d.
M23	*M. laxa*	tebuconazole	0.18	d.	d.
		thiophanate m	3.12	d.	d.
M15FC	*M. laxa*	tebuconazole	0.002	n.d.	n.d.
		thiophanate m	1.357	d.	n.d.
M27FC	*M. laxa*	tebuconazole	0.163	d.	d.
		thiophanate m	0.082	d.	d.
M7454	*M. laxa*	tebuconazole	0.125	d.	d.
		thiophanate m	0.032	d.	n.d.
MCL4	*M. laxa*	tebuconazole	0.037	n.d.	n.d.
		thiophanate m	0.51	d.	n.d.
MCL5	*M. laxa*	tebuconazole	0.02	n.d.	n.d.

		thiophanate m	0.60	d.	n.d.
MCL10	*M. fructicola*	tebuconazole	0.059	n.d.	d.
		thiophanate m	34.08	d.	d.
MCL12	*M. fructicola*	tebuconazole	0.051	n.d.	d.
		thiophanate m	37.05	d.	d.
MCL13	*M. fructicola*	tebuconazole	0.067	n.d.	d.
		thiophanate m	22.74	d.	d.
MCL16	*M. fructicola*	tebuconazole	0.037	n.d.	n.d.
		thiophanate m	0.18	d.	n.d.
MCL17	*M. fructicola*	tebuconazole	0.051	d.	n.d.
		thiophanate m	0.61	d.	n.d.
MCL19	*M. fructicola*	tebuconazole	0.04	n.d.	d.
		thiophanate m	3.69	d.	d.
MCL9311	*M. fructicola*	tebuconazole	0.025	n.d.	d.
		thiophanate m	10.05	d.	d.
MCG2	*M. fructigena*	tebuconazole	0.172	d.	n.d.
		thiophanate m	0.23	d.	n.d.
MCG3	*M. fructigena*	tebuconazole	0.124	d.	n.d.
		thiophanate m	0.27	d.	n.d.
MCG5	*M. fructigena*	tebuconazole	0.155	d.	n.d.
		thiophanate m	0.36	n.d.	n.d.

[a]n.d.: no determined
[b]d.: determined

3.6 Data analysis

The statistical analysis of peach fruit susceptibility during the phenological stages and the EC_{50} computations were performed with Statistics for Statgraphic (Centurion), with a significance level of 95%.

The EC_{50} from amended medium test and AB assay was calculated by the linear regression of the probit-transformed inhibition percentage

and the log_{10}-transformed fungicide concentration. EC_{50} values correspond to the regression line intercept (zero probit = 50% reduction).

The EC_{50} values (μg ml^{-1}) derived from SGD assay were automatically calculated by SGE software. The following parameters were entered into the SGE software using the program option for single antimicrobial plates: plate size (15 cm Petri dishes), length of incubation (2 days), fungicide stock concentration applied to the plate (400 μl $ml^{-1}$and 50 μl ml^{-1} thiophanate methyl and tebuconazole, respectively), and mode of application (exponential) for "antimicrobial". The molecular weights of the fungicides had been entered earlier into the antimicrobial database of the program, to calculate stock concentrations of the test substance. The radial distance (in millimeters) between the zone of 50% inhibition of growth and the center of the plate was entered as ending radius (RE) for each plate between 1 and 15 in the "data entry area". The local fungicide concentration was then automatically displayed as ending concentration (EC). Data sheets for each plate were then exported to Excel (Microsoft Corp.). For each fungus-fungicide combination, the data were averaged over the replicate Petri dishes for the repeated experiments.

Data for each fungus-temperature growth combination *in vitro* and *in vivo* test were averaged. The influence of temperature in *in vitro* and *in vivo* trials was computed by one-way ANOVA with a significance level of 95%. Means were separated using the Duncan test. The data were processed using the statistics for Statgraphics (Centurion). All experiments were carried out in a completely randomized block design.

Chapter 4

Results

4.1 Isolation and identification of *Monilinia* species

The identification of *Monilinia* species based on symptoms, host, morphology, and culture characteristics is currently very difficult. Frequently the conidial dimension and the sporulation are similar in *M. laxa* and *M. fructicola* and the germ tube is similar among *M. fructigena* and *M. fructicola* (table 8and Figures 18, 19 and 20). In this study, through the colony characteristics on agar medium fifteen different morphotypes were recognized: six for *M. laxa*, six *M. fructicola* and three for *M. fructigena* (Figures 21, 22 and 23)

Table 8: Main differences between *Monilinia* species: conidia dimension , germ tube elongation and sporulation

Species	Conidia dimension[a]	Germ tube elongation[a]	Sporulation conidia ml^{-1} [b]
M. laxa	12-17x8-11 µm	Short and branching near spore, long without breaching, long with breaching far the spore or with double germ tube	10^7
M. fructicola	14-17x10-11 µm	Long and branching far from spore, long without breaching or with double germ tube	10^7 with microconidia
M. fructcigena	18-22x11-13 µm	Long and branching far from spore, long with breaching near the spore or with double germ tube	10^5

[a] Conidia dimensions and germe tube elongation were evaluated from conidia (10^5 conidia per ml) in sterile water by microscope (200x)

[b] Conidia were harvested from a colony on V8 agar after 7 days of incubation at 25°C by adding 5 ml of sterile water with Tween 80 (0.05%) and rubbing the sporulating mycelial mat with a sterile plastic loop. The conidial suspension was filtered through two layers of cheesecloth and then counted by hematocytometer

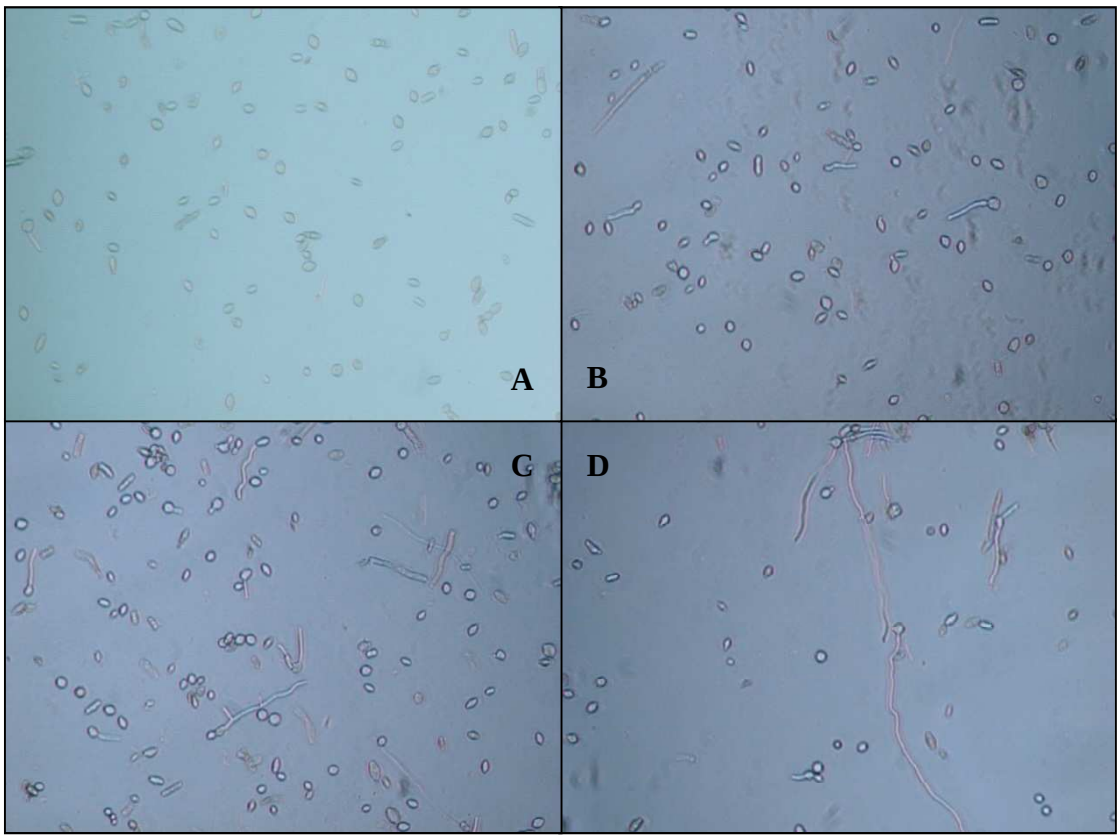


Figure 18: Conidia size and shape of the germ tube elongation of *M. laxa* were evaluated with microscope (200x) after 3, 6, 12 and 24 hours (A, B, C and D respectively) of incubation at 25°C in sterile water in the dark. The conidia dimensions were approximately 12-17x8-11 µm

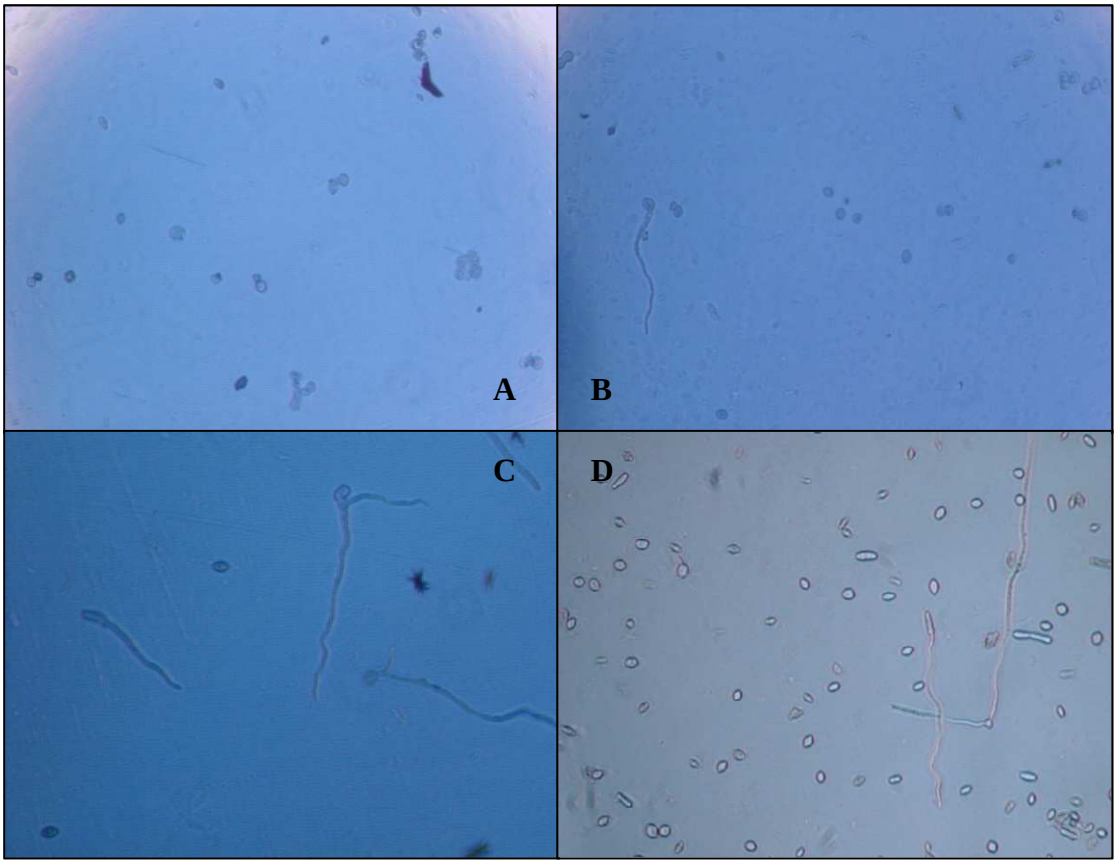


Figure 19: Conidia size and shape of the germ tube elongation of *M. fructicola* were evaluated with microscope (200x) after 3, 6, 12 and 24 hours (A, B, C and D respectively) of incubation at 25°C in sterile water in the dark. The conidia dimensions were approximately 14-17x10-11 μm

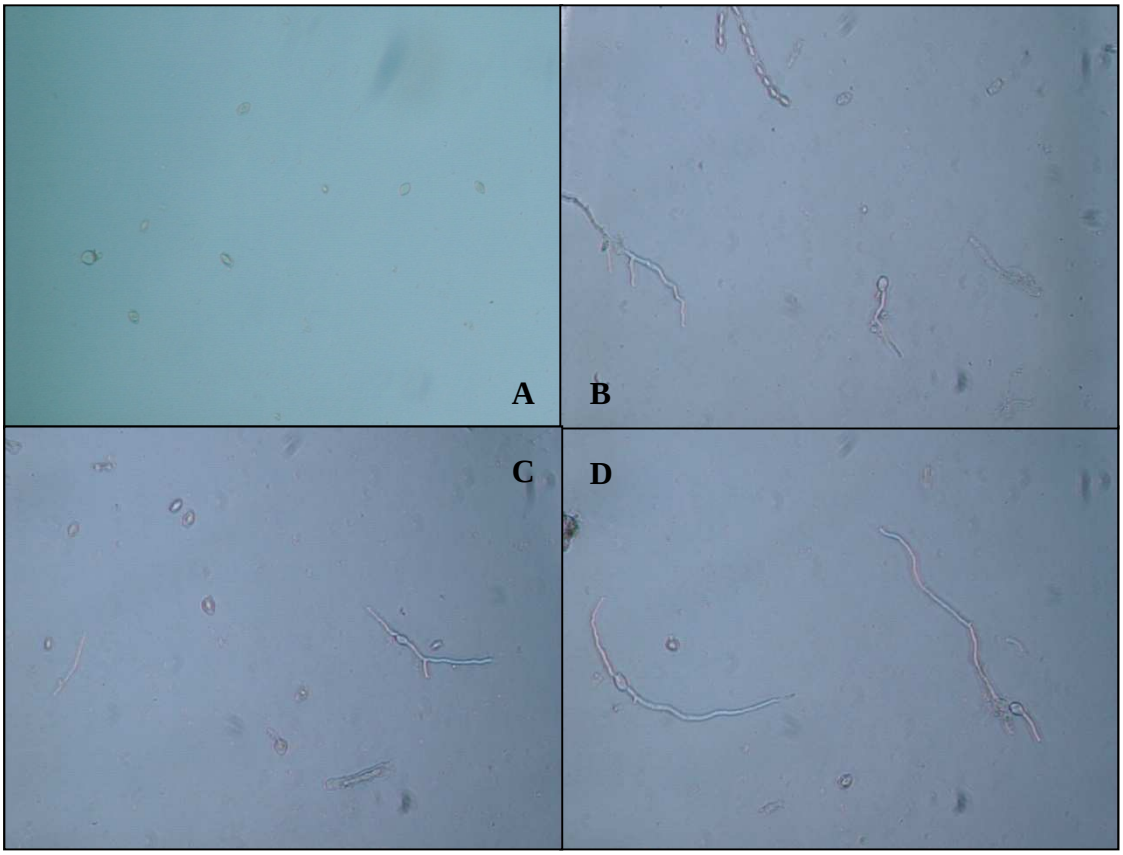
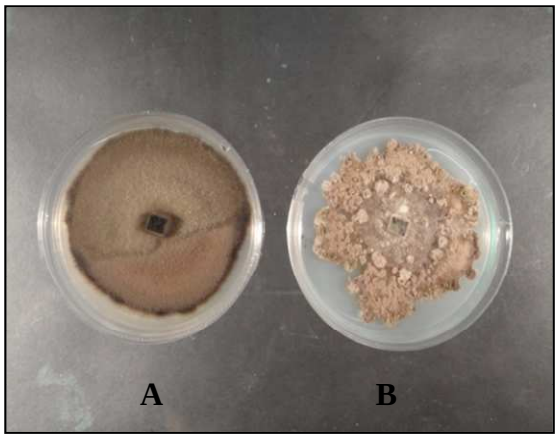
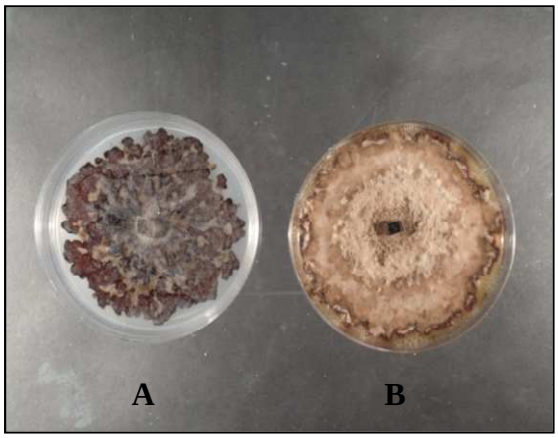
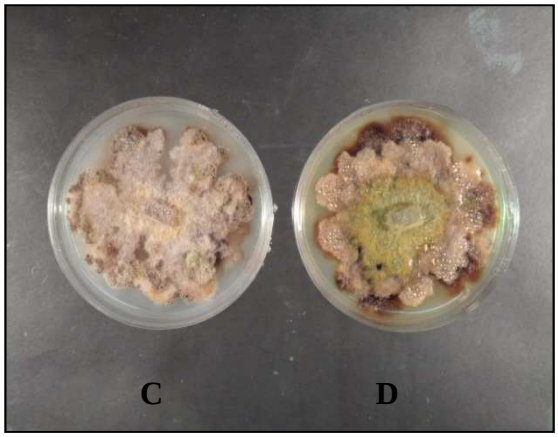
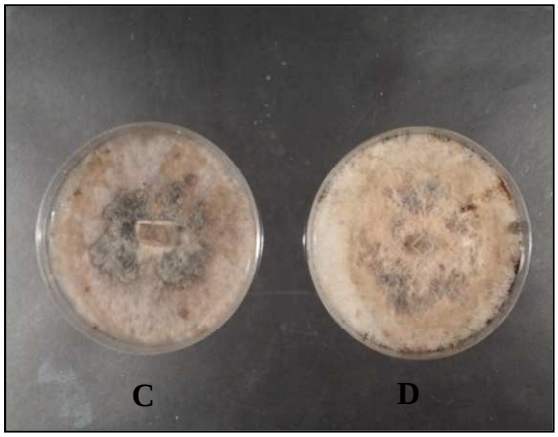


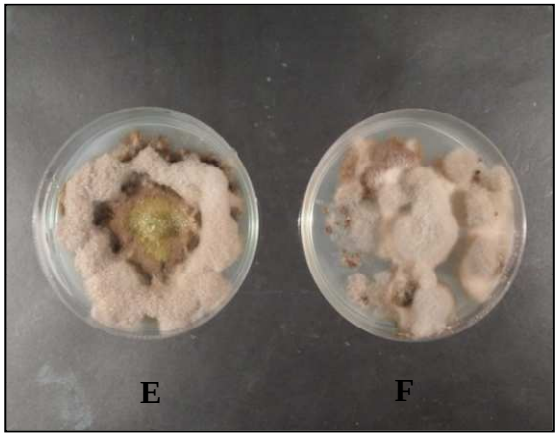
Figure 20: Conidia size and shape of the germ tube elongation of *M. fructicola* were evaluated with microscope (200x) after 3, 6, 12 and 24 hours (A, B, C and D respectively) of incubation at 25°C in sterile water in the dark. The conidia dimensions were approximately 18-22x11-13 μm











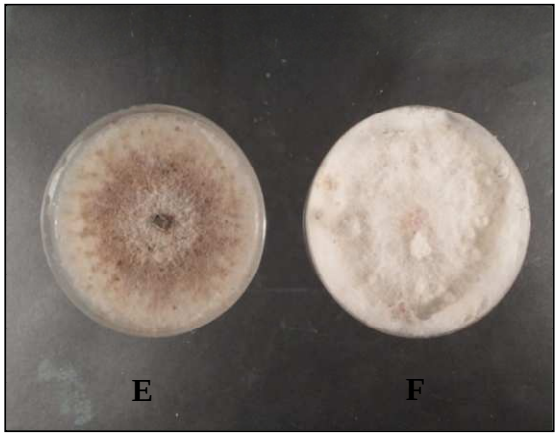


Figure 21: Different *M. laxa* phenotypes individuated through the morphological identification

Figure 22: Different *M. fructicola* phenotypes individuated through the morphological identification

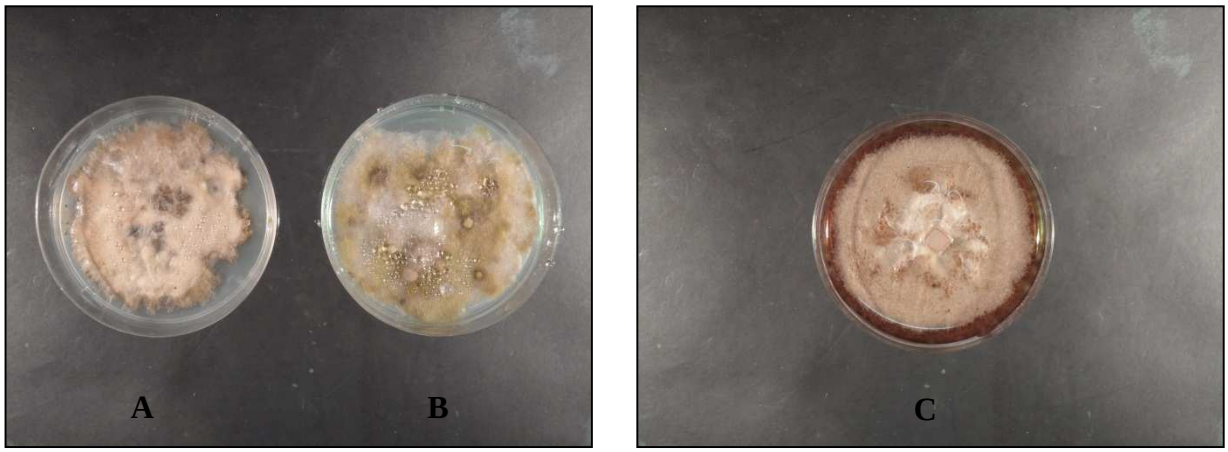


Figure 23: Three different *M. fructigena* phenotypes individuated through the morphological identification

M. laxa (Figure 21) grown on PDA at 25°C for 7 days in the dark in general showed a lobed mycelial growth (B, C, D, E). In this study, another phenotype without lobed mycelial growth, characterized by a dark color, and similar to *M. fructicola* mycelium was observed (A). On the contrary, the phenotype F appeared very irregular, presented a lobed mycelial growth and its mycelium was light color. On the phenotype D the colony formed rosettes and, in the middle, the color is olive green.

M. laxa conidia were sparsely produced on PDA, while on the V8 agar medium, rich in carbon and nitrogen, the sporulation was abundant (Figure 24).

Figure 24: *M. laxa* conidia were sparsely produced on PDA (on the left), while the sporulaton was more abundant when cultivated on V8 agar (on the right)

M. fructicola grown on PDA at 25°C for 7 days in the dark, formed colonies with active growing, entire margins and fluffy (C, D, E, F). The bands were usually light buff in color (E and F). However, in the phenotypes A and B the colonies showed lobed margins like *M. laxa*, dark in the phenotype A and brown in B. Black crusts normally develop only after the colony has covered all the surface, however, in morphotype A, the crusts appeared early, during mycelium growth. In the phenotype B numerous, small, black microconidial clusters near the edge of the Petri dish can be observed and the culture showed zonation. C and D phenotypes showed different zonation in color and shape. There was considerable variation between isolates of *M. fructicola*, particularly in the amount of stromatal tissues on media as PDA. The stromatal tissues of *M. fructicola* were normally grayish or hazel in color. Large numbers of conidia were produced on V8 agar (Figure 22).

M.fructicola showed faster growth rates than *M. laxa* (about 50% faster) (Figure 25).

Figure 25: Conidia of *M. fructicola* (on the left) and *M. laxa* (on the right) produced on V8 agar, after seven days at 25°C in the dark

M. fructigena grown on PDA at 25°C in the dark showed mycelium with entire margins (C). The mycelium yellowish or buff-colored enlarged uniformly to the edge of the plate (A and B)(Figure 23).

Conidia were not produced on PDA and were sparsely produced on V8 agar (Figure 26). Conidia of *M. fructigena* were generally larger than those of *M. fructicola* and *M. laxa* (18-22 x 11-13 μm) (Figure 20).

Figure 26: conidia of *M. fructicola* sparsely produced on V8 agar

In order to distinguish *M. laxa* from *M. fructicola* and *M. fructigena* the internal Transcribed Spacer (ITS) between the 18S and 28S rRNA gene was amplified using specie-specific primers ITS1-Mlx1 and ITS4-Mlx1 for *M. laxa*, ITS1-Mfc1 and ITS4-Mfc1 for *M. fructicola*, and ITS1-Mfgn1 and ITS4-Mfgn1 for *M. fructigena*. A total of 297 isolates were assayed and a single 380-bp-long DNA fragment long was amplified using each of the three pairs of primers (Figure 27).

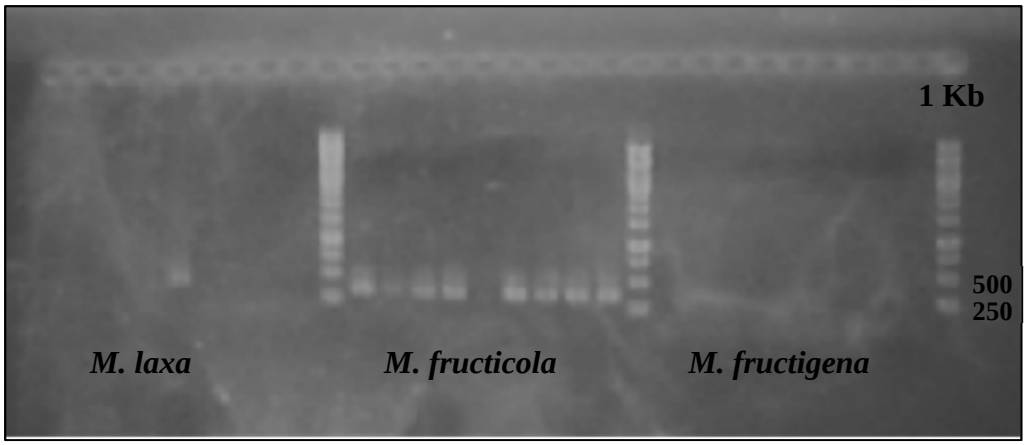


Figure 27: Amplification products using specie-specific primers. the PCR products of 380 bp ,were stained with ethidium bromide and photographed under UV light

The amplification results showed that the fungus *M. laxa* was isolated in 63% (61 isolates) and 39% (79 isolates) of the rotted fruits in 2010 and 2011, respectively. *M. fructicola* was isolated in 32% (31 isolates) and 56% (112 isolates) of the rotted fruits collected in 2010 and 2011, respectively. The pathogen *M. fructigena* was responsible for approximately 5% (5 and 9 isolates for 2010 and 2011, respectively) of rotted fruits, especially apples, in both years.

Two samples (MCL21 and 3c) were isolated from 'Gala' and 'Pink Lady' apples stored in the regional packaging-house (Apofruit, Cesena).

The 76% of the samples (228 isolates) was isolated from peaches, 8% (25 isolates) from nectarines, 6% (15 isolates) from plums, 4% (12 isolates) from apples, 3% (10 isolates) from cherries, 2% (5 isolates) from apricot, one isolate was from pear and one, CBS, was bought at CBS-KNAW (Fungal Biodiversity Center, Utrecht, Nederland) (Figure 28 and Appendix 3)

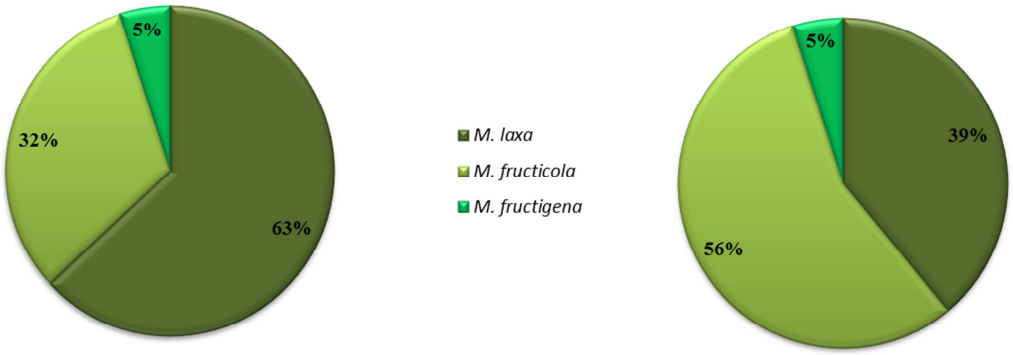


Figure 28: Subdivision of isolates of *Monilinia* spp. in 2010 (on the left) and 2011 (on the right)

4.2 Susceptibility of peach fruit to *Monilinia* spp. during phenological stages

The seasonal pattern of peach was 12 weeks.

The fruits diameter [mm] (Figure 29), weight [g] (Figure 30) and the percentage of soluble solids (Figure 31) showed the typical double sigmoid pattern with the four-stage standard growth (S1-S4):

- **S1 phase** in which the pericarp enlarges (from 4th to 6th week after full bloom)
- **S2 phase** in which pit hardening occurs and fruit stops the growth (from 7th to 9th week after full bloom)
- **S3 phase**, the pre-climateric phase, during which the mesocarp increases (from 10th to 11th week after full bloom)
- **S4 phase** the climacteric phase, in which fruit ripening occurs.

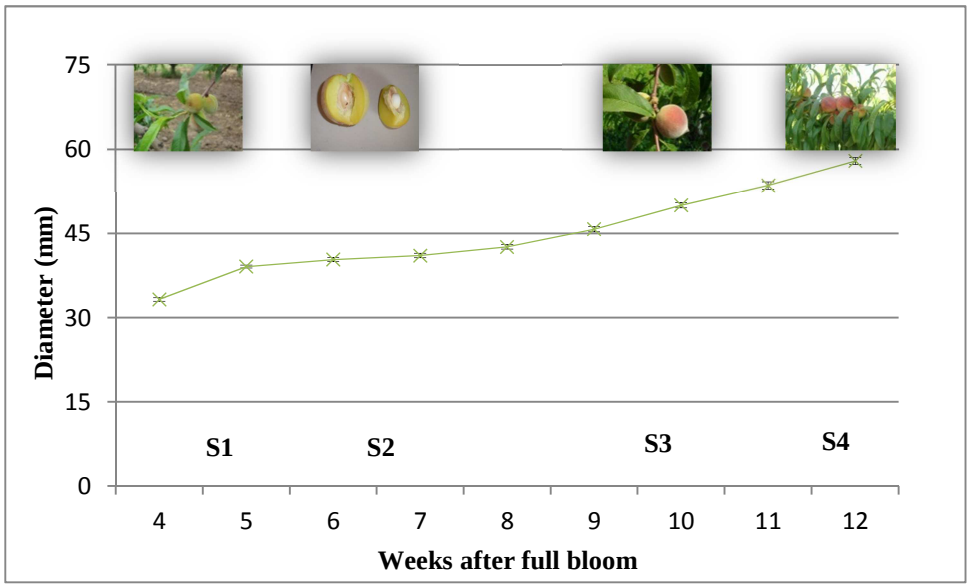


Figure 29: Peach fruit diameter curve. Variation on fruit caliber during weekly sampling times from the 14th May (4th weeks after full bloom, to 16th July (12th week after full bloom). Data are the average of 20 samples ± st err

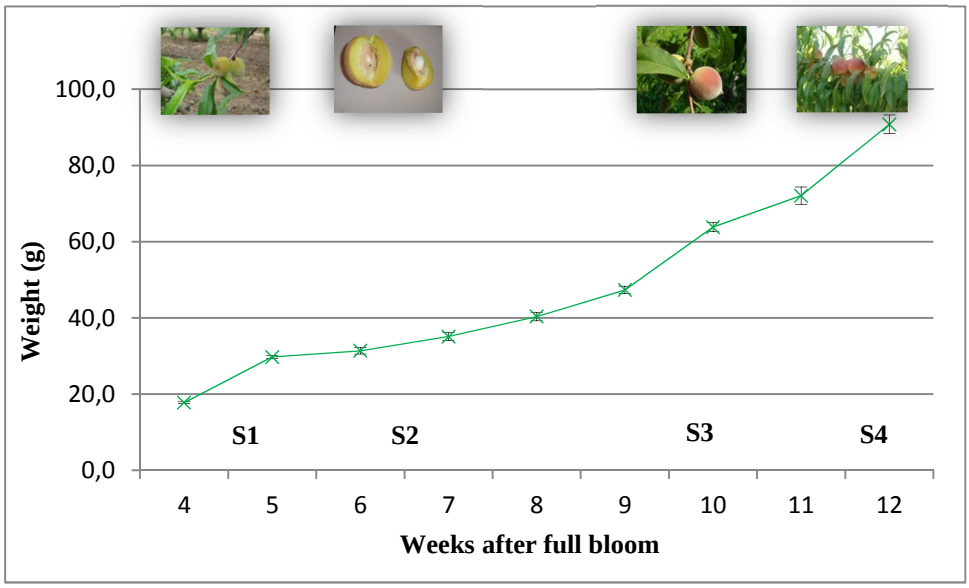


Figure 30: Peach fruit weight curve. Variation on fruit weight during weekly sampling times from the 4th weeks after full bloom, to 12th week after full bloom. Data are the average of 20 samples ± st err

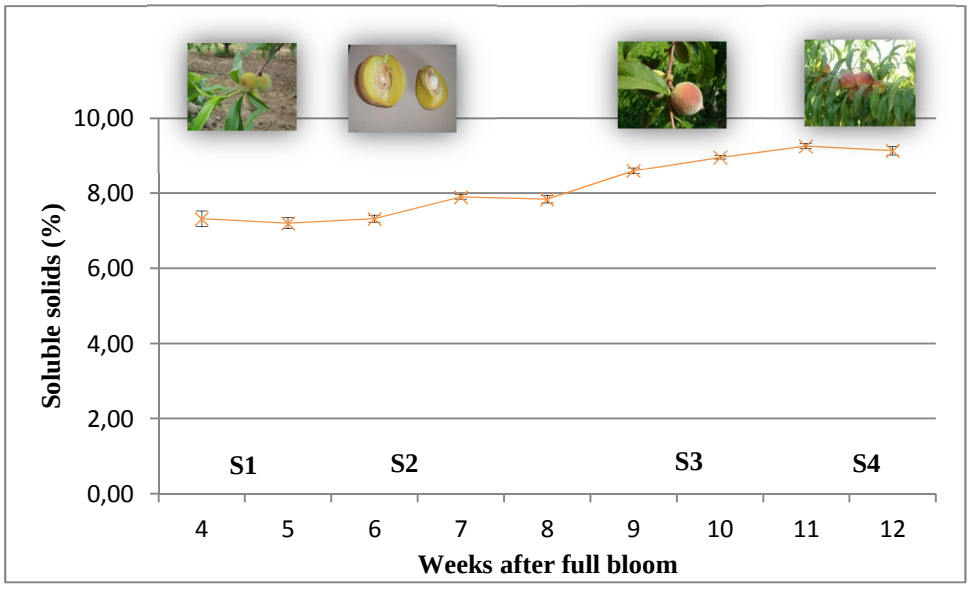


Figure 31: Soluble solids curve. Variation on fruit weight during weekly sampling times from the 4th weeks after full bloom, to 12th week after full bloom Data are the average of 5 samples + St. err.

The susceptibility to *M. laxa*, *M. fructicola* and *M. fructigena* was weekly evaluated after artificial inoculation in wounded fruits as percentage of infected wounds and in unwounded fruits as percentage of infected fruits. Pit hardening occurred after six or seven weeks after full bloom, depending on the year. It can be noticed that the seasonal pattern of peach susceptibility to *Monilinia* spp. is almost the same for the three species and it could be divided in three phases. The first, until six or seven weeks after full bloom, showed a susceptibility ranging from <40% of infected wounds in 2010 and >40% of infected wounds in 2012. The highest percentage of infected wounds (63%) was observed in 2012. The second phase from 2-3 weeks to 6-8 weeks after full bloom was characterized by a high resistance to *Monilinia* rots. In the second phase the pit hardening occurred and this phase corresponded to S2. During the third phase, 7-8weeks from full bloom to harvest, the susceptibility to *Monilinia* spp. increased and reached almost 100% of wound infections. (Figure 32).

No substantial differences between unwounded and wounded fruits were observed in phase two, brown rot was not detected on the artificially inoculated fruits(Figure 33).

No significant correlation was found between diameter, weight, percentage of soluble solids and susceptibility to *M. laxa*, *M. fructicola* and *M. fructigena*.

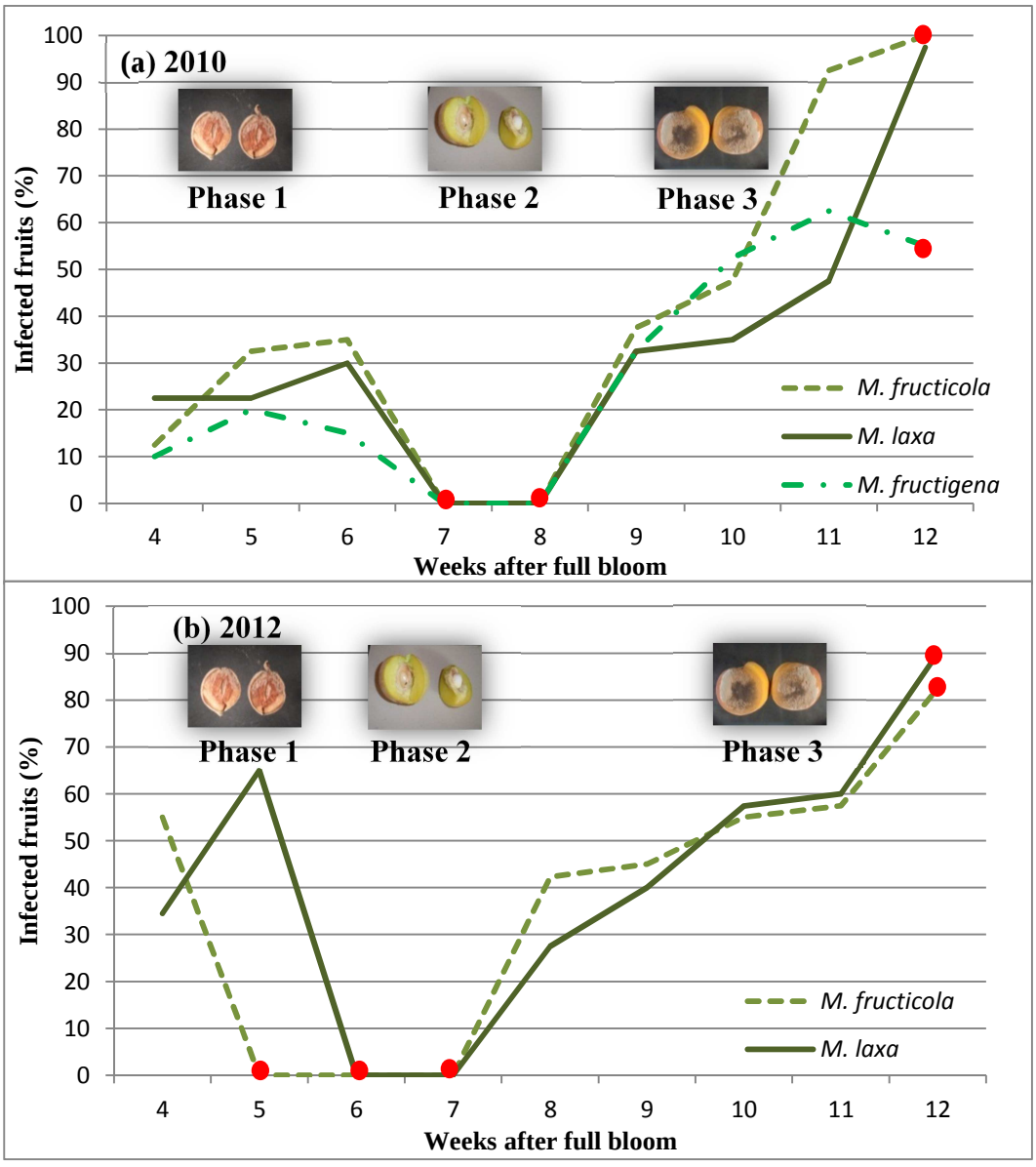


Figure 32: Variation 'Red Heaven' peach susceptibility to *M. laxa*, *M. frucicola* and *M. fructgena* infections in 2010(a), and *M. laxa*, and *M. fructicola* in 2012(b). Fruits were weekly harvested, wounded and inoculated by dipping in a conidia suspension ($10^5 ml^{-1}$) for one minute. Infections were recorded after one week of incubation at 20°C and the percentage of infected fruit was calculated. The red dots indicate the change of phase

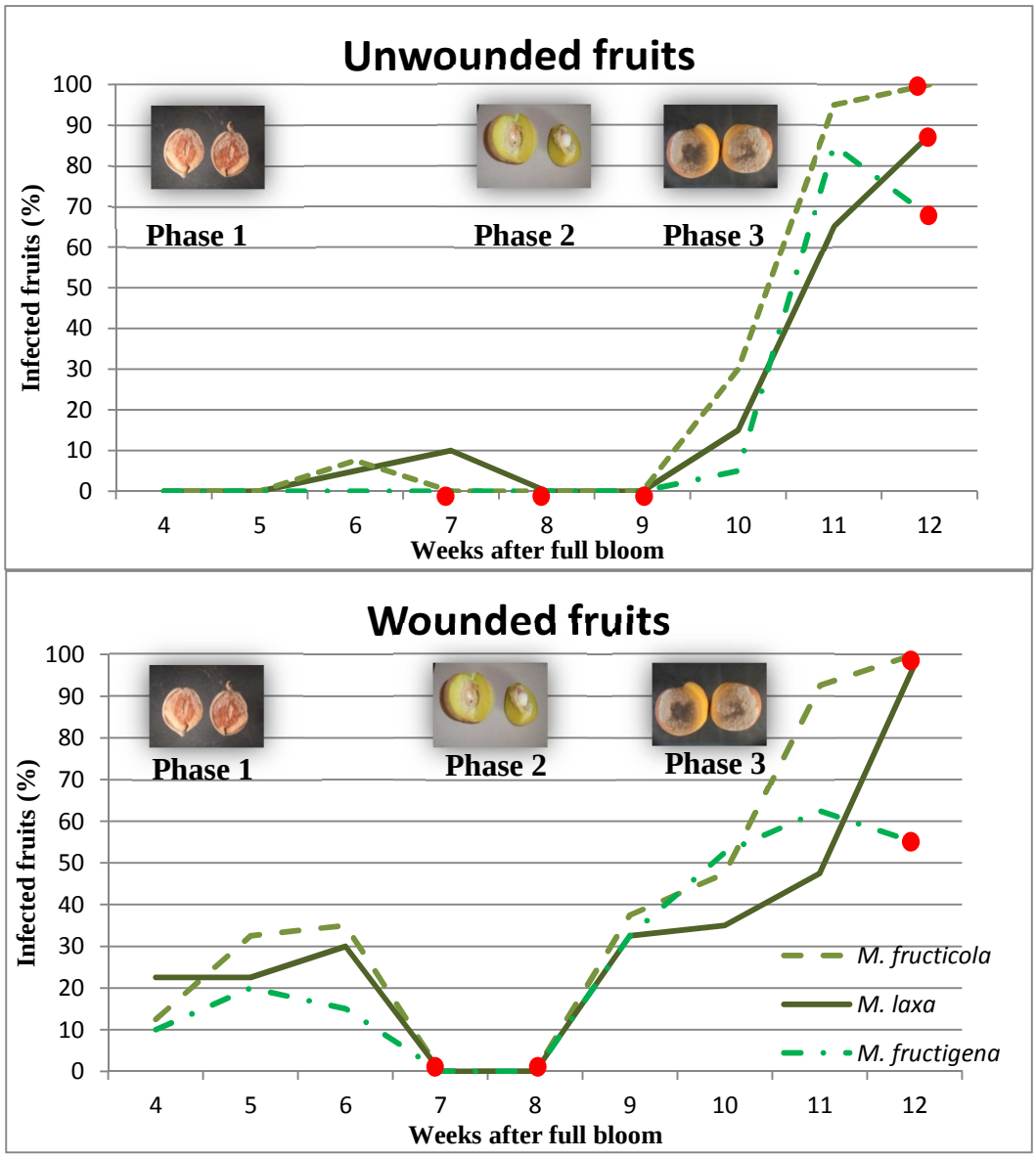


Figure 33: Variation 'Red Heaven' peach susceptibility to *M. laxa*, *M. fructigena* and *M. fructicola* infections in 2010. Two hundred fruits harvested weekly were divided in two equal lots. Fruit of the first lot was wounded with a sterile nail (one wound per fruit). The fruit on the second lot were unwounded. Peaches were dipped in a conidia suspension (10^5 conidia ml^{-1}) for one minute, control fruit were dipped in water. Infections were recorded after one week of incubation at 20°C and percentage of infected fruit was calculated. The red dot denotes the change between stages and the other. The red dot indicates the change of phase

4.3 Preformed antifungal compounds in peach peel extracts at different developmental stages

Crude ethanol extracts from the peel of 'Maycrest' 'Red Heaven', and 'Tardibelle' peaches during S1, S2 and S4 stages showed different degrees of antifungal activity in a 1D TLC bioassay. Fungal inhibition was observed when either 5, 10, 20 and 30 µl (data not shown) or 40 and 100 µl of extracts (0.2 ml g^{-1} FW) were applied to the TLC plates (Figure 34).

Solvent systems 60:40:20 and 60:40:30 [hexame:ethyl acetate:methanol (v/v/v)] provided the greatest separation of antifungal compounds.

The fungal inhibition was classified in three classes: feeble inhibition (FI) characterized by rarefied mycelium and some small areas without mycelium; inhibition (I) characterized by rarefied and uniformly distributed mycelium; marked inhibition (MI) characterized by absence of mycelium.

The peel extract from fruits of S1 stage contained fewer antifungal compounds than that of S2 stage fruits with two and four antifungal zones in solvent system 60:40:30 (Table 9). Only one FI spot was present in S1 stage (six stains) and S4 stage (two stains) while in S2 stage, eleven spots were observed: two FI, six I and three MI. (Table 10).

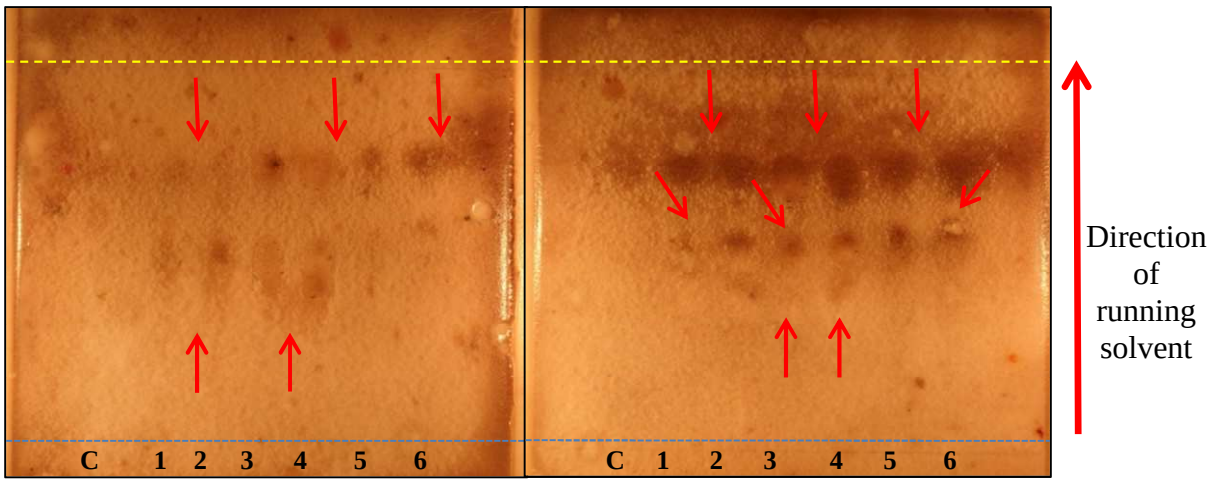


Figure 34: One- dimensional TLC bioassays of crude ethanol extract portioned into an organic dichloromethane phase of S1 stage (1 and 2), S2 stage (3 and 4) and S4 stage (5 and 6) 'Red Heaven' (40 and 100 µl; 0.2 ml g^{-1}) and run in solvent system of increasing polarity [hexane:ethyl acetate:methanol (v/v/v); C=60:40:20 (left) and D=60:40:30 (right)]. C is negative control with ethanol (99% v/v). Dotted line=origin. Dashed line=solvent front

TLC bioassay on 'Maycrest', 'Red Heaven', and 'Tardibelle' peaches showed a similar antifungal fingerprint with R_f 73.3 and 53.3 in the S1 stage with solvent systems 60:40:20 and 60:40:30. During S2 stage the solvent system 60:40:30 highlighted two different inhibition spots (R_f 26.6 and 13.3). A feeble inhibition spot at values Rf 13.3 was present in 'Tardibelle' and 'Red Heaven' peaches, while no inhibition spot with the same R_f was observed in 'Mycrest' peaches. Peel extract from 'Maycrest', 'Red Heaven' and 'Tardibelle' peaches in S2 phase had a similar fingerprint (R_f 26.6) showing the greatest antifungal activity in solvent system 60:40:30. No inhibition spots were detected on 'Maycrest' and 'Tardibelle' peaches in S4 stage, very feeble inhibition spots were found in 'Red Heaven' peaches with R_f values 73.3 and 53.3 (Figure 34) (Table 10).

Auto-fluorescence to UV light suggested that all R_f contained phenolics, and the dark blue color appeared after FolinCiocalteu reaction confirmed this hypothesis for R_f 73.3, 53.3 and 26.6. Only for

one of the inhibition spots corresponding to a R_f 13.3 the presence of phenolic compounds was not confirmed (Figure 35).

Table 9: One-dimensional TLC bioassay (*M. laxa*) R_f value of peel crude hetanol extract of 'Red Heaven' peach portioned into dichloromethane and run in solvent systems of increasing polarity (hexane:ethyl acetate:methanol (v/v/v); A=60:40:1, B=60:40:10, C=60:40:20 and D=60:40=30. The fungal inhibition was classified in three classes: feeble inhibition (FI) rarefied mycelium, with some little areas without mycelium; inhibition (I) rarefied mycelium uniformly distributed in the area; marked inhibition (MI), absence of mycelium

Solvent system A			Solvent system B			Solvent system C			Solvent system D		
S1	S2	S4	S1	S2	S4	S1	S2	S4	S1	S2	S4
-[a]	73.3	-	73.3	73.3	73.3	73.3	73.3	73.3	73.3	73.3	73.3
53.3	53.3	53.3	53.3	53.3	-	53.3	53.3	53.3	53.3	53.3	53.3
-	-	-	-	26.6	-	-	26.6	-	-	26.6	-
-	-	-	-	-	-	-	-	-	-	13.3	-

[a]-: no inhibition zone

Table 10: 1D TLC bioassay (*M. laxa*) of crude ethanol extract portioned into an organic dichloromethane phase of S1, S2 and S4 stages 'Maycrest', 'Red Heaven', and 'Tardibelle' peach peel (20 and 40 µl spot; 0.2 ml g^{-1} FW) and run in hexane:ethyl acetate:methanol (60:40:30 (v/v/v)). Inhibition spot for each for fruit development stage

Cultivars	R_f x100	S1	S2	S4
'Maycrest'	73.3	Feeble[b]	Inhibition[c]	- [a]
	53.3	Feeble	Inhibition	-
	26.6	-	Marked[d]	-
	13.3	-	-	-
'Red Heaven'	73.3	Feeble	Inhibition	Feeble
	53.3	Feeble	Inhibition	Feeble
	26.6	-	Marked	-
	13.3	-	Feeble	-
'Tardibelle'	73.3	Feeble	Inhibition	-
	53.3	Feeble	Inhibition	-
	26.6	-	Marked	-
	13.3	-	Feeble	-

[a] No inhibition zone

[b] Feeble inhibition rarefied mycelium

[c] Inhibition rarefied, uniformly distributed mycelium

[d] Marked inhibition, absence of mycelium

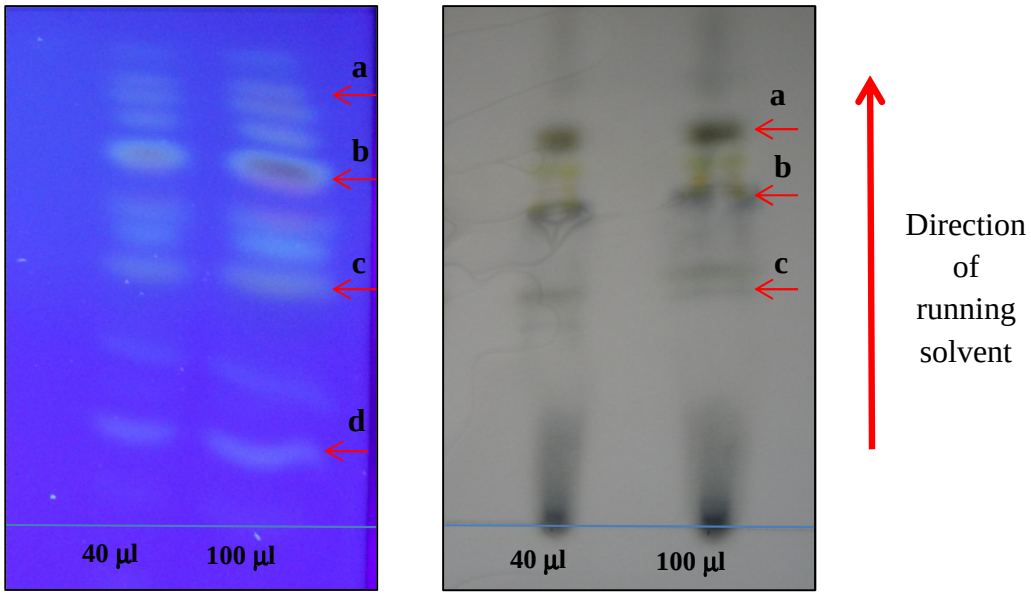


Figure 35:1D TLC. Crude ethanol extract from 'Red Heaven' peach in S2 phase, separated in solvent system 60:40:30 under UV light (254 nm) using fluorescent TLC plates (on the left) and subsequently stained with Folin Ciocalteu reagent (on the right)

4.4 *In vitro* sensitivity of *Monilinia* isolates to fungicide

4.4.1 Sensitivity of *M. fructicola* and *M. fructigena* isolates to tebuconazole and thiophanate methyl: amended medium method

The sensitivity to fungicides was evaluated on 32 isolates (see the Appendix 6) 27 of *M. fructicola* and 8 of *M. fructigena* collected during 2010. In the case of *M. fructicola* the EC_{50} values of tebuconazole ranged from 0.008 to 0.115µgml^{-1} and in the case of thiophanate methyl from 0.18 to 37.05µg ml^{-1}. All *M. fructicola* isolates can be considerate sensitive to tebuconazole since all EC_{50} values were lower than 0.1µgml^{-1}. Twenty one *M. fructicola* isolates exhibited resistance to thiophanate methyl with the EC_{50} values ranging from 1.25 to 37.05 µg ml^{-1} (higher than 1 µg ml^{-1}and lower than 50 µg ml^{-1}).

The *M. fructigena* EC_{50} values for tebuconazole ranged from 0.124 to 0.172 μg ml^{-1} and for thiophanate methyl from 0.23 to 0.36 μg ml^{-1}. The isolates MCG2 (0.172 μg ml-1), MCG4 (0.132 μg ml-1) and (MCG5 0.155 μg ml-1) can be considered resistant to tebuconazole and all isolates can be considered sensitive to thiophante methyl.

4.4.2 Sensitivity of *M. laxa* to tebuconazole and thiophanate methyl: SGD method

In 2010,the EC_{50} values of 59 isolates of *M. laxa* were determined with SGD method. For tebuconazole and thiophanate methyl the EC_{50} values ranged between values of 0.004 to 0.32 μg $ml^{-1}$and from 0.03 to 3.12μg ml^{-1}, respectively. Six *M. laxa* isolates were considered resistant to tebuconazole with EC_{50} values of 0.13, 0.14, 0.16, 0.18, 0.31, 0.32μg ml^{-1} and 54 isolates were considered sensitive with EC_{50} values ranging from 0.004 to 0.087μg ml^{-1}. Nine *M. laxa* isolates exhibited low resistance to thiophanate methyl with EC_{50} values between 0.781 and 3.12 μg ml^{-1} and 51 isolates were sensitive to thiophanate methyl with EC_{50} values ranging from 0.006 to 0.691 μg ml^{-1}. Isolates ML8B (0.32 and 2.27 μg ml^{-1}), M23 (0.18 and 3.12 μg ml^{-1}), EBR1 (0.31 and 3.126 μg ml^{-1}) and Quince 2010 (0.139 and 0.969 μg ml^{-1}) showed a resistant phenotype for tebuconazole and thiophanate methyl (see the Appendix 7).

4.4.3 Relationship between Alamar blue relative percentage reduction and resistance to fungicide

The relative percentage of AB reduction in the control was computed using equation (a) in section 3.4.3. *M. laxa* isolates characterized by

thyophanate methyl EC_{50} values greater than 1 μg ml^{-1}(resistant) showed the AB reductions higher than 35%. The highest EC_{50} value (28.29 μg ml^{-1}) corresponded to the highest AB reduction 91.5% (C34), but the same pattern was not observed when considering the lowest EC_{50} value (0.0004 μg ml^{-1}) that corresponded to AB reduction of 48% (C30). Eighty one percent of resistant isolates that showed an EC_{50} greater than 1 μg ml^{-1} had an AB reduction over 50%. Fifty eight of sensible isolates showed an AB reduction less than 50%.

In the case of *M. fructicola*, the highest thyophanate methyl EC_{50} values are not associated with largest AB reductions (e.g., G062 105 C/2 with EC_{50} 57.59μg ml^{-1} and AB reduction 61%). The percentage of AB reduction was between 11.9% and 99%. The lowest value of AB reduction in resistant isolates was 20% with an EC_{50} of 11.31 μg ml^{-1}. Forty percent of resistant isolates showed an AB reduction over 90.6% and in 13 isolates the AB degradation was 99%. The sensitive isolates showed a percentage of reduction between 11.95 and 96%, most of them (52%) had an AB reduction lower than 50%.(see the Appendix 9).

The three *M. laxa* isolates considered resistant to tebuconazole (inhibitory dose > 0.1 μg ml^{-1}) showed reductions over 40%. The *M. fructicola* isolates that showed resistance to tebuconazole had an AB reductions over 20% and most of the sensitive isolates showed an AB reduction higher than 21% (see the Appendix 10).

4.4.4 Sensitivity of *M. laxa* and *M. fructicola* isolates to fungicides: AB method

The conidia concentration of 10^5 conidia ml^{-1}in half-dose PDB with the addition of different concentrations of tebuconazole and

thiophanate methyl produced consistent visual result after 12 h. In the wells where the conidia respiration occurred the color of suspension turned into a pink colour indicating a certain resistance to fungicide, while violet wells indicated little or no respiration and consequently fungicide sensitivity (Figure 36 and 37).

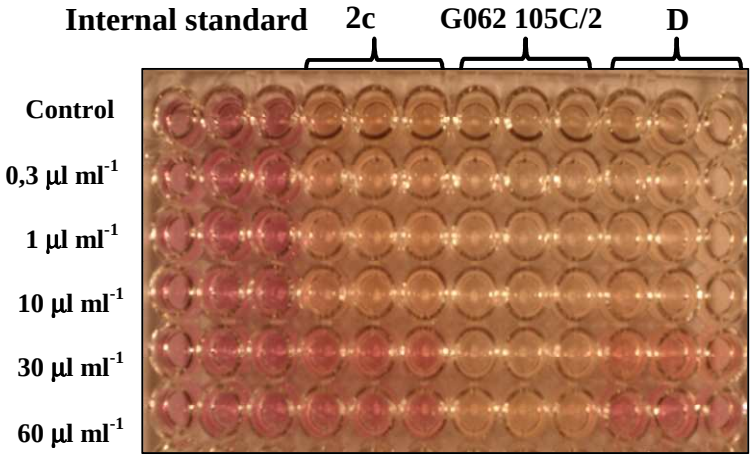


Figure 36: Dose response reaction of select isolate of *M. fructicola* to thiophanate methyl. Light pink colored wells indicated fungal respiration (AB reduction), while violet colored wells indicated feeble respiration

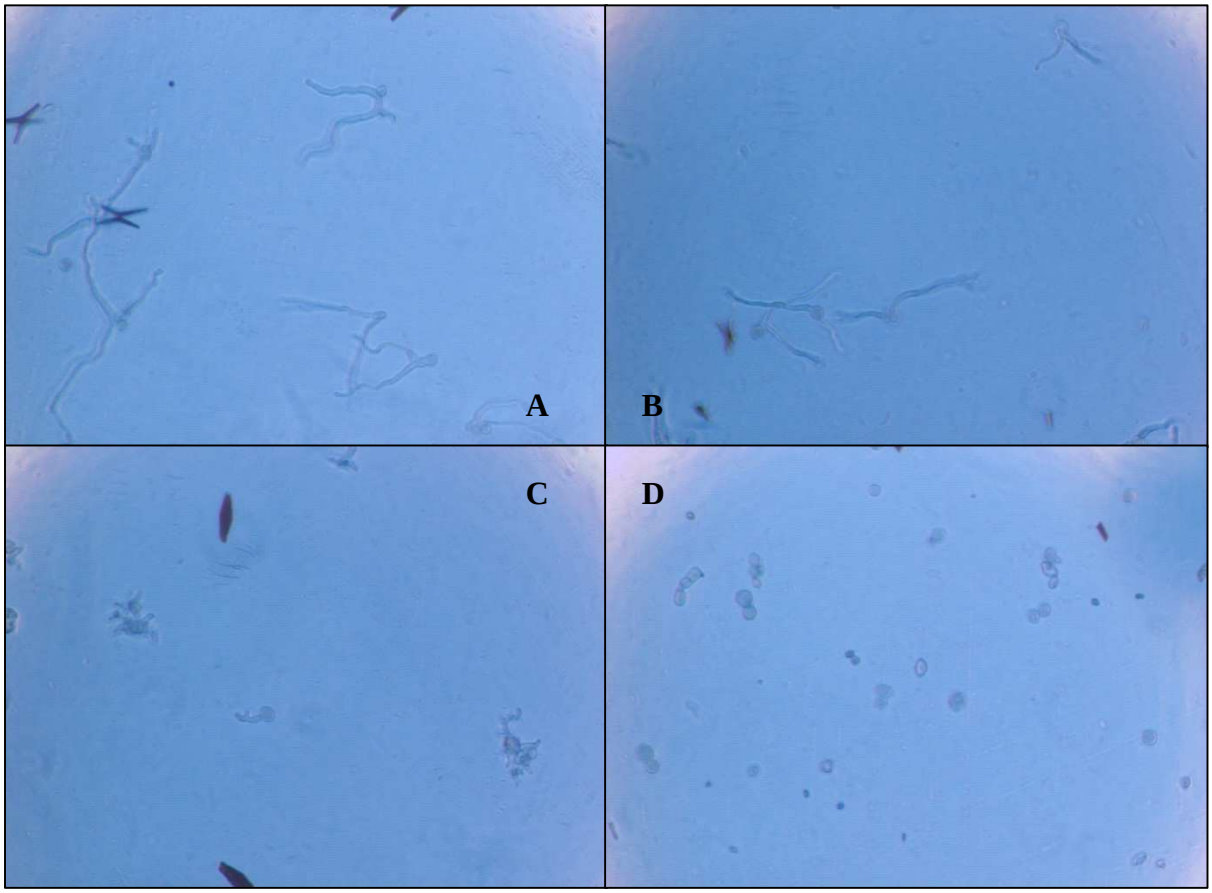


Figure 37: Conidia germination of *M. fructicola* 2c isolate in half-dose PDB amended with AB and different concentrations of thiophanate methyl; A: 0.3 µg ml^{-1}, B:1 µg ml^{-1}, C:10 µg ml^{-1}and D:30 µg ml^{-1}. The isolate was incubated for 12 h at 25°C. All assays were performed in 96-well, rounded-bottom. Each well contained half-dose PDB (80 µl) and conidial suspension (10^5 µl) of *M. laxa* or *M. fricticola*. An additional 100 µl of PDB amended with tebuconazole at the final concentration of 0.001, 0.01, 0.1, 1, 10, 100 µg ml^{-1} or thiophanate methyl at 0.3, 1, 10, 30, 60, 120, 240 µg ml^{-1} was added to each well, followed by of AB (20 µl). The total volume was 200 µl

Three out of four isolates of *M. laxa* MD5, C2 and TR800.3 were considered resistant to tebuconazole with EC_{50} value of 0.43 for MD5 and C2, 0.28 μg ml^{-1} for TR800.3, exceeding the inhibitory dose of 0.1 μg ml^{-1}. The C8 isolate with an EC_{50} value of 0.05 μg ml^{-1} was considered sensitive to tebuconazole. Among the *M. fructicola* isolates tested with tebuconazole, only two samples (6074.2 and G062 105 C/2) can be considerate resistant since their EC_{50} values were 0.18 and 0.13 μg ml^{-1}, respectively. The other six isolates showed EC_{50} values ranging from 0.04 to 0.001 μg ml^{-1}(Figure 38).

To determine the thyophanate methyl EC_{50}, 28 *M. laxa* and 64 *M. fructicola* isolates were tested with AB method. The EC_{50} values for *M. laxa* isolates that exceed the inhibitory dose (1 μg ml^{-1}) were 57% of the total, with EC_{50} ranging from 1.06μg ml^{-1} (C28)to 28.29 μg ml^{-1} (C34). The EC_{50} values of sensitive *M. laxa* isolates ranged from 0.0004 μg ml^{-1} (C30) to 0.76 μg ml^{-1} (RE 4/D) (Figure 39).

The *M. fructicola* results showed that the 69% of the isolates were resistant with EC_{50} values higher than the inhibitory dose (1 μg ml^{-1}) with values between 0.99 μg ml^{-1}(AZ2/2) and 57.59 μg ml^{-1}(G062 105 C/2) (Figure 40).

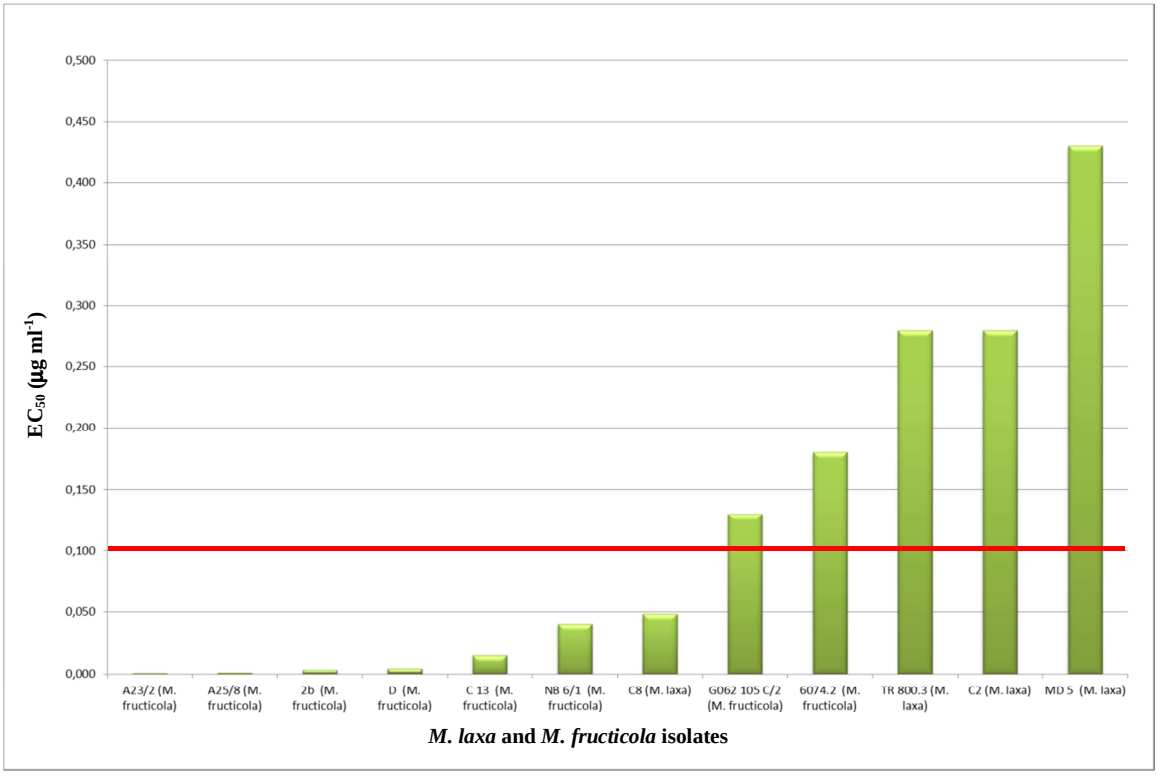


Figure 38: *M. laxa* and *M. fructicola* isolates tested with tebuconazole. Forty one percent of them can be considered resistant with EC_{50} value ranging from 0.13 to 0.43 µg ml^{-1} exceeding the inhibitory dose of 0.1 µg ml^{-1} indicated in the graph by the red line

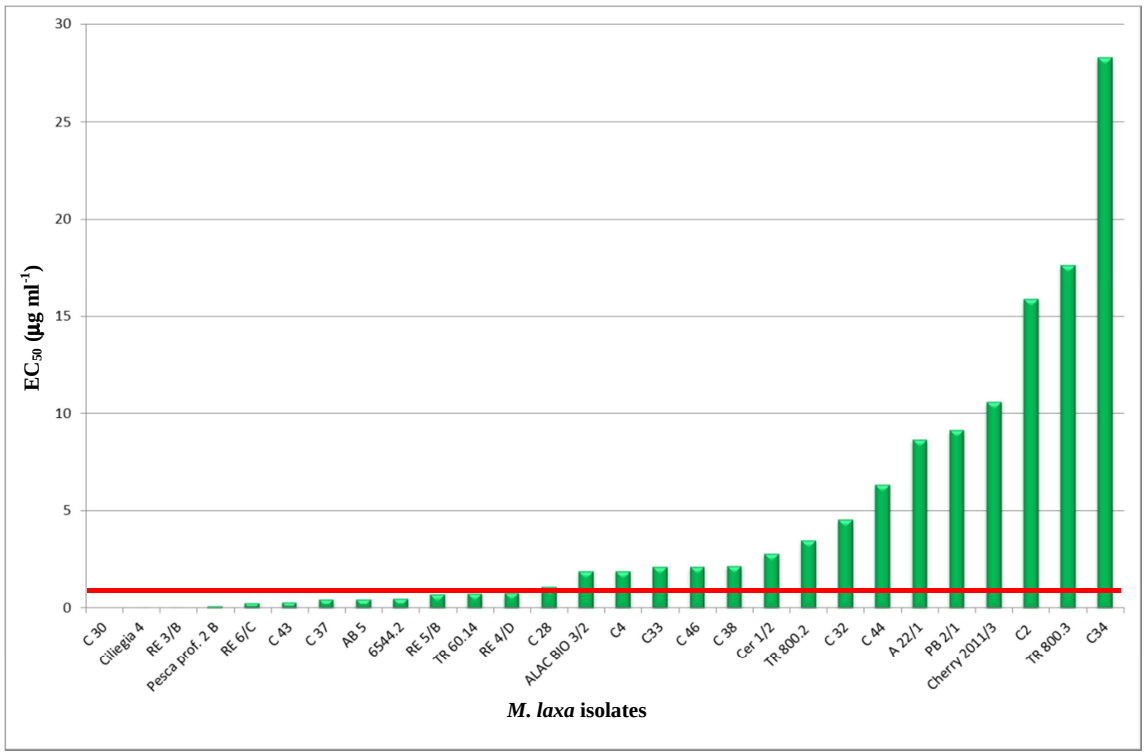


Figure 39:*M. laxa* isolates tested with thiophante methyl. Fifty seven percent of the isolates can be considered resistant with EC_{50}ranging from 1.06 to 28.29µg ml^{-1} exceeding the inhibitory dose of 1 µg ml^{-1} indicated in the graph by the red line

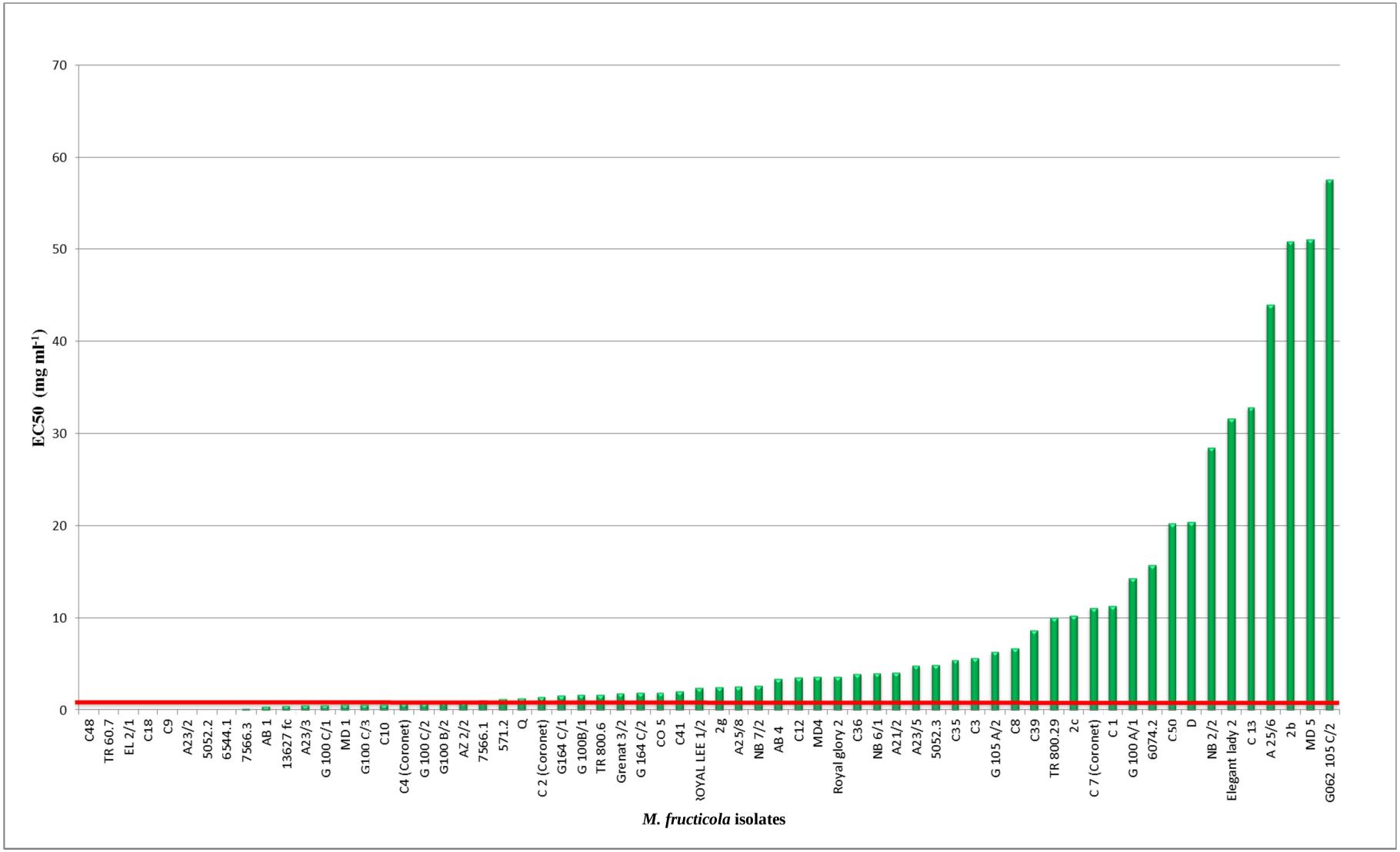


Figure 40: *M. fructicola* isolates tested with thiophante methyl. Sixty nine percent of the total isolates can be considered resistant. The EC_{50} values range from 0.002 µg ml^{-1} (C8) to 57.59 µg ml^{-1} (G062 105 C/2). The red line on the graph indicates the inhibitory dose of 1 µg ml^{-1}

4.4.5 Comparison between *M. laxa* and *M. fructicola* EC_{50} values obtained with amended medium and AB methods

The EC_{50} values determined on 28 isolates of *Monilinia* spp. with amended medium and AB methods showed a significant correlation with a R^2 values between 0.93 and 0.99 ($P<0.0001$) (figure 41). The two methods showed agreement when applied to identify sensitive/resistant isolates of *M. laxa* and *M. fructicola* (see the Appendix 8).

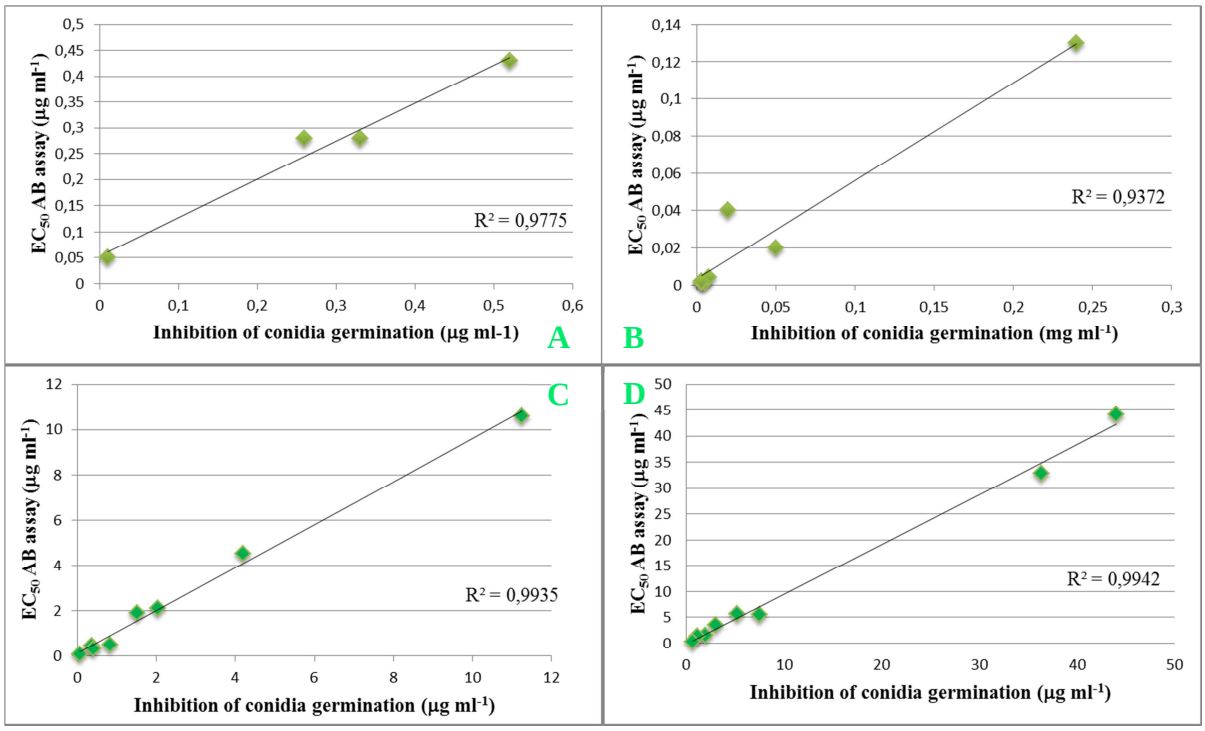


Figure 41: Relationship between fungicide sensitivity determined by the AB method and by inhibition of conidia germination for *M.laxa* (A and C) and *M. fructicola* (B and D) treated with tebuconazole (A and B) and thiophanate methyl (C and D). Three replicated-plates were used for each isolates on agar dilution method at different fungicide concentration, and three replicated-wells were used for each isolates on AB method at different fungicide concentration

4.5 *In vivo* sensitivity of *Monilinia* isolates to fungicides

For five of the fourteen phenotype isolates resistant to tebuconazole (ML8B, M15FC, M23, MCG2, MCG5) and ten of the sixteen phenotype isolates resistant to thiophanate methyl (ML5, ML8B, ML11, ML13, ML7454, M23, Quince2010, MCL12, MCL13 and MCL19) the treatment did not offered a significant disease control (P=0.05) when compared to the water-treated control.

Disease caused by isolates ML8B and M23 was not controlled by tebuconazole and thiophanate methyl as *in vitro* assay.

Both of fungicides provided essentially complete control of disease caused by the isolates that showed phenotype sensitivity to tebuconazole (ML7, ML10B, ML12BO, ML7454, ML7784, M20FC, MCG1, MCG3 and MCG4) and thiophanete methyl (ML7, ML12BO, M20FC, MCL4 and MCL9311) in the *in vitro* assay (Table 11).

Table 11: *In vivo* effectiveness of tebuconazole and thiophanate methyl against *M. laxa*, *M. fructicola* and *M. fructigena*

Isolates	Fungicides			
	Tebuconazole (36 ml 10 l^{-1})[a]		Thiophanate methyl (92.5 ml 100 l^{-1})	
	Control (mm)	Treatment (mm)	Control (mm)	Treatment (mm)
ML5	n.d.	n.d.	72.6 a	58.4 a
ML8B	30.3 a[b]	28.4 a[c]	41.0 a	32.3 a
ML7	23.8 a	0.6 b	19.1 a	1.5 b
ML10B	21.5 a	0.5 b	n.d.[d]	n.d.
ML11	n.d.	n.d.	34.7 a	28.7 a
ML12BO	24.1 a	0.7 b	11.8 a	0.4 b
ML13	n.d.	n.d.	24.9 a	23.3 a
ML7454	20.3 a	1,0 b	23.2 a	20.7 a
ML7784	26.6 a	1.7 b	n.d.	n.d.
M15FC	27.7 a	23.9 a	n.d.	n.d.
M20FC	27.0 a	1.3 b	23.2 a	4.7 b
M27FC	n.d.	n.d.	24.3 a	20.5 b
M23	34.1 a	32.8 a	21.8 a	18.5 a
Quince 2010	n.d.	n.d.	13.7 a	6.7 a
MCL4	n.d.	n.d.	14.5 a	1.4 b
MCL12	n.d.	n.d.	49.5 a	45.6 a
MCL13	n.d.	n.d.	18.4 a	12.2 a
MCL19	n.d.	n.d.	20.6 a	17.5 a
MCL9311	n.d	n.d	35.2 a	15.5 b
MCG1	27.7 a	3.8 b	n.d	n.d
MCG2	29.1 a	21.1 a	n.d	n.d
MCG3	22.1 a	0.5 b	n.d	n.d
MCG4	21.1 a	6.3 b	n.d	n.d
MCG5	31.3 a	26.3 a	n.d	n.d

[a] Half dose recommended by producer

[b] Values are the means of two experiments (30 peaches, three replicates of 10 fruit each were inoculated); results were similar (F exps< F table), thus the data were combined

[c] The same letters indicate no significant difference according to the Duncan test ($P < 0.005$)

[d] n.d.: not determined

4.6 Analysis of partial sequences of the β-tubulin gene

4.6.1 Partial sequences of the β-tubulin gene *M. laxa*

In order to investigate the molecular mechanisms behind the benzimidazole resistance, the analysis of point mutations of β-tubulin gene was performed using the primers TubA and TubR1 on each of the ten *M. laxa* isolates, five sensitives (S) (ML7784, ML7, M20FC ML12BO and ML7454) and five resistants (R) (ML13, ML5, M15FC, M23 and ML8B). A single DNA fragment of 1634-bp was amplified and sequenced: the β-tubulin gene contained six introns and seven exons. The deduced amino-acid sequence of the β-tubulin gene from *M. laxa*isolates showed 99% of similarities to the sensitive *M. laxa* on GenBank (accession number AY349149). The alignments of nucleotidic and aminacidic sequences of five S and five R isolates show that all ten isolates contained the sequence CTC (Lecucine) at the codon 240 in the β-tubulin gene and no mutations were present in the rest of the sequences (Figure 42 and 43) (75).

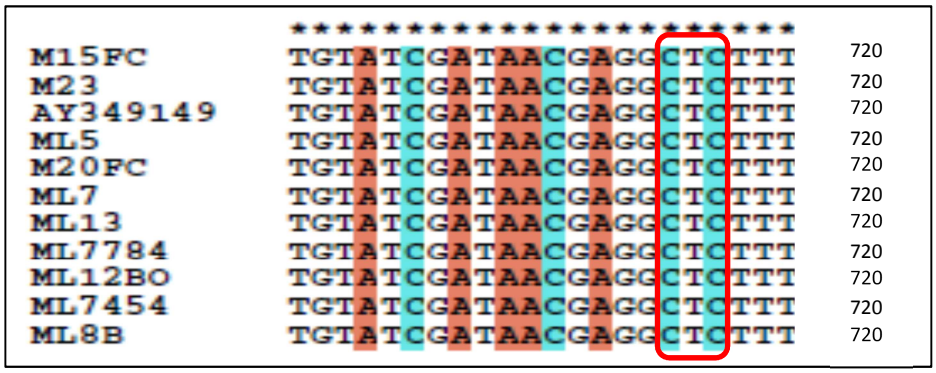


Figure 42: Alignment of DNA sequences of β-tubulin genes from *M. laxa*. The nucleotidic sequence of the β-tubulin gene from *M. laxa* isolates showed similarities of 99% to that sensitive *M. laxa* on GenBank (accession number AY349149). All five S and five R isolates have not a point mutation causing substitution of CTC (Leucine) in TTC (Phenylalanine)

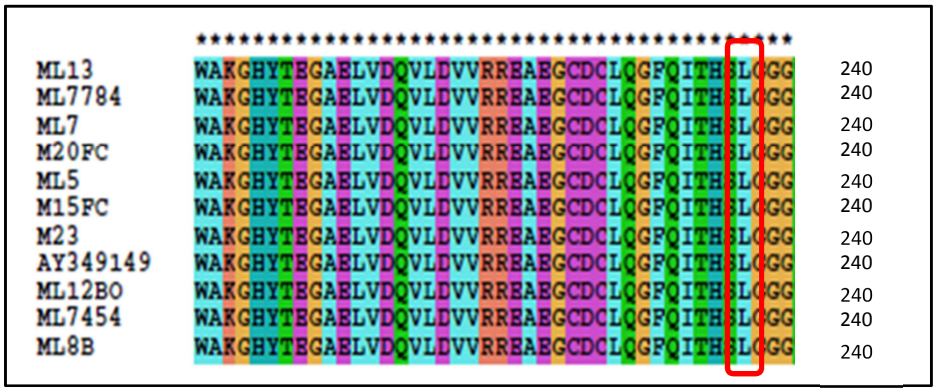


Figure 43: Alignment of amino acid sequences of β-tubulin genes from *M. laxa*. All five S and five R isolates contained a Leucine at the codon 240

4.6.2 Partial sequences of the β-tubulin gene *M. fructicola*

A single DNA fragment (1634 bp) was amplified with the primer pair TubA and TubR1 from each of four representative isolates, one sensitive S isolate (MCL4)and three resistants R isolates (MCL10, MCL13 and MCL19). The amplified section of β-tubulin gene included all positions known to affect the sensitivity to benzimidazoles in *Monilinia*. (REF Ma et al.). The analysis of DNA sequences of the β-tubulin gene showed that the R isolates of *M. fructicola* (MCL10, MCL13 and MCL19) had a punctual allelic change at codon 198, GCA (alanine, A) instead of GAA (glutaminic acid, E). On the other hand, the sensitive (S) isolate MCL4 of *M. fructicola* presented instead the sequence GAA (glutamic acid) in the same point of the β-tubulin gene explaining the different behavior observed during the resistance tests (Figure 44 and 45).

Figure 44: Alignment of DNA sequences of β-tubulin genes from *M. fructicola*. MCL4 was classified sensitive to thiophanate methyl. All three R isolates have a point mutation causing substitution of GCA (Alanine) in GAA (Glutaminic acid)

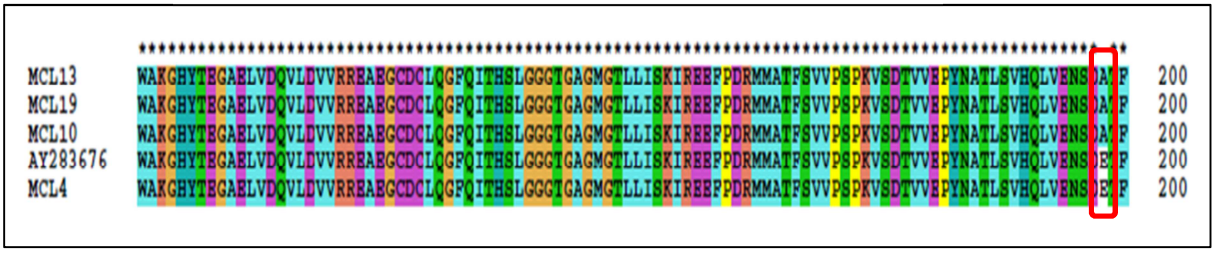


Figure 45: Alignment of amino acid sequences of β-tubulin genes from *M. fructicola.* The R isolates of *M. fructicola* had a punctual allelic change at codon 198, GCA (alanine) instead of GAA (glutaminic acid)

4.7 Influence of temperature on *Monilinia* spp

4.7.1 Influence on mycelial growth: *in vitro* trials

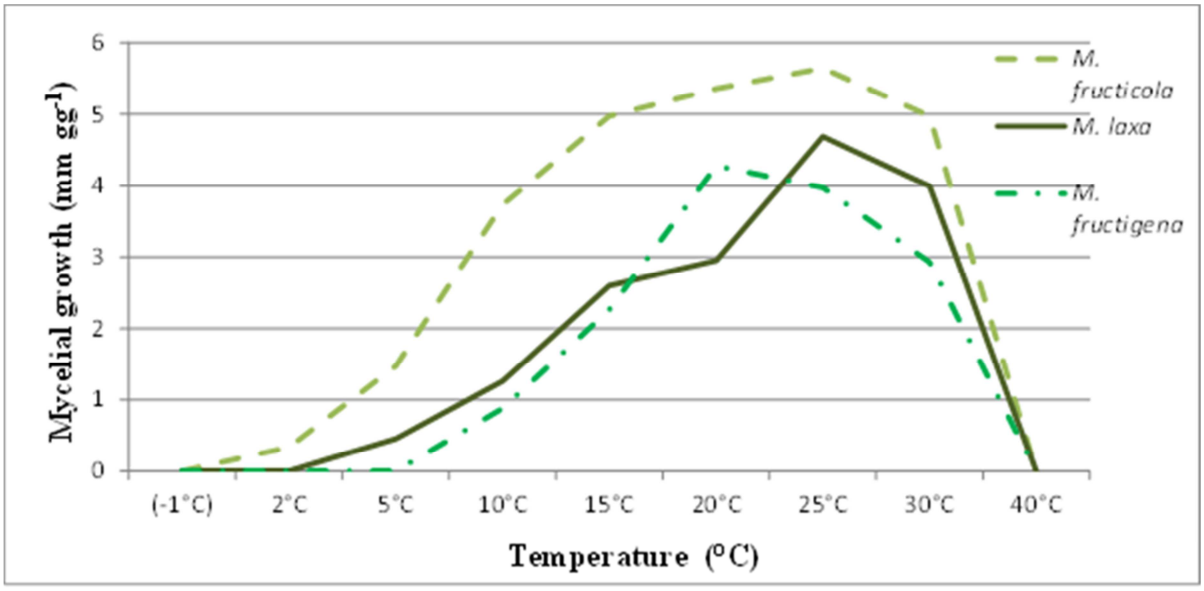


Figure 46: Effect of temperature on mycelial growth (mm gg^{-1}) of *M. laxa*, *M. fructicola and M. fructigena* in a range from -1° to 40°C. The values are the mean of three replicates

The results showed that the optimal temperatures for mycelial growth of *M. laxa*, and *M. fructicola* and *M. fructigena* were 25°C for *M. laxa* and *M. fructicola* and 20°Cfor *M. fructigena*. The mycelial growth stopped at 40°C for all three species. (Figure 46).

Data reported in Figure 47 shows in detail the mycelial growth of fungal pathogen at the typical fruit storage temperatures, from 0°C to 5°C for 30 days. At 0°C no mycelial growth occurred after 30 days of incubation for all the three pathogens; in particular, *M. fructigena* did not grow below 2.5°C. The faster growth was observed in *M. fructicola*, with a rate of approximately 0.4 mm gg^{-1}. The growth rate of *M. laxa* and *M. fructigena* were approximately 0.2 mm $gg^{-1}$and 0.1 mm gg^{-1}, respectively.

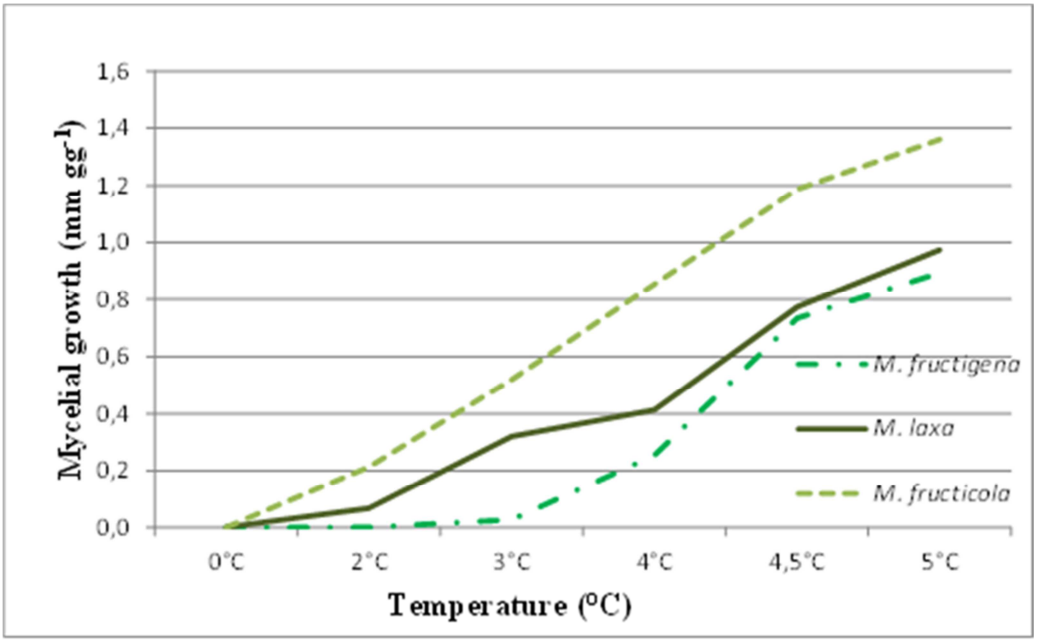


Figure 47: Effect of low temperatures on mycelial growth (mm gg^{-1}) of *M. laxa*, *M. fructicola* and *M. fructigena* after 30 days of incubation. The values are the mean of three replicates

4.7.2 Influence of the temperature on spore production and germination

The three species of *Monilinia* were able to produce spores after 30 days of incubation above 3°C. The highest conidia concentration was reported for *M. fructicola* ($6x10^5$ conidia ml^{-1}) at 5°C, while *M. fructigena* seems to be less active since the number of spores was constantly below 10^5conidia ml^{-1}. (Figure 48).

After 30 days of incubation, the three species of *Monilinia* were able to germinate over 4°C, although the percentage of germination expressed as culturable conidia was low, particularly for *M. laxa* (that started to germinate at 4°C). No germination occurred at 0°C and 3°C in *M. fructicola*. Culturable conidia of *M. fructigena* increased approximately three times (from 12% to 31%) moving from 4°C to 5°C. A similar trend was observed for *M. fructicola*: the percentage of conidia germination increased two times, from 16% at 4°C to 26% at 5°C.

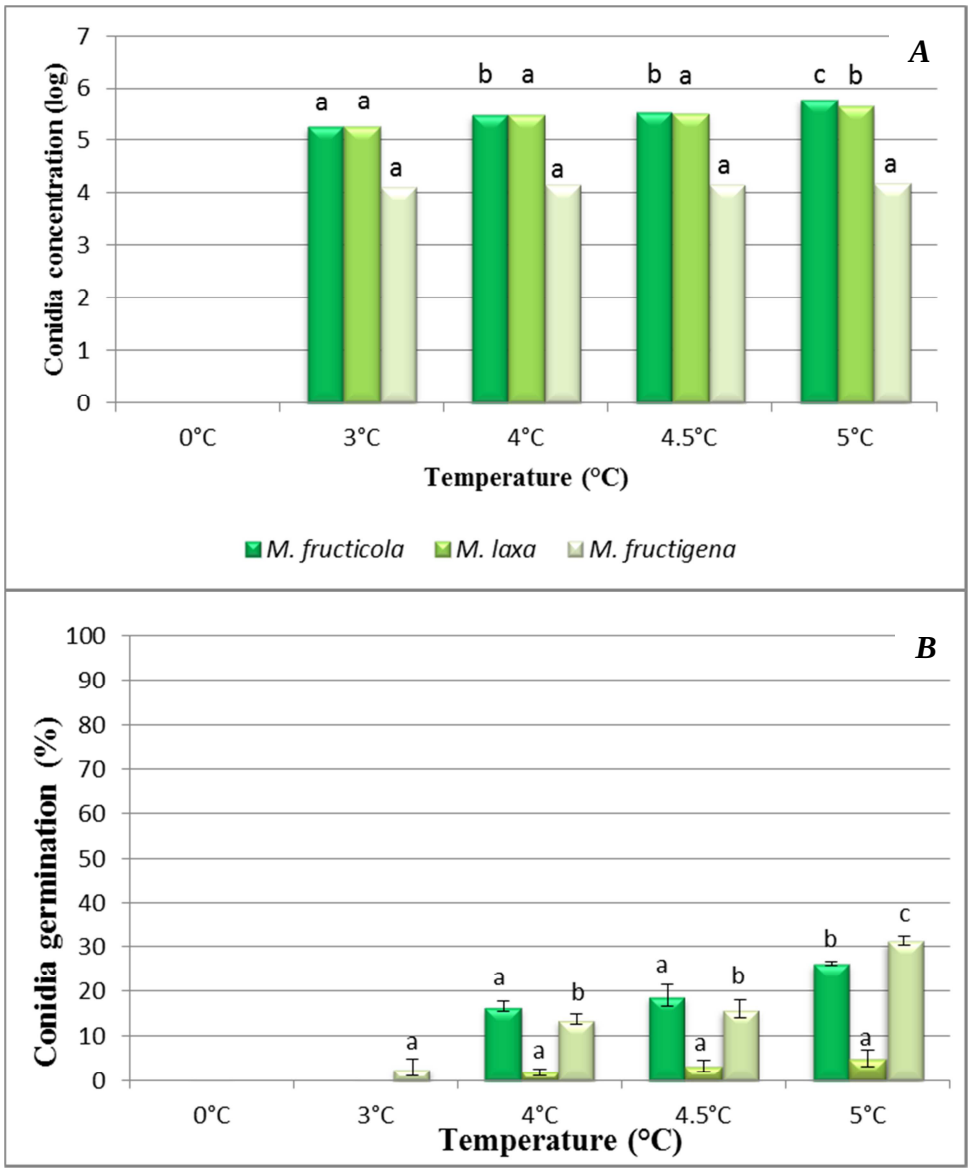


Figure 48:*A*-Effect of temperature on culturable conidia (%) of *M. laxa*, *M. fructicola* and *M. fructigena* after 30 days of incubation. *B*-Effect of temperature on spore density of *M. laxa*, *M. fructicola* and *M. fructigena* after 30 days of incubation. The values are the mean of three replicates. Whiskers represent the standard error. Within the same species different letters denote a significant differences for *P*<0.05 (Duncan Test)

4.7.3 Influence of the temperature on the resistance and sensitivity of *M. laxa, M. fructicola* and *M. fructigena* isolates to fungicides. *In vitro* and *in vivo* trials

Tebuconazole

In *in vitro* assay, *M. laxa* isolates were considered resistant to tebuconazole if EC_{50}>0.1 μg ml^{-1}. The fungicide treatment against resistant isolates incubated at 20°C produced a significant growth reduction (-78%). Sensitive isolates, incubated at 15°C were inhibited more than the same isolates incubated at the other tested temperatures. This suggests that the incubation temperature affects the treatment efficacy. Considering for example the ML34 isolate, the inhibition dropped from -36°C at 15°C to 0% at 30°C. For *in vivo* assay, fruits were treated and then inoculated with the *M. laxa* resistant isolates: M23, M27C and M7454. The optimal temperature that produced the highest inhibition was 20°C; at a lower temperature (15°C) the efficacy dropped by 55% for isolates M23 and M27C, while isolate M7454 showed the lowest inhibition at 20°C (-50%). The isolate ML8B appeared resistant in the case of treated fruits incubated at 30°C and sensitive when the fruits were incubated at 15°C.

The sensitive isolate (MCL17) of *M. fructicola* was inoculated on substrate amended with tebuconazole, and after incubation at different temperatures the highest inhibition was observed at 30°C (-76%) while at 25° the pathogen reduction was -45%.

Isolates of *M. fructigena* MCG2 and MCG5 were considered resistant if EC_{50}>0.1 μg ml^{-1}. In *in vitro* assay, tebuconazole was more effective at 15°C, showing inhibition ranging from -100% to -90% for MCG2 and MCG5, respectively. While the sensitive MCG3 showed the highest inhibition at 20°C (-70%). The fungicide efficacy decreased in

both resistant and sensitive isolates when the temperature increased to 30°C (Table 12).

Table 12: *M. laxa*, *M. fructicola* and *M. fructigena* isolates used in the *in vitro* and *in vivo* temperature assay with tebuconazole. *In vitro* assay PDA plates were amended with the EC_{50} value determined for each isolate. Plates were incubated at 15, 20, 25, 30°C for seven days in the dark, and mycelial growth was recorded. *In vivo* assay peaches were wounded and inoculated with a spore suspension (10^3conida ml^{-1}) of *M. laxa* or *M. fructicola*. Six hour before the inoculum the fungicide at a concentration of half the label rate (92.5 ml 100 l^{-1}) was applied with a hand sprayer. Peaches were stored at 15, 20, 25 and 30°C for 7 days

Isolate	Specie	R/S	INIBITHION (%)[a]							
			In vitro				*In vivo*			
			15°C	20°C	25°C	30°C	15°C	20°C	25°C	30°C
M23	*M. laxa*	R	48±0.4[b]	**74**±0.5	66±1.3	**42**±0.6	**55**±1.9	**83**±1.3	65±0.9	69±1.4
M27FC	*M. laxa*	R	44±0.2	**78**±1.4	55±1.3	**20**±0.9	**65**±1.4	**85**±1.9	75±1.7	79±0.7
M7454	*M. laxa*	R	55±0.5	**83**±0.3	66±1.2	46±0.9	84±1.7	**91**±1.4	**50**±1.2	77±0.8
ML8B	*M. laxa*	R	70±1.4	**83**±1.1	54±0.6	**23**±0.9	**100**±0	69±1.3	77±0.9	**60**±0.8
ML9B	*M. laxa*	S	**62**±0.1	45±0.4	37±0.7	**16**±0.3	n.d.[c]	n.d.	n.d.	n.d.
ML21	*M. laxa*	S	**58**±0.9	19±0.5	46±0.4	**15**±0.9	n.d.	n.d.	n.d.	n.d.
ML34	*M. laxa*	S	**36**±0.5	30±1.1	20±0.7	**0**	n.d.	n.d.	n.d.	n.d.
MCL17	*M. fructicola*	S	48±1.1	59±1.7	**45**±0.8	**76**±0.6	n.d.	n.d.	n.d.	n.d.
MCG2	*M. fructigena*	R	**100**±0	23±0.8	51±0.6	**18**±0.1	n.d.	n.d.	n.d.	n.d.
MCG5	*M. fructigena*	R	**90**±0.01	68±0.1	64±0.7	**54**±0.1	n.d.	n.d.	n.d.	n.d.
MCG3	*M. fructigena*	S	70±0.8	**83**±0.1	57±0.6	**44**±0.1	n.d.	n.d.	n.d.	n.d.

[a] Inhibition percentage= [(diameter of control)-(diameter of treatment)/(diameter of control)]*100

[b] standard error

[c] n.d.: not determined

Thiophanate methyl

The results obtained in *in vitro* assay showed that for both resistant (EC_{50}>1 μg ml^{-1}) and sensitive (EC_{50}<1 μg ml^{-1}) *M. laxa* isolates the largest pathogen reduction was achieved when isolates were incubated at 30°C (ML8B and ML9B presented complete inhibition). A different behavior was observed for ML11 (R), M27FC (R) and M7454 (S), the treatment resulted more effective at 15°C. The results of the *in vivo* tests confirmed the same pattern. The thiophanate methyl efficacy tended to drop at temperatures lower than 30°C: 15°C and 20°C for the sensitive isolates and 15°C and 25°C for the resistant ones. Only for the M27FC isolate, the efficacy dropped at 30°C.

In the *in vitro* trial on *M. fructicola*, the fungicide efficacy generally reached its maximum at 30°C for both resistant and sensitive isolates. Only MCL4 (S) and MCL5 (S) presented different optimal temperatures, 15°C and 25°C, respectively. The inhibition dropped at 20°C, with the exception of MCL13 (R) for which the minimum inhibition was observed at 15°C. Similar results were observed in *in vivo* tests, with the smallest inhibition occurring at 15°C (Table 13).

Table 13: *M. laxa* and *M. fructicola* isolates used in the *in vitro* and *in vivo* temperature assay with thiophanate methyl. *In vitro* assay PDA plates were amended with the EC_{50} value determined for each isolate. Plates were incubated at 15, 20, 25, 30°C for seven days in the dark, and mycelial growth was recorded. *In vivo* assay peaches were wounded and inoculated with a spore suspension (10^3conida ml^{-1}) of *M. laxa* or *M. fructicola*. Six hour before the inoculum the fungicide at a concentration of half the label rate (36 ml 10 l^{-1}) was applied with a hand sprayer. Peaches were stored at 15, 20, 25 and 30°C for 7 days

Isolate	Specie	R/S	INIBITHION (%)[a]							
			In vitro				*In vivo*			
			15°C	20°C	25°C	30°C	15°C	20°C	25°C	30°C
ML5	*M. laxa*	R	**52**±0.08[b]	53±1.1	58±0.6	**79**±0.6	**20**±1.2	24±1.2	28±0.8	**45**±0.9
ML8B	*M. laxa*	R	62±0.7	62±0.5	**60**±0.8	**100**±0.5	**12**±0.5	36±1.1	21±1.3	**52**±1.3
ML11	*M. laxa*	R	**73**±0.9	63±1.5	**60**±1.1	66±0.6	**21**±1.7	25±1.5	34±1.3	**47**±1.2
ML13	*M. laxa*	R	67±0.9	72±1.2	**63**±0.9	**85**±0.9	n.d. [c]	n.d.	n.d.	n.d.
M23	*M. laxa*	R	66±1.5	**63**±0.1	75±0.9	**80**±0.7	14±1.3	**4**±0.8	35±0.1	**42**±0.9
M27FC	*M. laxa*	R	**83**±0.2	77±0.4	66±0.5	**54**±0.1	**100**±0	32±0.3	55±0.4	**26**±0.1
ML21	*M. laxa*	S	14±0.8	**32**±0.9	31±0.7	**49**±0.7	n.d.	n.d.	n.d.	n.d.
ML9B	*M. laxa*	S	**5**±0.3	40±0.5	36±0.8	**100**±0	n.d.	n.d.	n.d.	n.d.
ML34	*M. laxa*	S	**6**±0.6	31±0.9	37±0.7	**66**±0.7	n.d.	n.d.	n.d.	n.d.
M7454	*M. laxa*	S	**95**±0.9	83±0.4	76±1.1	**66**±0.3	n.d.	n.d.	n.d.	n.d.
MCL10	*M. fructicola*	R	49±1.8	**46**±0.8	55±1.7	**57**±0.9	**3**±0.5	8±0.7	12±0.7	**29**±0.5
MCL12	*M. fructicola*	R	68±1.4	**50**±0.7	56±0.6	**86**±0.5	**8**±0.5	8±0.7	15±1.3	**19**±0.6
MCL13	*M. fructicola*	R	**36**±0.8	42±0.6	57±0.4	**63**±0.9	**4**±0.9	7±0.3	18±0.6	**26**±0.4
MCL19	*M. fructicola*	R	26±0.5	**25**±0.9	47±0.1	**88**±0.8	**3**±0.9	14±0.2	19±0.3	**28**±0.6
MCL4	*M. fructicola*	S	**85**±0.1	**45**±0.8	63±0.4	51±0.9	n.d.	n.d.	n.d.	n.d.
MCL5	*M. fructicola*	S	43±0.2	**17**±0.3	**54**±0.1	42±0.9	n.d.	n.d.	n.d.	n.d.
MCL16	*M. fructicola*	S	25±0.5	**19**±0.6	41±0.1	**80**±0.5	n.d.	n.d.	n.d.	n.d.
MCL17	*M. fructicola*	S	44±1.2	**21**±0.2	54±0.2	**62**±0.2	n.d.	n.d.	n.d.	n.d.

[a] Inhibition percentage= [(diameter of control)-(diameter of treatment)/(diameter of control)]*100

[b] standard error

[c] n.d.: not determined

Chapter 5

Discussion

5.1 *Monilinia* spp. population in Italy

European peach and nectarine import/export is predominant, with 75% and 82% of the overall fruit imports and exports, respectively. Among the top 15 exporting countries in the world, 11 are European, with Spain and Italy at the top positions (105). Peach and nectarine export is growing at an higher rate than the production (34) and for this reason it is important to monitor the *Monilinia* population present in Europe and particularly in Italy since new specie could spread in our Country.

In this dissertation specific primers for *M. laxa*, *M. fructicola* and *M. fructigena* (55, 121) were used to individuate which species of *Monilinia* were present on Italian stone and pome fruits. Considering that since few years ago *M. fructicola* belonged to the list of quarantine pathogens, the very similar percentage (47% and 48%, respectively) of *M. laxa* and *M. fructicola* isolates found in Italy in two years in this indentification screening, confirms the relevant role of *M. fructicola* in causing serious fruits rot of peaches in Europe (25, 26, 64) and, in particular, in Italy (100). *M. fructigena* was detected only in 5% of the cases during the species screening, especially on apple fruits, and this pathogen cannot not be considered an important threat for the peach growers.

Interestingly, *M. fructicola* was found on stored apple (*Malus domestica* Borkh.) during a survey for fungal postharvest pathogens, fruit that showed brown rot symptoms belonged to the ‘Gala’ and

‘Pink Lady’. The colonies developed a gray mass of spores in concentric rings with the reverse side black, and further studied by molecular tools using specific primers. The BLAST search in GenBank revealed the highest similarity (99%) to *M. fructicola* sequences, representing the first report of the fungus *M. fructicola* on apple in Italy. Further studies are necessary to determine geographic distribution, prevalence and economic importance of this quarantine organism in Italy.

5.2 Susceptibility of peach fruit during phenological stages

The variation of susceptibility to *M. laxa* in peach fruit during its development has been studied for several years and under different conditions. First investigations revealed that green fruits are more resistant than the ripe ones to brown rot (18) and recently studies revealed that peach susceptibility strongly decreased during S2 (pit hardening) and increased thereafter (38, 77, 83, 93, 108, 127). The same behavior, was observed in this study for all three species. (Figure 33). The difference of *Monilinia* rot incidence (Figure 32) across fruit development stages may be due to the following resistance factors: mechanical resistance of green fruits, presence of inhibitory substances and /or diverse availability of nutrients (4, 35, 38, 48, 63, 77, 116). In this study a biochemical approach was undertaken to investigate the possible antifungal compounds ascribed in this temporary loss of susceptibility.

5.3 Preformed antifungal compounds in peach peel tissues extracted from fruit at different developmental stage

The presence of antifungal compounds in crude extracts derived from peel of ‘Maycrest’ ‘Red Heaven’, and ‘Tardibelle’ peach assayed at different stages (S1, S2 and S4) was demonstrated in 1D TLC bioassay. Greater inhibition of brown rot was observed in peel extracts of fruit during the pit hardening (S2) as compared to S1 and S4 stage.

TLC bioassay showed similar antifungal fingerprint with R_f 73.3 and 53.3 in the S1 stage for all three fruit species. During S2 stage the solvent system 60:40:30 (hexane:ethyl acetate: methanol) showed other two more inhibition spots evident with R_f 26.6 and 13.3. A feeble inhibition spot, at values R_f 13.3, was observed in ‘Tardibelle’ and ‘Red Heaven’ peach, but no inhibition spot with the same R_f was revealed in ‘Mycrest’ peach. No inhibition spots were detected on ‘Maycrest’ and ‘Tardibelle’ peach in S4 stage, while very feeble inhibition spots were found in ‘Red Heaven’ peach with R_f values 73.3 and 53.3 (Table 10). These results confirm the presence of inhibitory compounds in the peel of peach fruit during the pit hardening, probably associated to the variation of gene expression of phenylpropanoid pathway key genes as explained by Lee and Bostock (16, 63) and Zubini (137).

Total phenolics highlighted with auto-fluorescence after exposure to UV light were analyzed by the Folin-Ciocalteu reaction to confirm the presence of total free phenolic groups for R_f 73.3, 53.3 and 26.6. An increase in the total phenolic compounds in peach peel during the pit hardening stage (S2) was already shown; in particular, cafeolic acid (CGA; 5-O-caffeoylquinic acid), catechin and chlorogenic acid were found as major phenolic compounds (8, 108). CGA, caffeic acid (CA; 3,4-dihydroxycinnamic acid), catechin, chloregenic acids, cinnamic

acids, flavan-3-ols, flavonols and cyaniding-3-glucoide are located mainly in the peel tissue and their concentrations decrease during fruit development and ripening (after S2 stage) (8, 45, 63), especially CGA that significantly declines during the transition from S2 to S4 (45, 63). Bostock et al. (1999) in their study (16) demonstrated how *M. fructicola* spore germination and mycelial growth were not affected by CA and GCA at a concentration similar to or greater than the one in the exocarp (i.e., cuticle, epidermis, and hypodermis) of immature resistant fruit. Eight years later Lee and Bostock clarified the CGA and CA role on inhibition of polygalacturonase (PG) and appressorium formation by *M. fructicola* at concentrations at which there is no inhibition of fungal growth and at the concentrations that are within the physiological range that occurs in fruit peels (61). However, it is still unclear if these two phenols (CGA and CA) determined the presence of the inhibition spots, since nevertheless the TLC bioassay showed the inhibition of *Monilinia* growth, their presence has not been verified. Chlorogenic acid is the main hydroxycinnamic and catechin is generally the main monomeric flavon-3-ol derivate present in 'Red Heaven' peel before S4 stage (8). More investigations are needed to clarified the role of this phenol in *Monilinia* inhibition.

As mentioned above, another feeble inhibition spot at R_f 13.3 was present in 'Tardibelle' and 'Red Heaven' (Table 10). Folin-Ciocalteu reaction did not confirm the presence of phenols in that position suggesting that alkaloids or terpens could be present with feeble inhibitory effect (116). Hirai et al. proposed by TLC analysis the presence of the inducible triterpenes, found in green strawberry fruit and conferring resistance to *Collototrichum fragariae*. This compound

probably corresponds to the phytoalexin found in strawberry roots (50, 89).

The TLC technique was previously used in other similar studies in order to investigate on the antifungal compounds present in mango, avocado, strawberry fruits, Geraldton waxflower, and soybeans (4, 27, 60, 115, 116, 126). The TLC can be used both as an analytical and a preparative technique, and traditionally regarded as a simple, rapid, and inexpensive method for the separation, identification tentative, and visual semi-quantification of a wide range of substances. Further studies investigating on the presence of antifungal compounds in stone fruit during the pit hardening stage are in progress.

5.4 Sensitivity of *M. laxa, M. fructicola* and *M. fructigena* to tebuconazole and thiophanate methyl

Demethylation inhibitor fungicides (DMI) appeared in 1980s are widely used against brown rot and have effectively replaced methyl benzimidazole carbammates (MBC). Their repetitive use has selected fungicide-resistant populations, since they are used almost exclusively for *M. laxa*, *M. fructicola* and *M. fructigena* control during flowering and pre-harvest period (20, 106)

Our *in vitro* results indicate a good effectiveness of tebuconazole against *M. laxa* and *M. fructicola* in both of year (2010 and 2011) (Appendixes 6 and 7, figure 38). These data are in agreement with Tonini et al. (120), and Adaskaveg et al. (3), who reported that tebuconazole applied 15 days before harvest was an effective fungicide against pre- and postharvest brown rot peaches, while the fungicide residue resulted below the half of Italian legal limit.

The intensive use of DMI fungicides could lead to either an accelerated rate of selection for resistance (7, 51) however Schnabel et

al. (113) reported a slow selection resistance process on *M. fructicola* isolates treated with propiconazole

In the past, Italian *M. laxa* and *M. fructigena* were controlled by chemical treatments based on MBCs, but three decades ago DMIs were introduced in substitution of this class of fungicides because resistant strains developed (though, thiophanate methyl is still used against cankers on peach tree branches caused by *Fusicoccum amygdali*) (20, 26).

The results obtained in this work indicated that the percentage of resistant *M. laxa* isolates increased from 15% to 57% as well as the EC_{50} resistance values (0.78-3.12 μg ml^{-1} 2010 and 1.06-28.29 μg ml^{-1} in 2011). These data are in agreement with Ma et al. (75) and Brent et al. (17) who reported that the EC_{50} values of *M. laxa* isolates from California and in Europe have only one resistance level: the low resistance (EC_{50} between 1 μg ml^{-1} and 50 μg ml^{-1}). In contrast, Sharma (110) and Thomidis et al. (117) indicated that thiophanate methyl was effective fungicide against fruit rots in peaches caused by *M. laxa* in warmer areas.

Interestingly, the percentage of the *M. fructicola* resistant to thiophanate methyl isolates was almost the same in both of years (2010 and 2011) with a percentage of resistant isolate (EC_{50}>1μg ml^{-1}) was more than 65%. Their EC_{50} value ranged from 0.99 and 57.59 μg ml^{-1} (Appendix 6 and figure 40). The same results to benzimidazole-resistant phenotypes isolates were obtained by Cox et al., Luo and Schnabel, Riley at al., and Ma et al. (21, 66, 72) in different American States.

The authors demonstrated as none of the isolates of *M. fructicola* was resistant to both tebuconazole and thiophanate methyl. Similarly Yoshimura et al. (132) tested the sensitivity of *M. fructicola* isolates to

thiophanate methyl and tebuconazole and found that the isolates resistant to thiophanate methyl were sensitive to tebuconazole. So far, Holb and Schnabel (51) indicated differential performances among triazoles (tebuconazole, fenbuconazole and propiconazole) against *M. fructicola* isolates with reduced sensitivity to DMIs but no cross resistance with MBC was observed.

In most cases, resistance is associated with point mutations in the β-tubulin gene which result in altered amino acid sequences at the benzimidazole binding site (61)

Analysis of DNA sequences of the β-tubulin gene showed that all resistant isolates had a single pair mutation at the codon 198 of the β-tubulin gene resulting in an alanine (GCA) replacing the glutamic acid (GAA) (74). This kind of mutation at the codon 198 in benzimidazole-resistant isolates was found in several other phytopatogenic fungi such as *Botrytis cinerea*, *Helminthosporium solani*, *Penicillium* spp., *Sclerotinia homoeocarpa*, *Venturia inaequalis* and *V. pirina*, *Tapesia yallundae* and *T. acutormis* (table 1). The direct involvement of these mutation at the codon 198 in conferring resistance to benzimidazole has been confirmed using site-direct mutagenesis by Fujimura and collegues (41). The PCR results showed that the resistant Italian *M. fructicola* testes isolates had not a point mutation at the codon 6 that cause the replacement of the amino acid histidine by tirosin typical of the low resistant isolates of Ma et al. (74) suggesting that in our resistant isolates the different level of resistance (weak or moderate) is not associated to a genetic variation at the codon 6 of the β-tubulin gene, further investigations are need to understand the causes of this different level of resistance to thiophanate methyl resistant isolates. Moreover, in this study the EC_{50} method was used to classified the isolates in different levels of resistance to tiophanate methyl, while

Ma et al. (74) indicated a different scale of resistance related to mycelial growth.

The analysis of the β-tubulin gene sequence in *M. laxa* isolates resistant phenotype showed no mutation at the codon position 240 typical of the low resistant isolates found by Ma et al. (75). This means that the resistance in this case was not related to a genetic variations on the β-tubulin gene, suggesting that the phenotypic resistance found in our resistant isolates could be influenced by environmental stimuli such as the humidity and temperature (19).

Ma et al. (75) reported that in *in vitro* assay *M. laxa* low resistant grew poorly on PDA amended with 1 μg ml^{-1} of benomyl at 28°C, while it grew significantly at temperature range 8-24°C. The results obtained in this work confirmed this thesis on *M. laxa* and *M. fructicola* resistant isolates treated with thiophanate methyl and incubated at different temperatures (15, 20, 25 and 30°C). In fact, *in vitro* trials the incubation at 30°C showed the largest inhibition of mycelium.

The methylation status or deletion of the Mona element in a portion of the MfCYP51 gene could cause the highest resistance to tebuconazole at 30°C showed on resistant isolates of *M. laxa* and *M. fructicola* in this work. It is possible that neither the deletion of Mona element nor the methylation of Mona or MfCYP51 gene implicate the different expression of the resistance. Some studies suggest that other genes or other parts of the genome were methylated and involved in propiconazole resistant instability (11, 67).

The influence of the temperature on the fungicide effectiveness with respect to pathogen isolates can have a direct impact on the strategies for brown rot management. Louise et al. (72) proposed that, in some cases, tebuconazole does not have effect in subtropical, conditions, making the management of the pathogen ever more problematic. The

association of favorable conditions like the presence of multiple species and a warm and humid climate, may increase the pathogen aggressiveness, necessitating frequent fungicide applications.

An important consideration for fungicides resistance management is the amount of inoculum (10, 14, 20, 78, 123). The efficacy of DMI treatments decreased under high inoculum in peach orchards in China (65), Georgia (6, 7, 113), South Carolina(7, 51, 62, 68, 134, 136), New York (125, 126), California (3, 82, 84, 86) Brazil (72), Greece (76, 117, 118), and Spain (87), suggesting that DMI fungicides by themselves cannot provide adequate control in cases of high pressure disease. To achieve acceptable brown rot control with fungicide applications, measures to reduce inoculum levels (38, 42, 53), such as the alternation of active ingredients during the entire season, must be implemented in commercial peach orchards (7, 51, 68, 72).

Chapter 6

6.1 Contributions

The list of contributions in this work includes:

- The identification of the composition of the *Monilinia* populations from different Italian region that produce stone fruits.
- First report of *M. fructicola* on apple fruit from storage.
- A clarification of the relationship between the presence of phenols and their antifungal properties.
- A systematic quantitative assessment of *M. laxa* and *M. fructicola* sensitivity/resistance to DMI and MBC fungicides through the EC_{50} values.
- The definition of a protocol for the utilization of AB (Alamar blue) assay for the rapid assessment of *M. laxa* and *M. fructicola* resistance to tebuconazole and thiophanate methyl.
- A potentially more accurate assessment of the temperature effect on the phenotype resistance to DMI and MBC fungicides based on the actual EC_{50} values and not on the inhibitory dose.

APPENDIXES

APPENDIX 1

Substrates

LB	Luria Bertani	30 g of LB (Difco, Franklin lakes, NJ), 20 g of Agar Technical (Oxoid, Rodano, Milan, Italy), 1 litre distilled water
LB liquid	Luria Bertani liquid	30 g of LB (Difco, Franklin lakes, NJ), 1 litre distilled water
MEA	Malt extract agar	50 g of MEA (Oxoid, Rodano, Milan, Italy), 1 litre distilled water
PDA	Potato dextrose agar	39 g of PDA (Oxoid, Rodano, Milan Italy), 1 litre distilled water
PDB	Potato dextrose broth	24 g of PDB (Sigma Aldrich, St. Louis, MO), 1 litre distilled water
PDB half dose	Potato dextrose broth half dose	12 g of PDB (Sigma Aldrich, St. Louis, MO), 1 litre distilled water
V8 agar	V8 juice agar	250 ml of tomato source (Pomì, Rivarolo del Re, CR, Italy), 20 g of Agar Technical (Oxoid, Rodano, Milan, Italy), 750 ml distilled water

APPENDIX 2

Antibiotic solutions

Neomicyn	Fluka, Buchs, SG, Switzerland	0.1 g on 1 litre of substrate at temperature 50°C
Streptomicyn	Sigma Aldrich, St. Louis, MO	0.1 g on 1 litre of substrate at temperature 50°C

APPENDIX 3

Isolates: ***M. laxa, M. fructcicola*** **and** ***M. fructigena*** **isolates from rotted fruits and identified with specific primers for each specie** (53, 119)

	Isolate	Species	Region come from	Host	Cultivar	Isolation year
1	**ML 1**	*M. laxa*	Romagna	Nectarine	n.d.[a]	2010
2	**ML 2**	*M. laxa*	Romagna	Plum	Angeleno	2009-2010
3	**ML 3**	*M.laxa*	Romagna	Plum	Angeleno	2009-2010
4	**ML 4**	*M. laxa*	Emilia	Peach	Red Heaven	2010
5	**ML 5**	*M. laxa*	Romagna	Plum	Angeleno	2009-2010
6	**ML 6**	*M. laxa*	Romagna	Plum	Angeleno	2009-2010
7	**ML 7**	*M. laxa*	Romagna	Plum	Angeleno	2009-2010
8	**ML8**	*M. laxa*	Romagna	Peach	Royal Majestic	2010
9	**ML9**	*M. laxa*	Emilia	Cherry	Bigarlou	2010
10	**ML10**	*M. laxa*	Romagna	Nectarine	Big Top	2010
11	**ML 11**	*M. laxa*	Romagna	Peach	Royal glory	2010
12	**ML12**	*M. laxa*	Romagna	Peach	Royal glory	2010
13	**ML13**	*M. laxa*	Romagna	Peach	Red Moon	2010
14	**ML14**	*M. laxa*	Romagna	Peach	Springbelle	2010
15	**ML15**	*M. laxa*	Romagna	Plum	Angeleno	2009
16	**ML16**	*M. laxa*	Romagna	Plum	Angeleno	2009
17	**ML17**	*M. laxa*	Romagna	Peach	n.d.	2009
18	**ML18**	*M. laxa*	Romagna	Peach	n.d.	2010
19	**ML19**	*M. laxa*	Romagna	Peach	n.d.	2010
20	**ML20**	*M. laxa*	Romagna	Peach	n.d.	2010
21	**ML21**	*M. laxa*	Romagna	Peach	n.d.	2010
22	**ML22**	*M. laxa*	Romagna	Peach	n.d.	2010
23	**ML23**	*M. laxa*	Romagna	Peach	n.d.	2010
24	**ML24**	*M. laxa*	Romagna	Peach	Springbelle	2010
25	**ML25**	*M. laxa*	Romagna	Peach	Springbelle	2010
26	**ML26**	*M. laxa*	Romagna	Peach	Springbelle	2010
27	**ML27**	*M. laxa*	Romagna	Peach	Springbelle	2010
28	**ML28**	*M. laxa*	Romagna	Peach	Springbelle	2010
29	**ML29**	*M. laxa*	Romagna	Peach	Royal Glory	2010
30	**ML30**	*M. laxa*	Romagna	Peach	Springbelle	2010
31	**ML31**	*M. laxa*	Romagna	Peach	Super Crinson	2010
32	**ML32**	*M. laxa*	Romagna	Peach	n.d.	2010
33	**ML33**	*M. laxa*	Romagna	Plum	President	2010
34	**ML34**	*M. laxa*	Romagna	Plum	President	2010
35	**ML 8B**	*M. laxa*	Romagna	Peach	Red Moon	2010
36	**ML 9B**	*M. laxa*	Romagna	Peach	Red Moon	2010

37	**ML 10B**	*M. laxa*	Romagna	Peach	Red Moon	2010
38	**ML 12B**	*M. laxa*	Romagna	Peach	Red Moon	2010
39	**ML13B**	*M. laxa*	Romagna	Apricot	n.d.	2010
40	**M4FC**	*M. laxa*	RPPC[b]	Peach	n.d.	2010
41	**M5FC**	*M. laxa*	RPPC	Peach	n.d.	2010
42	**M7FC**	*M. laxa*	RPPC	Peach	n.d.	2010
43	**M8FC**	*M. laxa*	RPPC	Peach	n.d.	2010
44	**M9FC**	*M. laxa*	RPPC	Peach	n.d.	2010
45	**M15FC**	*M. laxa*	RPPC	Peach	n.d.	2010
46	**M20FC**	*M. laxa*	RPPC	Peach	n.d.	2010
47	**M21FC**	*M. laxa*	RPPC	Apricot	n.d.	2010
48	**M27FC**	*M. laxa*	RPPC	Peach	n.d.	2010
49	**M10BO**	*M. laxa*	RPPC	Peach	n.d.	2010
50	**M12BO**	*M. laxa*	RPPC	Peach	n.d.	2010
51	**M19RA**	*M. laxa*	RPPC	Peach	n.d.	2010
52	**M4**	*M. laxa*	RPPC	Peach	n.d.	2010
53	**M23**	*M. laxa*	RPPC	Peach	n.d.	2010
54	**M24**	*M. laxa*	RPPC	Apricot	n.d.	2010
55	**M7454**	*M. laxa*	RPPC	Peach	n.d.	2010
56	**M7537**	*M. laxa*	RPPC	Peach	n.d.	2010
57	**M7636**	*M. laxa*	RPPC	Peach	n.d.	2010
58	**M7782**	*M. laxa*	RPPC	Peach	n.d.	2010
59	**M7784**	*M. laxa*	RPPC	Peach	n.d.	2010
60	**M7785**	*M. laxa*	RPPC	Peach	n.d.	2010
61	**5052.1**	*M. laxa*	RPPC	Peach organic	n.d.	2011
62	**571.1**	*M. laxa*	RPPC	Peach	Sweet Lady	2011
63	**6544.2**	*M. laxa*	RPPC	Nectarine organic	Venus	2011
64	**A 25/4**	*M. laxa*	Romagna	Peach	n.d.	2011
65	**A22/1**	*M. laxa*	Romagna	Peach	n.d.	2011
66	**AB 5**	*M. laxa*	Veneto	Nectarine	Andross	2011
67	**A. bio 1/2**	*M. laxa*	Romagna	Peach organic	Ambra	2011
68	**A. bio 3/1**	*M. laxa*	Romagna	Peach organic	Ambra	2011
69	**A. bio 3/2**	*M. laxa*	Romagna	Peach organic	Ambra	2011
70	**Alb. reale**	*M. laxa*	Romagna	Apricot	Reale	2011
71	**Amb. 2/1**	*M. laxa*	RPPC	Peach	n.d.	2011
72	**Amb. 3/1**	*M. laxa*	RPPC	Peach	Ambra	2011
73	**C 2**	*M. laxa*	Emilia	Peach	n.d.	2011
74	**C4**	*M. laxa*	Emilia	Peach	n.d.	2011
75	**C5**	*M. laxa*	Emilia	Peach	n.d.	2011
76	**C 13**	*M. laxa*	Emilia	Peach	n.d.	2011
77	**C14**	*M. laxa*	Emilia	Peach	n.d.	2011
78	**C17**	*M. laxa*	Emilia	Peach	n.d.	2011
79	**C19**	*M. laxa*	Emilia	Peach	n.d.	2011
80	**C21**	*M. laxa*	Emilia	Peach	n.d.	2011
81	**C24**	*M. laxa*	Emilia	Peach	n.d.	2011
82	**C25**	*M. laxa*	Emilia	Peach	n.d.	2011

83	**C27**	*M. laxa*	Emilia	Peach	n.d.	2011
84	**C28**	*M. laxa*	Emilia	Peach	n.d.	2011
85	**C30**	*M. laxa*	Emilia	Peach	n.d.	2011
86	**C32**	*M. laxa*	Emilia	Peach	n.d.	2011
87	**C32.1**	*M. laxa*	Emilia	Peach	n.d.	2011
88	**C33**	*M. laxa*	Emilia	Peach	n.d.	2011
89	**C34**	*M. laxa*	Emilia	Peach	n.d.	2011
90	**C35**	*M. laxa*	Emilia	Peach	n.d.	2011
91	**C37**	*M. laxa*	Emilia	Peach	n.d.	2011
92	**C38**	*M. laxa*	Emilia	Peach	n.d.	2011
93	**C41**	*M. laxa*	Emilia	Peach	n.d.	2011
94	**C43**	*M. laxa*	Emilia	Peach	n.d.	2011
95	**C44**	*M. laxa*	Emilia	Peach	n.d.	2011
96	**C45**	*M. laxa*	Emilia	Peach	n.d.	2011
97	**C46**	*M. laxa*	Emilia	Peach	n.d.	2011
98	**C47**	*M. laxa*	Emilia	Peach	n.d.	2011
99	**C49**	*M. laxa*	Emilia	Peach	n.d.	2011
100	**C52**	*M. laxa*	Emilia	Peach	n.d.	2011
101	**C57**	*M. laxa*	Emilia	Peach	n.d.	2011
102	**C58**	*M. laxa*	Emilia	Peach	n.d.	2011
103	**CER 1.2**	*M. laxa*	Romagna	Peach	n.d.	2011
104	**CER 1.3**	*M. laxa*	Romagna	Peach	n.d.	2011
105	**C. 2011/2**	*M. laxa*	Romagna	Cherry	n.d.	2011
106	**C. 2011/3**	*M. laxa*	Romagna	Cherry	n.d.	2011
107	**Ciliegia 4**	*M. laxa*	Emilia	Cherry	n.d.	2011
108	**Ciliegia 5**	*M. laxa*	Emilia	Cherry	n.d.	2011
109	**Ciliegia 6**	*M. laxa*	Emilia	Cherry	n.d.	2011
110	**Ciliegia 10**	*M. laxa*	Emilia	Cherry	n.d.	2011
111	**Holb 1**	*M. laxa*	USA	Peach	n.d.	2011
112	**Holb 2**	*M. laxa*	USA	Peach	n.d.	2011
113	**MD 2**	*M. laxa*	Veneto	Nectarine	Maria Dolce	2011
114	**NB 5/1**	*M. laxa*	Veneto	Nectarine organic	n.d.	2011
115	**NT 1**	*M. laxa*	Emilia	Peach organic	n.d.	2011
116	**P 2010**	*M. laxa*	Emilia	Peach	Prince	2011
117	**PB 1/1**	*M. laxa*	Lombardia	Plum organic	Stanley	2011
118	**PB 1/2**	*M. laxa*	Lombardia	Plum organic	Stanley	2011
119	**PB 2/1**	*M. laxa*	Lombardia	Plum cv Stanley organic	Stanley	2011
120	**PB 2/2**	*M. laxa*	Lombardia	Plum organic	Stanley	2011
121	**PBio 1/1**	*M. laxa*	Lombardia	Plum organic	Stanley	2011
122	**P.P.2B**	*M. laxa*	Emilia	Peach	n.d.	2011
123	**Big Bang**	*M. laxa*	Romagna	Peach	Big Bang	2011
124	**RE 1/C**	*M. laxa*	Emilia	Peach	Red heaven	2011
125	**RE 2/A**	*M. laxa*	Emilia	Peach	Red heaven	2011
126	**RE 3/B**	*M. laxa*	Emilia	Peach	Red heaven	2011
127	**RE 4/D**	*M. laxa*	Emilia	Peach	Red heaven	2011

128	**RE 5/B**	*M. laxa*	Emilia	Peach	Red heaven	2011
129	**RE 5/C**	*M. laxa*	Emilia	Peach	Red heaven	2011
130	**RE 5/D**	*M. laxa*	Emilia	Peach	Red heaven	2011
131	**RE 6/B**	*M. laxa*	Emilia	Peach	Red heaven	2011
132	**RE 6/C**	*M. laxa*	Emilia	Peach	Red heaven	2011
133	**R.H 6/d**	*M. laxa*	Emilia	Peach	Red heaven	2011
134	**TR 60.4**	*M. laxa*	Emilia	Peach	n.d.	2011
135	**TR 60.9**	*M. laxa*	Emilia	Peach	n.d.	2011
136	**TR 60.10**	*M. laxa*	Emilia	Peach	n.d.	2011
137	**TR 60.14**	*M. laxa*	Emilia	Peach	n.d.	2011
138	**TR 800.2**	*M. laxa*	Emilia	Peach	n.d.	2011
139	**TR 800.3**	*M. laxa*	Emilia	Peach	n.d.	2011
140	**CBS**	*M. laxa*	AB75654		n.d.	
141	**MCL 1**	*M. fructicola*	Romagna	Peach	n.d.	2010
142	**MCL 2**	*M. fructicola*	Romagna	Peach	n.d.	2010
143	**MCL 3**	*M. fructicola*	Romagna	Peach	n.d.	2010
144	**MCL 4**	*M. fructicola*	Romagna	Peach	n.d.	2010
145	**MCL 5**	*M. fructicola*	Romagna	Peach	n.d.	2010
146	**MCL 6**	*M. fructicola*	Romagna	Peach	n.d.	2010
147	**MLC7**	*M. fructicola*	Romagna	Peach	Springbelle	2010
148	**MCL8**	*M. fructicola*	Romagna	Peach	Springbelle	2010
149	**MCL9**	*M. fructicola*	Emilia	Cherry	Bigarlou	2010
150	**MCL 10**	*M. fructicola*	Romagna	Peach	n.d.	2010
151	**MCL11**	*M. fructicola*	Romagna	Peach	n.d.	2010
152	**MCL12**	*M. fructicola*	Romagna	Peach	n.d.	2010
153	**MCL13**	*M. fructicola*	Romagna	Peach	n.d.	2010
154	**MCL14**	*M. fructicola*	Romagna	Peach	n.d.	2010
155	**MCL15**	*M. fructicola*	Romagna	Peach	n.d.	2010
156	**MCL16**	*M. fructicola*	Romagna	Peach	Springbelle	2010
157	**MCL17**	*M. fructicola*	Romagna	Apricot	n.d.	2010
158	**MCL18**	*M. fructicola*	Emilia	Peach	n.d.	2010
159	**MCL19**	*M. fructicola*	Emilia	Peach	n.d.	2010
160	**MCL20**	*M. fructicola*	Romagna	Peach	n.d.	2010
161	**MCL21**	*M. fructicola*	Emilia	Apple	Gala	2009
162	**Mcl6989**	*M. fructicola*	RPPC	Peach	n.d.	2010
163	**Mcl9285**	*M. fructicola*	RPPC	Peach	n.d.	2010
164	**Mcl9310**	*M. fructicola*	RPPC	Peach	n.d.	2010
165	**Mcl9311**	*M. fructicola*	RPPC	Peach	n.d.	2010
166	**Mcl9341**	*M. fructicola*	RPPC	Peach	n.d.	2010
167	**Mcl9345**	*M. fructicola*	RPPC	Peach	n.d.	2010
168	**Mcl9346**	*M. fructicola*	RPPC	Peach	n.d.	2010
169	**Mcl9348**	*M. fructicola*	RPPC	Peach	n.d.	2010
170	**Mcl9375**	*M. fructicola*	RPPC	Peach	n.d.	2010
171	**Mcl9561**	*M. fructicola*	RPPC	Peach	n.d.	2010
172	**2 a.1**	*M. fructicola*	Emilia	Nectarine	Orion Organic	2011

173	**2a**	*M. fructicola*	Emilia	Nectarine	Orion Organic	2011
174	**2b**	*M. fructicola*	Emilia	Peach	Sweet Lady	2011
175	**2c**	*M. fructicola*	Emilia	Peach	Fayette	2011
176	**2g**	*M. fructicola*	Emilia	Peach	Fayette	2011
177	**3c**	*M. fructicola*	Emilia	Apple	Pink Lady	2011
178	**5052. 2**	*M. fructicola*	RPPC	Nectarine organic	Venus	2011
179	**5052.3**	*M. fructicola*	RPPC	Peach	Royal Lee	2011
180	**571.2**	*M. fructicola*	RPPC	Peach	Royal Lee	2011
181	**6074.2**	*M. fructicola*	RPPC	Peach	n.d.	2011
182	**6074.3**	*M. fructicola*	RPPC	Peach	n.d.	2011
183	**6544.1**	*M. fructicola*	RPPC	Peach	n.d.	2011
184	**7566.1**	*M. fructicola*	RPPC	Peach	Royal Lee	2011
185	**7566.3**	*M. fructicola*	RPPC	Peach	Royal Lee	2011
186	**A21/1**	*M. fructicola*	Romagna	Peach	n.d.	2011
187	**A21/2**	*M. fructicola*	Romagna	Peach	n.d.	2011
188	**A21/4**	*M. fructicola*	Romagna	Peach	n.d.	2011
189	**A23/2**	*M. fructicola*	Romagna	Peach	n.d.	2011
190	**A23/3**	*M. fructicola*	Romagna	Peach	n.d.	2011
191	**A23/5**	*M. fructicola*	Romagna	Peach	n.d.	2011
192	**A23/5**	*M. fructicola*	Romagna	Peach	n.d.	2011
193	**A25/1**	*M. fructicola*	Romagna	Peach	n.d.	2011
194	**A25/3**	*M. fructicola*	Romagna	Peach	n.d.	2011
195	**A25/6**	*M. fructicola*	Romagna	Peach	n.d.	2011
196	**A25/8**	*M. fructicola*	Romagna	Peach	n.d.	2011
197	**AB 1**	*M. fructicola*	Romagna	Nectarine	Andross	2011
198	**AB 4**	*M. fructicola*	Romagna	Nectarine	Andross	2011
199	**Amb 1/2**	*M. fructicola*	RPPC	Peach	n.d.	2011
200	**AZ 2/2**	*M. fructicola*	Romagna	Peach	n.d.	2011
201	**BG1**	*M. fructicola*	Romagna	Peach organic	Baby Gold	2011
202	**BG2**	*M. fructicola*	Romagna	Peach organic	Baby Gold	2011
203	**BG4**	*M. fructicola*	Romagna	Peach organic	Baby Gold	2011
204	**C1**	*M. fructicola*	Emilia	Peach	n.d.	2011
205	**C2 (Cor)**	*M. fructicola*	USA	Peach	Coronet	2011
206	**C3**	*M. fructicola*	Emilia	Peach	n.d.	2011
207	**C4 (Cor)**	*M. fructicola*	USA	Peach	Coronet	2011
208	**C7 (Cor)**	*M. fructicola*	USA	Peach	Coronet	2011
209	**C8**	*M. fructicola*	Emilia	Peach	n.d.	2011
210	**C8 (Cor)**	*M. fructicola*	USA	Peach	Coronet	2011
211	**C9**	*M. fructicola*	Emilia	Peach	n.d.	2011
212	**C10**	*M. fructicola*	Emilia	Peach	n.d.	2011
213	**C12**	*M. fructicola*	Emilia	Peach	n.d.	2011
214	**C13**	*M. fructicola*	Emilia	Peach	n.d.	2011
215	**C18**	*M. fructicola*	Emilia	Peach	n.d.	2011
216	**C23**	*M. fructicola*	Emilia	Peach	n.d.	2011
217	**C35**	*M. fructicola*	Emilia	Peach	n.d.	2011

218	**C36**	*M. fructicola*	Emilia	Peach	n.d.	2011
219	**C36.1**	*M. fructicola*	Emilia	Peach	n.d.	2011
220	**C39**	*M. fructicola*	Emilia	Peach	n.d.	2011
221	**C41**	*M. fructicola*	Emilia	Peach	n.d.	2011
222	**C48**	*M. fructicola*	Emilia	Peach	n.d.	2011
223	**C50**	*M. fructicola*	Emilia	Peach	n.d.	2011
224	**C51**	*M. fructicola*	Emilia	Peach	n.d.	2011
225	**C56**	*M. fructicola*	Emilia	Peach	n.d.	2011
226	**CER 1.1**	*M. fructicola*	Romagna	Peach	n.d.	2011
227	**C. 2011**	*M. fructicola*	Romagna	Cherry	n.d.	2011
228	**C. 2011/2**	*M. fructicola*	Romagna	Cherry	n.d.	2011
229	**CO 5**	*M. fructicola*	USA	Peach	Contender	2011
230	**D**	*M. fructicola*	Sardegna	Peach	Guglielmina	2011
231	**EL 2/1**	*M. fructicola*	Sardegna	Peach	Elegant Lady	2011
232	**EL 2/2**	*M. fructicola*	Sardegna	Peach	Elegant Lady	2011
233	**EL 2**	*M. fructicola*	Sardegna	Peach	Elegant Lady	2011
234	**G 062 B/2**	*M.fructicola*	Veneto	Peach	n.d.	2011
235	**G 062 C/1**	*M. fructicola*	Veneto	Peach	n.d.	2011
236	**G 100 A/1**	*M. fructicola*	Veneto	Peach	n.d.	2011
237	**G 100 C/1**	*M. fructicola*	Veneto	Peach	n.d.	2011
238	**G 100 C/2**	*M. fructicola*	Veneto	Peach	n.d.	2011
239	**G 100B/1**	*M.fructicola*	Veneto	Peach	n.d.	2011
240	**G 105 A/2**	*M. fructicola*	Veneto	Peach	n.d.	2011
241	**G 159 B/2**	*M. fructicola*	Veneto	Peach	n.d.	2011
242	**G 164 C/2**	*M. fructicola*	Veneto	Peach	n.d.	2011
243	**G062 105 A/2**	*M. fructicola*	Veneto	Peach	n.d.	2011
244	**G062 105 A/3**	*M. fructicola*	Veneto	Peach	n.d.	2011
245	**G062 105 C/2**	*M. fructicola*	Veneto	Peach	n.d.	2011
246	**G062 B/1**	*M. fructicola*	Veneto	Peach	n.d.	2011
247	**G062 C/2**	*M. fructicola*	Veneto	Peach	n.d.	2011
248	**G100 B/2**	*M. fructicola*	Veneto	Peach	n.d.	2011
249	**G100 C/2**	*M. fructicola*	Veneto	Peach	n.d.	2011
250	**G100 C/3**	*M. fructicola*	Veneto	Peach	n.d.	2011
251	**G164 A/2**	*M. fructicola*	Veneto	Peach	n.d.	2011
252	**G164 C/1**	*M. fructicola*	Veneto	Peach	n.d.	2011
253	**Grenat 2/1**	*M. fructicola*	Romagna	Nectarine	Maria Dolce	2011
254	**Grenat 2/2**	*M. fructicola*	Romagna	Nectarine	Maria Dolce	2011
255	**Grenat 3/2**	*M. fructicola*	Romagna	Nectarine	Maria Dolce	2011
256	**MD 1**	*M. fructicola*	Romagna	Nectarine organic	n.d.	2011
257	**MD 5**	*M. fructicola*	Romagna	Nectarine organic	n.d.	2011
258	**MD 4**	*M. fructicola*	Romagna	Nectarine organic	n.d.	2011

259	**NB 1/1**	*M. fructicola*	Veneto	Nectarine organic	n.d.	2011
260	**NB 2/2**	*M. fructicola*	Veneto	Nectarine organic	n.d.	2011
261	**NB 4/2**	*M. fructicola*	Veneto	Nectarine organic	n.d.	2011
262	**NB 6/1**	*M. fructicola*	Veneto	Nectarine organic	n.d.	2011
263	**NB 7/2**	*M. fructicola*	Veneto	Nectarine organic	n.d.	2011
264	**Nett. bio**	*M. fructicola*	Veneto	Nectarine organic	n.d.	2011
265	**NT2**	*M. fructicola*	Emilia	Peach	n.d.	2011
266	**Q**	*M. fructicola*	Sardegna	Peach	n.d.	2011
267	**RE 3/A**	*M. fructicola*	Emilia	Peach cv	Red Heaven	2011
268	**RE 7/A**	*M. fructicola*	Emilia	Peach	Red Heaven	2011
269	**RE 7/D**	*M. fructicola*	Emilia	Peach	Red Heaven	2011
270	**Royal glory 1**	*M. fructicola*	Romagna	Peach	Royal Glory	2011
271	**Royal glory 2**	*M. fructicola*	Romagna	Peach	Royal Glory	2011
272	**Royal lee 1/2**	*M. fructicola*	Romagna	Peach	Royal Lee	2011
273	**Royal lee 2/1**	*M. fructicola*	Romagna	Peach	Royal Lee	2011
274	**Royal lee 2/2**	*M. fructicola*	Romagna	Peach	Royal Lee	2011
275	**TR 60.2**	*M. fructicola*	Emilia	Peach	n.d.	2011
276	**TR 60.6**	*M. fructicola*	Emilia	Peach	n.d.	2011
277	**TR 60.7**	*M. fructicola*	Emilia	Peach	n.d.	2011
278	**TR 60.9**	*M. fructicola*	Emilia	Peach	n.d.	2011
279	**TR 800.1**	*M. fructicola*	Emilia	Peach	n.d.	2011
280	**TR 800.6**	*M. fructicola*	Emilia	Peach	n.d.	2011
281	**TR 800.15**	*M. fructicola*	Emilia	Peach	n.d.	2011
282	**TR 800.29**	*M. fructicola*	Emilia	Peach	n.d.	2011
283	**Venus 2/2**	*M. fructicola*	Romagna	Nectarine	Venus	2011
284	**MCG 1**	*M. fructigena*	Emilia	Pear	n.d.	2009
285	**MCG 2**	*M. fructigena*	Emilia	Apple	Golden	2010
286	**MCG 3**	*M. fructigena*	Emilia	Apple	Golden	2010
287	**MCG 4**	*M. fructigena*	Emilia	Plum	Stanley	2010
288	**MCG 5**	*M. fructigena*	Emilia	Peach	Red Heaven	2010
289	**1A**	*M. fructigena*	Lombardia	Apple	pollinator	2011
290	**3A**	*M. fructigena*	Lombardia	Apple	Pink Lady	2011
291	**4A**	*M. fructigena*	Lombardia	Apple	Pink Lady	2011
292	**2B**	*M. fructigena*	Lombardia	Apple	pollinator	2011
293	**NP 4/1**	*M. fructigena*	Romagna	Nectarine	n.d.	2011
294	**PL 10**	*M. fructigena*	Lombardia	Apple	Pink Lady	2011
296	**PL 11**	*M. fructigena*	Lombardia	Apple	Pink Lady	2011
297	**PL 13**	*M. fructigena*	Lombardia	Apple	Pink Lady	2011

298	**PL 7**	*M. fructigena*	Lombardia	Apple	Pink Lady	2011

[a] n.d.: not determined [b] RPPC: Regional Plant Protection Centre

APPENDIX 4

MOA	TARGET SITE AND CODE	GROUP NAME	CHEMICAL GROUP	COMMON NAME	COMMENTS	FRAC CODE
D:AMINO ACIDS AND PROTEIN SYSTEM	D1: Methionine biosynthesis (proposed) (Cgs gene)	Al-fungicides (Anilino-Pyrimidines)	Aniline-pyrimidines	Cyprodinil Mepanipyrim pyrimethanil	Resistance know in botrytis and Venturia, sporadically in Oculimacula. Medium risk	9
G: STEROL BIOSYNTHESIS IN MEMBRANES	G3: 3-keto-reduc-tase, C4-de-metylation (erg27)	Hydroxyanilides (SBI:classic)	hydroxyanilides	Fenhexamid	Low to medium risk. Resistance management require	17
	G1: C14-demethylase in sterol biosynthesis (erg11/cyp51)	DMI-fungicides (De Methylation Inhibitors)(SBI:clasI)	Triazole	Tebuconazole Fenbuconazole etc.	There are differencesin the activity spectra of DMI fungicides. Resistance in known in varius fungal species. Several resistance mechanisms are known incl. target site mutations in cyp51 (erg11) gene. Generally wise to accept that cross resistance	3

					is present between DMI fungicides, active against the same fungus. DMI fungicides are Sterol Biosynthesis Inhibitors (SBIS) but show no cross resistance to other SBI closses	
C: RESPIRATION	C2 complex II: succinate-dehydro-fenase	SDHI (succinate dehydrogenase inhibitors	Pyridine-caboxamides	boscalid	Resistance known for several fungal species in field populations and lab mutants. Target site mutations in sdh gene. Resistance management required. Medium risk	7
	C3 complex III: cytochrome bc1 (ubiquinol oxidase) at Qosite (cyt b gene)	QoI-fungicides (Quinone outside Inhibitors)	Methoxy-carbamates	Pyraclostrobin Trifloxystrobin Etc.	Resistance to known in varius fungal species. Target site mutations in cytb gene (G143A, F129L) and addizional mechanisms. Cross resistance shown between all members of the QoI group	11

E: SIGNAL TRANSDUCTION	E2 MAP/Histidine-kinase in osmotic signal transduction (os-2, HOG1)	PP-fungicides (Phenyl Pyrroles)	phenylpirroles	Fenpiclonil fludioxonil	Resistance found sporadically, mechanism speculative. Low to medium risk. Resistance management required	12
	E3 MAP/Histidine-kinase in osmotic signal transduction (os-1, daf1)	dicarboximides	dicarboximides	Procymidone Iprodione	Resistance common in Botrytis and some other pathogens. Several mutation in OS-1 mostly 1365 S. Cross resistance common between the group members. Medium to high risk.	2

APPENDIX 5

Fungicides used in this study

Active ingredient (a.i.)	Chemical name	Commercial name	% of a.i.	Produced by
Tebuconazole	Alpha-[2-(4-chlorophenyl)ethyl]-alpha-(1,1-dimethylethyl)-1H-1,2,4-triazole-1-ethanol	Folicur SE	4.35%	Bayer CropScience, Lyon, France
Tebuconazole	Alpha-[2-(4-chlorophenyl)ethyl]-alpha-(1,1-dimethylethyl)-1H-1,2,4-triazole-1-ethanol	Elite	45%	Bayer CropScience, NC
Thiophanate methyl	(dimethyl[1,2-phenylene)-bis(iminocarbonothioyl)]bis[carbamate])	Enovit Metil FL	38.3%	Sipcam, Pero, Milan, Italy
Thiophanate methyl	(dimethyl[1,2-phenylene)-bis(iminocarbonothioyl)]bis[carbamate])	Topsin-M 70WP	70%	Cerexagri-Nisso LLC, PA

APPENDIX 6

Amended medium method: values of EC_{50} (μg ml^{-1}) for *M. fructicola* and *M. fructigena* mycelial growth in isolates of 2010 and determined by the agar amended method. For each concentration and for each fungicide, four plates (replicates) were used

Isolate no.	Pathogen	Fungicide	EC_{50} □g ml^{-1} [a]	r^2	R/S
MCL 1	*M.fructicola*	-Tebuconazole	0.053	0.95	S
		-Thiophanate m.	19.55	0.90	R
MCL 2	*M.fructicola*	-Tebuconazole	0.063	0.92	S
		-Thiophanate m.	22.7	0.88	R
MCL 3	*M. fructicola*	-Tebuconazole	0.02	0.94	S
		-Thiophanate m.	14.81	0.89	R
MCL 4	*M. fructicola*	-Tebuconazole	0.037	0.97	S
		-Thiophanate m.	0.51	0.87	S
MCL 5	*M.fructicola*	-Tebuconazole	0.02	0.89	S
		-Thiophanate m.	0.60	0.8	S
MCL 6	*M.fructicola*	-Tebuconazole	0.02	0.92	S
		-Thiophanate m.	6.94	0.86	R
MLC7	*M.fructicola*	-Tebuconazole	0.041	0.89	S
		-Thiophanate m.	20.15	0.87	R
MCL8	*M.fructicola*	-Tebuconazole	0.008	0.9	S
		-Thiophanate m.	18.71	0.92	R
MCL9	*M.fructicola*	-Tebuconazole	0.037	0.9	S
		-Thiophanate m.	20.36	0.86	R
MCL 10	*M.fructicola*	-Tebuconazole	0.059	0.94	S
		-Thiophanate m.	34.08	0.97	R
MCL11	*M.fructicola*	-Tebuconazole	0.019	0.99	S
		-Thiophanate m.	23.98	0.94	R

MCL12	*M.fructicola*	-Tebuconazole	0.051	0.94	S
		-Thiophanate m.	37.05	0.81	R
MCL13	*M.fructicola*	-Tebuconazole	0.067	0.96	S
		-Thiophanate m.	22.74	0.97	R
MCL14	*M.fructicola*	-Tebuconazole	0.05	0.91	S
		-Thiophanate m.	1.25	0.97	R
MCL15	*M.fructicola*	-Tebuconazole	0.115	0.98	S
		-Thiophanate m.	1.61	0.88	R
MCL16	*M.fructicola*	-Tebuconazole	0.037	0.99	S
		-Thiophanate m.	0.18	0.88	S
MCL17	*M.fructicola*	-Tebuconazole	0.051	0.97	S
		-Thiophanate m.	0.61	0.92	S
MCL19	*M.fructicola*	-Tebuconazole	0.04	0.89	S
		-Thiophanate m.	3.69	0.99	R
MCL20	*M.fructicola*	-Tebuconazole	0.089	0.99	S
		-Thiophanate m.	36.32	0.96	R
MCL21	*M.fructicola*	-Tebuconazole	0.089	0.99	S
		-Thiophanate m.	n.d.	n.d.	n.d.
MCL6989	*M.fructicola*	-Tebuconazole	0.043	0.9	S
		-Thiophanate m.	10.58	0.95	R
MCL9285	*M.fructicola*	-Tebuconazole	n.d.	n.d.	
		-Thiophanate m.	11.75	0.9	R
MCL9310	*M.fructicola*	-Tebuconazole	0.043	0.97	S
		-Thiophanate m.	n.d	n.d.	n.d.
MCL9311	*M.fructicola*	-Tebuconazole	0.025	0.98	S
		-Thiophanate m.	10.05	0.99	R
MCL9341	*M.fructicola*	-Tebuconazole	0.037	0.91	S
		-Thiophanate m.	18.51	0.97	R
MCL9348	*M.fructicola*	-Tebuconazole	0.06	0.91	S
		-Thiophanate m.	10.78	0.97	R
MCL9375	*M.fructicola*	-Tebuconazole	0.08	0.98	S
		-Thiophanate m.	19.17	0.95	R
MCG 1	*M. fructigena*	-Tebuconazole	0.118	0.91	S
		-Thiophanate m.	n.d.	n.d.	n.d.
MCG 2	*M. fructigena*	-Tebuconazole	0.172	0.88	R
		-Thiophanate m.	0.23	0.99	S

MCG 3	*M. fructigena*	-Tebuconazole	0.124	0.93	S
		-Thiophanate m.	0.27	0.97	S
MCG 4	*M. fructigena*	-Tebuconazole	0.132	0.94	R
		-Thiophanate m.	n.d.	n.d.	n.d.
MCG 5	*M. fructigena*	-Tebuconazole	0.155	0.99	R
		-Thiophanate m.	0.36	0.97	S

[a] EC_{50} is the effective concentration of fungicide for a 50%. inhibition of mycelial growth. All values are means of two experiments, with four replicate petri dishes each

APPENDIX 7

SGD method: EC_{50} values for tebuconazole and thiophanate methyl of *M. laxa* 2010 isolates. Mycelial growth inhibition determined by the spiral gradient dilution (SGD) method. Four replicated-plates were used for each isolates and fungicide

Isolate no.	Fungus	Fungicide	EC_{50} ($\mu g\ ml^{-1}$)[a]	R/S
CBS	*M. laxa*	-Tebuconazole	n.d.	n.d
		-Thiophanate m	0.006±0.001[b]	S
ML 1	*M. laxa*	-Tebuconazole	0.018±0.008	S
		-Thiophanate m	0.333±0.005	S
ML 2	*M. laxa*	-Tebuconazole	0.022±0.028	S
		-Thiophanate m	0.422±0.011	S
ML 3	*M.laxa*	-Tebuconazole	0.01±0.002	S
		-Thiophanate m	0.093±0.002	S
ML 4	*M. laxa*	-Tebuconazole	0.033±0.005	S
		-Thiophanate m	0.085±0.012	S
ML 5	*M. laxa*	-Tebuconazole	0.03±0.001	S
		-Thiophanate m	2.231±0.004	R
ML 6	*M. laxa*	-Tebuconazole	0.014±0.006	S
		-Thiophanate m	0.141±0.032	S
ML 7	*M. laxa*	-Tebuconazole	0.008±0.065	S
		-Thiophanate m	0.544±0.008	S
ML8	*M.laxa*	-Tebuconazole	0.027±0.001	S
		-Thiophanate m	0.555±0.006	S
ML9	*M.laxa*	-Tebuconazole	0.03±0.014	S
		-Thiophanate m	0.318±0.057	S
ML10	*M.laxa*	-Tebuconazole	0.007±0.017	S

		-Thiophanate m	0.206±0.077	S
ML 11	*M.laxa*	-Tebuconazole	0.017±0.005	S
		-Thiophanate m	0.876±0.067	R
ML12	*M.laxa*	-Tebuconazole	0.008±0.078	S
		-Thiophanate m	0.359±0.054	S
ML13	*M.laxa*	-Tebuconazole	0.026±0.001	S
		-Thiophanate m	0.781±0.005	R
ML14	*M.laxa*	-Tebuconazole	0.01±0.043	S
		-Thiophanate m	0.626±0.041	S
ML15	*M.laxa*	-Tebuconazole	0.015±0.013	S
		-Thiophanate m	0.105±0.045	S
ML17	*M.laxa*	-Tebuconazole	0.022±0.037	S
		-Thiophanate m	0.457±0.008	S
ML18	*M.laxa*	-Tebuconazole	0.025±0.016	S
		-Thiophanate m	0.257±0.002	S
ML19	*M.laxa*	-Tebuconazole	0.034±0.046	S
		-Thiophanate m	0.129±0.018	S
ML20	*M.laxa*	-Tebuconazole	0.026±0.024	S
		-Thiophanate m	0.691±0.007	S
ML21	*M.laxa*	-Tebuconazole	0.027±0.065	S
		-Thiophanate m	0.331±0.013	S
ML22	*M.laxa*	-Tebuconazole	0.022±0.043	S
		-Thiophanate m	0.431±0.064	S
ML23	*M.laxa*	-Tebuconazole	0.019±0.005	S
		-Thiophanate m	0.616±0.001	S
ML27	*M.laxa*	-Tebuconazole	0.004±0.016	S
		-Thiophanate m	0.144±0.01	S
ML28	*M.laxa*	-Tebuconazole	0.012±0.076	S
		-Thiophanate m	0.266±0.014	S
ML29	*M.laxa*	-Tebuconazole	0.033±0.076	S
		-Thiophanate m	0.294±0.037	S
ML30	*M.laxa*	-Tebuconazole	0.023±0.003	S
		-Thiophanate m	0.247±0.016	S

ML31	*M.laxa*	-Tebuconazole	n.d.	n.d.
		-Thiophanate m	0.601±0.028	S
ML32	*M.laxa*	-Tebuconazole	0.035±0.017	S
		-Thiophanate m	0.241±0.057	S
ML33	*M.laxa*	-Tebuconazole	0.024±0.001	S
		-Thiophanate m	0.233±0.032	S
ML34	*M.laxa*	-Tebuconazole	0.020±0.009	S
		-Thiophanate m	0.211±0.087	S
ML 8B	*M.laxa*	-Tebuconazole	0.32±0.012	R
		-Thiophanate m	2.27±0.005	R
ML 9B	*M.laxa*	-Tebuconazole	0.021±0.008	S
		-Thiophanate m	0,061±0.075	S
ML 10B	*M.laxa*	-Tebuconazole	0.01±0.007	S
		-Thiophanate m	0.216±0.074	S
ML 12B	*M. laxa*	-Tebuconazole	0.019±0.016	S
		-Thiophanate m	0.084±0.006	S
ML13B	*M.laxa*	-Tebuconazole	0.02±0.076	S
		-Thiophanate m	0.391±0.089	S
M4FC	*M. laxa*	-Tebuconazole	0.063±0.089	S
		-Thiophanate	0.261±0.017	S
M5FC	*M. laxa*	-Tebuconazole	0.021±0.034	S
		-Thiophanate	0.521±0.057	S
M7FC	*M. laxa*	-Tebuconazole	0.012±0.005	S
		-Thiophanate m	0.412±0.008	S
M8FC	*M. laxa*	-Tebuconazole	n.d.	n.d.
		-Thiophanate m	0.227±0.039	S
M9FC	*M. laxa*	-Tebuconazole	0.005±0.038	S
		-Thiophanate m	0.359±0.027	S
M10BO	*M. laxa*	-Tebuconazole	0.004±0.004	S
		-Thiophanate m	0.478±0.007	S
M12BO	*M. laxa*	-Tebuconazole	0.019±0.045	S
		-Thiophanate m	0.084±0.062	S
M15FC	*M. laxa*	-Tebuconazole	0.002±0.005	S

		-Thiophanate m	1.357±0.004	R
M19RA	*M. laxa*	-Tebuconazole	0.014±0.007	S
		-Thiophanate m	0.402±0.043	S
M20FC	*M. laxa*	-Tebuconazole	0.026±0.014	S
		-Thiophanate m	0.210±0.053	S
M21FC	*M. laxa*	-Tebuconazole	0.011±0.054	S
		-Thiophanate m	0.306±0.065	S
M27FC	*M. laxa*	-Tebuconazole	0.163±0.012	R
		-Thiophanate m	0.082±0.034	S
M4	*M. laxa*	-Tebuconazole	0.016±0.076	S
		-Thiophanate m	0.375±0.026	S
M23	*M. laxa*	-Tebuconazole	0.18±0.001	R
		-Thiophanate m	3.12±0.005	R
M7454	*M. laxa*	-Tebuconazole	0.125±0.008	R
		-Thiophanate m	0.032±0.058	S
M7537	*M. laxa*	-Tebuconazole	0.029±0.056	S
		-Thiophanate m	0.331±0.087	S
M7636	*M. laxa*	-Tebuconazole	0.015±0.007	S
		-Thiophanate m	0.15±0.076	S
M7782	*M. laxa*	-Tebuconazole	0.027±0.005	S
		-Thiophanate m	0.364±0.023	S
M7784	*M. laxa*	-Tebuconazole	0.03±0.054	S
		-Thiophanate m	0.412±0.067	S
M7785	*M. laxa*	-Tebuconazole	0.03±0.018	S
		-Thiophanate m	0.412±0.089	S
EBR 1	*M. laxa*	-Tebuconazole	0.310±0.014	R
		-Thiophanate m	3.126±0.006	R
Holb 1	*M. laxa*	-Tebuconazole	0.087±0.041	S
		-Thiophanate m	0.251±0.045	S
Holb 2	*M. laxa*	-Tebuconazole	0.067±0.005	S
		-Thiophanate m	0.826±0.008	S
Quince 2010	*M. laxa*	-Tebuconazole	0.139±0.003	R
		-Thiophanate m	0.969±0.009	R

APPENDIX 8

Comparison between amended medium and AB method: values for 50% effective concentration (EC_{50}) for inhibition of *M. laxa* and M. *fructicola* conidia germination in strains isolated in 2011 as determined by the agar dilution and AB method. Three replicated-plates were used for each isolates on agar dilution method at different fungicide concentration, and three replicated-wells were used for each isolates on AB method at different fungicide concentration

Fungicide	Fungus	Isolate no.	EC_{50} (μg ml^{-1})	
			Inhibition of conidia germination[a]	AB method[b]
-Tebuconazole	*M. laxa*	C2	0.26	0.28
-Tebuconazole	*M. laxa*	C8	0.01	0.05
-Tebuconazole	*M. laxa*	MD5	0.52	0.43
-Tebuconazole	*M. laxa*	TR800.3	0.33	0.28
-Tebuconazole	*M.fructicola*	A23/2	0.005	0.001
-Tebuconazole	*M.fructicola*	A25/8	0.003	0.001
-Tebuconazole	*M.fructicola*	C13	0.05	0.02
-Tebuconazole	*M.fructicola*	D	0.008	0.004
-Tebuconazole	*M.fructicola*	G064 105 C/2	0.24	0.13
-Tebuconazole	*M.fructicola*	NB 6/1	0.02	0.04
-Tebuconazole	*M.fructicola*	2b	0.003	0.002
-Tebuconazole	*M.fructicola*	6074.2	0.11	0.18
-Thiophanate m.	*M. laxa*	AB 5	0.36	0.45
-Thiophanate m.	*M. laxa*	Alac BIO 3/2	1.51	1.88

-Thiophanate m.	*M. laxa*	Cherry 2011/3	11.26	10.59
-Thiophanate m.	*M. laxa*	C 32	4.20	4.54
-Thiophanate m.	*M. laxa*	C 38	2.04	2.12
-Thiophanate m.	*M. laxa*	C 43	0.40	0.3
-Thiophanate m.	*M. laxa*	Pesca prof.2 B	0.052	0.079
-Thiophanate m.	*M. laxa*	6544.2	0.83	0.48
-Thiophanate m.	*M.fructicola*	A25/6	44.02	44.06
-Thiophanate m.	*M.fructicola*	C3	5.20	5.67
-Thiophanate m.	*M.fructicola*	C12	3.03	3.57
-Thiophanate m.	*M.fructicola*	C13	36.40	32.91
-Thiophanate m.	*M.fructicola*	C35	7.50	5.45
-Thiophanate m.	*M.fructicola*	G164 C/1	1.97	1.60
-Thiophanate m.	*M.fructicola*	Q	1.13	1.30
-Thiophanate m.	*M.fructicola*	7566.3	0.64	0.34

[a] EC_{50} is the effective concentration of the fungicide at which conidial germination was inhibited by 50%. All values are means of two experiments, with three replicate petri dishes (agar dilution) or wells (AB assay) per experiment.

[b] EC_{50} values (µg ml-1) were calculated by regressing the percentages of the relative conidial germination against the logarithm of inhibitor concentrations

APPENDIX 9

Inhibition values and fungal respiration with thiophanate methyl: were determined by measuring the absorbance of the dye at 570 nm (λ1 red light reflectance, blue light absorbance) and 600 nm (λ2 blue light reflectance, red light absorbance) on a tunable microplate reader. The percentage reduction in AB control for thiophanate methyl and the percentage difference between treated and control were calculated by equations *a* and *b* in section 3.4.3 for *M. laxa* and *M. fructicola*

M. laxa	Reduction of AB on the Control(%)[a]	EC_{50} $\mu g\ ml^{-}_{1b}$	R/S	*M. laxa*	Reduction of AB on the Control (%)	EC_{50} $\mu g\ ml^{-}_{1}$	R/S
RE 6/C	28.0	0.23	S	**A 22/1**	60.0%	8.67	R
RE 3/B	34.0	0.05	S	**RE 4/D**	64.5%	0.76	S
C 44	35.0	6.36	R	**TR 800.3**	74.0%	17.65	R
C4	37.4	1.88	R	**C2**	76.0%	15.87	R
AB 5	42.0	0.45	S	**Pescaprof2B**	76.0%	0.08	S
Ciliegia 4	43.0	0.0008	S	**Alacbio 3/2**	76.6%	1.88	R
C 32	44.0	4.54	R	**C 28**	77.0%	1.06	R
C 30	48.0	0.0004	S	**TR 60.14**	77,0%	0.74	S
C 43	49.7	0.29	S	**Cer 1/2**	79.3%	2.81	R
C 37	50.0	0.43	S	**6544.2**	84.0%	0.48	S
RE 5/B	50.2	0.71	S	**C 38**	84.2%	2.12	R
C33	51.3	2.09	R	**C 46**	85.4%	2.10	R
TR 800.2	53.1	3.40	R	**Cherry2011/3**	87.4%	10.59	R
PB 2/1	58.1	9.14	R	**C34**	91.5%	28.29	R
M. fructicola	Reduction of AB on the Control (%)	EC_{50} $\mu g\ ml^{-1}$	R/S	*M. fructicola*	Reduction of AB on the Control (%)	EC_{50} $\mu g\ ml^{-}_{1}$	R/S
TR60.7	11.9	0.002	S	**C2 (Coronet)**	65.8%	1.45	R
C 1	20.0	11.31	R	**Royalglory 2**	66.0%	3.70	R
6544.1	21.0	0.11	S	**2c**	69.6%	10.31	R
A25/8	21.0	2.59	R	**G 164 C/2**	70.7%	1.90	R
C12	23.0	3.57	R	**Royal lee 1/2**	74.5%	2.49	R
C4 (Coronet)	27.0	0.78	S	**C3**	82.3%	5.67	R
C48	28.0	0.002	S	**Q**	86.0%	1.30	R
AB 1	30.7	0.41	S	**G164 C/1**	86.4%	1.60	R
G100 C/3	34.0	0.63	S	**C 13**	87.7%	32.91	R

EL 2/1	35.0	0.01	S	**A21/2**	90.0%	4.14	R
CO5	43.0	1.95	R	**13627 fc**	90.0%	0.49	S
G 105 A/2	44.0	6.34	R	**C10**	96.0%	0.68	S
C41	44.2	2.09	R	**C39**	90.6%	8.68	R
G 100 C/1	49.0	0.60	S	**TR800.29**	91.2%	10.03	R
MD4	49.3	3.67	R	**MD 5**	97.0%	51.10	R
6074.2	49.3	15.81	R	**C36**	97.7%	3.95	R
C9	50.2	0.07	S	**NB 7/2**	99.0%	2.72	R
G 100 A/1	50.2	14.34	R	**2g**	99.0%	2.50	R
AZ 2/2	51.8	0.99	R	**571.2**	99.0%	1.27	R
A23/2	54.0	0.07	S	**7566.1**	99.0%	1.10	R
C 7 (Coronet)	55.8	11.14	R	**A 25/6**	99.0%	44.06	R
A23/5	56.1	4.89	R	**C35**	99.0%	5.45	R
5052.3	56.1	4.98	R	**C50**	99.0%	20.32	R
A23/3	57.0	0.57	S	**Elegant lady 2**	99.0%	31.17	R
C8	59.7	6.73	R	**G 100B/1**	99.0%	1.71	R
D	60.0	20.46	R	**Grenat3/2**	99.0%	1.83	R
AB 4	60.3	3.43	R	**NB 2/2**	99.0%	28.51	R
C18	61.0	0.05	S	**NB 6/1**	99.0%	4.05	R
G062 105 C/2	61.0	57.59	R	**TR800.6**	99.0%	1.73	R
G 100 C/2	61.9	0.81	R	**7566.3**	n.d.[c]	0.24	R
MD 1	63.5	0.62	S	**5052.2**	n.d.	0.09	R
2b	63.8	50.88	R	**G100B/2**	n.d.	0.95	R

[a]Reduction percentage of AB = {[(O2 x A1)-(O1 x A2)]/[(R1 x N2)-(R2 x N1)]}x100 (section 3.4.3)

[b] Percent difference between treated and control conidia={[(O2 x A1)-(O1 x A2)]/[(O2 x P1)-(O1 x P2)]}x100.The EC_{50} values (μg ml^{-1}) were calculated by the regression between probit and inhibition percentage using log_{10}-transformed fungicide concentrations. EC_{50} values of fungicide concentrations were obtained from the regression line intercept (50% reduction).

[c]n.d.: not determined

APPENDIX 10

Inhibition values and fungal respiration with tebuconazole: were determined by measuring the absorbance of the dye at 570 nm (λ1 red light reflectance, blue light absorbance) and 600 nm (λ2 blue light reflectance, red light absorbance) on a tunable microplate reader. The percentage reduction in AB control wells for tebuconazole and the percentage difference between treated and control were calculated by equations *a* and *b* in section 3.4.3 for *M. laxa* and *M. fructicola*

M. laxa	**Reduction of AB on the Control (%)[a]**	**EC_{50} μg ml^{-} [1b]**	**R/S**	***M. laxa***	**Reduction of AB on the Control (%)**	**EC_{50} μg ml^{-1}**	**R/S**
C8	20.0%	0.05	S	**C2**	54.0%	0.28	R
MD5	40.0%	0.43	R	**TR800.3**	90.0%	0.28	R
M. fructicola	**Reduction of AB on the Control (%)**	**EC_{50} μg ml^{-1}**	**R/S**	***M. fructicola***	**Reduction of AB on the Control (%)**	**EC_{50} μg ml^{-1}**	**R/S**
A23/2	16.0%	0.001	S	**G062 105 C/2**	39.0%	0.13	R
6074.2	20.0%	0.18	R	**NB 6/1**	46.0%	0.04	S
A25/8	21.0%	0.001	S	**2b**	58.0%	0.002	S
D	24.0%	0.004	S	**C13**	80.0%	0.02	S

[a]Reduction percentage of AB = {[(O2 x A1)-(O1 x A2)]/[(R1 x N2)-(R2 x N1)]}x100
[b]Percent difference between treated and control conidia={[(O2 x A1)-(O1 x A2)]/[(O2 x P1)-(O1 x P2)]}x100.The EC_{50} values (μg ml^{-1}) were calculated by the regression between probit and inhibition percentage using log_{10}-transformed fungicide concentrations. EC_{50} values of fungicide concentrations were obtained from the regression regression line intercept (50% reduction)

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